Pesticide residues in food – 2010

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2010

Part II — Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food — 2010

Toxicological evaluations

Sponsored jointly by FAO and WHO

Joint Meeting of the
FAO Panel of Experts on Pesticide Residues
in Food and the Environment
and the
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* First full evaluation

** Evaluated within the periodic review programme of the Codex Committee on Pesticide Residues

2010 Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

Rome, 21–30 September 2010

LIST OF PARTICIPANTS

FAO Panel of Experts on Pesticide Residues in Food and the Environment

Dr Ursula Banasiak, Federal Institute for Risk Assessment, Berlin, Germany

Mr Stephen Funk, Health Effects Division, Environmental Protection Agency, Washington, DC, USA

Mr Denis J. Hamilton, Principal Scientific Officer Biosecurity, Department of Primary Industries and Fisheries, Brisbane, Australia

Mr David Lunn, Senior Programme Manager (Residues–Plants), Export Standards Group, New Zealand Food Safety Authority, Wellington, New Zealand (*FAO Rapporteur*)

Dr Dougal MacLachlan, Australian Quarantine and Inspection Service, Australian Government Department of Agriculture, Fisheries and Forestry, Canberra, ACT, Australia

Dr Bernadette Ossendorp, Centre for Substances and Integrated Risk Assessment, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*FAO Chair*)

Dr Yukiko Yamada, Deputy Director-General, Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan

WHO Core Assessment Group on Pesticide Residues

Professor Alan R. Boobis, Experimental Medicine & Toxicology, Division of Investigative Science, Faculty of Medicine, Imperial College London, London, England

Dr Les Davies, Chemical Review, Australian Pesticides and Veterinary Medicines Authority, Kingston, ACT, Australia

Dr Vicki L. Dellarco, Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, USA (*WHO Rapporteur*)

Professor Angelo Moretto, Department of Environmental and Occupational Health, University of Milan, International Centre for Pesticides and Health Risk Prevention, Luigi Sacco Hospital, Milan, Italy (*WHO Chair*)

Dr Roland Solecki, Chemical Safety Division, Steering of Procedures and Overall Assessment, Federal Institute for Risk Assessment, Berlin, Germany

Dr Maria Tasheva, Consultant, National Service for Plant Protection, Ministry of Agriculture and Food, Sofia, Bulgaria

Secretariat

Ms Catherine Adcock, Health Evaluation Directorate, Pest Management Regulatory Agency, Ottawa, Ontario, Canada (*WHO Temporary Adviser*)

Dr Árpád Ambrus, Hungarian Food Safety Office, Budapest, Hungary (*FAO Temporary Adviser*)

Mr Kevin Bodnaruk, West Pymble, NSW, Australia (*FAO Editor*)

Dr Ian Dewhurst, Chemicals Regulation Directorate, York, England (*WHO Temporary Adviser*)

Dr William Donovan, Environmental Protection Agency, Washington, DC, USA (*FAO Temporary Adviser*)

Mr Makoto Irie, Plant Product Safety Division, Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan (*FAO Temporary Adviser*)

Dr Debabrata Kanungo, Directorate General of Health Services, Ministry of Health and Family Welfare, New Delhi, India (*WHO Temporary Adviser*)

Dr Douglas B. McGregor, Toxicity Evaluation Consultants, Aberdour, Scotland (*WHO Temporary Adviser*)

Dr Francesca Metruccio, International Centre for Pesticides and Health Risk Prevention (ICPS), Luigi Sacco Hospital, Milan, Italy (*WHO Temporary Adviser*)

Dr Rudolf Pfeil, Toxicology of Pesticides and Biocides, Federal Institute for Risk Assessment, Berlin, Germany (*WHO Temporary Adviser*)

Dr Xiongwu Qiao, Shanxi Academy of Agricultural Sciences, Shanxi, China (*FAO Temporary Adviser*)

Ms Jeannie Richards, Saint Remy, France (*FAO Temporary Advisor*)

Dr Prakashchandra V. Shah, Environmental Protection Agency, Washington, DC, USA (*WHO Temporary Adviser*)

Dr Weili Shan, Residues Division, Institute for Control of Agrochemicals, Ministry of Agriculture, Beijing, China (*FAO Temporary Adviser*)

Ms Marla Sheffer, Orleans, Ontario, Canada (*WHO Editor*)

Mr Christian Sieke, Federal Institute for Risk Assessment, Berlin, Germany (*FAO Temporary Adviser*)

Dr Angelika Tritscher, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Joint Secretariat*)

Ms Trijntje van der Velde, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*FAO Temporary Adviser*)

Dr Philippe Verger, GEMS/Food Programme, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Joint Secretariat*)

Dr Gerrit Wolterink, Centre for Substances & Integrated Risk Assessment, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*WHO Temporary Adviser*)

Ms Yong Zhen Yang, Plant Protection Service, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Joint Secretary*)

Dr Midori Yoshida, Section Chief, Division of Pathology, Biological Safety Research Center, National Institute of Health Sciences, Ministry of Health, Labour and Welfare, Tokyo, Japan (*WHO Temporary Adviser*)

Dr Jürg Zarn, Nutritional and Toxicological Risks Section, Swiss Federal Office of Public Health, Zurich, Switzerland (*WHO Temporary Adviser*)

Abbreviations used

2,4-DNHPC	2,4-dinitro-6-(1-methylheptyl)phenyl crotonate
2,6-DNHPC	2,6-dinitro-6-(1-methylheptyl)phenyl crotonate
4-EH	2,6-dinitro-4-(1-ethylhexyl)phenyl crotonate
4-PP	2,6-dinitro-4-(1-propylpentyl)phenyl crotonate
6-EH	2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate
6-PP	2,4-dinitro-6-(1-propylpentyl)phenyl crotonate
ACT	5-aminomethyl-2-chlorothiazole
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AFC	antibody-forming cell
a.i.	active ingredient
ALT	alanine aminotransferase
ANDM	aminopyrine-*N*-demethylase
ANOVA	analysis of variance
AP	alkaline phosphatase
ARfD	acute reference dose
AST	aspartate aminotransferase
ATG-Ac	*N*′-[amino(2-chlorothiazol-5-ylmethylamino)methylene] acetohydrazide
ATMG-Pyr	*N*′-[(2-chlorothiazol-5-ylmethylamino)(methylamino)methylene]-2-oxopropanohydrazide
AUC	area under the (concentration–time) curve
BA	benzamide
BAP	benzo[*a*]pyrene
BF	bile fistulation
BrdU	5-bromo-2′-deoxyuridine
BROD	7-benzyloxyresorufin *O*-debenzylase
BUN	blood urea nitrogen
bw	body weight
CAR	constitutive androstane receptor
CAS	Chemical Abstracts Service
CCPR	Codex Committee on Pesticide Residues
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CL	clearance
C_{max}	maximum concentration
COH	coumarin 7-hydroxylase
COX-2	cyclooxygenase-2
CPK	creatine phosphokinase

cpm	counts per minute
CPS	cyclophosphamide
CTCA	2-chlorothiazole-5-carboxylic acid
CYP	cytochrome P450
DCGA	3,6-dichlorogentisic acid
DCHBA	3,6-dichloro-2-hydroxybenzoic acid
DCSA	3,6-dichlorosalicylic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	dinitrochlorobenzene
DNHPC	(2,4- or 2,6-)dinitro-6-(1-methylheptyl)phenyl crotonate
DNOPC	(2,4- or 2,6-)dinitro-6-(2,3,4-octyl)phenyl crotonate
dUTP	2′-deoxyuridine 5′-triphosphate
EC	European Commission
EEC	European Economic Community
EH	epoxide hydrolase
EMS	ethylmethanesulfonate
Eq	equivalent
EROD	7-ethoxyresorufin *O*-deethylase
F	female; filial generation
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
GA	glucuronic acid
GD	gestation day
GGT	γ-glutamyl transferase (transpeptidase)
GLP	good laboratory practice
GSD	geometric standard deviation
GSH	glutathione
GST	glutathione *S*-transferase
Hb	haemoglobin
HD	high dose
HDL	high-density lipoprotein
HGPRT	hypoxanthine–guanine phosphoribosyl transferase
HID	highest ineffective dose
HPLC	high-performance liquid chromatography
ID-I	iodothyronine deiodinase type I
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
ip	intraperitoneal
IPCS	International Programme on Chemical Safety (WHO)

ISO	International Organization for Standardization
IU	international unit
IUPAC	International Union of Pure and Applied Chemistry
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
K_m	Michaelis-Menten constant
LA 11-OH	lauric acid 11-hydroxylase
LA 12-OH	lauric acid 12-hydroxylase
LC_{50}	median lethal concentration
LD	lactation day; low dose
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LED	lowest effective dose
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOEL	lowest-observed-effect level
LOQ	limit of quantification
M	male
MAI	3-methylamino-1H-imidazo[1,5-c]imidazole
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
mEH	microsomal epoxide hydrolase
MG	methylguanidine
MMAD	mass median aerodynamic diameter
MMF	mean mutant frequency
MNG	*N*-methyl-*N*′-nitroguanidine
MNPC	micronucleated polychromatic erythrocyte
MOA	mode of action
MOLD	multiple oral low dose
mRNA	messenger ribonucleic acid
MROD	7-methoxyresorufin *O*-demethylase
MRT	mean residence time
MS	mean survival
MTCA	2-methylthiothiazole-5-carboxylic acid
MTD	maximum tolerated dose
n.a.	not analysed
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NCE	normochromatic erythrocyte
NCPR	NADPH–cytochrome P450 reductase

n.d.	not detected
nd	not determined
ND	not determined; not detected
NDMA	*N*-nitrosodimethylamine
NMR	nuclear magnetic resonance
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NODM	*p*-nitroanisole-*O*-demethylase
NOEC	no-observed-effect concentration
NOEL	no-observed-effect level
NR	not reported
NTG	nitroguanidine
OECD	Organisation for Economic Co-operation and Development
OH	hydroxy
OPPTS	Office of Prevention, Pesticides and Toxic Substances (USEPA)
P	parental generation; dose-normalized equivalent concentration
PB	phenobarbital
PCE	polychromatic erythrocyte
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PCV	packed cell volume
PEG	polyethylene glycol
P_{max}	maximum dose-normalized equivalent concentration
PND	postnatal day
po	per os (by mouth)
PPARα	peroxisome proliferator-activated receptor alpha
ppm	part per million
PROD	7-pentoxyresorufin *O*-dealkylase (*O*-depentylase)
PT	pretreatment
PXR	pregnane X receptor
QA	quality assurance
RBC	red blood cell
RRR	residual radioactive residue
RT-PCR	reverse transcriptase polymerase chain reaction
S9	9000 × *g* rat or mouse liver supernatant
SA	sulfate
SDH	succinate dehydrogenase
SOHD	single oral high dose
SOLD	single oral low dose
SRBC	sheep red blood cell
$t_{½}$	half-life

T_3	triiodothyronine
T_4	thyroxine
THMN	*N*-2-chlorothiazol-5-ylmethyl-*N*-hydroxy-*N*′-methyl-*N*′′-nitroguanidine
TLC	thin-layer chromatography
T_{max}	time to maximum concentration
TMG	*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylguanidine; thiazolmethylguanidine
TMHG	*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-hydroxy-*N*′′-methylguanidine
TNFα	tumour necrosis factor alpha
TOCP	tri-*ortho*-cresylphosphate
TPO	thyroid peroxidase
TRR	total radioactive residue
TSH	thyroid stimulating hormone
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
TZG	2-chlorothiazol-5-ylmethylguanidine; thiazolguanidine
TZMU	*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylurea; thiazolylmethylurea
TZNG	*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine; thiazolylnitroguanidine
TZU	2-chlorothiazol-5-ylmethylurea; thiazolylurea
U	unit; uniform
UDPGT	uridine diphosphate-glucuronosyltransferase
UDS	unscheduled DNA synthesis
UL	uniformly labelled
USEPA	United States Environmental Protection Agency
UV	ultraviolet
v/v	volume per volume
V_{max}	maximum rate
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

Introduction

The toxicological monographs and monograph addenda contained in this volume were prepared by a WHO Core Assessment Group on Pesticide Residues that met with the FAO Panel of Experts on Pesticide Residues in Food and the Environment in a Joint Meeting on Pesticide Residues (JMPR) in Rome, Italy, on 21–30 September 2010.

Nine of the substances evaluated by the WHO Core Assessment Group (chlorothalonil metabolite R611965, clothianidin, cyproconazole, dicamba, etoxazole, flubendiamide, fluopyram, meptyldinocap and thiamethoxam) were evaluated for the first time. Two compounds (dithianon and tebuconazole) were re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Reports and other documents resulting from previous Joint Meetings on Pesticide Residues are listed in Annex 1.

The report of the Joint Meeting has been published by the FAO as *FAO Plant Production and Protection Paper 200*. That report contains comments on the compounds considered, acceptable daily intakes established by the WHO Core Assessment Group and maximum residue limits established by the FAO Panel of Experts. Monographs on residues prepared by the FAO Panel of Experts are published as a companion volume, as *Evaluations 2010, Part I, Residues*, in the FAO Plant Production and Protection Paper series.

The toxicological monographs and monograph addenda contained in this volume are based on working papers that were prepared by temporary advisers before the 2010 Joint Meeting. A special acknowledgement is made to those advisers and to the Members of the Joint Meeting who reviewed early drafts of these working papers.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological properties or toxicity of the compounds included in this volume should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Meeting on Pesticide Residues, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva, Switzerland.

TOXICOLOGICAL MONOGRAPHS AND MONOGRAPH ADDENDA

CHLOROTHALONIL METABOLITE R611965 (addendum)

First draft prepared by
G. Wolterink[1] and V. Dellarco[2]

[1] Centre for Substances and Integrated Risk Assessment, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[2] Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, United States of America (USA)

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Explanation

R611965 (3-carbamyl-2,4,5-trichlorobenzoic acid, formerly known as SDS-46851) is a chlorothalonil metabolite that is formed in the soil and taken up through the roots by crops. The present Meeting evaluated R611965 for the first time.

Chlorothalonil (Chemical Abstracts Service [CAS] No. 1897-45-6) and SDS-3701, a chlorothalonil metabolite that is found in plants, soil and ruminants, were last evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2009.

The present Meeting evaluated the newly submitted studies on R611965 at the request of the 2009 JMPR, to address the toxicological relevance of this soil degradation product.

All critical studies complied with good laboratory practice (GLP).

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

A single dose of ^{14}C-ring-labelled R611965 (radiochemical purity 98.6%) and unlabelled R611965 (purity 99.3%; Lot No. 0207) was administered to Sprague-Dawley (Crl:CD Br VAF/Plus) rats (five of each sex per dose) at 10 or 1000 mg/kg body weight (bw). Two males and two females received vehicle only (0.75% methylcellulose). Expired air was collected at 6, 12 and 24 hours after dosing. Urine and faeces were collected at 6 (urine only), 12 and 24 hours and daily thereafter. After 7 days, the animals were killed, and levels of radioactivity were determined in a range of organs and tissues. Statements of adherence to quality assurance (QA) and GLP were included.

Average recovery ranged from 91% to 99%. Following administration, 90% of the administered dose was excreted within 48 hours (10 mg/kg bw) or 96 hours (1000 mg/kg bw). At both doses, the major route of excretion was faeces (68–77% of the dose), with 16–27% being excreted in urine. Less than 0.02% of the administered dose was expired, and after 7 days, less than 0.3% of the dose was recovered from tissues and carcass. At termination, highest concentrations were found in liver (0.02–0.05% of the dose). No obvious sex differences were observed (Ho, Marciniszyn & Killeen, 1990).

2. Toxicological studies

2.1 Acute toxicity

The results of studies of acute toxicity with R611965 are summarized in Table 1.

2.2 Short-term studies of toxicity

Mice

In a range-finding study, Crl:CD-1(ICR) VAF/Plus mice (five of each sex per dose) were fed diets containing R611965 (purity 99.3%; Lot No. 0207) at 0, 250, 500, 1000, 5000 or 10 000 parts per million (ppm), equal to 0, 46, 96, 188, 963 and 2028 mg/kg bw per day in males and 0, 54, 108, 217, 1023 and 2112 mg/kg bw per day in females, for 28 days. Clinical observations were made daily, and a complete physical examination was performed weekly. Body weights and feed consumption were measured weekly. At termination after 28 days, haematology was performed, and the animals were necropsied. Selected organs were weighed, and lungs, liver, kidneys, testes and ovaries were examined histologically. Statements of adherence to QA and GLP were included.

The only finding in this study was slight to mild hyperplasia of the renal tubule epithelium in males at 250 (1/5), 1000 (1/5) and 10 000 ppm (2/5), but not at 500 or 5000 ppm. The severity of the lesion did not increase with dose, and there was no dose–response relationship. Moreover, in an 18-month dietary study in mice of the same strain, using 60 animals of each sex per dose, no treatment-related effects on kidneys were found at doses up to about 1000 mg/kg bw per day (Lucas & Laveglia, 1994). Therefore, it is concluded that the slight to mild hyperplasia observed in the present study is likely to be an incidental finding. Other parameters were also not affected by treatment (Mizens & Killeen, 1991).

Table 1. Results of studies of acute toxicity with R611965

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50} (mg/kg bw)	Reference
Rat	Sprague-Dawley	Male/female	Oral	0.5% methylcellulose	~95	> 5000	Shults & Killeen (1985)[a,b]

LD_{50}, median lethal dose

[a] Statements of adherence to GLP and QA were included.

[b] Only two animals of each sex per dose were used. Soft stool and mucus in the stool were noted in two rats dosed at 1000 mg/kg bw. Soft stool, mucus and tan-coloured particles in the stool were noted in all rats dosed at 5000 mg/kg bw. One rat dosed at 5000 mg/kg bw also exhibited anogenital staining. Red nasal discharge and/or chromodacryorrhoea were observed in one male on day 7 and in one female on day 10. There was no effect on body weight, and no compound-related gross pathological changes were noted.

In a 90-day dietary study, Crl:CD-1 (ICR) VAF/Plus mice (10 of each sex per dose) received diets containing R611965 (purity 99.3%; Lot No. 0207) at 0, 250, 750, 2200 or 7500 ppm, equal to 0, 41, 122, 368 and 1270 mg/kg bw per day for males and 0, 47, 145, 456 and 1532 mg/kg bw per day for females. The animals were observed daily for mortality and clinical signs and were given a detailed physical examination weekly. Body weights and feed consumption were measured weekly. At the end of the treatment period, blood was collected for haematology. At the end of the scheduled period, the animals were killed and necropsied. Brain, liver, kidneys and testes were weighed. An extensive range of tissues was examined histologically. Statements of adherence to QA and GLP were included.

One low-dose moribund female was killed. The poor physical state of the animal was considered not related to treatment. There were no treatment-related effects on clinical signs, body weight, body weight gain, feed consumption, organ weights or macroscopic abnormalities. Isolated statistically significant differences in haematological parameters between control and treated groups were not dependent on dose and were considered not treatment related. Slight to moderate renal tubular epithelial hyperplasia was observed in some male mice in the 250 (1/10), 750 (2/10) and 7500 ppm (4/10) dose groups, but not in controls or at 2200 ppm. There was no evidence of a dose–response relationship for this effect or an increase in the severity of the lesion with increasing dose. Given that no treatment-related effects on kidneys were found at doses up to about 1000 mg/kg bw per day in an 18-month dietary study in mice of the same strain, using 60 animals of each sex per dose (Lucas & Laveglia, 1994), it is concluded that the renal hyperplasia observed in the present study is likely to be an incidental finding.

The no-observed-adverse-effect level (NOAEL) was 7500 ppm, equal to 1270 mg/kg bw per day, the highest dose tested (Fillmore, Mizens & Killeen, 1991).

Rats

In a range-finding study, CD Sprague-Dawley rats (five of each sex per dose) were fed diets containing R611965 (purity > 94%; Lot No. 0202) at concentrations of 0, 125, 250, 500, 1000 or 2000 mg/kg bw per day (the dietary concentrations were adjusted based on body weight and feed consumption to maintain the target doses) for at least 14 days. Clinical observations (daily), complete physical examinations, body weights and feed consumption (weekly), haematology, clinical chemistry and urinalysis (at termination) were assessed. At the end of the scheduled period, the animals were killed and subjected to a full postmortem examination. Selected organs were weighed, and a limited range of specified tissues was taken for subsequent histopathological examination. Statements of adherence to QA and GLP were included.

The animals consumed between 83% and 90% of the target doses of R611965. There were no treatment-related effects on mortality, clinical findings, body weight, body weight gain, feed consumption, haematology, urinalysis or organ weights, and there were no macroscopic or histopathological abnormalities. Isolated statistically significant differences, observed in some clinical chemistry

parameters at 125 and 250 mg/kg bw per day, were small and not observed at higher doses and were considered not to be treatment related (Wilson, Sadler & Killeen, 1985).

CD Sprague-Dawley rats (five of each sex per dose) received R611965 (purity > 94%; Lot No. 0202) in the diet at 0, 500 or 2000 mg/kg bw per day (the dietary concentrations were adjusted based on body weight and feed consumption to maintain the target doses) for at least 30 days. Clinical observations (daily), complete physical examinations, body weights and feed consumption (weekly), haematology, clinical chemistry and urinalysis (at termination) were assessed. At the end of the scheduled period, the animals were killed and subjected to a full postmortem examination. Selected organs were weighed, and an extensive range of tissues was examined histologically. Statements of adherence to QA and GLP were included.

The animals consumed between 92% and 105% of the target doses of R611965. There were no treatment-related effects on mortality, clinical findings, body weight, body weight gain, feed consumption, haematology, clinical chemistry, urinalysis or organ weights, and there were no macroscopic abnormalities. Relative liver weights were increased in both sexes at 500 mg/kg bw per day (10–13%, statistically significant for females) and 2000 mg/kg bw per day (17–18%, statistically significant in both sexes). It was noted, however, that the relative liver weights of controls were below the historical control range and that only the relative liver weights of males at 2000 mg/kg bw per day exceeded the historical control range. Very slight to slight centrilobular hypertrophy was seen in all high-dose animals and in one male at 500 mg/kg bw per day. In the absence of histopathological damage and relevant clinical chemistry changes, the increased relative liver weight and hepatocellular hypertrophy should not be considered adverse effects.[1]

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (Wilson & Ignatoski, 1986).

A combined 90-day toxicity and single-generation reproductive toxicity study with R611965 was performed in which 35 rats of each sex per dose were given feed containing R611965. Twenty-five rats of each sex per dose were used to study the reproductive effects of R611965. The findings of the 90-day study are summarized here. Sprague-Dawley rats (10 of each sex per dose) received R611965 (purity > 99%; Lot No. 46851-0204) in the diet at 0, 250, 750 or 2000 mg/kg bw per day (the dietary concentrations were adjusted based on body weight and feed consumption to maintain the target doses) for a duration of 90 days. The animals were subjected to clinical observations (daily), physical examinations, body weight and feed consumption measurements (weekly), haematology and clinical chemistry (weeks 6 and 13), urinalysis (weeks 5 and 12) and ophthalmoscopy (day 75). At the end of the scheduled period, the animals were killed and necropsied. Selected organs were weighed, and an extensive range of tissues was examined histologically. Statements of adherence to QA and GLP were included.

From week 7 of treatment onward, occasional soft stools were observed in animals fed 2000 mg/kg bw per day, more commonly in males than in females. However, induction of soft stools by R611965 was not reported in a 2-year study in rats (Killeen, Laveglia & Serrone, 1993) and in studies of reproductive toxicity (Serrone & Killeen, 1988; Lucas & Killeen, 1993), and therefore these findings are considered not toxicologically significant. An increase (19%) in prothrombin levels was seen in high-dose males at week 6, but not at week 13. At the high dose, increases in blood glucose in males (29%) and potassium in females (9%) and a decrease in alanine aminotransferase (ALT) activity in females (21%) were significantly different from control at week 6, but not at week 13. These findings in blood are therefore considered fortuitous and not related to treatment. No toxicologically

[1] Guidance on the interpretation of hepatocellular hypertrophy, General consideration 2.7 in Annex 1, reference 107.

relevant macroscopic or microscopic findings were observed. Statistically significant increases in relative liver weights (9–10%) and relative kidney weights (10–12%) in mid- and high-dose males, in relative liver weights (10%) in high-dose females and in relative adrenal weights (20–27%) in females of all dose groups were observed. These weight changes were not accompanied by histological changes. In the absence of associated histopathological findings, these changes were considered not to be adverse.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (Serrone & Killeen, 1988).

Dogs

In a preliminary feeding study, R611965 (purity 98%; Lot No. 0202) was administered by capsule to Beagle dogs (two of each sex per dose) at 0, 100, 500 or 1000 mg/kg bw per day for up to 38 days. All animals were observed daily for mortality and clinical signs. Detailed physical examinations were conducted weekly. Body weight was measured weekly, and feed consumption was measured daily. Ophthalmoscopy was performed prior to the start of the study and prior to termination. Blood and urine samples were collected pretest and at the end of the treatment period for haematology, clinical chemistry and urinalysis. At termination, the dogs were killed and subjected to gross examinations. Selected organs were weighed. An extensive range of organs was examined histologically. Statements of adherence to QA and GLP were included.

There were no mortalities. At 1000 mg/kg bw per day, one male and one female were lethargic during week 5, with red faeces and red emesis seen in the female and tarry faeces seen in the male. Both of these animals, in particular the male, lost weight during the study. The male dog showed an increased haemoglobin, haematocrit and total erythrocyte count and low sodium, potassium and chloride levels, indicative of a dehydrated condition and a disturbed electrolyte balance. One mid-dose male showed body weight loss, and one mid-dose female had a low weight gain that was outside the normal limits of variability. Overall, the feed consumption coincided with the body weight pattern of the dogs. In high-dose dogs, urinary pH was decreased from around 8.0 in controls to 6.0. Reductions in absolute (60%) and relative (38%) testes weights in the two high-dose dogs were found. Relative liver weight was increased in all dogs at 1000 mg/kg bw per day (43–61%) and in one dog of each sex at 500 mg/kg bw per day (31% and 75%). Microscopic examination showed a dose-related centrilobular to diffuse hypertrophy in the livers of all treated dogs. The testes of both dogs at 1000 mg/kg bw per day showed diffuse hypoplasia of the seminiferous tubules with aspermatogenesis. Both dogs at 500 mg/kg bw per day had reduced spermatogenesis compared with controls. The prostates of both dogs at 1000 mg/kg bw per day had moderately severe diffuse hypoplasia. The prostates of both dogs at 500 mg/kg bw per day and of one dog at 100 mg/kg bw per day had minimal to slight diffuse hypoplasia. At 1000 mg/kg bw per day, the ovaries and uterus of the female dog that lost body weight during the study had moderately severe diffuse hypoplasia. It is unclear whether these effects were related to treatment or were a secondary consequence of body weight loss (Serrone & Killeen, 1989a).

In a 90-day study, performed in accordance with European Commission guideline 87/302/EEC B.27, which resembles Organisation for Economic Co-operation and Development (OECD) guideline 409, groups of four male and four female Beagle dogs were dosed daily via gelatine capsules with R611965 (purity > 99%; Lot No. 0207) at 0, 5, 15, 50 or 500 mg/kg bw per day. All animals were observed daily for mortality and clinical signs. Detailed physical examinations were conducted weekly. Body weight was measured weekly, and feed consumption was measured daily. Ophthalmoscopy was performed prior to the start of the study and prior to termination. Blood and urine samples were collected pretest, after about 45 days of treatment and at the end of the treatment period for haematology, clinical chemistry and urinalysis. At termination, the dogs were killed and subjected to gross examinations. Selected organs were weighed. An extensive range of organs was examined histologically. Statements of adherence to QA and GLP were included.

There were no mortalities. Increased incidences of watery stools were seen in both sexes at 500 mg/kg bw per day (observed in four of four males and females, 88 observations, compared with three of four males and females in the control group, 25 observations). At the high dose, watery stools were first observed at day 9 of treatment. Body weight gain was reduced in males at the top dose to 1.6 kg (0.5–3.2 kg) compared with 3.2 kg (2.2–3.9 kg) in controls (body weight gain in three high-dose males was outside the control range), but no such effect was observed in females. The reduction in body weight gain in the three high-dose males was most marked during the first 6 weeks of treatment. Feed consumption and haematological and urinalysis parameters were not affected by treatment. Relative liver weights were increased in males and females at the high dose by 28% and 23%, respectively, reaching statistical significance in males only. Absolute liver weights at this dose were increased by 10% and 23% (not statistically significant), respectively. In the absence of changes in clinical chemistry parameters and microscopic changes, the increased liver weights are considered an adaptive response.[1] Increases in glucose levels were observed at doses of 15 mg/kg bw per day and higher at week 8, but not at week 13. The differences were small, not always dose dependent and reported to be within the historical control range (data not shown), and they are not considered toxicologically relevant. No treatment-related findings on ophthalmoscopy or macroscopic and microscopic examination were reported. The effects on the testes reported in the preliminary dog study were not confirmed.

The NOAEL was 50 mg/kg bw per day, based on reduced body weight gain in males and watery stools in both sexes at 500 mg/kg bw per day (Serrone & Killeen, 1990).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month carcinogenicity feeding study, performed in accordance with European Commission guideline 87/302/EEC, which resembles OECD guideline 451, groups of Crl:CD-1 (ICR) BR VAF/Plus mice (60 of each sex per dose) were fed diets containing R611965 (purity 99.3%; Lot No. 0207) at 0, 1000, 3500 or 7000 ppm. Based on the lowest measured weekly compound intake, this is equal to 0, 136, 488 and 1022 mg/kg bw per day in males and 0, 155, 566 and 1123 mg/kg bw per day in females. Animals were checked twice daily for clinical signs, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly for the first 13 weeks and every other week thereafter. Differential leukocyte counts were conducted in 10 mice of each sex of the control and high-dose groups at 12 months and at termination. At termination, gross examination was performed on all animals. Brain, liver, kidneys and testes of 10 animals of each sex per dose were weighed. An extensive number of tissues were examined microscopically. Statements of adherence to QA and GLP were included.

No treatment-related effects on mortality or body weight gain were observed. The observed slight increases in aggression, activity and anogenital staining in high-dose males might be treatment related. Feed consumption in the high-dose group tended to be higher than in controls, which may reflect the lower nutritional value due to the high concentration of R611965. No effects of treatment on differential leukocyte count, necropsy, organ weights or histology (including neoplastic changes) were observed.

The NOAEL was 1022 mg/kg bw per day, the highest dose tested (Lucas & Laveglia, 1994).

Rats

In a combined chronic toxicity and carcinogenicity feeding study, R611965 (purity 99.3%; Lot No. 0207) was administered in the diet to Sprague-Dawley (Crl CD VAF/Plus) rats (60 rats of each sex per dose) for 105 weeks. Concentrations of R611965 in the diet were adjusted based on body

[1] Guidance on the interpretation of hepatocellular hypertrophy, General consideration 2.7 in Annex 1, reference *107*.

weight and feed consumption data in order to achieve desired intakes of 0, 80, 200, 500 and 1000 mg/kg bw per day. The animals were checked daily for clinical signs. The physical condition of the animals was monitored weekly. Body weight and feed consumption were measured weekly for the first 13 weeks and once every month thereafter. Ophthalmoscopy was performed prior to treatment and at months 12, 18 and 24. For haematological and clinical chemistry examinations and urinalysis, blood and urine samples were collected from 10 animals of each sex per group after 3, 6, 12 and 18 months and at termination. All rats were subjected to gross pathology, and liver, kidneys, brain and testes were weighed. A wide range of tissues was evaluated microscopically. Statements of adherence to QA and GLP were included.

Overall mean compound intake was greater than 95% of target intake for all doses. No treatment-related effects on mortality, clinical signs, ophthalmoscopy, body weight gain or organ weights were observed. Slightly higher feed consumption in treated animals was considered to be compensating for the lower nutritional value of the feed. Incidental findings on haematological, clinical chemistry and urinalysis parameters were considered not treatment related. Histological examination revealed increased incidences of bilateral retinal atrophy. For the 0, 80, 200, 500 and 1000 mg/kg bw per day groups, the incidences out of 60 rats were 1, 1, 2, 3 and 6 for males and 1, 2, 2, 6 and 9 for females, respectively. The increased incidences in high-dose males and in females at 500 and 1000 mg/kg bw per day are considered related to treatment. The incidences at 80 and 200 mg/kg bw per day are considered not to be different from control. In males of all treatment groups, slight, not dose-dependent increases in the incidence of severe chronic progressive nephropathy were observed, which could be related to treatment. However, chronic progressive nephropathy is a common finding in ageing rats that is not considered toxicologically relevant for humans (Hard & Khan, 2004). No other treatment-related histological changes, including neoplastic changes, were seen.

The NOAEL was 200 mg/kg bw per day, based on bilateral retinal atrophy observed at 500 mg/kg bw per day (Killeen, Laveglia & Serrone, 1993).

2.4 Reproductive toxicity

(a) Multigeneration studies

Rats

In a one-generation study of reproductive toxicity, as part of a combined study with a 90-day toxicity study (see section 2.2), Sprague-Dawley rats (25 of each sex per dose) were fed diets containing R611965 (purity > 99%; Lot No. 0204) at 0, 250, 750 or 2000 mg/kg bw per day (the dietary concentrations were adjusted based on body weight and feed consumption to maintain the target doses) during the 14-week premating phase. From the start of the reproduction phase to the end of the lactation phase, males received diets with constant concentrations of 0, 5200, 15 500 or 40 000 ppm in males, and females received diets with concentrations of 0, 4000, 12 000 or 32 000 ppm (i.e. the dietary concentrations fed during week 13 of the premating phase). The parental clinical condition, body weights and feed consumption were monitored throughout the treatment period. Mating, fertility and gestation indices, length of gestation, number of implants, number of live and dead pups, sex of pups, pup weights and pup abnormalities were recorded. At postnatal day (PND) 4, litters were culled to 10 pups. At the end of the scheduled period, the animals were killed and necropsied. Statements of adherence to QA and GLP were included.

In the parental animals, no effects of treatment on clinical signs, body weights, feed consumption, fertility or reproductive performance, or necropsy were observed. At PND 21, high-dose pups showed a small (9%) reduction in body weight compared with controls. As pups will have started eating test diet, this may be the result of the decreased nutritional value of the food as a result of the high test compound concentration. Moreover, this finding was not observed in the two-generation study of

reproductive toxicity (see below). Therefore, the slight reduction in body weight in high-dose pups is not considered toxicologically relevant. No other effects on pups were observed.

The NOAEL for parental, reproductive and offspring toxicity was 2000 mg/kg bw per day, the highest dose tested (Serrone & Killeen, 1988).

In a dietary two-generation study of reproductive toxicity, performed in accordance with European Commission guideline 87/302/EEC B.35, which resembles OECD guideline 416, R611965 (purity 99.3%; Lot No. 0207) was administered to Sprague-Dawley (CD-VAF/Plus) rats (35 rats of each sex per dose) at dietary concentrations of 0, 2000, 6000 or 20 000 ppm. Based on the lowest weekly compound intake, this is equal to 0, 93, 276 and 943 mg/kg bw per day in males and 0, 121, 370 and 1246 mg/kg bw per day in females of the P generation and 0, 88, 269 and 911 mg/kg bw per day in males and 0, 131, 390 and 1323 mg/kg bw per day in females of the F_1 generation. The rats were observed daily for clinical signs. Detailed physical examinations were performed weekly for all males throughout the study and for all females prior to mating and during the resting week in between weaning of the F_{1a} generation and mating for the F_{1b} generation. Body weights and feed consumption were recorded weekly in males, except during the mating periods. In females, body weights and feed consumption were measured weekly prior to mating and during the resting periods. Body weights were also recorded on gestation days (GDs) 0, 7, 14 and 20 and PNDs 0, 7, 14 and 21 for the P and F_1 females. On PND 4, litters were culled to eight pups. Until weaning at PND 21, litters were examined for number of live and dead pups, litter weight, pup weight and sex, clinical signs and external alterations. After the F_{1b} or F_{2b} litter production, all P and F_1 parents were subjected to gross pathology. The reproductive organs, liver, kidneys, lungs and all gross lesions of all P parents of all dose groups and F_1 parents of the control and high-dose groups were examined histologically. In addition, kidneys, stomachs and gross lesions of parental animals of the low- and mid-dose groups, the epididymis, prostate and seminal vesicles of the P adults and the testes of the F_1 adults were examined. Statements of adherence to QA and GLP were included.

In parental animals (P and F_1), there were no treatment-related effects on mortality, clinical signs, body weight, feed consumption, reproductive parameters or macroscopic and microscopic parameters. In mid-dose and high-dose P females, an increase in incidence, but not severity, of regenerative tubular epithelium was observed, which was statistically significant for high-dose females only. This increase was not found in F_1 females or in P or F_1 males. It was noted that the incidence in females of the P control group may have been unusually low. It is concluded that the kidney findings in the P females were not treatment related. In the offspring, no treatment-related effects on any of the evaluated parameters were noted.

The NOAEL for parental, reproductive and offspring toxicity was 20 000 ppm, equal to 911 mg/kg bw per day, the highest dose tested (Lucas & Killeen, 1993).

(b) Developmental toxicity

Rats

In a developmental toxicity study performed in accordance with European Commission guideline 87/302/EEC B.31, which resembles OECD guideline 414, R611965 (purity 99%; Lot No. 0204) was administered in 1% aqueous methylcellulose by oral gavage to pregnant Sprague-Dawley rats (25 per dose group) on GDs 6–15 at doses of 0, 500, 1000 or 2000 mg/kg bw per day. Animals were examined daily for clinical signs. A detailed physical examination and body weight measurements were performed on GDs 0, 6, 9, 12, 16 and 20. Feed consumption was recorded over the intervals between body weight measurements. At termination on GD 20, numbers of live and dead fetuses, corpora lutea, implantation sites, and early and late resorptions were recorded, live fetuses were weighed and sexed, and external alterations and intrauterine locations were recorded. Approximately one half of the fetuses from each

litter were examined for skeletal alterations. All remaining live fetuses were examined for visceral abnormalities. Dams were necropsied. Statements of adherence to QA and GLP were included.

No effects of treatment on any of the examined maternal parameters were noted. No treatment-related effects on any of the fetal parameters tested were noted.

The NOAEL for maternal and developmental toxicity was 2000 mg/kg bw per day, the highest dose tested.

No teratogenic properties of R611965 were identified (Chun, Wilson & Killeen, 1989).

Rabbits

In a developmental toxicity study performed in accordance with European Commission guideline 87/302/EEC B.31, which resembles OECD guideline 414, R611965 (purity > 99%; Lot No. 0202) was administered in 0.5% aqueous methylcellulose by gavage to pregnant New Zealand White rabbits (20 per dose) on GDs 7–19 at doses of 0, 250, 500 or 1000 mg/kg bw per day. Animals were examined daily for clinical signs. On GDs 0, 7, 13, 16, 19, 24 and 29, a detailed physical examination was performed, and body weights were recorded. Feed consumption was recorded over the intervals between body weight measurements. At termination on GD 29, numbers of live and dead fetuses, fetal resorptions, implantation sites and corpora lutea were recorded. Live fetuses were weighed and sexed, and external/visceral alterations and intrauterine locations were recorded. All live fetuses were subsequently examined for skeletal alterations. Dams were necropsied. Statements of adherence to QA and GLP were included.

In the low-, mid- and high-dose groups, two, one and seven dams were killed, respectively, after showing signs of abortion, compared with none in the control group. Three deaths in the control group and one death in the low-dose group were attributed to gavage error. Two high-dose animals were found dead, and one moribund animal was killed. These deaths were not attributed to a specific pathological finding or dosing error.

Clinical observations are presented in Table 2. Increased incidences of mucus-like faeces, few or no faeces, soft faeces, anorexia and thinness (increases in both number of animals and total number of clinical observations) were found in all treated groups. No historical control data were provided.

Effects on body weight gain are presented in Table 3. During the treatment period (GDs 7–19), the high dose group animals showed a body weight loss (0.27 kg), whereas control animals gained weight (0.11 kg) over the same period. In the low-, mid- and high-dose groups, overall body weight gains (0.16, 0.18 and 0.13 kg, respectively) were lower compared with controls (0.28 kg), although the differences were not statistically significant. Over the entire treatment period, feed consumption of the high-dose group was decreased (1062 g) compared with control (1930 g), low-dose (1956 g) and mid-dose animals (1826 g). At 500 mg/kg bw per day, feed consumption was decreased during GDs 17–20. Necropsy showed gall bladder lesions, enlarged gall bladder, reddened and gaseous intestines, pale liver and foci and erosions of the gastric mucosa in the high-dose group. At 1000 mg/kg bw per day, the number of live fetuses per litter (n = 6.6, 82% of total number of fetuses) and fetal weight (34.7 g) were slightly decreased (not statistically significantly) compared with control (n = 8.5, 98% of total number of fetuses, and 39.2 g, respectively). No other effects were observed upon fetal examination.

A NOAEL for maternal toxicity could not be determined. The lowest-observed-adverse-effect level (LOAEL) is 250 mg/kg bw per day, based on abortions, clinical signs (increased incidences of mucus-like faeces, few or no faeces, soft faeces, anorexia and thinness) and slightly reduced body weight gain.

The NOAEL for fetal toxicity is 500 mg/kg bw per day, based on a decreased number of live fetuses and decreased fetal weight observed at 1000 mg/kg bw per day, in the presence of maternal toxicity.

No teratogenic properties of R611965 were identified (Serrone & Killeen, 1989b).

Table 2. Clinical observations in pregnant rabbits treated from GD 7 to GD 19 with R611965

	Dose (mg/kg bw per day)			
	0	250	500	1000
Found dead or moribund	3[a]	1[a]	0	3
Abortions	0	2	1	7
Mucus-like faeces	0 (0)[b]	0 (0)	3 (6)	13 (39)
Few or no faeces	10 (34)	17 (91)	19 (143)	20 (183)
Soft faeces	2 (6)	6 (20)	6 (17)	16 (65)
Anorexia	0 (0)	6 (35)	10 (56)	18 (153)
Thinness	0 (0)	1 (1)	1 (1)	6 (20)

From Serrone & Killeen (1989b)
[a] Deaths due to gavage error.
[b] Number of animals showing effect out of 20 (total number of days observed).

Table 3. Body weight gain in rabbits during pregnancy

Gestation days	Body weight gain (kg)			
	Dose (mg/kg bw per day)			
	0	250	500	1000
0–7	0.14	0.16	0.14	0.18
7–13	0.02	0.01	0.03	−0.11
13–16	0.05	0.01	0.02	−0.07
16–19	0.04	0.01	−0.03	−0.09
19–24	0.05	0.03	−0.02	−0.07
24–29	−0.03	−0.04	0.01	0.03

From Serrone & Killeen (1989b)

2.5 Genotoxicity

The results of genotoxicity tests with R611965 are summarized in Table 4.

Comments

Biochemical aspects

Following a single oral dose of R611965 at 10 or 1000 mg/kg bw in rats, at least 16–27% is absorbed (based on urinary excretion within 48–96 hours). Excretion is rapid, with 90% being excreted within 48–96 hours, predominantly in faeces (68–77% of the dose). Biliary excretion was not assessed. Seven days after administration, less than 0.3% of the administered dose was found in tissues and carcass; highest levels were observed in liver. No obvious sex differences in kinetics were observed.

Toxicological data

The acute oral toxicity of R611965 in rats is low (LD_{50} > 5000 mg/kg bw). No data on acute dermal or inhalation toxicity, eye or skin irritation or skin sensitization were available.

Repeated-dose toxicity studies showed that R611965 had low oral toxicity in mice, rats and dogs. The overall NOAEL in short-term (28 and 90 days) and chronic studies (18 months) in mice was 1022 mg/kg bw per day, the highest dose tested in an 18-month carcinogenicity study. In rats, the

Table 4. Results of studies on the genotoxicity of R611965[a]

End-point	Test object	Concentration	Purity (%)	Results	Reference[b]
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	39–3900 µg/plate (±S9)[c]	99.4	Negative	Jones & Killeen (1985a)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	100–10 000 µg/plate (±S9)[d]	99.4	Negative	Jones & Killeen (1985b)
Gene mutation	Mouse lymphoma L5178Y TK+/− cells	75–1000 µg/ml (±S9)[e]	≥ 98	Negative	Mizens & Killeen (1988)
Sister chromatid exchange	K1-BH4 cells (Chinese hamster ovary)	200–2000 µg/ml (±S9)[f]	99.4	Negative	Jones & Killeen (1985c)
Unscheduled DNA synthesis	Rat hepatocyte primary cultures	0.008–240 µg/well[f]	99.4	Negative	Jones & Killeen (1985d)
In vivo					
Micronucleus formation	Mouse bone marrow (male and female)	500, 2500 or 5000 mg/kg bw (by gavage)[g]	≥ 92.3	Negative	Mizens, Killeen & Siou (1985)

DNA, deoxyribonucleic acid; S9, 9000 × *g* rat liver supernatant

[a] Positive and negative (solvent) controls were included in all studies.

[b] Statements of adherence to QA and GLP were included for all studies.

[c] Lot No. 0202. The highest dose was selected on the basis of a preliminary assay using TA100 and TA1538, in which slight toxicity was observed at 5000 µg/plate.

[d] Lot No. 0202. S9 homogenate was prepared from kidneys from Aroclor 1254–treated rats.

[e] Lot No. 0202. In a preliminary assay, toxicity was observed at 10 000 µg/ml, but not at doses at or below 1000 µg/ml.

[f] Lot No. 0202.

[g] Lot No. 0202. In a pilot study at 10 000 mg/kg bw, no signs of toxicity were noted. At 20 000 mg/kg bw, diarrhoea was observed. In the main study, two males and one female at 2500 mg/kg bw and one female at 5000 mg/kg bw died. The treatment of mice with the test substance did result in slight, irregular increases in the number of micronuclei in the initial test. The increases were small, within the acceptable solvent control range and not considered indicative of a clastogenic effect. Polychromatic erythrocyte/normochromatic erythrocyte ratio was statistically significantly decreased at 5000 mg/kg bw 72 h after dosing. A repeat test, in which two females at 5000 mg/kg bw died, did not show the test substance–induced micronuclei.

overall NOAEL in short-term (14, 30 and 90 days) and chronic studies (2 years) was 200 mg/kg bw per day, based on bilateral retinal atrophy observed at 500 mg/kg bw per day in a 2-year combined chronic toxicity and carcinogenicity study.

In a 90-day study in dogs, the NOAEL was 50 mg/kg bw per day, based on reduced body weight gain in males and watery stools in both sexes at 500 mg/kg bw per day.

In an 18-month study in mice and a 2-year study in rats, no carcinogenic effects of R611965 were observed. The Meeting concluded that R611965 is not carcinogenic in rodents.

R611965 was tested in an adequate range of studies of genotoxicity in vitro and in vivo. There was no evidence for genotoxicity. The Meeting concluded that R611965 is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that R611965 is unlikely to pose a carcinogenic risk to humans.

In one-generation and two-generation studies of reproductive toxicity with R611965 in rats, the overall NOAEL for parental, reproductive and offspring toxicity was 20 000 ppm, equal to 911 mg/kg bw per day, the highest dose tested.

In a study of developmental toxicity in rats, the NOAEL for maternal and developmental toxicity was 2000 mg/kg bw per day, the highest dose tested. In a study of developmental toxicity in rabbits, a NOAEL for maternal toxicity could not be determined. The LOAEL for maternal toxicity was 250 mg/kg bw per day, the lowest dose tested, on the basis of abortions, clinical signs (increased incidences of few or no faeces, soft faeces, anorexia and thinness) and slightly reduced body weight

gain. The NOAEL for fetal toxicity was 500 mg/kg bw per day, based on a decreased number of live fetuses and decreased fetal weight observed at 1000 mg/kg bw per day.

No neurotoxicity studies with R611965 were available. In acute and repeated-dose oral studies in mice, rats, rabbits and dogs in which R611965 was administered in the diet, by gavage or by capsule, no neurotoxic signs were observed.

No data on R611965 in humans were provided.

The Meeting concluded that the existing database on R611965 was sufficient to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting noted that the soil metabolite R611965 is considerably less toxic than the parent compound chlorothalonil (e.g. NOAELs of 200 versus 1.8 mg/kg bw per day in 2-year rat studies, respectively). R611965 is not acutely toxic by the oral route. The metabolite induced adverse effects in only a few repeated-dose oral toxicity studies in rats and dogs, at levels of 250–500 mg/kg bw per day. In the majority of the repeated-dose studies in rodents, no effects were observed at doses of 911–2000 mg/kg bw per day.

In view of the lower toxicity of the metabolite R611965 in comparison with the parent compound chlorothalonil (acceptable daily intake [ADI] = 0–0.02 mg/kg bw; acute reference dose [ARfD] = 0.6 mg/kg bw), the Meeting considered it unnecessary to derive a separate ADI and ARfD for this metabolite for risk management purposes.

Levels relevant for risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of carcinogenicity	Carcinogenicity	1022 mg/kg bw per day[a]	—
Rat	Two-year study of toxicity and carcinogenicity[b]	Toxicity	200 mg/kg bw per day	500 mg/kg bw per day
		Carcinogenicity	1000 mg/kg bw per day[a]	—
	One- and two-generation studies of reproductive toxicity[b]	Parental toxicity	20 000 ppm, equal to 911 mg/kg bw per day[a]	—
		Offspring toxicity	20 000 ppm, equal to 911 mg/kg bw per day[a]	—
		Reproductive toxicity	20 000 ppm, equal to 911 mg/kg bw per day[a]	—
	Developmental toxicity[c]	Maternal toxicity	2000 mg/kg bw per day[a]	—
		Fetotoxicity	2000 mg/kg bw per day[a]	—
Rabbit	Developmental toxicity[c]	Maternal toxicity	—	250 mg/kg bw per day[d]
		Fetotoxicity	500 mg/kg bw per day	1000 mg/kg bw per day
Dog	Ninety-day study[e]	Toxicity	50 mg/kg bw per day	500 mg/kg bw per day

[a] Highest dose tested.
[b] Dietary administration.
[c] Gavage administration.
[d] Lowest dose tested.
[e] Capsule administration.

Estimate of acceptable daily intake for humans

Unnecessary

Estimate of acute reference dose

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of exposures in humans

Critical end-points for setting guidance values for exposure to chlorothalonil metabolite R611965

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of absorption	Rapid; at least 16–27% at 10 and 1000 mg/kg bw (rat)
Distribution	Highest concentration in liver (rat)
Potential for accumulation	Low (rat)
Rate and extent of excretion	90% excretion within 48–96 h (rat)
Metabolism in animals	No data
Toxicologically significant compounds (in animals, plants and the environment)	R611965
Acute toxicity	
LD_{50}, oral	> 5000 mg/kg bw (rat)
LD_{50}, dermal	No data
LC_{50}, inhalation	No data
Dermal irritation	No data
Ocular irritation	No data
Dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Body weight, clinical signs (dog)
Lowest relevant oral NOAEL	50 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Bilateral retinal atrophy (rat)
Lowest relevant NOAEL	200 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	No reproductive effects (rat)
Lowest relevant reproductive NOAEL	20 000 ppm, equal to 911 mg/kg bw per day, highest dose tested (rat)

Developmental target	Decreased number of live fetuses, decreased fetal weight in the presence of maternal toxicity (rabbit); not teratogenic
Lowest relevant developmental NOAEL	500 mg/kg bw per day (rabbit)

Neurotoxicity/delayed neurotoxicity

Neurotoxicity	No data; no indication of neurotoxic potential in acute and repeated-dose oral studies

Medical data

	No data

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CLOTHIANIDIN

First draft prepared by
F. Metruccio[1] and A. Boobis[2]

[1] International Centre for Pesticides and Health Risk Prevention, Milan, Italy
[2] Experimental Medicine & Toxicology,
Faculty of Medicine, Imperial College London, London, England

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Explanation

Clothianidin is the International Organization for Standardization (ISO)–approved name for (*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine (International Union of Pure and

Applied Chemistry [IUPAC]) (Chemical Abstracts Service [CAS] No. 210880-92-5). Clothianidin is a neonicotinoid insecticide that controls insects by acting as an agonist at the nicotinic acetylcholine receptor, affecting the synapses in the insect central nervous system.

Clothianidin has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All pivotal studies with clothianidin were certified to be compliant with good laboratory practice (GLP) and met the basic requirements of the relevant Organisation for Economic Co-operation and Development (OECD) or national test guideline.

Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion of clothianidin were investigated in rats, goats and hens.

The structural formulae of the [nitroimino-^{14}C]- and [thiazolyl-2-^{14}C]-labelled compounds are given in Figure 1.

1.1 Absorption, distribution, metabolism and excretion

(a) Oral administration

Rats

The toxicokinetic behaviour of clothianidin in the rat, including distribution using whole-body autoradiography and metabolism, was studied using a protocol in compliance with test method B.36 of European Commission directive 87/302/EEC. Sprague-Dawley Crl:CD BR rats (196–228 g, 7–8 weeks of age) received a single oral dose of clothianidin in 0.5% aqueous tragacanth solution as follows:

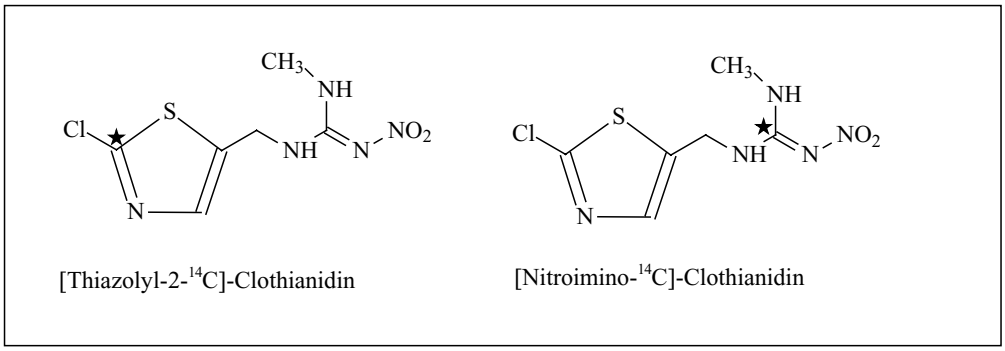
Preliminary study:

- (Test 1) Four male rats received [nitroimino-^{14}C]clothianidin at 2.5 mg/kg body weight (bw) for the assessment of expired air, urine, faeces and carcass.

Definitive studies:

- (Tests 2–3) Four rats of each sex received [nitroimino-^{14}C]clothianidin at 2.5 mg/kg bw (low dose) for the assessment of urine, faeces, organs and blood plasma.
- (Test 4) Four male rats received [nitroimino-^{14}C]clothianidin at 250 mg/kg bw (high dose) for the assessment of urine, faeces, organs and blood plasma.
- (Test 5) Four male rats received [nitroimino-^{14}C]clothianidin at 25 mg/kg bw, subsequent to a pretreatment period of 14 days with unlabelled clothianidin at 25 mg/kg bw, for the assessment of urine, faeces, organs and blood plasma.
- (Test 6) Six male rats received [nitroimino-^{14}C]clothianidin at 5.0 mg/kg bw for whole-body autoradiographical assessment.
- (Test 7) Four male rats received [thiazolyl-2-^{14}C]clothianidin at 2.5 mg/kg bw for the assessment of urine, faeces, organs and blood plasma.

Figure 1. Structural formulae of [nitroimino-^{14}C]- and [thiazolyl-2-^{14}C]-labelled compounds



* Position of label

Between 95.5% and 99.7% of the administered radioactivity was recovered in expired air, urine, faeces, organs and tissues at termination. From the proportion of radioactivity excreted in urine, the extent of absorption of clothianidin was at least 90% (Table 1). Both renal excretion and plasma kinetic data indicated almost complete absorption, with a short onset and rapid absorption rate at the low dose. Absorption was somewhat delayed at higher doses, in a dose-dependent manner.

Distribution was assessed by measuring the concentration of radioactivity in the blood plasma and by whole-body autoradiography.

Peak radioactivity in the plasma (C_{max}) was observed at about 1.5 hours in both males and females at the low dose. At the high dose, the time to C_{max} (T_{max}) was about 6–8 hours; plasma levels then decreased at 24 hours, with apparent rebound at about 32 hours (probably due to a second phase of absorption of clothianidin during the passage of non-absorbed active substance through the small intestine). Pretreatment for 14 days before administration of active ingredient at 25 mg/kg bw (intermediate dose) shifted the T_{max} to about 3 hours. Plasma concentrations appeared to show good proportionality with administered dose.

From the plasma low-dose and pretreatment data, various pharmacokinetic parameters were calculated (Table 2). Owing to the biphasic plasma concentration–time curve after high-dose administration, calculation of parameters for this dose group was not meaningful.

The total radioactive residue (TRR) in organs and tissues (measured by liquid scintillation counting) after 72 hours was low. A total of between 0.085% and 0.253% of the administered dose was found in the body (excluding gastrointestinal tract). The highest concentration was detected in the liver and in the kidneys, irrespective of dose level, sex or pretreatment. When results in low-dose males using the two different ^{14}C-labelled forms were compared, levels of radioactivity with the thiazolyl-labelled form were about 2- to 3-fold those with the nitroimino-labelled form. Levels of radioactivity with the former were also the highest in the kidney, which was indicative of the slower absorption, biotransformation and excretion of the putative metabolites detected by this labelled precursor (Table 3).

The residue distribution was comparable to that obtained with quantitative whole-body autoradiography.

Urinary bladder, nasal mucosa, kidney and liver were the sites with the highest radioactive concentration at 1 hour after administration. The level in the blood (3 µg/g) was similar to that found in both liver and kidney. This finding, as well as the initial high level in the excretory organs, illustrates the fairly rapid absorption and start of excretion immediately after administration of the compound. For most organs, the radioactivity decreased gradually at 4, 8, 24, 48 and 72 hours after administration. At 72 hours, trace concentrations of radioactivity were observed in the liver (0.021 µg/g), nasal mucosa (0.022 µg/g) and vitreal body of the eye (0.020 µg/g). Lower but quantifiable levels were also present in the renal cortex and the adrenals (Table 4).

Table 1. Cumulative excretion of radioactivity after administration of clothianidin to rats[a]

Compartment	Time (h)	Cumulative excretion of radioactivity (% of dose)						
		Dose (mg/kg bw)						
		2.5	2.5		250	25[b]	2.5[c]	25[c,d]
		Test 1	Tests 2–3		Test 4	Test 5	Test 7	Test 7
		Male	Male	Female	Male	Male	Male	Male
Expired air	4	0.005	—	—	—	—	—	—
	8	0.005	—	—	—	—	—	—
	24	0.013	—	—	—	—	—	—
	48	0.015	—	—	—	—	—	—
	72	0.017	—	—	—	—	—	—
Urine	4	27.12	34.90	43.09	3.01	16.32	32.65	15.24
	8	53.97	71.62	78.20	21.31	56.62	66.47	65.11
	24	90.60	88.21	93.32	57.17	91.33	87.85	94.16
	48	91.24	88.80	94.21	88.86	92.57	88.88	95.00
	72	91.48	89.10	94.56	90.47	92.99	89.25	95.19
Faeces	24	6.56	5.99	2.90	3.49	6.18	7.02	7.09
	48	6.80	6.18	3.20	7.82	6.35	7.34	7.32
	72	6.89	6.27	3.29	8.58	6.60	7.79	7.53
Total (mass balance)		98.39	95.37	97.85	99.05	99.58	97.04	102.7

From Weber (2000)

[a] Values reported are averages of four animals, except where noted otherwise.

[b] Preceded by 14-day pretreatment period with 25 mg/kg bw per day.

[c] ^{14}C label on thiazolyl-2.

[d] Only one animal.

Table 2. Calculated plasma kinetics parameters after administration of clothianidin to rats

Parameter	Dose (mg/kg bw)			
	2.5		25[a]	2.5[b]
	Tests 2–3		Test 5	Test 7
	Male	Female	Male	Male
AUC (µg·h/ml)	10.3	7.3	116.0	10.2
t_{lag} A (h)	0.045	0.050	0.060	0.078
$t_{½}$ A (h)	0.51	0.27	0.022	0.0036
$t_{½}$ Eα (h)	1.20	1.49	1.89	0.88
$t_{½}$ Eβ (h)	54.1	22.6	28.3	37.0
CL (µg/ml)	4.1	5.7	3.6	4.1
T_{max} (h)	1.5	1.4	2.7	2.1
C_{max} (µg/ml)	1.8	1.3	15.0	1.3
MRT_{disp} (h)	5.9	5.5	4.3	9.2

From Weber (2000)

AUC, area under the concentration–time curve; CL, total clearance of drug from plasma; C_{max}, highest observed concentration; MRT_{disp}, mean disposition residence time; $t_{½}$ A, absorption half-life; $t_{½}$ E, elimination half-life (α, rapid phase; β, slow phase); t_{lag} A, lag time between gavage and onset of absorption; T_{max}, time of highest concentration after gavage

[a] Preceded by 14-day pretreatment period with 25 mg/kg bw.

[b] Position of ^{14}C label on thiazolyl-2 instead of nitroimino.

Table 3. Total radioactive residue in tissues measured by liquid scintillation counting, 72 hours after administration of clothianidin to rats[a]

Sample	TRR (µg/g)				
	Dose (mg/kg bw)				
	2.5		250	25[b]	2.5[c]
	Tests 2–3		Test 4	Test 5	Test 7
	Male	Female	Male	Male	Male
Red blood cells	0.0056	0.0044	0.789	0.0537	0.0119
Plasma	0.0033	0.0027	0.361	0.0257	0.0079
Spleen	0.002	< LOQ	0.263	< LOQ	0.0047
Gastrointestinal tract	0.0021	0.0031	0.245	< LOQ	0.0234
Liver	0.0313	0.0167	2.880	0.2077	0.0329
Kidney	0.0093	0.0070	0.864	0.0693	0.0380
Testes	0.0011	—	0.167	0.0124	0.0031
Skeletal muscle	< LOQ	< LOQ	0.235	< LOQ	0.0028
Bone (femur)	< LOQ	< LOQ	< LOQ	< LOQ	0.0041
Heart	0.0022	0.0022	0.318	0.0219	0.0046
Lung	0.0042	0.0037	0.560	0.0400	0.0084
Brain	< LOQ	< LOQ	< LOQ	< LOQ	0.0018
Skin	0.0035	0.0034	0.317	< LOQ	0.0104
Carcass	0.0020	0.0021	0.254	0.0267	0.0037

From Weber (2000)
LOQ, limit of quantification
[a] Values reported in equivalent concentration (µg/g or parts per million [ppm]) are averages of four animals; radioactivity levels in adrenals, adrenal fat, thyroid, ovaries and uterus are less than the LOQ.
[b] Preceded by 14-day pretreatment period with 25 mg/kg bw.
[c] Position of ^{14}C label on thiazolyl-2 instead of nitroimino.

Data from liquid scintillation counting measurements and whole-body autoradiography pointed towards a rapid and homogeneous distribution of the radioactive tracer across the various organs. Despite the delayed elimination of radioactivity from both the nasal mucosa and vitreal eye body, the accumulation was transient, and there was no evidence of accumulation at 72 hours after administration, with most, but not all, values decreasing below the limit of detection (LOD) or limit of quantification (LOQ).

Most of the radioactivity was rapidly excreted in the urine, with about 90% at 24 hours at both the low dose and after repeated dosing and with about 57% at the high dose.

At the low dose, renal excretion was slightly more important in females than in males at all sampling times, accounting for 94.6% (females) and 89.1% (males) at termination (see Table 1).

The possible involvement of biliary excretion was not assessed, but the presence of biphasic kinetics in the plasma after administration of a high dose of clothianidin suggests limited enterohepatic circulation. This finding was in line with the presence of radioactivity in both liver and gut at that time, as visualized by whole-body autoradiography. At termination, urinary and faecal elimination combined accounted for about 95–99% of the administered dose. At the high dose, a slight delay of excretion was observed, but excretion was also essentially complete at 72 hours.

For quantification of metabolites, pooled urine samples (from tests 2, 3, 4, 5 and 7) and faecal extract (from tests 2, 3 and 7) were chromatographed by high-performance liquid chromatography

Table 4. Quantitative whole-body autoradiography time course of total radioactive residue in organs and tissues after administration of clothianidin at 5.0 mg/kg bw to rats[a]

Organ/tissue	Time of termination (h)					
	1	4	8	24	48	72
Adrenal	2.943	1.925	0.641	0.025	0.010	0.010
Blood	3.000	1.140	0.376	0.010	0.004[b]	0.005[b]
Bone marrow	0.908	0.687	0.240	0.004[b]	0.001[c]	0.001[c]
Brain	0.338	0.250	0.116	0.002[c]	0.000[c]	0.000[c]
Brown fat	1.854	1.330	0.331	0.018[b]	0.004[c]	0.004[c]
Eye (vitreal body)	0.009	0.550	0.324	0.112	0.048	0.020
Heart	1.993	1.174	0.428	0.007	0.002[b]	0.001[c]
Kidney (cortex)	4.075	2.472	0.778	0.020	0.009	0.008
Kidney (medulla)	2.892	4.389	0.870	0.019	0.004	0.002[b]
Liver	3.424	2.229	0.821	0.065	0.026	0.021
Lung	0.492	0.148	0.215	0.006	0.001[c]	0.001[c]
Muscle	1.877	1.132	0.392	0.006[b]	0.001[c]	0.001[c]
Nasal mucosa	5.641	3.816	4.306	0.072	0.04	0.022
Pineal body	1.348	0.878	0.318	0.007	n.a.	n.a.
Pituitary gland	1.878	1.082	0.354	0.006	n.a.	n.a.
Salivary gland	2.215	1.497	0.544	0.008	0.002[c]	0.002[c]
Skin	2.239	1.337	0.556	0.013	0.004[b]	0.003[b]
Spinal marrow	0.276	0.205	0.105	0.002[c]	0.000[c]	0.000[c]
Spleen	1.532	0.959	0.316	0.006	0.002[c]	0.001[c]
Testes	0.656	0.553	0.211	0.004	0.001[c]	0.000[c]
Thymus	1.370	0.861	0.282	0.004[c]	0.001[c]	0.001[c]
Thyroid	2.340	1.531	0.472	0.006	0.003[b]	0.001[c]
Tongue	2.231	1.333	0.398	0.007	0.002[c]	0.001[c]
Urinary bladder	5.998	6.169	5.987	n.a.	0.018	0.002[b]

From Weber (2000)

n.a., not available (no value measured)

[a] Values reported in equivalent concentration (µg/g or parts per million [ppm]) at 1–72 h after administration (5.0 mg/kg bw, ^{14}C label on nitroimino, one male per time point).

[b] Radioactivity levels below limit of quantification.

[c] Radioactivity levels below limit of detection.

(HPLC). An overview of the radioactivity balance is given in Table 5 (data integrated from different analyses). The main compound detected in all tests was clothianidin itself, accounting for 55–74% of the administered dose. The compound should thus be considered metabolically moderately labile. In the urine, the main metabolites detected were TZNG, MNG, NTG and MTCA, whereas TMG was the major metabolite in the faecal extract.[1] Unidentified metabolites accounted for approximately 2–7% of the total administered dose. The higher value, 7%, was obtained after administration of ^{14}C-thiazolyl-2-labelled compound at a dose of 2.5 mg/kg bw. In the urine fractions 0–4 hours, 4–8 hours and 8–24 hours, 10 metabolites were unresolved. The metabolites are listed in Table 6. The nature and relative proportions of the metabolites did not change with increasing dose.

[1] See Table 6 for full chemical names of the abbreviations used.

Table 5. Metabolites excreted in urine and faeces after administration of clothianidin to rats

Metabolite	Metabolites excreted (% of dose)							
	Dose (mg/kg bw)							
	2.5		2.5		250	25[a]	2.5[b]	
	Test 1		Test 2		Test 4	Test 5	Test 7	
	M, urine	M, faeces	F, urine	F, faeces	M, urine	M, urine	M, urine	M, faeces
ACT[c]	—	—	—	—	—	—	1.03	0.28
CTCA[c]	—	—	—	—	—	—	0.88	0.06
MTCA[c]	—	—	—	—	—	—	8.52	0.02
Polar HPLC fraction[d]	—	0.6	—	0.34	—	—	—	—
Urea[e]	0.26	—	0.13	—	0.06	0.09	—	—
NTG[e]	3.92	—	1.42	—	3.49	1.92	—	—
MNG[e]	13.21	—	7.5	—	9.46	8.78	—	—
MG[e]	0.45	—	0.3	—	0.30	0.24	—	—
TZG[f]	—	0.36	—	0.19	—	—	—	0.54
TMG[f]	0.11	1.44	0.13	0.6	0.19	0.16	0.2	2.17
TZU[f]	0.52	0.12	0.49	0.07	0.64	0.67	0.21	0.2
THMN[f]	0.11	—	0.12	—	0.09	0.17	—	—
TZMU[f]	0.15	0.1	0.1	0.03	0.29	0.06	0.19	—
TZNG[f]	11.25	0.14	7.01	0.06	12.48	10.18	10.44	0.18
Clothianidin[f]	**54.66**	**0.9**	**73.49**	**0.53**	**59.98**	**66.53**	**59.83**	**1.51**
Total identified	84.64	3.66	90.94	1.82	86.99	88.82	81.31	4.95
Not identified	3.59	0.29	2.36	0.24	1.86	2.48	6.54	0.42
Total analysed	88.23	3.95	93.3	2.06	88.85	91.30	87.85	5.37
Total excreted	95.37		93.32		99.05	99.58	97.04	

From Weber (2000)
F, female; M, male
[a] Preceded by 14-day pretreatment period with 25 mg/kg bw.
[b] Position of ^{14}C label on thiazolyl-2 instead of nitroimino. Metabolites detectable.
[c] Only by thiazolyl label.
[d] Polar fraction in faeces, not further identified.
[e] Only by nitroimine label.
[f] By both thiazolyl and nitroimine label.

The main metabolic reactions of clothianidin are 1) oxidative demethylation to form TZNG and 2) cleavage of the nitrogen–carbon bond between the thiazolyl-methyl position and the nitroimino moiety (see Figure 1). About 10–18% of the metabolites detected result from this cleavage of clothianidin. The main detectable metabolites formed from the nitroimino-labelled compound are MNG and NTG, whereas with the thiazolyl-2-labelled compound, the main detectable metabolite formed is CTCA, which is further metabolized into MTCA. A minor catabolic pathway leads to ACT. The degradation of clothianidin by transformation of the nitroimino moiety into the urea compounds TZMU and TZU and into the guanidines TMG, TZG and THMN is considered a minor metabolic reaction.

The fate of clothianidin in the rat is illustrated in Figure 2.

In summary, clothianidin was almost completely absorbed from the gastrointestinal tract (90%) within 24 hours. The rate and extent of absorption were essentially independent of sex, dose or dose rate. The compound was widely and homogeneously distributed across various organs (T_{max} = 1.5

Table 6. Nomenclature of identified metabolites of clothianidin in the rat

Code	Chemical name
TI-435	(*E*)-1-(2-Chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine; clothianidin
ACT	5-Aminomethyl-2-chlorothiazole
CTCA	2-Chlorothiazole-5-carboxylic acid
MG	Methylguanidine
MNG	*N*-Methyl-*N*′-nitroguanidine
MTCA	2-Methylthiothiazole-5-carboxylic acid
NTG	Nitroguanidine
THMN	*N*-2-Chlorothiazol-5-ylmethyl-*N*-hydroxy-*N*′-methyl-*N*″-nitroguanidine; thiazolhydroxymethylnitroguanidine
TMG	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-methylguanidine; thiazolmethylguanidine
TZG	2-Chlorothiazol-5-ylmethylguanidine; thiazolguanidine
TZMU	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-methylurea; thiazolylmethylurea
TZNG	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine; thiazolylnitroguanidine
TZU	2-Chlorothiazol-5-ylmethylurea; thiazolylurea
Urea	Urea

From Weber (2000)

hours), with a rapid decrease in residues to levels near or at the LOQ at 72 hours. There was no evidence of accumulation, although up to 4 hours post-dosing, higher levels were detected in kidney and liver.

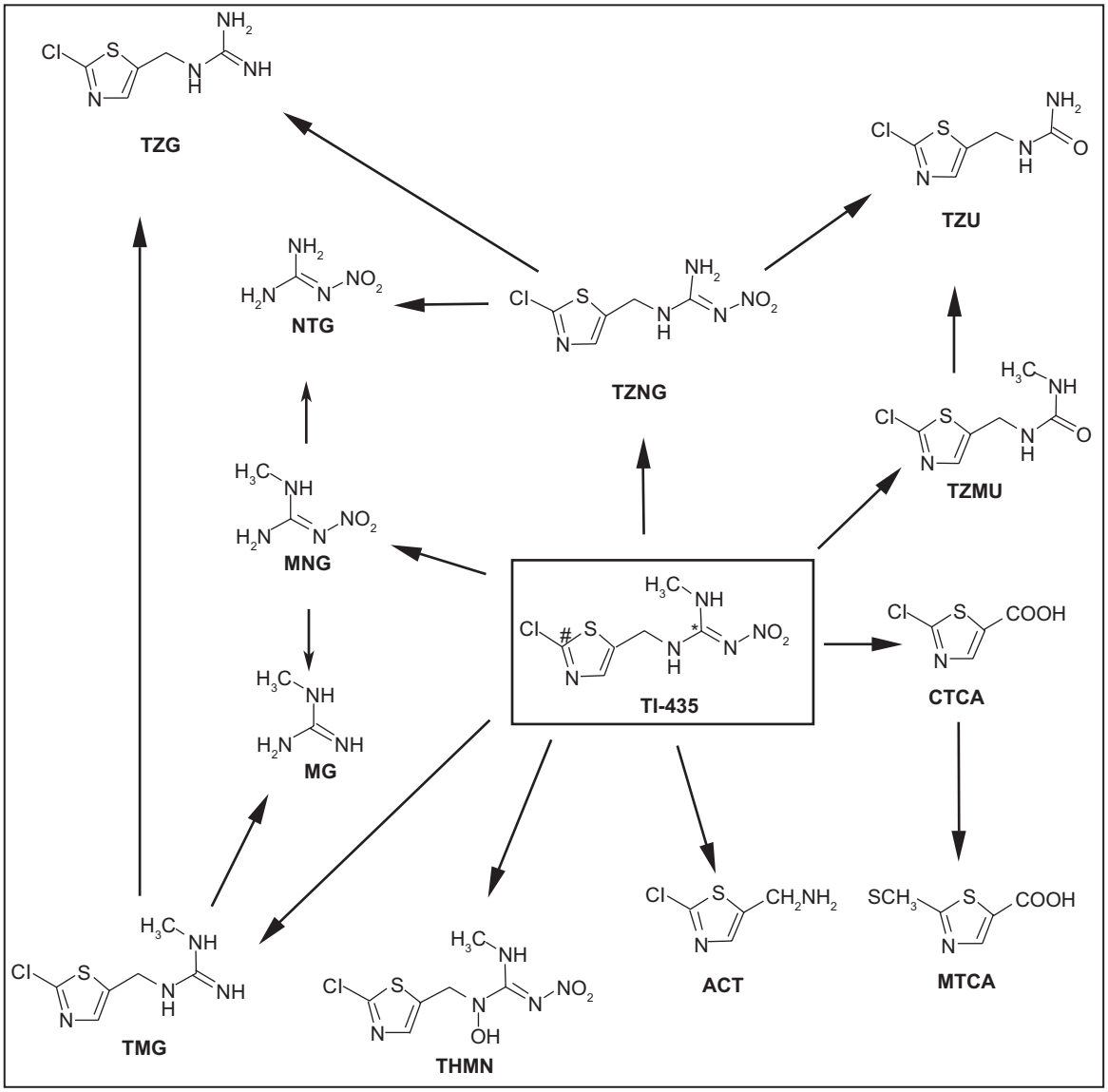
At 24 hours, about 94–96% of the compound was excreted. Urinary excretion was the major elimination route, with about 89% (males) and 95% (females) at termination (72 hours). Faecal elimination accounted for about 6% (males) and 3% (females). The excretion profile after high-dose administration at 72 hours (elimination half-life = 1.9 hours) was almost identical to that after low-dose administration (elimination half-life = 1.2 hours), although the plasma concentration exhibited biphasic kinetics, with concentration peaks at 6 hours and 32 hours, suggesting a moderate enterohepatic cycling.

Clothianidin was metabolized to a moderate extent, with 56–74% excreted unchanged over 72 hours. The main metabolic pathways were 1) oxidative demethylation and 2) cleavage of the nitrogen–carbon bond between the thiazolyl-methyl position and the nitroimino moiety. The main urinary metabolites recovered after low-dose testing were TZNG (7–11%), MNG (8–13%) and NTG (1–4%). In the faeces, MTCA (9%) and TMG (2%) were recovered. Other characterized metabolites were present at less than 2% of the dose (Weber, 2000).

Goat

The metabolism of clothianidin was studied in the goat using a study protocol partly in compliance with test method B.36 of European Commission directive 87/302/EEC. One lactating goat ("Bunte Deutsche Edelziege") (35.5 kg, 30 months of age) received [nitroimino-^{14}C]clothianidin by gavage at a dose of 10 mg/kg bw per day on 3 consecutive days. This dose corresponded to a concentration of 201 parts per million (ppm) in the feed. Urine, faeces and samples of blood and milk were collected. The animal was terminated 53 hours after the first administration. Specific radioactivity was 3.91 MBq/mg, radiochemical purity was greater than 98% and chemical purity was greater than 99%. The batch number of the non-labelled clothianidin was M00343 (purity 99.8%). Samples of liver, kidney, muscle and fat were taken. Radioactivity in samples was quantified, and metabolites were identified by thin-layer chromatography.

Figure 2. Metabolic transformation of clothianidin (TI-435) in the rat



*,# Position of radiolabel

From the time of administration to termination, the amounts of radiolabel excreted in urine, faeces and milk were 48.8%, 13.5% and 1.5%, respectively. However, only 70.4% of radioactivity was recovered, owing to the short time before termination (5 hours) after the last administration. Hence, the exact extent of absorption could not be determined. The radioactivity in edible tissues at termination was estimated to be 6.6% of the dose.

C_{max} (= 4.31 µg/ml) occurred at approximately 4 hours after the first dose. A rapid monophasic depletion was observed (elimination half-life = 5.3 hours), with an equivalent concentration of 0.69 µg/ml at 24 hours.

Eight hours subsequent to dosing, the residues in milk were 6.57 ppm (first dose) and 5.98 ppm (second dose). At termination (5 hours post-dosing), the value was 6.35 ppm. At 24 hours post-dosing, the residue levels declined to 0.97 and 0.92 ppm, respectively, corresponding to about 0.14% and 0.13% of the radioactivity.

Clothianidin was the main residue in milk (1.6 ppm, 51.2%), fat (0.8 ppm, 36.6%) and muscle (1.1 ppm, 25%), whereas it was undetectable in liver and kidney. Only two metabolites found in the goat were not covered by the rat metabolism: *N*-(2-chlorothiazol-5-ylmethyl)-*N′*-hydroxy-*N″*-methylguanidine (TMHG) (milk: 0.048 ppm, 1.6%) and *N′*-[(2-chlorothiazol-5-ylmethylamino)-(methylamino)methylene]-2-oxopropanohydrazide (ATMG-Pyr) (other compartments: range 0.14–0.97 ppm, 2.54–10.4%).

TMHG was formed by hydroxylation of the imino part of clothianidin, followed by denitrification and then oxidation of TMG. It is a more polar molecule and considered toxicologically less relevant than its precursors (see section 2.6(c)).

ATMG-Pyr was present in all tissues, but not in milk. In the rat, it was not acutely toxic by the oral route and negative for in vitro mutagenicity (see section 2.6(c)).

Characterized (on HPLC) but non-identified metabolites accounted for about 7% (milk), 25.6% (liver), 14.2% (muscle), 38.3% (kidney) and 8.6% (fat) of the administered dose (Spiegel & Weber, 2000).

Hen

The metabolism of clothianidin was studied in the hen using a study protocol partly in compliance with test method B.36 of European Commission directive 87/302/EEC. Six laying hens (*Gallus domesticus*, "White Leghorn") (1.53 kg, about 27 weeks of age) received [nitroimino-^{14}C]clothianidin by gavage at a dose volume of 1 ml/kg bw and at a dose of 10 mg/kg bw per day on 3 consecutive days. This dose corresponded to a concentration of 134 ppm in the feed (assuming a daily feed uptake of 7.5% of body weight). The animals were terminated 53 hours after the first administration. Samples of eggs, liver, kidney, muscle, skin (without subcutaneous fat) and fat were taken. Radioactivity in samples was quantified and then analysed by thin-layer chromatography.

From the time of administration to termination, the amount of radioactivity excreted was about 94.7%, with about 0.15% found in eggs. The estimated residue in prepared tissues was about 3.12%. The total radioactivity recovered was on average 98%. As the extent of absorption cannot be determined directly from the excretion values in birds, it was inferred from the relatively high residue values in both liver and kidney that gastrointestinal absorption had occurred. The excretion rate was relatively high, as about 38%, 35% and 22% of the radioactivity was excreted at 24, 48 and 53 hours, respectively.

In eggs, the cumulative radioactivity amounted to 0.201% of the TRR; the equivalent concentrations were 0.384, 0.753 and 0.943 ppm at 24, 48 and 53 hours, respectively (plateau phase was not achieved). At termination, the equivalent concentrations were determined in the various compartments: kidneys, 7.859 ppm (0.18% of dose administered); liver, 5.147 ppm (0.42%); dissected eggs, 1.835 ppm; breast and leg muscle, 1.735 ppm and 1.419 ppm, respectively; skin, 1.091 ppm; and subcutaneous fat, 0.193 ppm. The whole-body values for muscle, skin and fat (mean values from different locations) were 2%, 0.14% and 0.07%, respectively, and were calculated assuming relative weights of 40% (muscle), 4% (skin) and 12% (fat).

Metabolites were analysed in eggs, liver, muscle and fat. When the sum of identified and characterized radioactivity and solids (unresolved radioactivity) was taken as unity in order to normalize the data, the identified and characterized radioactive fractions were as follows: 98.6% and 0.56% (eggs), 75.4% and 13.56% (liver), 65.27% and 31.61% (muscle) and 74.45% and 18.11% (fat), respectively.

The identified metabolites at greater than 5% of TRR were distributed as follows: eggs, TZNG 0.504 ppm (87.52% of TRR) and clothianidin 0.122 ppm (21.21%); liver, TZNG 2.211 ppm (45.97%) and TZG 1.07 ppm (22.26%); muscle, *N′*-[amino(2-chlorothiazol-5-ylmethylamino)methylene] acetohydrazide (ATG-Ac) 0.551 ppm (35.12%), TZNG 0.124 ppm (7.91%) and TZG 0.091 ppm (5.78%); fat, ATG-Ac 0.048 ppm (31.32%), TZNG 0.036 ppm (23.71%), *N′*-[amino(2-chlorothiazol-5-ylmethylamino)methylene]-2-oxopropanohydrazide (ATG-Pyr) 0.011 (7.13%) and clothianidin 0.008 ppm (5.30%).

Two metabolites were found that were not recovered in the rat (ATG-Ac and ATG-Pyr), but the structurally equivalent goat metabolite ATMG-Pyr has been shown to be non-mutagenic and of a lower order of toxicity than clothianidin itself. Likewise, ATG-Ac was also tested and considered less toxic than clothianidin (Weber & Weber, 2000) (see section 2.6(c)).

(b) Exposure via lactation

Rats

In a study to determine whether pup exposure results through lactation when dams are exposed to clothianidin, a suspension of [nitroimino-^{14}C]clothianidin was administered by oral gavage to five female Sprague-Dawley rats each with a litter of four female and four male pups at an average dose of 257 mg of active ingredient per kilogram of body weight once daily for 4 consecutive days starting on day 9 postpartum. At 2, 4, 8 and 24 hours after the final dose, five pups of the same sex, one from each litter, were killed, homogenized and radioassayed to determine the exposure of the offspring to the test substance through lactation. Samples from the 4-hour and 24-hour sampling intervals were extracted, and the extracts were analysed by HPLC.

At 2, 4, 8 and 24 hours after the fourth oral dose to the dams, the TRRs (expressed as clothianidin equivalents) found in the female pups were 11.10, 11.22, 11.39 and 11.06 ppm, respectively. The TRRs found in the male pups at these sampling intervals were 12.08, 14.98, 11.07 and 11.32 ppm, respectively. The major residue found in pups killed at the 4- and 24-hour sampling intervals was clothianidin, the parent material. It was concluded that following four consecutive doses of [nitroimino-^{14}C]clothianidin to dams, the litters were exposed to the test substance through lactation, and the oral exposure of dams is sufficient to evaluate the potential effects of exposure to clothianidin for infants and children (Duah, Lopez & Nguyen, 2005).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In a test for acute oral toxicity, five mice (CRL:CD®1(ICR)BR) of each sex per dose received clothianidin in 5% weight per volume (w/v) aqueous gum arabic by gavage at a dose level of 304, 380, 475, 594 or 742 mg/kg bw (dose volume 10 ml/kg bw) following a preliminary range-finding study. The study was conducted in compliance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

Deaths occurred on days 1 and 2. No deaths occurred in either males or females treated with a dose of 304 mg/kg bw, whereas dose levels of 380 mg/kg bw and above caused mortalities in both sexes. Clinical signs included palpebral closure, decreased activity, ataxia and tremor. Lethargy and respiratory impairment were seen at the two highest doses. Onset of principal clinical signs was 0.5 hour post-dosing and persisted until day 2 in the survivors.

There were no consistent necropsy findings associated with active ingredient administration. The median lethal dose (LD_{50}) was 389 mg/kg bw in males and 465 mg/kg bw in females (Gardner, 1997b).

In a test of acute oral toxicity, five rats (CRL:CD®BR) of each sex per dose received clothianidin in 5% w/v aqueous gum arabic by gavage at a dose level of 1758, 2283, 2965, 3850 or 5000 mg/kg bw (dose volume 10 ml/kg bw). The study was conducted in accordance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

At all doses, palpebral closure, decreased activity and tremor, followed by hunched posture, wasted appearance and hair loss, were observed. Onset of principal clinical signs was on day 2 and persisted until day 15 at higher dose levels, whereas recovery from all clinical signs except hair loss occurred by day 15 at lower dose levels. Occasional appearance of ataxia, chromodacryorrhoea and discoloured urine was also observed, especially in females.

Significant and dose-related decreases in body weights and body weight gains of both males and females were observed at day 8 and day 15; these findings were more pronounced in the females. There was no consistent necropsy finding associated with dosing.

A single female rat treated with a dose of 2965 mg/kg bw died on day 2, and one dosed with 5000 mg/kg bw died on day 4. Two rats were killed on humane grounds on day 12 as a result of marked weight loss: a male at a dose of 5000 mg/kg bw and a female at a dose of 3850 mg/kg bw. The LD_{50} in both males and females is greater than 5000 mg/kg bw (Gardner, 1997a).

In a test of acute oral toxicity, five rats (Fischer 344 CDF (F-344)/BR) of each sex per dose received clothianidin in 5% w/v aqueous methylcellulose/0.4% Tween 80 by gavage (dose volume 10 ml/kg bw) at a dose of 250, 5000 or 1000 mg/kg bw (actual doses based on analytical results were 290, 523 and 1216 mg/kg bw). The study was conducted partially in accordance with test method B.1 of European Commission directive 92/69/EEC.

Deaths occurred beween days 1 and 4. Compound-related signs were evident in males and females that received a dose of 500 or 1000 mg/kg bw but not 250 mg/kg bw. Clinical signs in high-dose males and females included tremors, locomotor incoordination, hypoactivity, oral clear/red/brown stain and lacrimation. Clinical signs at 500 mg/kg bw were similar in nature and incidence (particularly for females) to the effects seen at the 1000 mg/kg bw dose level. Coolness to touch, gasping and moribundity were evident in animals that died at either dose level.

The earliest clinical signs were evident 3 hours after treatment, consisting of tremor and decreased activity. Many additional effects were evident at the 4-hour observation. Animals continued to exhibit marked evidence of toxicity 24 hours after treatment, with incomplete recovery at 72 hours.

Males and females at 500 mg/kg bw and above had reduced body weight or body weight gain; these findings were more pronounced in the females.

The survivors were killed on day 4 rather than, as more usually, on day 14, when recovery was evident, and when it was apparent that no additional animals would die as a result of treatment. No gross necropsy examination was performed, as the study was intended as a range-finding study for an acute neurotoxicity study (determination of time of peak for clinical signs). The acute oral LD_{50} was between 1216 and 2000 mg/kg bw for males and between 523 and 1216 mg/kg bw for females (Sheets, 2002).

In a test for acute dermal toxicity, five male and five female rats (CRL:CD®BR) were exposed to clothianidin moistened with 200 µl water at a dose level of 2000 mg/kg bw and a surface density of about 0.018–0.021 g/cm^2 by dermal semiocclusive application for 24 hours following a preliminary range-finding study. The protocol was in compliance with test method B.3 of European Commission directive 92/69/EEC and OECD Guideline No. 402 (Acute Dermal Toxicity; 24 February 1987). There were no deaths or local or systemic signs of toxicity. Two out of five females showed a slightly impaired body weight gain on day 8 and day 15. No abnormal findings were observed in any of the animals at necropsy. The acute dermal LD_{50} in males and females was greater than 2000 mg/kg bw (Gardner, 1997c).

In a head-only inhalation assay, five male and five female rats (CRL:CD®BR) were exposed to clothianidin as a micronized dust aerosol at a concentration of 5.538 ± 1.954 mg/l (by gravimetry) for 4.5 hours. The mass median aerodynamic diameter was 2.78 ± 2.38 µm (range 1.46–8.40 µm); about

55% of the particles were 1–4 µm. A concomitant control group of five rats of each sex was included. The study protocol was partly in compliance with test method B.2 of European Commission directive 92/69/EEC and OECD Guideline No. 403 (Acute Inhalation Toxicity; 12 February 1981). There were no mortalities during the study. Clinical signs in both sexes included hypothermia during exposure; ataxia, ptosis, hunched posture, stained body/eyes/nose and lethargy were observed up to day 4. There were no necropsy findings of note. Body weights of treated animals were decreased by about 9%, compared with controls. Owing to intermittent jamming of the aerosol generator during the first hour of exposure, with concomitant drop of the atmospheric concentration to about 1.6 mg/l, the test duration was extended by 0.5 hour. The acute inhalation median lethal concentration (LC_{50}) was greater than 5.538 mg/l (gravimetric determination for 4.5 hours) (Shepherd, 1998).

(b) Dermal and ocular irritation and skin sensitization

The skin irritation potential of clothianidin was tested in six rabbits (Crl: NZW Kbl©BR) (one female and five males). The study was conducted according to test method B.4 of European Commission directive 92/69/EEC and OECD Guideline No. 404 (Acute Dermal Irritation/Corrosion; 17 July 1992). The animals were exposed to 0.5 g clothianidin moistened with 100 µl water, applied to 6 cm^2 of back skin under a semiocclusive cover for 4 hours. No skin irritation or dermal reaction was observed from 24 hours through 3 days. Clothianidin was not irritating to the skin in this test (Gardner, 1997d).

The eye irritation potential of clothianidin was investigated in six male rabbits (Crl: NZW©BR) by instillation of 0.066 g, corresponding to 0.1 ml of the undiluted test substance, into the everted lid of the left eye. The study was conducted in accordance with test method B.5 of European Commission directive 92/69/EEC and OECD Guideline No. 405 (Acute Eye Irritation; 24 February 1987). Minor conjunctival reactions were observed on the treatment day in all rabbits. These reactions had resolved by 24 hours post-treatment. There were no ocular effects at any of the evaluation time points from 24 through 72 hours. Clothianidin is considered to be practically non-irritating to the eye in this test (Gardner, 1997e).

The skin sensitization potential of clothianidin was investigated using the maximization method in groups of 20 (control and test) female Dunkin-Hartley (Tif:DHP) guinea-pigs. The protocol was partly in compliance with test method B.6 of European Commission directive 92/69/EEC and OECD Guideline No. 406 (Skin Sensitisation; 17 July 1992). Clothianidin was dissolved in Alembicol D (fractionated coconut oil). Test concentrations were selected on the basis of preliminary testing and were 1% for intradermal injection (maximum practical concentration injectable with Freund's complete adjuvant and/or Alembicol D), 55% by occlusive application over 48 hours for dermal induction (seen to be slightly irritating) and 10% and 20% for application on one flank, with vehicle on the other flank, for 24 hours for challenge (highest non-irritant concentration).

Slight to well-defined erythema was observed at 1 day after intradermal injection in both control and test animals receiving Alembicol D alone (controls) or the test material in Alembicol D. On the next day after removal of percutaneous induction, slight to well-defined erythema was observed in the control animals, but no response was observed in the test animals.

At 24 hours after challenge, 10% and 15% of the test animals showed slight erythema after, respectively, 10% weight per weight (w/w) and 20% w/w test substance exposure, whereas 5% of the animals showed this response in the concomitant controls. At 48 hours after challenge, 5% of the controls showed slight erythema. None of the dermal reactions in the test animals was clearly more marked than those of the control group. Therefore, clothianidin was concluded not to be sensitizing in this study (Denton, 1997).

In summary, the acute toxicity by the oral, dermal and inhalation routes of exposure to clothianidin was low. Certain clinical signs observed after oral dosing of rats or mice suggest mild and

Table 7. Summary of acute toxicity, including irritancy and skin sensitization, of clothianidin

Type of test	Result	Purity (%)	Batch No.	Reference
Rat, oral	LD_{50} > 5000 mg/kg bw	96	30034708	Gardner (1997a)
Rat, oral	1216 < LD_{50} < 2000 mg/kg bw (males) 523 < LD_{50} < 1216 mg/kg bw (female)	96	30037120	Sheets (2002)
Mouse, oral	389 mg/kg bw (males) 465 mg/kg bw (females)	96	30034708	Gardner (1997b)
Rabbit, dermal	> 2000 mg/kg bw	96	30034708	Gardner (1997c)
Rat, 4 h inhalation	> 5.54 mg/l	96	30034708	Shepherd (1998)
Rabbit, skin irritation	Non-irritating	96	30034708	Gardner (1997d)
Rabbit, eye irritation	Practically non-irritating	96	30034708	Gardner (1997e)
Guinea-pig, skin sensitization (Magnusson and Kligman test)	Not sensitizing	96	30034708	Denton (1997)

transient neurotoxic effects (tremor, ataxia, hypoactivity, hunched posture, piloerection, respiratory impairment, hypothermia). These effects are considered to be due to overdosage of the compound. However, in both acute neurotoxicity and pharmacological studies, similar dose-dependent signs were observed, suggesting nicotinic central nervous system effects.

In a range-finding acute neurotoxicity study of the active ingredient formulated in aqueous carboxymethylcellulose rather than aqueous gum arabic, the oral LD_{50} value was between 500 and 1000 mg/kg bw (in the female rat). Both the clinical signs and recovery appeared earlier in the latter study, suggesting a more rapid and/or more complete absorption than in the former. Moreover, mice appeared more sensitive than rats when both mortality and clinical signs were considered. No dermal toxicity was observed. The compound is not a skin irritant, is practically non-irritating to the eye and is not a skin sensitizer.

The results of studies of acute toxicity of clothianidin administered orally, dermally or by inhalation are summarized in Table 7.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Groups of six mice (CRL:CD-1®(ICR)BR) of each sex per dose were fed a diet of clothianidin (purity 97.5%; batch No. 30033623) at dose levels of 0, 500, 1000, 2000 and 4000 ppm, which were equal to 0, 90, 190, 383 and 683 mg/kg bw per day for males and 0, 122, 248, 491 and 619 mg/kg bw per day for females, for a period of 4 weeks. Accuracy, homogeneity and stability of the administered diet were confirmed by chemical analysis. The protocol was partly in compliance with test method B.7 of European Commission directive 92/69/EEC and OECD Guideline No. 407 (Repeated Dose Oral Toxicity – Rodent; 12 May 1981). This study was a dose range–finding exercise for further study; limitations of the study design included the lack of performance of histopathology of intermediate-dose groups when effects were observed at the top dose and the unreliability of urinalysis data as a result of insufficient samples in the female control group.

Four males and all females died at 4000 ppm (week 2); two male deaths occurred among 2000 ppm animals as a result of the anaesthestic used for the blood sampling procedure. At the

top dose, lethargy, tremors, hunched posture, piloerection, emaciation, half-closed eyes, unsteady gait, hypothermia and extremity pallor were observed during week 2 and, to a lesser extent, during week 1; at 2000 ppm, emaciation and hunched posture (week 3 and beyond) were the principal clinical findings.

Feed consumption was impaired at 500 ppm. Body weight was decreased dose-dependently throughout the study at 2000 and 4000 ppm. Females treated at 1000 ppm also showed weight loss, and males treated at 1000 ppm showed decreased weight gain during week 1, but subsequently gained weight. Thus, there was a treatment-related reduction in the overall weight gains of both sexes at 1000 ppm, although there was no statistical significance ($P > 0.05$). The weight gains of animals at 500 ppm were unaffected by treatment. Overall, during the 4-week treatment period, feed consumption was significantly reduced among all treated male and female groups when compared with that of their respective controls, with animals receiving 2000 or 4000 ppm showing the largest reduction in feed consumption.

Feed utilization was slightly superior to that of controls for males receiving 500 ppm, whereas it was impaired among the remaining treated male groups and all the treated female groups when compared with that of their respective controls, reflecting the body weight changes noted among the treated groups.

At the top dose, packed cell volume, red blood cells, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and lymphocyte count were decreased in the two surviving males. At 1000 ppm and above, neutrophil counts increased in males. Minor decreases in MCV and mean corpuscular haemoglobin concentration (MCHC) and an increase in eosinophils were also observed in the females at 2000 ppm.

Increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were observed at 2000 ppm and above. Increases in sodium and chloride ion concentrations were observed at 2000 and 4000 ppm, and in females also at 500 and 1000 ppm. Potassium ion concentration was also increased in females at 1000 and 2000 ppm. At 2000 ppm and above, triglyceride and glucose levels were markedly decreased. These findings were consistent with the observed reduced feed intake and associated weight losses.

At 2000 and 4000 ppm, most organ weight changes were interpreted as secondary to the body weight loss due to underfeeding. At necropsy, size diminution was noted in the organs of the immune system (thymus, spleen), adipose tissue, thyroids, stomach and gonads at 4000 ppm. The majority of these animals died during the study.

Reduced white pulp cellularity and red pulp atrophy in spleens and thymic involution or atrophy were observed with increased incidence among animals at 2000 and 4000 ppm; these are considered to reflect induced stress and markedly reduced weight gain. In the adrenal cortex, vacuolation of cells in the zona fasciculata (males) and congestion of the zona reticularis (females) were observed at the top dose. In males at 4000 ppm, the incidence of atrophic change (testicular atrophy, reduced colloid in prostate or seminal vesicle) was increased. In females, reduced thickness of the uterine wall was found at the top dose, and an increased incidence of animals lacking corpora lutea in the ovary was reported at 2000 ppm. These changes in the reproductive system are considered to reflect induced stress and markedly reduced weight gain.

Selected clinical and histopathological findings from this 28-day toxicity study in mice are summarized in Tables 8 and 9, respectively.

On the basis of mortalities, clinical findings, reduction of body weight and reduction of body weight gain, it can be concluded that the maximum tolerated dose (MTD) was attained at 2000 ppm (383–491 mg/kg bw per day). The no-observed-adverse-effect level (NOAEL) was 1000 ppm (190 mg/kg bw per day), and the lowest-observed-adverse-effect level (LOAEL) was 2000 ppm (383 mg/kg bw per day), based on effects on body weight gain, clinical signs and clinical chemistry

Table 8. Oral 28-day toxicity of clothianidin in mice: selected clinical findings

	% change relative to controls									
	Dietary concentration (ppm)									
	0		500		1000		2000		4000	
	M	F	M	F	M	F	M	F	M	F
Mortality	0	0	0	0	0	0	2	0	4	6
Feed consumption[a]	—	—	↓21*	↓19*	↓16*	↓16*	↓26**	↓33**	↓53**	—
Body weight (week 4)	—	—	—	—	↓6	—	↓25	↓27	↓53	—
Body weight gain[a]	2.7	2.1	3.4	0.9	2.1	1.1	−2.6*	−2.1**	−5.2*	—
Haematology										
Haematocrit	—	—	—	—	—	—	—	—	↓31**[b]	[c]
Haemoglobin	—	—	—	—	—	—	—	—	↓27**[b]	[c]
Red blood cells	—	—	—	—	—	—	—	—	↓23**[b]	[c]
MCV	—	—	—	—	—	—	—	↓3*	↓11**[b]	[c]
MCH	—	—	—	—	—	—	—	↓4*	↓6*[b]	[c]
White blood cells	—	—	↑71	↓29	↑56	↓33	↓3	↑25	↓49	—
Neutrophils	—	—	—	—	↑81*	—	↑54*	↑180**[b]	↑98*[b]	[c]
Lymphocytes	—	—	—	—	—	—	—	—	↓73*[b]	[c]
Eosinophils	—	—	—	—	—	—	—	—	↑180**[b]	[c]
Blood chemistry										
ALT	—	—	—	—	—	—	↑55	↑100*	↑204[b]	[c]
AST	—	—	—	—	—	—	↑61*	↑30	↑285[b]	[c]
Na^+	—	—	—	—	—	↑1.3*	↑3.3**	↑3.3**	↑3.3[b]	[c]
K^+	—	—	—	—	—	↑20*	—	↑17*	—	[c]
Cl^-	—	—	—	↑1.8*	—	↑1.8*	↑3.6**	↑3.6**	↑3.6[b]	[c]
Glucose	—	—	—	—	—	—	—	↓31**	↓43[b]	[c]
Triglycerides	—	—	—	—	—	—	↓36	↓52*	↓73[b]	[c]

From Chambers (1997b)
F, female; M, male; * $P < 0.05$; ** $P < 0.01$ (Williams' test)
[a] Body weight gain and feed consumption in grams per mouse during weeks 1–4.
[b] Results on 2/6 (haematology, organ weights) or 1/6 (clinical chemistry, urinalysis) samples only.
[c] No results available (mortality).

changes. On the basis of these results, it was concluded that dose levels in excess of 2000 ppm, equal to 383 mg/kg bw per day, would not be suitable for future study (Chambers, 1997b).

In a 90-day study, 10 mice (Crl:CD BR) of each sex per dose were fed a diet of clothianidin (purity 95.8%; batch No. 30034708) at a dietary concentration of 0, 100, 500, 1000 or 1500 ppm (achieved doses: 0, 16, 82, 160 and 263 mg/kg bw per day for males and 0, 22, 107, 207 and 329 mg/kg bw per day for females). Animals were housed two per cage. Accuracy, homogeneity and stability of the diet were confirmed by chemical analysis. The protocol is partly in compliance with test method B.26 of European Commission directive 92/69/EEC. The report is GLP certified, but reporting of the study was not finalized and the study may be disregarded, because the test facility was in breach of GLP regulations at the time.

Twelve mice (nine male) distributed among the 500, 1000 and 1500 ppm groups were found dead following the stress of the routine blood sampling procedure during weeks 12 and 13.

Table 9. Oral 28-day toxicity of clothianidin in mice: selected histopathological findings

	No. of positive findings/no. of examined animals (decedent + terminal)									
	Dietary concentration (ppm)									
	0		500		1000		2000		4000	
	M	F	M	F	M	F	M	F	M	F
Thymus: involution/atrophy	—	—	—	—	—	—	1/1	3/3	2/2	—
Spleen: reduced white pulp cellularity	—	—	—	—	—	—	—	1/6	3/3	6/6
Spleen: red pulp atrophy	—	—	—	—	—	—	—	2/6	3/3	5/6
Liver: apparent reduction in hepatocyte size	—	—	—	—	—	—	—	—	1/2	—
Liver: slight centrilobular vacuolization	—	—	—	—	—	—	—	1/6	—	—
Adrenals: vacuolated cells zona fasciculata	—	—	—	—	—	—	—	—	1/3	—
Adrenals: congestion zona reticularis	—	—	—	—	—	—	—	—	—	3/3
Prostate: absent colloid	—	—	—	—	—	—	—	—	2/2	—
Seminal vesicles: reduced seminal colloid	—	—	—	—	—	—	—	—	1/2	—
Epididymis: spermatozoa absent	—	—	—	—	—	—	—	—	1/2	—
Epididymis: abnormal shape/reduced spermatozoa number	—	—	—	—	—	—	—	—	1/2	
Testes: seminiferous epithelial atrophy	—	—	—	—	—	—	—	—	2/2	—
Uterus: reduced mural thickness	—	—	—	—	—	—	—	5/6	5/6	—
Ovaries: absence/reduced number of corpora lutea	—	—	—	—	—	—	—	3/5	—	—

From Chambers (1997b)
F, female; M, male

There were no relevant ophthalmological findings. Vocalizations were noted from week 4 in both males and females at the top dose. Over the whole treatment period, reduced body weight gains were observed in both sexes at 1000 ppm and above. There were no relevant haematological findings. The alkaline phosphatase values were slightly increased for females receiving 1000 ppm and above. AST and phosphorus levels were also increased at 500 ppm and above, although without clear dose dependency at the top dose or any treatment-related histopathological findings. There were no relevant findings in urinalysis.

At necropsy, decreased relative kidney weight in males at 100, 500 and 1000 ppm was not considered biologically meaningful by the study director, because of the high control value of the absolute kidney weight (0.791 ± 0.090 g) compared with the mean value of the historical control (0.629 ± 0.110 g). The decreased relative kidney weight was not corroborated by any gross pathological or histopathological lesion. Weight decreases of other organs (absolute and relative lung weight in females and slight, but not significant, decrease of relative liver weight in both males and females) were considered secondary to the observed body weight loss.

During histological evaluation, "sparse numbers of corpora lutea" were found in the ovaries at the top dose (6/10 females). Detailed re-evaluation (Sangha, 2000) revealed decreases in the numbers of corpora lutea (−49%), large follicles (−54%) and degenerating follicles (−51%) in both ovaries at the top dose. In the uterus, an increased number of animals were in estrus, and the study pathologist described an increased prominence of uterine endometrial glands at the top dose. The change was described as an increase of enlarged, pale endometrial glandular cells, frequently with vesicular nuclei. In addition, varying degrees of glandular luminal dilatation with or without the presence of luminal eosinophilic material were reported.

In the view of the study director, however, these changes reflected normal structures within the range of cyclical change of the mouse reproductive tract, due to prolongation of the estrous stage of the cycle or cycle arrest in estrus. It is possible that the effects could also be secondary to the underfeeding and stressful state of the animals. The NOAEL was 100 ppm, equal to 16 mg/kg bw per day, based on increased AST activity at 500 ppm, equal to 82 mg/kg bw per day (Chambers, 1997d). The notifier considers the results of this study to be inconsistent with those of other studies, potentially compromised by poor GLP practices at the test facility, and should be discarded. The study is summarized here for information purposes only.

Rats

Groups of five rats (CRL:CD®BR) of each sex per dose were fed a diet of clothianidin (purity 97.5%; batch No. 30033623) at a concentration of 0, 1250, 2500, 5000 or 7500 ppm (0, 120, 249, 475 and 602 mg/kg bw per day for males and 0, 137, 228, 454 and 689 mg/kg bw per day for females) for 4 weeks. Accuracy, homogeneity and stability of the administered diet were confirmed by chemical analysis. The protocol was partly in compliance with test method B.7 of European Commission directive 92/69/EEC and OECD Guideline No. 407 (Repeated Dose Oral Toxicity – Rodent; 12 May 1981), with the exception that the study was a dose range–finding exercise for further study; hence, histopathology of intermediate-dose groups was performed only when there were macroscopic abnormalities in these animals.

There were no deaths. Clinical signs of toxicity at 5000 and 7500 ppm included half-closed eyes and occasional hair loss. At the top dose, brown nasal staining was observed. Findings are summarized in Table 10.

Body weights, body weight gains, feed consumption and feed efficiency were impaired at 2500 ppm and above, in a dose-related manner, in both males and females. Haematocrit, haemoglobin and red blood cell counts were increased at 2500 ppm and above, whereas reticulocytes were decreased at 5000 and 7500 ppm. In the males, white blood cell counts were lower at 5000 ppm and above; differential counts revealed a significant decrease in all cell populations. In the females, there was no corresponding marked decrease in white blood cell count, but eosinophils decreased in a dose-dependent way at 2500 ppm and above. An increased blood urea nitrogen at the top dose was related to effects in the kidneys, and urinalysis showed a decrease in specific gravity, decrease in urinary protein concentration and increase in pH. However, there were no corresponding changes in renal pathology, with no effect on organ weights, gross pathology or histopathology. Decreased chloride content at all doses and increased triglycerides at 2500 ppm and above are considered biologically irrelevant in the absence of a dose–effect relationship. Bilirubin levels were significantly increased at the top dose. Alkaline phosphatase activity was increased at 5000 ppm and above in females, whereas AST activity was increased at the top dose in both sexes. Although the alkaline phosphatase increase may be attributable to an effect on the liver, it may also be secondary to underfeeding of the animals.

A decrease in absolute weight and an increase in relative weight were detected for a number of organs at 2500 ppm and above and were attributed to the observed body weight decrease. Absolute weight of thyroids (females), thymus (males and females), liver (males and females), spleen (males and females), kidneys (males and females), adrenals (males and females) and testes (males) showed a dose-related decrease, but it did not attain statistical significance. The thymus and the liver showed a decrease in relative weight.

Histologically, atrophic change of spleen, thymus and reproductive tract at 5000 ppm and above was mostly in line with organ weight findings. The observed findings are most probably a secondary effect to the underfeeding of the animals (effects in reproductive organs) or to the induced stress as a consequence of this fact (thymus: involution and atrophy; spleen: reduced white pulp).

On the basis of reductions in body weight and body weight gain, it was concluded that dietary concentrations of 5000 ppm and above were unsuitable for further study and that the observed

Table 10. Oral 28-day toxicity of clothianidin in rats: selected clinical findings

	% change relative to controls									
	Dietary concentration (ppm)									
	0		1250		2500		5000		7500	
	M	F	M	F	M	F	M	F	M	F
Feed consumption[a]	—	—	↓4	—	↓12	↓21	↓33	↓31	↓51	↓37
Body weight (week 4)	—	—	—	↓5	↓12	↓12	↓35	↓25	↓53	↓37
Body weight gain[a]	155	84	160	76	116**	52**	37**	26**	−22**	−5**
Haematology										
Haematocrit	—	—	—	—	↑15**	↑12**	↑14**	↑12**	↑17**	↑17**
Haemoglobin	—	—	—	↑7*	↑16**	↑14**	↑14**	↑16**	↑19**	↑19**
Red blood cells	—	—	—	—	↑13**	↑12**	↑14**	↑14**	↑21**	↑20**
Reticulocytes[b]	—	—	—	—	—	—	↓59**	—	↓84**	↓67*
Platelets	—	—	—	—	—	—	—	—	—	↓22**
White blood cells	—	—	—	—	—	—	↓33*	—	↓68**	—
Neutrophils	—	—	—	—	—	—	↓74*	—	↓91**	—
Lymphocytes	—	—	—	—	—	—	—	—	↓57**	—
Eosinophils	—	—	—	—	—	↓58*	—	↓67*	↓92**	↓84**
Basophils	—	—	—	—	—	—	—	—	↓75**	—
Monocytes	—	—	—	—	—	—	—	—	↓86**	—
Large unstained cells	—	—	—	—	—	—	—	—	↓84**	—
Blood chemistry										
Blood urea nitrogen	—	—	—	—	—	—	↑50*	—	↑200**	↑71**
Alkaline phosphatase	—	—	—	—	—	—	—	↑57*	—	↑97**
AST	—	—	—	—	—	—	—	—	↑34**	↑25**
Urinalysis										
pH[c]	6.7	6.6	7.0	6.6	6.9	6.8	7.1**	6.7	7.5**	7.2**
Density	—	—	↓0.9**	—	↓0.7**	—	↓1.5**	—	↓1.2**	↓1.1**

From Chambers (1997a)
F, female; M, male; * $P < 0.05$; ** $P < 0.01$ (Williams' test or Student's *t*-test)
[a] Feed consumption and body weight gain in grams per rat during weeks 0–4.
[b] % change compared with actual reported.
[c] Actual study values.

haematological, biochemical, organ gravimetrical, gross pathological and histopathological findings were a consequence of the underfed status of the animals. The data from this study did not permit unequivocal identification of a specific target organ.

The NOAEL was 1250 ppm, equal to 120 mg/kg bw per day, and the LOAEL was 2500 ppm, equal to 249 mg/kg bw per day, based on effects on feed consumption, body weight and body weight gain (Chambers, 1997a).

In a dietary study in rats, 10 rats (CRL:CD®BR, Sprague-Dawley) of each sex per dose were fed a diet of clothianidin (purity 95.8%) at a dose level of 0, 100, 250, 1250 or 2500 ppm (achieved doses: 0, 7.7, 19.7, 96.0 and 189 mg/kg bw per day for males and 0, 9.4, 24.0, 119 and 232 mg/kg bw per day for females) for 13 weeks. Animals were housed five per cage. Accuracy, homogeneity and stability of the administered diet were confirmed by chemical analysis. The protocol was partly in

compliance with test method B.26 of European Commission directive 92/69/EEC. A methodological limitation was damage to the adrenal tissues during necropsy, which prevented a complete evaluation of possible effects on the adrenals. Reporting of this study was not finalized, and results may be disregarded, because the test facility was in breach of GLP regulations at the time.

One female at 1250 ppm was found dead on week 13 (cause of death not compound related). There were no relevant ophthalmological findings and no clinical signs. At the top dose, feed consumption was significantly reduced during week 1 in males, whereas in females, there was a non-statistically significant reduction in feed consumption at both the top dose and 1250 ppm. Slight decreases in feed consumption were noted in both sexes over the whole treatment period. During the first week of treatment, body weight gain compared with controls was appreciably reduced for both sexes receiving 2500 ppm and to a lesser extent among females receiving 1250 ppm. A marginal reduction in body weight gain was also apparent among males receiving 1250 ppm and in females receiving 250 ppm (both non-significant). For weeks 1–13, body weight gain for both sexes was considered to be similar to that of controls.

At the top dose, increases in haemoglobin in both sexes, red blood cells and haematocrit in males and MCHC in females were observed in comparison with controls. At 250 ppm and above, lower white blood cell counts were found in males, but without dose dependency. Differential counts indicated decreases in monocytes and large unstained cells in females receiving 2500 ppm.

The impaired feed intake was most likely responsible for the changes in a number of parameters (ALT, alkaline phosphatase, glucose, chloride ion, inorganic phosphorus, calcium ion).

Both absolute and relative uterine and ovarian weights at 1250 ppm were increased. Increases at 1250 ppm and/or the top dose in relative liver weight (males and females), relative lung weight (females), relative heart weight (females), relative adrenal weight (females) and relative spleen weight (females) were attributed to the decreased body weight at week 14. The histopathological analysis showed the presence of parenchymal inflammatory cells and sinusoidal dilatation/congestion in the liver of males.

At all doses, about 40% of females exhibited uterine fluid distension at necropsy. Microscopically, this was corroborated by evidence of uterine luminal dilatation. In a later re-evaluation of the histopathology, the stage of the reproductive cycle was determined, and in all treated animals, about 2–3 out of 10 were in proestrus (Table 11). According to the study author, this incidence was a chance occurrence and may be associated with cycle synchronization subsequent to co-housing of the animals (five per cage). When relative uterine weights were considered for animals that exhibited no cytological findings (such as proestrus, dilatation), a dose-dependent increase was apparent, attaining high significance at 1250 ppm and above. It was concluded by the study director that the increase could not be attributed entirely to uterus dilatation. In the ovaries, a slight increase of persistent corpora lutea was observed, but without statistical significance.

The NOAEL was 250 ppm, equal to 19.7 mg/kg bw per day, based on body weight and body weight gain at 1250 ppm, equal to 96.0 mg/kg bw per day (Chambers, 1997c).

In a 90-day toxicity study, 15 rats (Sprague-Dawley) of each sex per dose (low and intermediate doses) and 30 rats of each sex per dose (control and high doses) were fed diets containing clothianidin (purity 96%; batch No. 30037120) at concentrations of 0, 150 (low), 500 (intermediate) and 3000 (high) ppm (achieved doses: 0, 9.0, 27.9 and 202.0 mg/kg bw per day for males and 0, 10.9, 34.0 and 254.2 mg/kg bw per day for females). Following the exposure period, 15 rats of each sex of the 0 and 3000 ppm groups were placed on control feed for another 50 days (recovery group). Accuracy, homogeneity and stability of the administered diet were confirmed by chemical analysis. In addition to typical clinical chemistry tests, portions of liver were used to determine the activity of *p*-nitroanisole-*O*-demethylase (NODM), aminopyrine-*N*-demethylase (ANDM), 7-ethoxyresorufin *O*-deethylase (EROD), 7-pentoxyresorufin *O*-dealkylase (PROD) and cytochrome P450 (CYP) content. Thyroid

Table 11. Oral 90-day toxicity of clothianidin in rats: selected findings

	% change relative to controls				
	Dietary concentration (ppm)				
	0	100	250	1250	2500
Organ weight					
Liver absolute	—	—	—	—	—
Liver relative	—	—	—	—	14**
Adrenals absolute	—	—	—	—	12
Adrenals relative	—	—	—	—	24*
Uterus absolute	—	—	—	29	39*
Uterus relative[a]	18	22	22	25 (39*)	28 (55**)
Uterus relative[b]	17	17	18	21 (24**)	24 (41**)
Ovaries absolute	—	—	—	16	19
Ovaries relative	—	—	—	25*	32**
Gross pathology					
Uterine fluid distension[c]	0	3	3	3	4
Histopathology					
Uterus					
Uterus luminal dilatation[c]	1	3	2	3	4
Cervix					
Proestrus stage[c]	0	3	2	3	3
Ovaries					
Corpora lutea	18.7 ± 4.8	—	—	—	25.2 ± 9.0
"Large" follicles	9.7 ± 2.5	—	—	—	11.8 ± 4.2
Degenerating follicles	3.0 ± 1.9	—	—	—	3.4 ± 2.7

From Chambers (1997c)

* $P < 0.05$; ** $P < 0.01$ (Williams' test or Student's *t*-test)

[a] In all animals; first number = relative weight (g/10 kg bw).

[b] In animals with uteri without histological changes (neither dilatations nor proestrus); first number = relative weight (g/10 kg bw).

[c] Occurrence per 10 animals.

function was assessed by measuring triiodothyronine, thyroxine and thyroid stimulating hormone levels by radioimmunoassay. Additionally, special attention was given to the estrous cycle, by examining vaginal smears daily over a 21-day period prior to termination. Animals were selected for termination each day on the basis of being in the same estrous cycle (i.e. day 2 diestrus), thus alleviating any possible effects of non-synchronous estrous cycle stage on the various measured parameters (uterus weight, ovarian follicular population). The protocol was partly in compliance with test method B.26 of European Commission directive 92/69/EEC and OECD Guideline No. 408 (Repeated Dose Oral Toxicity – Rodent; 12 May 1981). During European Union review, methodological limitations noted included the lack of measurement of urine volume (stated in the methodology, but no results) and the absence of individual data for vaginal smears to control estrous cycling activity.

One male at 500 ppm was found dead on day 17 (cause of death not reported); no females died during the study. One incidence of cataract was found in the males at the top dose; none was found in the females. An increase of top-dose females with alopecia, dark red discharge, moderate to many amorphous phosphate crystals in urine and amber/cloudy urine appearance was also seen.

Lower body weights and body weight gains were observed at the top dose for both males and females. After recovery, minimal body weight decreases were still observable in both sexes,

whereas body weight gain had increased. Feed consumption was not affected. In males at the top dose, some slight increases of red blood cell mass were noted. In females, there was slight lengthening of prothrombin time (the differences were unremarkable after recovery). However, there was no corresponding increase in white blood cell counts or reticulocyte counts, nor was any evidence of haematopoiesis noted at microscopic examination of the tissues.

A dose-related increase in phosphate and total protein levels attained significance among top-dose males. Creatine kinase and lactate dehydrogenase activities were statistically significantly decreased in 3000 ppm recovery males and both 150 and 3000 ppm females and were increased in 3000 ppm recovery females. These changes were considered not treatment related. No significant changes were observed in females.

Small increases of *N*- and *O*-demethylase activities were observed in both males (approximately 1.5-fold) and females (approximately 1.3-fold) at the top dose; however, increases in females were not statistically significant. After recovery, the activities had returned to control levels. Slight, non-significant increases of CYP in 3000 ppm males and females were noted. In tissue homogenates, the activities of EROD (2.2-fold) and PROD (3-fold) were increased in the males at the top dose. Overall, these data suggest that clothianidin is a weak inducer of hepatic monooxygenase activities, the changes in enzyme activities indicating induction of CYP2B and possibly CYP1A enzymes.

Thyroid hormone levels did not change; the slight dose-related increases in thyroid stimulating hormone in treated female groups (+3%, +13%, +38%) did not attain statistical significance and were not considered relevant in the absence of any corroborating data.

No adverse findings in urinalysis were noted at any dose for either sex during treatment.

In males, decreases in absolute heart and liver weights (liver weight decrease persistent after recovery) as well as increases in relative brain, heart, kidney and testes weights were consistent with lower body weights at the top dose. In females, the observed decreases in absolute organ weights were not statistically significant and in all cases were less than 10%. Relative uterine weight in the high-dose group was increased by 16%. However, in contrast to an earlier study (Chambers, 1997c), neither this change nor the ovary weight of treated animals was statistically significantly altered at termination, compared with the controls.

One animal showed a testicular mass at the top dose; no histological correlate was reported. In the recovery group, one testicular cyst was detected at the top dose. At the top dose, an excess of spleen pigmentation was detected at study termination (but not after recovery) in the treated males, but not in the females. This histological observation was not supported by haematological, serum chemical, organ weight or gross necropsy findings of the spleen. The finding is suggestive for haemosiderin deposition subsequent to haemoglobin breakdown.

Concerning the ovary follicular count, no difference in follicular population distribution was noted in the top-dose group compared with the control group (Table 12). From the vaginal smear analysis, according to the study director, estrous cycle stages were equally distributed throughout all groups (raw data are missing, however).

The NOAEL was 500 ppm, equal to 27.9 mg/kg bw per day, based on body weight and body weight gain at 3000 ppm, equal to 202.0 mg/kg bw per day (Wahle, 2000).

Dogs

In a range-finding/palatability study, two female dogs (Beagle purebred) per dose were fed a diet of clothianidin (purity 95.2%; batch No. 30037120) at dose levels of 0 or 3000 ppm on days 1–3, 4000 ppm on days 4–8 and 5000 ppm on days 9–11, over a period of 11 days in total. Observations and measurements were limited to clinical signs, body weights, feed consumption and test article intake. The study was not conducted in compliance with GLP. The animals were not killed and were returned to stock.

Table 12. Oral 90-day toxicity of clothianidin in rats: selected clinical and histopathological findings

	% change relative to controls							
	Dietary concentration (ppm)							
	0		150		500		3000	
	Males	Females	Males	Females	Males	Females	Males	Females
Body weight:								
- day 91	—	—	—	—	—	—	11*	12*
- day 140 (recovery)	—	—	—	—	—	—	5	9.4*
Body weight gain:								
- day 91	—	—	—	—	—	—	23**	—
- day 140 (recovery)	—	—	—	—	—	—	—	—
Clinical signs								
Red nose discharge[a]:								
- day 90	—	0	—	0	—	1 (3)	—	2 (1, 6)
- day 139	—	0	—	—	—	—	—	1 (1)
Haematology								
Red blood cells	—	—	—	—	—	—	5.6[§]	—
Haemoglobin	—	—	—	—	—	—	6.1[§]	—
Haematocrit	—	—	—	—	—	—	4.9[§]	—
Prothrombin time	—	—	—	—	—	—	—	9.5*
Clinical chemistry								
Inorganic phosphorus	—	—	—	—	—	—	11*	—
CYP content	—	—	—	—	—	—	29	43
N-demethylase (ANDM)	—	—	—	—	—	—	32*	—
O-demethylase (NODM)	—	—	—	—	—	—	50*	—
EROD:								
- day 90	—	—	—	—	—	—	127[†]	46
- day 139 (recovery)	—	—	—	—	—	—	25	50
PROD:								
- day 90	—	—	—	—	—	—	211[†]	36
- day 139 (recovery)	—	—	—	—	—	—	36	48
Histopathology								
Spleen pigmentation[a]:								
- day 89	6	14	6	13	8	14	13*	15
- day 139 (recovery)	12	—	—	—	—	—	11	—
Ovary follicle counts (left/right):								
- “non-anthral” follicles	—	27.3/27.5	—	—	—	—	—	24.1/28.9
- “anthral” follicles	—	12.7/11.3	—	—	—	—	—	9.9/11.3
- corpora lutea	—	20.6/18.8	—	—	—	—	—	19.4/19.0

From Wahle (2000)

* $P < 0.05$ or ** $P < 0.01$ (ANOVA + Dunnett’s); [§] $P < 0.05$ (Kruskal-Wallis + Mann-Whitney); [†] $P < 0.05$ (Student’s *t*-test)

[a] Occurrence per 15 animals; number of days with finding in parentheses.

There were no deaths and no relevant clinical signs. The mean daily feed consumption was decreased by 35% (3000 ppm period), 57% (4000 ppm period) and 63% (5000 ppm period), compared with that of the control dogs. The average daily consumption of active ingredient at days 1–3, days 4–8 and days 9–11 was, respectively, 68, 60 and 66 mg/kg bw per day (dog 1) and 34, 42 and 38 mg/kg bw per day (dog 2). No differences in body weights were observed between treated and control groups. For the period days 1–9, a treatment-related body weight change was observed in the treated group (−0.85 kg) compared with the controls (−0.25 kg). During days 9–11, the difference was −0.25 kg, compared with −0.10 kg.

Within the time frame of this study, it was not possible to determine whether improved acceptance of diet concentrations up to 5000 ppm would occur over longer periods of time, as in a longer-term study (Moore, 1998).

In a 30-day dietary study, three dogs (Beagle purebred) of each sex per dose were offered clothianidin (purity 95.2%; batch No. 30037120) at dose levels of 0, 1250, 2500 or 5000 ppm in diet, which are equivalent to 0, 36.3, 35.8 and 62.4 mg/kg bw per day for males and 0, 35.6, 52.3 and 57.4 mg/kg bw per day for females (data calculated for days 1–15 of the testing period). Accuracy, homogeneity and stability of the diet were confirmed by chemical analysis. The protocol was partly in compliance with test method B.7 of European Commission directive 92/69/EEC. Limitations included compromised interpretation of certain haematological, clinical chemistry and body weight/organ weight results due to high mortality at the intermediate and high doses. The achieved test article intake for the 2500 ppm (males) and 5000 ppm treatment groups was lower than expected; 2500 ppm is considered the MTD. However, dose–response evaluation is impeded for all effects at termination.

Because of the exclusion of some 2500 and 5000 ppm group data points in the statistical evaluation (missing samples in unscheduled termination, statistics only when $n = 3$), non-statistically significant changes as well as changes at the interim sample time (week 3) are also reported in Table 13 where appropriate.

At the top dose, one male died (day 18), and all remaining dogs in this group were terminated early (days 19–22) due to declining health. At 2500 ppm, one male and one female were terminated on day 32. Other animals survived to scheduled termination (day 33). At the top dose, commencing from approximately day 8, prostration, hunched posture, dyspnoea, emaciation, moderate dehydration, no/few/mucoid/discoloured faeces, salivation or hyperthermia were observed in the decedents. At 2500 ppm, commencing from day 18, animals ultimately terminated early exhibited prostration, hypoactivity, pale gums, hypothermia, moderate dehydration, polypnoea, salivation, chromorhinorrhoea/dacryorrhoea, discoloured/mucoid faeces and emaciation. Other animals from this dosing group showed clinical signs occasionally.

Feed consumption was decreased in males at 2500 ppm on weeks 2, 3 and 4 and in females at 5000 ppm on weeks 3, 4 and 5. At 1250 ppm, lower values of both feed consumption and body weight gain were noted in both sexes, but without attaining statistical significance at termination. Owing to the low feed consumption, test article intakes were not meaningfully different between males offered 2500 and 5000 ppm or females offered 1250 and 2500 ppm. Body weight gain was impaired at 2500 ppm, whereas dogs at 5000 ppm lost weight.

Decreased erythrocytic mass, white blood cell count and segmented neutrophils (which may additionally be explained by an observed pulmonary bacterial infection in mid- and high-dose animals) were correlated with the observed depletion of lymphoid cells in bone marrow, lymph node, thymus and spleen (Table 14).

An increase in alkaline phosphatase and cholesterol levels was in general not accompanied by hepatic histological findings, although enlarged liver volume was detected on occasion. Changes

Table 13. Oral 28-day toxicity of clothianidin in dogs: effects on body weight and feed consumption

	Dietary concentration (ppm)							
	0		1250		2500		5000	
	M	F	M	F	M	F	M	F
Active ingredient intake (mg/kg bw per day)	0	0	36.3	35.6	35.8	52.3	62.4	57.4
Mortality (/3)	0	0	0	0	1	1	3	3
Feed consumption (% of control):								
- weeks 1–2	—	—	↓2.8%	↓21%*	↓49%**	↓26%*	↓59%**	↓57%**
- weeks 1–3	—	—	↓3.6%	↓19%	↓50%**	↓20%	—	—
- weeks 1–4	—	—	↓4.5%	↓18%	↓47%*	↓18%	—	—
Body weight gain (kg):								
- weeks 1–3	0.5	0.7	0.3	0.2	−0.4*	0.0*	−0.8**	−0.8**
- weeks 1–5[a]	0.8	0.7	0.6	0.2	−0.7	0.1	—	—
Body weight (week 4) (% of control)	—	—	—	—	—	—	↓28%	↓12%

From Moore (2000)
F, female; M, male
* $P < 0.05$; ** $P < 0.01$
[a] Data analysed following rank transformation.

in other parameters were most likely explained by the severe nutritional deprivation and starvation status of treated animals.

No meaningful changes were detected in most organ weights of tested animals. Enlarged liver was noted in females at 2500 ppm and in males at 5000 ppm. At the top dose, increased liver weight of the surviving males occurred in association with cytoplasmic vacuolization, possibly suggestive of severe nutritional deprivation, although the observed alkaline phosphatase increase points towards a hepatotoxic effect.

The observed enlargement of kidney was not supported by histopathological findings. One caecum infarct was observed in females at the top dose.

Mottling of the lung, sometimes accompanied by signs of congestion and/or haemorrhage and alveolar oedema, was explained by a bacterial infection. The presence of dark mucosa was observed in different parts of the gastrointestinal tract (ileum, jejunum, duodenum, colon, caecum) in males at the top dose and in females at 2500 ppm, but was not clearly associated with histopathological findings. One caecum infarct was observed among males at 5000 ppm. At the middle and high doses, histological changes were observed in several immunocompetent or haematopoietic organs, such as bone marrow (hypocellularity and congestion/haemorrage), thymus (lymphoid depletion), spleen, ileum and mesenteric lymph node (lymphoid depletion). In the same animals, duodenum showed crypt gland dilatation and/or gland epithelial necrosis (necrosis particularly in females). Colon gland dilatation was also observed in one top-dose male.

It was concluded that the dietary level of 2500 ppm (equal to 36 mg/kg bw per day in males) exceeded the maximum feasible dose for a longer-term study.

The dietary concentration of 1250 ppm, also equal to an intake of 36 mg/kg bw per day in males, was concluded to be a NOAEL, based on mortalities, clinical signs and body weight decreases at 5000 ppm, equal to 62.4 mg/kg bw per day (Moore, 2000).

In a 90-day study, four Beagle dogs of each sex per dose were fed a diet of clothianidin (purity 95.2%; batch No. 30037120) at dietary concentrations of 0**,** 325, 650, 1500 and 2250 ppm for 90 days.

Table 14. Oral 28-day toxicity of clothianidin in dogs: haematology

	% change relative to controls							
	Dietary concentration (ppm)							
	0		1250		2500		5000	
	M	F	M	F	M	F	M	F
Red blood cells:								
- week 3	—	—	—	—	—	—	↓5.7	—
- week 4	—	—	—	—	—	—	↓8.6	—
Haemoglobin:								
- week 3	—	—	—	—	—	↓15	↓4.7	↓4.9
- week 4	—	—	—	—	—	↓11	↓9.2	↓3.6
Haematocrit:								
- week 3	—	—	—	—	—	↓16	↓6.5	↓6.4
- week 4	—	—	—	—	—	↓11	↓8.2	↓3.4
Platelets:								
- week 3	—	—	—	—	↓5	↓5	↓46	↓52
- week 4	—	—	—	—	—	—	—	↓48
White blood cells:								
- week 3	—	—	—	—	↓35	↓45	↓56	↓73
- week 4	—	—	—	—	↓47	↓31	↓31	↓77
Segmented neutrophils (absolute counts):								
- week 3	—	—	—	—	↓41	↓54	↓60	↓91
- week 4	—	—	—	—	↓58	↓25	↓37	↓93
Lymphocytes:								
- week 3	—	—	—	—	↓22	↓20	↓53	↓32
- week 4	—	—	—	—	↓21	↓26	↓17	↓44

From Moore (2000)
F, female; M, male

Achieved doses were 0, 9.2, 19.3, 40.9 and 58.2 mg/kg bw per day for males and 0, 9.6, 21.2, 42.1 and 61.8 mg/kg bw per day for females. Accuracy, homogeneity and stability of the administered diet were confirmed by chemical analysis. The protocol was partly in compliance with test method B.27 of European Commission directive 92/69/EEC and OECD Guideline No. 409 (Subchronic Oral Toxicity Non-rodent 90-day; 12 May 1981).

There were no deaths and no adverse ophthalmological findings. Emaciation was the main clinical finding in dogs of both sexes at the high-middle and high doses. In males at 1500 ppm and above, one of four animals showed excessive salivation. In females at 650 ppm and above, one of four animals showed food-containing emesis. Neither feed consumption nor body weight was significantly affected, but body weight change from week 1 to week 13 was slightly decreased among top-dose males.

In males, slight decreases in haematocrit at the top dose and in haematocrit, haemoglobin and red blood cells at the high-middle dose were noted. At 1500 ppm (high-middle dose), the slight anaemia was exacerbated by the presence of one male animal showing interdigital cysts (chronic bleeding), whereas platelet decrease was consistent with observed enlarged spleen; in the same animal, chronic inflammation in several organs (lung, kidney and liver) was seen in parallel with neutropenia,

whereas thymic atrophy and necrosis in lymph nodes explained lymphopenia on weeks 9–13 (other males not affected). In the top-dose females, white blood cell decrease was observed at weeks 5, 9 and 13, attaining significance only on week 5. In the females at the top dose, there was a trend towards decreased white blood cells, neutrophils and lymphocytes during weeks 5–13. Because one top-dose female animal showed marked leukocytosis, neutrophilia and lymphocytosis on weeks 5 and 9, differences were not statistically significant (the animal was treated with antibiotics during weeks 5–6 for lymphadenitis; the temporary increase is consistent with the acute inflammation status and was confirmed by a diagnostic sampling at week 8). Excluding these outliers revealed a mean drop in observed values (see footnote b in Table 15). There were no relevant findings in the organ weights or gross necropsy. Lymphoid necrosis was observed in ileum, colon, caecum and rectum in a single female at the top dose. Lymphoid hyperplasia was observed in males at 650 ppm and above and in females at 1500 ppm and above. However, these findings were consistent with commonly occurring lesions of spontaneous etiology, and none was considered to be treatment related.

The report indicated that the erythropoietic system was the probable target organ in this study, despite an absence of histological change in spleen and bone marrow.

The NOAEL was 650 ppm, equal to 19.3 mg/kg bw per day, based on clinical signs, red blood cell and white blood cell parameters and lymphoid hyperplasia at 1500 ppm, equal to 40.9 mg/kg bw per day) (Bernier, 2000a).

In a 1-year study, four Beagle dogs of each sex per dose were fed a diet of clothianidin (purity 95.2%; batch No. 30037120) at dose levels of 0, 325, 650, 1500 or 2000 ppm, which convert to dose levels of 0, 7.8, 16.6, 36.3 and 46.4 mg/kg bw per day in males and 0, 8.5, 15.0, 40.1 and 52.9 mg/kg bw per day in females. Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. The protocol was in compliance with test method B.30 of European Commission directive 92/69/EEC and OECD Guideline No. 452 (Chronic Toxicity Studies; 12 May 1981).

There were no deaths, relevant clinical signs or ophthalmological findings. Significant body weight changes were seen in week 1 of treatment in males at the top dose. Slight, non-statistically significant reductions in body weight and growth rate were observed in females at the top dose at study termination. Slight decreases in red blood cell mass indices, haematocrit, haemoglobin and red blood cell count were observed in the females at the top dose. These signs of slight anaemia may be related to the observed decreased body weight gain. In the same group, transient (week 5, week 39) decreases in total leukocytes, essentially caused by neutropenia, were observed. On week 5, these changes were accompanied by decreased monocyte and eosinophil counts, whereas at week 13, only monocytaemia was observed. The lymphopenia observed in the males at 650 ppm and above on week 5 was neither time nor dose related (pre-study sampling showed slightly lower values, whereas study control was relatively high).

A slight increase in cholesterol level was observed in males at the top dose. A decrease in ALT activity in both males and females at 650 ppm and above was noted. There was a clear dose–effect relationship and a clear time–effect relationship at the high-middle and high doses. These findings were not associated with any gross or microscopic hepatic change. There were no relevant findings from the urinalysis.

The increased adrenal weight in the females at the top dose, attaining statistical significance only when expressed relative to body weight, was considered treatment related (Table 16). A slight dose-related effect was apparent. Other observed changes were not statistically significant or dose related.

There were no relevant findings in the gross pathology. Notable occasional findings of inflammation (lung) and of thymus lymphoid depletion (males: 1, 0, 0, 0, 1; females: 0, 1, 0, 0, 1) were not dose related or also occurred in the control group. One animal per dose showed minimal prostate

Table 15. Oral 90-day toxicity of clothianidin in dogs: selected clinical and histopathological findings

	% relative to controls									
	Dietary concentration (ppm)									
	0		325		650		1500		2250	
	M	F	M	F	M	F	M	F	M	F
Clinical signs (/4)										
Emaciation	0	1	0	0	0	0	2	1	2	3
Ptyalism	0	0	0	0	0	0	1	0	1	0
Body weight gain[a]	1.1	1.0	2.1*	0.9	2.5*	1.6	0.5	0.9	0.3*	0.7
Haematology										
Haematocrit	—	—	—	—	—	—	↓13	↓12**	↓6.5*	—
Haemoglobin	—	—	—	—	—	—	↓13	↓11**	↓4.2	↓2.8
Red blood cells	—	—	—	—	—	—	↓14	↓9.2*	↓5.2	—
MCHC	—	—	—	—	—	—	—	—	↑2.7*	—
Platelets:										
- week 5	—	—	—	—	—	—	↓6	↓25	↓3	↓40*
- week 9	—	—	—	—	—	—	↓14	↓13	↓8	↓24
- week 13	—	—	—	—	—	—	↓15	↓15	↓15	↓32
White blood cells:										
- week 5	—	—	—	—	—	—	—	—	↓40*	↓61[b]
- week 9	—	—	—	—	—	—	—	—	↓24	↓33[b]
- week 13	—	—	—	—	—	—	—	—	↓30	↓52*
Neutrophils:										
- week 5	—	—	—	—	—	—	—	—	↓38	↓69[b]
- week 9	—	—	—	—	—	—	—	—	↓24	↓30[b]
- week 13	—	—	—	—	—	—	—	—	↓23	↓61*
Lymphocytes:										
- week 5	—	—	—	—	—	—	—	—	↓42**	↓32[b]
- week 9	—	—	—	—	—	—	—	—	↓23	↓30[b]
- week 13	—	—	—	—	—	—	—	—	↓39	↓30
Monocytes:										
- week 13	—	—	—	—	—	—	—	—	—	↓75**
Clinical chemistry										
ALT:										
- week 5	—	—	—	—	—	—	↓26	↓53*	↓55*	↓72**
- week 13	—	—	—	—	—	—	↓38	↓55*	↓38	↓65**
Protein	—	—	—	—	—	—	—	↓8.5	—	↓17*
Albumin:										
- week 5	—	—	—	—	—	—	—	—	—	↓13*
- week 13	—	—	—	—	—	—	—	↓11*	—	↓7.9*
Histopathology (/4)										
Jejunum: lymphoid hyperplasia	0	0	0	0	1	0	1	1	2	1

From Bernier (2000a)

F, female; M, male; * $P < 0.05$; ** $P < 0.01$ (Dunnett's *t*-test)

[a] Body weight gain in kilograms per dog during weeks 0–13.

[b] Values of 3/4 animals, excluding dog with antibiotic therapy at weeks 5–6 following lymphadenitis.

Table 16. Oral 1-year toxicity of clothianidin in dogs: selected findings

	% of control values									
	Dietary concentration (ppm)									
	0		325		650		1500		2000	
	M	F	M	F	M	F	M	F	M	F
Body weight	—	—	—	—	—	—	—	—	—	↓5.5
Body weight gain[a]	1.3	1.5	1.7	1.8	3.3	1.9	1.8	1.7	1.2	0.8
Haematology										
Haematocrit	—	—	—	—	—	—	—	—	—	↓18*
Haemoglobin	—	—	—	—	—	—	—	—	—	↓17*
Red blood cells	—	—	—	—	—	—	—	—	—	↓15*
White blood cells:										
- week 5	—	—	—	—	—	—	—	—	↓34	↓46*
- week 39	—	—	—	—	—	—	—	—	↓6	↓27*
- week 52	—	—	—	—	—	—	—	—	↓9	↓22
Neutrophils:										
- week 5	—	—	—	—	—	—	—	—	↓33	↓59
- week 26	—	—	—	—	—	—	—	—	↓23	↓31
- week 39	—	—	—	—	—	—	—	—	—	↓40*
- week 52	—	—	—	—	—	—	—	—	↓4	↓27
Lymphocytes:										
- week 5	—	—	—	—	↓24*	—	↓43*	—	↓36*	—
Monocytes:										
- week 5	—	—	—	—	—	—	—	—	—	↓83*
- week 13	—	—	—	—	—	—	—	—	—	↓67*
Eosinophils:										
- week 5	—	—	—	—	—	—	—	—	—	↓25*
Clinical chemistry										
Cholesterol:										
- week 13	—	—	—	—	—	—	—	—	↑37*	—
ALT:										
- week 13	—	—	—	—	—	↓32*	↓36*	↓45*	↓60*	↓63*
- week 26	—	—	—	—	↓29	—	↓44*	↓54*	↓63*	↓68*
- week 39	—	—	—	—	—	—	↓42*	↓51*	↓63*	↓71*
- week 52	—	—	—	—	—	—	↓38*	↓49*	↓55*	↓73*
Organ weight										
Adrenal:										
- absolute	—	—	—	—	—	—	—	—	—	↑27
- relative	—	—	—	—	—	—	—	—	—	↑45*

From Bernier (2000b)
F, female; M, male; * $P < 0.05$ (Dunnett's *t*-test)
[a] Body weight gain in kilograms per dog during weeks 0–52.

inflammation at 650 ppm and higher. Testicular atrophy and seminiferous tubular vacuolization (moderate) were observed in one animal at the top dose. However, in the absence of other animals in this treatment group with similar findings, the relationship with test substance administration is doubtful.

The NOAEL was 1500 ppm, equal to 36.3 mg/kg bw per day, based on decreased white blood cells and decreased neutrophils. The LOAEL was 2000 ppm, equal to 46.4 mg/kg bw per day (Bernier, 2000b).

The overall NOAEL for subchronic toxicity in the dog was 36.3 mg/kg bw per day.

(b) Dermal application

In a 28-day dermal toxicity study, 10 rats (Crl:CD®(SD)IGS BR) of each sex per dose were exposed to clothianidin (purity 95.2%; batch No. 30037120) at a dose level of 0, 100, 300 or 1000 mg/kg bw per day for 29 days (6 hours per day, 7 days per week). The protocol was in compliance with test method B.9 of European Commission directive 92/69/EEC and OECD Guideline No. 410 (Repeated Dose Dermal Toxicity 21/28 Day Study; 12 May 1981).

One female at the low dose (cause of death not evident) and one at the highest dose (collar caught in cage) died on day 15. There were no relevant clinical signs during the study; no evidence of irritancy or other changes at the application site were found. Male animals treated at 1000 mg/kg bw per day showed a statistically significant reduction in weight gain during week 1 (−61%), but thereafter showed weight gains comparable to those of all other groups. At the end of treatment, there was a slight decrease in body weight gain, by −21%, reflecting the change in week 1. Therefore, the initial lower weight gain is considered incidental to treatment.

Slight increases in red blood cells, haemoglobin, haematocrit and prothrombin time were observed in both sexes at the middle dose, but not at the top dose, and are considered not treatment related. There were no relevant findings in clinical chemistry, necropsy, organ weights or histopathology. The systemic NOAEL is 1000 mg/kg bw per day (Weiler, 2000).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a carcinogenicity study, 50 mice (Crl:CD-1®(ICR) BR-VAF/Plus®) of each sex per dose were fed a diet containing clothianidin (purity 95.2–95.5%; batch No. 30037120) at initial dose levels of 0, 100 (low dose), 350 (low-middle dose), 700 (high-middle dose) or 1250 (high dose) ppm for 18 months. However, in order to ensure exposure to an MTD, the high-middle dose was increased so that this group eventually received the highest dose. In order to achieve this, several adjustments to the high-middle dose were necessary, as follows: weeks 5–10, from 700 to 2000 ppm; weeks 11–34, from 2000 to 2500 ppm; weeks 35–78, males, from 2500 to 2000 ppm, and females, from 2500 to 1800 ppm. Thus, the administered dietary levels are cited in this report as 0, 100 (low dose), 350 (low-middle dose), 1250 (high-middle dose), 2000 ppm (high dose, males) and 1800 ppm (high dose, females). Accuracy, homogeneity and stability of administered diets were confirmed by chemical analysis. Achieved test article intakes were 0, 13.5, 47.2, 171.4 and 251.9 mg/kg bw per day for males and 0, 17.0, 65.1, 215.9 and 281.1 mg/kg bw per day for females (the intake for the highest dose was calculated on the basis of the final dose level, i.e. weeks 35–78). Blood smears were prepared for all doses at 53 weeks and evaluated in control and high-dose groups only. The protocol was partly in compliance with test method B.32 of European Commission directive 92/69/EEC and OECD Guideline No. 451 (Carcinogenicity Studies; 12 May 1981).

An increase in mortality was noted in females at 350 ppm and at the top dose. In the pathology report, the absence of microscopic findings explaining the preterminal death of the top-dose females

was partly explained by "accidental asphyxiation with CO_2 following blood collection procedure" (17/27 cases). For the high-middle-dose females, the main cause of death was malignant lymphoma and amyloidosis. In males, the main cause of death was amyloidosis. Noticeable was the early onset of mortality in the top-dose females, with a steep increase, starting on week 20. Mortality of 25% was attained between 30 and 40 weeks at the top dose, whereas for the high-middle-dose females, this percentage was attained between 70 and 80 weeks. It is probable that mortality in this dose group was consequent to the increased concentration of test article in the diet from 2000 to 2500 ppm during weeks 11–34.

Both body weights and body weight gains were significantly decreased at 1250 ppm and above in males and females. At 1250 ppm, the changes were observed throughout a major part of the study in the females and between weeks 12 and 30 in the males. Feed consumption was decreased only at the top dose, mainly during weeks 10–45 (males) and 11–33 (females) and on occasions thereafter. Average feed efficiency (proportion body weight gain/feed consumption) was altered at the top dose in both sexes, when calculated for the period between weeks 5 and 33. On the basis of the observed rebound of feed efficiencies in females and to a lesser extent in males, it was concluded that the administration of 2500 (males) or 2000 (females) ppm clearly exceeded the MTD.

Dose-dependent vocalization was observed in both sexes at 1250 ppm and above. A slight increase in neutrophil fraction (and concomitant decrease in lymphocyte fraction) was observed on the smears of animals at the top dose at scheduled termination. The finding may be correlated with the stressed and underfed status of this group.

Significant organ weight reductions were recorded in the heart and kidneys of males given 350, 1250 or 2000 ppm, and this trend extended to the kidneys of males given 100 ppm. However, in the kidney, no related histological findings were observed. In heart, weight changes were minimal, but histopathological findings (e.g. myocardial degeneration) were observed. The organ weight alterations observed at the higher dose levels may have been, at least in part, secondary to the observed decreases in body weight (Table 17).

There were no significant gross pathological findings. Histologically, in both sexes, an increased incidence of hepatocellular hypertrophy was detected in animals treated with 1250 ppm and above.

In females, an increase in fibromuscular hyperplasia of the cervix in the 1250 and 1800 ppm groups was observed both at scheduled termination and on unscheduled death. Although non-neoplastic uterine and cervical lesions are common in nulliparous ageing females, an association with clothianidin administration cannot be excluded, in view of the apparent dose–response relationship. Moreover, there was no correlation between the incidence of hyperplasia and mortality. In the ovary, a greater incidence of supernumerary corpora lutea was observed in the top-dose animals.

Other histopathological findings included an increased incidence of myocardial heart degeneration, non-glandular stomach hyperkeratosis, adrenal cortex congestion and lung congestion (Table 18).

Clothianidin is not carcinogenic in these experimental conditions.

The toxicity NOAEL was 350 ppm, equal to 47.2 mg/kg bw per day, and the LOAEL was 1250 ppm, equal to 171.4 mg/kg bw per day, based on body weight effects, clinical signs, and heart and cervical lesions (Biegel, 2000b).

Rats

In a combined chronic toxicity/carcinogenicity study in the rat, 80 rats (Crl:CD(SD)IGS BR-VAF/Plus®) of each sex per dose were fed diets containing clothianidin (purity 95.2–95.5%; batch No. 30037120) at dietary concentrations of 0, 150 (low dose), 500 (low-middle dose), 1500 (high-middle dose) and 3000 (high dose) ppm for 103/104 weeks. Analytical verification revealed that dose levels were 89–109% of nominal values. Compound stability for 4 weeks in the diet at ambient

Table 17. Carcinogenicity study of clothianidin in mice (78 weeks): clinical findings

	Dietary concentration (ppm)									
	0		100		350		1250		2000	1800
	M	F	M	F	M	F	M	F	M	F
Mortality (%)	32	20	16	14	40	42*	20	24	32	54**
Feed consumption[a]	—	—	—	—	—	—	—	—	↓8–22%*,** (weeks 10–45)	↓16–24%*,** (weeks 11–33)
Body weight[a]	—	—	—	—	—	—	↓5%*,** (weeks 12–30)	↓5–13%*,** (weeks 13, 14, 22–78)	↓5–18%** (weeks 6–78)	↓4–18%** (weeks 10–78)
Body weight gain[a]	—	—	↓6.8%	↓1.6%	↓9.9%	↓13.5%	↓9%	↓26%**	↓24%**	↓35%**
Clinical signs[b]										
Vocalizations	0	0	1	0	2	3	22	46	45	42
Haematology (differential count)[a]										
Neutrophils	—	—	—	—	—	—	—	—	—	↑29%*
Lymphocytes	—	—	—	—	—	—	—	—	—	↓21%*
Organ weight[a]										
Heart:										
- absolute	—	—	↓5.9%	↑3%	↓9.0%*	↑5.3%	↓9.3%**	↓4%	↓13%**	↓10.1%
- relative	—	—	↓4.2%	↑4.9%	↓7.1%	↑11.5%	↓8.6%	↑9%	↓4.2%	↑4.5%
Kidney:										
- absolute	—	—	↓8.7%*	↓2%	↓8.9%**	↑0.6%	↓11%**	↓11%	↓17%**	↓20%*
- relative	—	—	↓6.8%	↑1.3%	↓6.8%	↑7.1%	↓10%*	↑1.7%	↓8.8%*	↓5.6%
Liver:										
- absolute	—	—	↓3%	↑0.3%	↓0.2%	↓2.8%	↓2.5%	↓16%*	↓0.4%	↓17.5%*
- relative	—	—	↑2.3%	↑2.4%	↑2.6%	↑2.4%	↓1.4%	↓4%	↑5.4%	↓3%

From Biegel (2000b)

F, female; M, male; * $P < 0.05$, ** $P < 0.01$ (mortality: Cox-Tarone test; body/organ weights: one-way analysis of variance [ANOVA]).

[a] % relative to control.

[b] Incidences in 50 animals (observations noted from weeks 7 to 80 in middle-high-dose and high-dose animals and during week 69 for the other dose level animals.

temperature and compound homogeneity proved to be satisfactory. Achieved test article intakes were 0, 8.1, 27.4, 82.0 and 157 mg/kg bw per day for males and 0, 9.7, 32.5, 97.8 and 193 mg/kg bw per day for females. Additional to the daily standard clinical observations, 10 rats of each sex per dose were randomly selected during weeks 66–67 and subjected to a functional observational battery (FOB). Animals with physical abnormalities, possibly precluding proper assessment of functional neurological abnormalities, were excluded from the test and replaced by suitable animals. Twenty rats of each sex per dose were scheduled for interim termination (53 weeks). The protocol was partly in compliance with test method B.33 of European Commission directive 92/69/EEC and OECD Guideline No. 453 (Combined Chronic Toxicity/Carcinogenicity Studies; 12 May 1981). Limitations included low survival (below 50%) at 88 weeks in the control, 150 ppm and 500 ppm groups. However, survival was in line with OECD Guideline No. 453 at 1500 and 3000 ppm up to week 96. In the absence of test article–related neoplasms at the top dose, the deviation was unlikely to have altered the integrity of the study.

Table 18. Carcinogenicity study of clothianidin in mice (78 weeks): histopathological findings[a]

	Incidence in 50 animals									
	Dietary concentration (ppm)									
	0		100		350		1250		1800	2000
	M	F	M	F	M	F	M	F	M	F
Liver										
Hepatocellular hypertrophy§§	17	5	34**	5	15	3	27*	11**	40**	15**
Cervix										
Fibromuscular hyperplasia§§	—	14	—	12	—	12	—	27**	—	29**
Heart										
Myocardial degeneration	8	6	10	10	9	9	20**	5	8	1
Lung										
Congestion	2	0	0	0	1	2	2	0	3	13**
Adrenal cortex										
Congestion§§	0	1	0	0	1	3	0	0	0	11**
Non-glandular stomach										
Hyperkeratosis	3	3	8	4	7	12*	8	8	5	9
Ovary										
Supernumerary corpora lutea	—	0	—	0	—	0	—	0	—	4

From Biegel (2000b)
F, female; M, male; * $P < 0.05$ and ** $P < 0.01$ (Fisher-Irwin exact test for groupwise comparisons); § $P < 0.05$ and §§ $P < 0.01$ (Cochran-Armitage test for trends)

The survival rate of top-dose animals was better than that of controls. Among unscheduled deaths, the cause of death was most commonly the presence of pituitary neoplasms, followed by mammary gland carcinoma. The incidence of neoplasms was not related to dosage. Owing to high mortality in rats from the lower-dose groups (controls, 150 ppm and 500 ppm), females were terminated at week 103, whereas males continued on study for the full 104 weeks.

There were no relevant ophthalmological findings. Thin appearance was observed in a few females at the top dose, in the terminal group as well as among the unscheduled deaths, but not in the interim termination group.

There were no relevant findings in the FOB. Decreases in feed consumption were observed at 500 ppm and above in females and at 1500 ppm and above in both sexes. At 1500 ppm and above, decreases in both body weight and body weight gain were observed. These decreases were of greater magnitude in males than in females and essentially confined to weeks 1–53. Feed efficiency was not affected.

Slight increases in red blood cell parameters were observed in males at the top dose on most interim bleeding times, but not on scheduled termination. Increased inorganic phosphorus levels were consistently observed in males at the top dose and were associated with the observed renal effects. The observed changes in creatinine (+13%), calcium (+4.7%) and potassium (+13%) levels in the top-dose females were confined to terminal animals.

Only at the interim sampling time of week 13 was a decrease in both urine volume and specific gravity observed in the females at the top dose; the decreases at intermediate doses were dose related. No change was observed on later sampling times.

On termination, the statistically significant increases in relative organ weights (adrenal, brain, heart, kidney, lung) in the females at the top dose were attributed to the observed body weight decrease

(of about 20%, $P < 0.01$). The increase in relative liver weight in females at 1500 and 3000 ppm on interim termination and at the top dose on final termination was supported by histology. Absolute and relative uterine weights were slightly increased at the top dose at interim termination, but not at final termination (Table 19).

In males with both scheduled termination and unscheduled deaths, granular material or calculi were observed in the renal pelvis at 1500 ppm and above. The degree of mineralization was dose dependent. In the interim termination group, the same observation was made at the same dose levels, but only in the males (unilateral mineralization). Increases in erosion/ulceration (males) and dark foci/areas (both sexes) in the glandular stomach were observed at the top dose. Dark foci/areas were also found with greater incidences at this dose in both sexes of the interim termination group. The incidence of mottled livers was increased in the males at the top dose and was related to various subtle histopathological findings. In the interim group, mottling was restricted to males fed 1500 and 3000 ppm, with the histological correlate of periportal hepatocellular vacuolization. A slight increase in diffusely red/fluid-filled mass within the uteri was observed at the top dose. A slight increase in mottled lungs/light foci/areas was observed in the females at the top dose, which was histologically related to an increase in cases of chronic inflammation, in both unscheduled deaths and the terminal group.

At interim termination, stifle joint (hindlimb) articular cartilage cavitation was slightly increased at the top dose, mainly in males, but also in females (males: 1, 3, 2, 2, 6; females: 2, 3, 3, 4, 5), and observed in association with epiphysial cysts/fibrosis. The finding was unremarkable at termination. In addition, the changes in stifle joints of males given test material did not increase in incidence and severity in a dose-related manner, which could be expected if the changes were test material related. Consequently, the increase in slight joint changes seen at the interim termination in males was considered an incidental finding unrelated to administration of test material.

At the top dose, males exhibited renal pelvic mineralization, directly associated with secondary transitional cell hyperplasia and pelvic angiectasis. In females, both tubular ectasia and pelvic angiectasis were significantly increased in incidence and severity. These effects were associated with urinalysis findings in females.

The emergence of eosinophilic hepatocellular foci at the top dose in both sexes was the most prominent non-neoplastic finding. The lymphohistiocytic infiltrate in the liver at both high-middle and high doses in the females was of uncertain biological significance. Other observed non-neoplastic findings, such as mixed-cell foci, telangiectasis (hepatic sinusoid dilatation), centrilobular hypertrophy and biliary cyst, were considered of unclear biological significance because of lack of a dose–effect relationship or minimal occurrence. Hepatocellular carcinomas in males were found in the low-middle dose group (one at scheduled termination and two in unscheduled deaths) and at the top dose (four in unscheduled deaths). According to the pathology report and owing to the absence of any dose–effect relationship and the low incidence (only slightly exceeding laboratory historical control data and falling within the historical control level reported by other laboratories), the tumours are unlikely to be related to test substance administration.

The observed chronic inflammations in the lung of females at the top dose were most prominent in the scheduled termination group and may be explained by a generally lower health status at this stage.

The slightly increased incidence of thymus hyperplasia in females at the top dose was probably not biologically significant, because the increases were merely confined to the terminal group and may thus be attributed to the increased survival of the treated animals.

An increase in thyroid C-cell adenoma was observed in the females at 1500 and 3000 ppm, when compared with controls. Combined incidence (adenoma + carcinoma) showed an increase in females at 1500 ppm, but not at the top dose. C-cell adenomas and carcinomas represent a continuous biological spectrum, with adenomas progressing to carcinomas. Consequently, the most appropriate

Table 19. Carcinogenicity study of clothianidin in rats (males 104 weeks, females 103 weeks): selected clinical findings

	% relative to control[a]									
	Dietary concentration (ppm)									
	0		150		500		1500		3000	
	M	F	M	F	M	F	M	F	M	F
Mortality (%)	72	75	71	73	70	80	60	62	45**	50**
Body weight	—	—	—	—	—	↓5%	↓5%	↓5–10%	↓10%	↓15–20%
Body weight gain:										
- weeks 1–13	—	—	—	—	—	↓5%	↓5%	↓12%**	↓12%**	↓25%**
- weeks 1–50	—	—	—	—	—	—	↓6%	↓13%**	↓13%**	↓28%**
- weeks 1–102	—	—	—	—	—	—	—	↓5%	↓5%	↓30%**
Haematology										
Red blood cells:										
- week 13	—	—	—	—	—	—	—	—	↑3.5%	—
- week 27	—	—	—	—	—	—	—	—	↑5.9%	—
- week 53	—	—	—	—	—	—	—	—	↑7.6%	—
- week 79	—	—	—	—	—	—	—	—	↑11%*	—
- week 104/103	—	—	—	—	—	—	—	—	—	—
Haemoglobin:										
- week 13	—	—	—	—	—	—	—	—	↑6.3%*	—
- week 27	—	—	—	—	—	—	—	—	↑6.2%*	—
- week 53	—	—	—	—	—	—	—	—	↑4.7%	—
- week 79	—	—	—	—	—	—	—	—	↑7.8%*	—
- week 104/103	—	—	—	—	—	—	—	—	—	—
Haematocrit:										
- week 13	—	—	—	—	—	—	—	—	↑4.6%*	—
- week 27	—	—	—	—	—	—	—	—	↑6.2%	—
- week 53	—	—	—	—	—	—	—	—	↑5.9%	—
- week 79	—	—	—	—	—	—	—	—	↑8.4%*	—
- week 104/103	—	—	—	—	—	—	—	—	—	—
Blood chemistry										
Inorganic phosphorus:										
- week 13	—	—	—	—	—	—	—	—	↑13%*	↑10%
- week 27	—	—	—	—	—	—	—	—	↑9.3%	↑9.8%
- week 53	—	—	—	—	—	—	—	—	↑15%*	—
- week 79	—	—	—	—	—	—	—	—	↑23%*	↑14%
- week 104/103	—	—	—	—	—	—	—	—	↑20%*	↑15%
Urinalysis (week 13)										
Urine volume	—	—	—	—	—	—	—	—	—	↓60%*
Specific gravity	—	—	—	—	—	—	—	—	—	↓1.3%*
Organ weight										
Liver, relative										
- week 53	—	—	—	—	—	—	—	—	↑10%*	↑24%**
- week 104/103	—	—	—	—	—	—	—	—	—	↑23%**
Uterus, week 53:										
- absolute	—	—	—	—	—	—	—	—	—	↑21%
- relative	—	—	—	—	—	—	—	—	—	↑53%*

From Biegel (2000a)

F, female; M, male; * $P < 0.05$, ** $P < 0.01$ (mortality: Cox-Tarone test; others, except haematology/clinical chemistry: one-way ANOVA)

[a] Except where otherwise noted.

analysis is to compare the combined incidence of C-cell adenomas and carcinomas. As the combined incidence of C-cell adenomas and carcinomas in females at the top dose was not significantly greater when compared with controls and as there was no positive trend with dose, the greater incidence of C-cell neoplasms was not considered to be related to the administration of the test material.

The irritating effect of the substance in the stomach at the top dose was reflected by glandular erosion, glandular necrosis and oedema, mostly observed adjacent to the eroded focal lesions. The effect was more pronounced in the females, but the presence of haemorragic foci in the males showed that the effect was also present in this sex.

Histological uterine congestion confirmed the macroscopic observations (diffuse reddening). Suppurative inflammation was increased in the high-dose 2-year group. Statistically significant increases in the incidence of interstitial hyperplasia of the ovaries occurred in females treated at 500, 1500 and 3000 ppm. The increased incidences of this lesion are considered not to be a direct effect of treatment, but a secondary effect related to the normal ageing process, as females at 1500 and 3000 ppm survived longer than the corresponding controls. All other neoplastic changes and incidences were consistent with spontaneously occurring lesions expected in Sprague-Dawley-derived rats (Table 20).

Clothianidin was not carcinogenic in these experimental conditions. The NOAEL for chronic toxicity was 150 ppm, equal to 9.7 mg/kg bw per day, and the LOAEL was 500 ppm, equal to 32.5 mg/kg bw per day, based on decreased feed consumption and body weight effects (Biegel, 2000a).

2.4 Genotoxicity

A summary of genotoxicity studies is given in Table 21, and details of the studies are given in the text.

(a) In vitro

Clothianidin was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella typhimurium* and *Escherichia coli* WP2*uvrA*. The study satisfied test methods B.13–14 of European Commission directive 2000/32/EEC. Two tests were performed (range-finding and main). Overnight-grown bacteria (0.1 ml) from the different characterized strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537) or *E. coli* (WP2*uvrA*) were exposed to clothianidin (purity 99.7%; batch No. 12256321) dissolved in dimethyl sulfoxide (DMSO) at concentrations up to 5000 µg/plate ±S9 mix for 48 hours and in duplicate.

No toxicity to any of the bacterial strains used was exhibited. In all experiments, clothianidin provoked no increase of revertant colonies compared with negative controls. Clothianidin is not mutagenic under these experimental conditions (Otsuka, 1990a).

Clothianidin was investigated for its potential to induce mutations in an assay for gene mutation in *S. typhimurium.* The study satisfied test method B.14 of European Commission directive 2000/32/EC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 26 May 1983). Two tests were performed (plate incorporation and preincubation). Bacteria from the different characterized strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, TA1537) were exposed to clothianidin (purity 95.2%; batch No. 30037120) dissolved in DMSO at concentrations up to 5000 µg/plate (all strains ±S9 mix) and 0, 16, 32, 48, 64, 80, 96 and 112 µg/plate (TA102 +S9 mix) for 48 hours and in triplicate. In test 1, 0.1 ml of an overnight culture of cells was exposed to the active ingredient and plated, whereas in test 2, the cells were preincubated at 37 °C for 20 minutes in the presence of the active ingredient.

Table 20. Carcinogenicity study of clothianidin in rats (males 104 weeks, females 103 weeks), all intervals (interim, terminal and unscheduled deaths): selected histopathological findings

	Dietary concentration (ppm)										Trend	
	0		150		500		1500		3000			
	M	F	M	F	M	F	M	F	M	F	M	F
Number examined	*80*	*80*	*80*	*80*	*80*	*80*	*80*	*80*	*80*	*80*		
Kidney												
Pelvic mineralization	34	77	30	76	34	74	43	75	52**	79	§§	§
Pelvic angiectasis	10	9	5	12	7	5	9	7	16	21*	§	§
Transitional cell hyperplasia	33	60	30	58	29	59	29	65	51**	65	§§	—
Tubular ectasia	3	4	1	4	0	8	2	4	4	13*	—	§
Tubular epithelial vacuolization	0	1	0	3	2	3	2	1	4	4	§§	—
Liver												
Lymphohistiocytic infiltration	48	42	45	45	49	53	53	55*	54	55*	—	§§
Eosinophilic hepatocellular focus	6	9	12	14	6	6	8	17	16*	29**	§	§§
Mixed hepatocellular focus	2	4	4	0	7	4	8*	2	7	8	§	—
Telangiectasis	4	9	7	14	9	5	13*	12	11	14	§	—
Biliary cyst	1	0	0	0	1	0	2	0	4	0	§	—
Centrilobular hypertrophy	0	0	0	0	0	0	1	0	2	0	§	—
Carcinoma (secondary)	1	0	0	0	0	0	0	0	0	2[a]	—	—
Hepatocellular carcinoma[b]	0	0	0	0	3	0	0	0	4*	0	—	—
Lung												
Chronic inflammation	2	2	5	2	3	5	5	4	4	11**	—	§§
Chronic active inflammation	5	1	5	1	2	3	5	4	5	4	—	§
Thymus												
Epithelial hyperplasia	3	0	2	0	0	1	0	0	1	5*	—	§§
Thyroid												
Follicular cyst	20	17	20	24	24	18	26	25	26	30*	—	§
C-cell adenoma	8	7	13	13	17*	9	16*	17*	5	16*	—	—
C-cell carcinoma	5	2	1	2	1	1	1	1	3	1	—	—
Combined (adenoma/carcinoma)	13	9	14	14	18	10	17	18*	8	17	—	—
Glandular stomach												
Cyst	38	39	35	40	48	43	48	46	46	45	§	—
Glandular erosion	5	2	6	0	3	2	5	7	8	11**	—	§§
Glandular necrosis	5	1	2	2	1	1	2	3	2	4	—	—
Haemorrhage	0	0	0	0	0	0	1	1	5*	1	§§	—
Oedema	1	0	1	2	0	2	1	2	8*	6*	§§	§§
Uterus												
Suppurative inflammation	—	0	—	0	—	0	—	2	—	3	—	§§
Dilatation week 53	—	0	—	1	—	4	—	4	—	2	—	—
Dilatation week 105	—	0	—	1	—	4	—	5	—	3	—	§
Congestion	—	0	—	0	—	0	—	0	—	5*	—	§§
Ovary												
Interstitial gland hyperplasia	—	5	—	10	—	13*	—	20**	—	33**	—	§§

From Biegel (2000a)

F, female; M, male; * $P < 0.05$ and ** $P < 0.01$ (Fisher-Irwin exact test for groupwise comparisons); § $P < 0.05$ and §§ $P < 0.01$ (Cochran-Armitage test for trends)

[a] Two secondary tumours, found in unscheduled deaths (animal C87073, + week 54, metastasis from adrenal cortex, and animal C87096, + week 95, metastasis from uterus).

[b] Historical control of test laboratory (2/60 = 3.3%, 1036 males, 1990–1993).

Table 21. Summary of genotoxicity studies of clothianidin

Type of test	Result	Purity (%); batch No.	Reference
In vitro			
Salmonella typhimurium (TA98, TA100, TA1535, TA1537), plate incorporation assay, ±S9, DMSO	Negative	99.7; 12256321	Otsuka (1990a)
S. typhimurium (TA98, TA100, TA102, TA1535, TA1537), plate incorporation and preincubation assay, ±S9, DMSO	Negative	95.2; 30037120	Herbold (1999a)
S. typhimurium (TA98, TA100, TA1535, TA1537) and *Escherichia coli* (WP2*uvrA*), plate incorporation assay, ±S9, DMSO	Positive in TA1535 (+S9) only	96.0; 30034708	Thompson (2000)
S. typhimurium (TA1535), plate incorporation and preincubation assay, ±S9, DMSO	Negative	98.6; NLL6100-3 96.0; 30034708	Herbold (1999b)
S. typhimurium (TA98, TA100, TA102, TA1535, TA1537), plate incorporation ±S9, and preincubation assay, +S9, DMSO	Negative	99.8; PF0000256K	Sokolowski (2003)
Bacillus subtilis (H17, M45), rec assay, ±S9, DMSO	Negative	99.7; 12256321	Otsuka (1990b)
L5178Y/TK$^{+/-}$, mouse lymphoma gene mutation assay, ±S9, DMSO	Positive (small colonies indicating clastogenicity)	96.0; 30034708	Durward (2000a)
V79, CHL gene mutation assay, ±S9, DMSO	Negative	95.2; 30034708	Brendler-Schwaab (1999a)
V79, CHL gene mutation assay, ±S9, DMSO	Negative	96.4; 30037120	Poth (2003a)
V79, CHL gene mutation assay, ±S9, DMSO	Negative	99.8; PF0000256K	Poth (2003b)
CHL, chromosomal aberration assay, ±S9, DMSO	Positive	96.0; 30034708	Wright (2000)
V79, CHL chromosomal aberration assay, ±S9, DMSO	Positive	96.4; 30037120	Schulz (2003a)
V79, CHL chromosomal aberration assay, ±S9, DMSO	Negative	99.8; PF0000256K	Schulz (2003b)
In vivo			
Mouse, bone marrow, micronucleus assay (oral, 25, 50, 100 mg/kg bw, arachis oil)	Negative	96.0; 30034708	Durward (2000b)
Mouse, bone marrow, micronucleus assay (ip, 50, 100, 200 mg/kg bw, 0.5% Cremophor)	Negative	96.4; 30037120	Honarvar (2003a)
Mouse, bone marrow, micronucleus assay (ip, 75, 150, 300 mg/kg bw, 0.5% Cremophor)	Negative	99.8; PF0000256K	Honarvar (2003b)
Rat, liver, unscheduled DNA synthesis assay (oral, 2500, 5000 mg/kg bw, aqueous Cremophor)	Negative	95.2–96.2; 30034708	Brendler-Schwaab (1999b)
Rat, liver, unscheduled DNA synthesis assay (oral, 1000, 2000 mg/kg bw, 0.5% Cremophor)	Negative	96.4; 30037120	Honarvar (2003c)
Rat, liver, unscheduled DNA synthesis assay (oral, 1000, 2000 mg/kg bw, 0.5% Cremophor)	Negative	99.8; PF0000256K	Honarvar (2003d)

CHL, Chinese hamster lung; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; ip, intraperitoneal; S9, 9000 × *g* rat liver supernatant

No toxicity was exhibited to any of the bacterial strains used. In all experiments, clothianidin provoked no increase of revertant colonies compared with negative controls. The confirmative experiment was also negative. Clothianidin is not mutagenic under these experimental conditions (Herbold, 1999a).

Clothianidin (purity 96.0%; batch No. 30034708) dissolved in DMSO was investigated for its potential to induce mutations in a plate incorporation assay for gene mutation in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2*uvrA*, both with and without metabolic activation (±S9 mix), at concentrations up to 5000 µg/plate for 48 hours and in triplicate. The protocol was in compliance with test methods B.13–14 of European Commission directive 2000/32/EC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 26 May 1983).

No toxicity to any of the bacterial strains used was exhibited. In all experiments, clothianidin provoked no increase of revertant colonies compared with negative controls in the test strains TA98, TA100, TA1537 and WP2*uvrA*. In contrast, in test strain TA1535, a slight increase of revertants was observed in the presence of metabolic activation at 1500 µg/plate (experiment 1) and 5000 µg/plate (experiments 1 and 2) (Table 22). A weaker response was observed with the same test strain in the absence of metabolic activation. However, the increase was less than 2-fold. Clothianidin is weakly mutagenic in TA1535 with metabolic activation under these experimental conditions (Thompson, 2000).

Clothianidin was investigated for its potential to induce mutations in an assay for gene mutation in *S. typhimurium.* The study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 26 May 1983). Two tests were performed (plate incorporation and preincubation). Overnight-grown bacteria (0.1 ml) from the characterized strain of *S. typhimurium* TA1535 were exposed to clothianidin of two different batches (batch 1: purity 98.6%, batch No. NLL6100-3; and batch 2: purity 96.0%, batch No. 30034708) dissolved in DMSO. In test 1 (plate incorporation), final concentrations were up to 5000 (batch 1) or 7000 (batch 2) µg/plate (±S9 mix). In test 2 (preincubation), 0.1 ml of bacteria was additionally preincubated at 37 °C in the presence of the active ingredient at final concentrations up to 8000 µg/plate for both test materials.

Based upon bacterial counts, it was observed that the active ingredient caused bacteriotoxicity at 5000 µg/plate (plate incorporation) or 6000 µg/plate (preincubation) and above in the absence of S9 mix. At 8000 µg/plate (preincubation), the compound precipitated. No increase of revertants above control levels was observed in any experiment. Clothianidin is not mutagenic in strain TA1535 with or without metabolic activation under these experimental conditions (Herbold, 1999b). No confirmation of the positive response in TA1535 with metabolic activation (Thompson, 2000) was obtained with the same batch (batch No. 30034708).

Clothianidin (purity 99.8%; batch No. PF0000256K) dissolved in DMSO was investigated for its potential to induce mutations in an assay for gene mutation in *S. typhimurium* with and without metabolic activation. The study satisfied test methods B.13–14 of European Commission directive 2000/32/EEC.

Clothianidin did not induce significant bacteriotoxicity in any strain, except in TA102, for which a decrease of the number of revertants was observed at the top dose of 5000 µg/plate (±S9 in the plate incorporation assay, +S9 in the preincubation assay). Compared with solvent control, the test article did not increase the number of spontaneous revertants in any test strain, either in the presence or in the absence of S9. Clothianidin is not mutagenic under these experimental conditions (Sokolowski, 2003).

Clothianidin (purity 99.7%; batch No. 12256321) was investigated for its potential to induce mutations in a spore rec assay for gene mutation in *Bacillus subtilis.*

The compound provoked no growth inhibition of germinating bacterial spores, either in the presence or in the absence of metabolic activation (±S9), at concentrations of 0, 375, 750, 1500,

Table 22. Mean number of revertants in test strain TA1535 treated with clothianidin

	S9 mix	Concentration (μg/plate)					
		0	50	150	500	1500	5000
Experiment 1	+	11	12	15	13	20*	28***
	−	20	27	21	26	30*	32*
Experiment 2	+	12	13	15	12	12	26***
	−	19	19	25	24	29	37***

From Thompson (2000)
* $P < 0.05$; *** $P < 0.005$

3000 or 6000 μg/plate. Clothianidin is not mutagenic under these experimental conditions (Otsuka, 1990b).

Clothianidin (purity 96.0%; batch No. 30034708) was investigated for its potential to induce mutations in a test with mouse lymphoma cells L5178Y/TK$^{+/-}$. The study was in compliance with test method B.17 of European Commission directive 2000/32/EC and OECD Guideline No. 476 (Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests; 4 April 1984).

In the first experiment, the test material did not induce any statistically significant or dose-related increases in the mutant frequency either in the presence or in the absence of metabolic activation. In the second experiment, in the absence of S9, an increased mutant frequency was observed at 1600 μg/ml; in the presence of S9, an increased mutant frequency was observed at 2000 μg/ml and above, in conditions of cytotoxicity. In addition, a significant linear dose–effect trend was noted ($P < 0.01$). There was a dose-related increase of the proportion of small colonies, indicating clastogenic activity, both in the presence and in the absence of metabolic activation (Table 23).

Clothianidin is mutagenic under these experimental conditions at concentrations inducing cytotoxicity. The emergence of small colonies is an indication of the clastogenic nature of the mutation (Durward, 2000a).

Clothianidin (purity 95.2%; batch No. 30034708) dissolved in DMSO was investigated for its potential to induce gene mutations in a test with the V79 Chinese hamster cell line. The study was in compliance with test method B.17 of European Commission directive 92/69/EEC and OECD Guideline No. 476 (Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests; 4 April 1984).

No reproducible increase in mutant frequency could be observed, in either the presence or the absence of a metabolic activation system. Clothianidin is not mutagenic under these experimental conditions (Brendler-Schwaab, 1999a).

Clothianidin (purity 96.4%; batch No. 30037120) dissolved in DMSO was investigated for its potential to induce gene mutations in a test with the V79 Chinese hamster cell line. The study was in compliance with test method B.17 of European Commission directive 2000/32/EC and OECD Guideline No. 476 (Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests; 21 July 1997).

The dosing of clothianidin was without significant effect on the mutation frequency. Up to the highest investigated concentration (2500 μg/plate in experiment I and 1500 μg/plate in experiment II), no relevant increase of the mutation frequency was observed in either the presence or the absence of metabolic activation (Poth, 2003a).

Table 23. Mutagenic activity of clothianidin in the mouse lymphoma assay

Dose (µg/ml)	−S9				+S9			
	Relative survival (%)	Relative total growth (%)	Mutant frequency[a]	Small colony mutants (%)[b]	Relative survival (%)	Relative total growth (%)	Mutant frequency[a]	Small colony mutants (%)[b]
Experiment 1								
0	100	100	108.76	61	100	100	85.14	51
312.5	97.72	109	133.17	61	89.56	89	75.79	50
625	94.42	112	72.70	68	99.34	126	68.56	39
1250	72.88	82	137.44	74	91.97	113	49.87	41
1667	22.46	14	179.65	86	56.78	87	133.77	71
2500	1.43	1	123.42	100	8.77	5	493.31[c]	74
Experiment 2								
0	100.00	100	93.5	31	100	100	94.09	29
300	126.91	113	87.08	37	—	—	—	—
600	101.84	95	109.21	34	83.22	96	108.22	33
1200	79.01	95	177.66	56	88.25	100	115.86	31
1600	31.28	30	272.04*	52	79.24	93	94.72	33
2000	3.42	2	179.62	69	57.81	67	217.03*	55
2400	—	—	—	—	19.03	26	307.28*	57

From Durward (2000a)

* $P < 0.05$

[a] Mutant frequency: trifluorothymidine-resistant mutants/10^6 viable cells (48 h treatment).

[b] % of total observed colonies.

[c] Data excluded from statistical analysis due to excessive heterogeneity.

Clothianidin (purity 99.8%; batch No. PF0000256K) dissolved in DMSO was investigated for its potential to induce gene mutations in a test with the V79 Chinese hamster cell line. The study was in compliance with test method B.17 of directive 2000/32/EC and OECD Guideline No. 476 (Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests; 21 July 1997).

The dosing of clothianidin was without significant effect on the mutation frequency. Up to the highest investigated concentration (2500 µg/plate), no relevant increase of the mutation frequency was observed in either the presence or absence of metabolic activation (Poth, 2003b).

Clothianidin (purity 96.0%; batch No. 30034708) dissolved in DMSO was investigated for its potential to induce chromosomal aberrations with Chinese hamster lung cells. The study was in compliance with test method B.10 of European Commission directive 2000/32/EC and OECD Guideline No. 473 (Genetic Toxicology: *In vitro* Mammalian Cytogenetic Test; 26 May 1983). Cells were exposed over 12 hours (−S9), 4 hours (+S9) with 8-hour recovery, 6 hours (±S9) with 18-hour recovery, 24 hours (−S9) or 48 hours (−S9).

In the cells treated for 12 hours (−S9), reductions of mitotic index to 41% and of cell count to 55% of control were observed at 937.5 µg/ml. In the presence of S9, a reduction of mitotic index to 39% of control was observed at 1562.5 µg/ml and to 7% of control at the top dose, whereas cell count at these doses was not markedly affected.

Clothianidin produced statistically significant ($P < 0.001$) increases in the frequency of cells with aberrations at the highest dose level (1875 µg/ml) in the 6-hour (with 18-hour recovery) exposure

group with metabolic activation and at 625 µg/ml in the 48-hour continuous exposure group. Smaller, but statistically significant ($P < 0.05$ or $P < 0.01$) increases in the incidence of aberrant cells also occurred at the highest dose level (1250 µg/ml) in the 6-hour (with 18-hour recovery) group with metabolic activation and in both of the 12-hour harvest groups treated at 937.5 µg/ml without activation and 1562.5 µg/ml with activation. A weak response also occurred at 625 µg/ml in the 24-hour exposure group. Clothianidin did not induce an increased incidence of polyploidy at any dose level in any of the exposure groups.

Clothianidin is clastogenic in cells treated at near-cytotoxic or cytotoxic doses, for both short and long treatment periods. In the absence of S9, there was both a time- and dose-dependent increase of cells with aberrations. No increase of polyploid cell number was observed, however (Wright, 2000).

Clothianidin (purity 96.4%; batch No. 30037120) dissolved in DMSO was investigated for its potential to induce chromosomal aberrations in a test with the V79 Chinese hamster cell line. The study was in compliance with test method B.10 of European Commission directive 2000/32/EC and OECD Guideline No. 473 (Genetic Toxicology: *In vitro* Chromosome Aberration Test; 21 July 1997).

In the experiment (4 hours + 14-hour recovery), statistically significant and biologically relevant increases in the number of cells carrying structural chromosomal aberrations were observed in the absence of S9 mix. However, in the presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed. No relevant increase in the frequency of polyploid metaphases was found after treatment with the test item compared with the frequencies of the controls.

Clothianidin was clastogenic in the absence of metabolic activation under these experimental conditions (Schulz, 2003a).

Clothianidin (purity 99.8%; batch No. PF0000256K) dissolved in DMSO was investigated for its potential to induce chromosomal aberrations in a test with the V79 Chinese hamster cell line. The study was in compliance with test method B.10 of European Commission directive 2000/32/EC and OECD Guideline No. 473 (Genetic Toxicology: *In vitro* Chromosome Aberration Test; 21 July 1997). Clothianidin was tested in the V79 cells up to 1500 µg/ml, equivalent to a dose causing some cytotoxicity (–S9) and/or compound precipitation (±S9).

In the first experiment (4 hours + 14-hour recovery), a slight, statistically significant increase in aberration frequency was observed at the top dose (1500 µg/ml) in the presence of S9 mix. However, the value was equal to that of the untreated control. In the second experiment, in the presence of S9 mix (4 hours + 24-hour recovery), the aberration frequency was statistically increased above the solvent control level. However, the incidence was still within the historical control range (historical control: aberrant cells 0–7% inclusive of gaps, 0–4% exclusive of gaps, 0–2% with exchanges).

Clothianidin was not clastogenic under these experimental conditions (Schulz, 2003b).

(b) In vivo

Clothianidin (purity 96.0%; batch No. 30034708) was investigated for its potential to induce chromosomal aberrations in an in vivo mouse micronucleus test. The study was in compliance with test method B.12 of European Commission directive 92/69/EEC and OECD Guideline No. 474 (Genetic Toxicology: Micronucleus Test; 23 May 1983). Five mice (CD1) of each sex per dose per time point were exposed to clothianidin dissolved in arachis oil at the nominal dose levels of 0, 25, 50 and 100 mg/kg bw by gavage; the dose volume was 10 ml/kg bw. Animals were killed at 24, 48 or 72 hours after dosing.

At the top dose, three of five females died at the 48-hour sampling time. Clinical signs were observed at all doses and included hunched posture, decreased/laboured respiration, ptosis and lethargy. For neither dosage nor sampling point was the polychromatic erythrocyte/normochromatic erythrocyte ratio (PCE/NCE) different from the solvent control value. The frequency of micronucleated polychromatic erythrocytes (MNPC) was not altered in either dose group compared with the concurrent solvent controls. Clothianidin is not clastogenic under these experimental conditions (Durward, 2000b).

Clothianidin (purity 96.4%; batch No. 30037120) was investigated for its potential to induce chromosomal aberrations in an in vivo mouse micronucleus test. The study was in compliance with test method B.12 of European Commission directive 2000/32/EC and OECD Guideline No. 474 (Genetic Toxicology: Micronucleus Test; 21 July 1997). Twelve (top dose) or six (other doses) mice (NMRI) of each sex per dose were exposed to clothianidin dissolved in 0.5% w/v Cremophor at a dose level of 50, 100 or 200 mg/kg bw by intraperitoneal injection at a dose volume of 10 ml/kg bw. Animals were killed 24 hours (all doses) or 48 hours (top dose) after dosing.

Two male animals died 6 hours after dosing at the top dose of 200 mg/kg bw. These animals also had the following clinical signs within 24 hours of treatment: reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and tremor (one male). For neither dosage nor sampling point was the PCE/NCE ratio different from the solvent control value. The frequency of MNPC was not altered in either dose group compared with the concurrent solvent controls. Clothianidin is not clastogenic under these experimental conditions (Honarvar, 2003a).

Clothianidin (purity 99.8%; batch No. PF0000256K) was investigated for its potential to induce chromosomal aberrations in an in vivo mouse micronucleus test. The study was in compliance with test method B.12 of European Commission directive 2000/32/EC and OECD Guideline No. 474 (Genetic Toxicology: Micronucleus Test; 21 July 1997). Twelve (top dose) or six (other doses) mice (NMRI) of each sex per dose were exposed to clothianidin dissolved in 0.5% w/v Cremophor at a dose level of 75, 150 or 300 mg/kg bw by intraperitoneal injection at a dose volume of 10 ml/kg bw. Animals were killed 24 (all doses) or 48 hours (top dose) after dosing.

Two males and one female died within 24 hours of dosing. These animals also had the following clinical signs within 6 hours of treatment: reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and tremor (one female). For neither dosage nor sampling point was the PCE/NCE ratio different from the solvent control value. The frequency of MNPC was not altered in either dose group compared with the concurrent solvent controls. Clothianidin is not clastogenic under these experimental conditions (Honarvar, 2003b).

Clothianidin (purity 95.2–96.2%; batch No. 30034708) was investigated for its potential to induce gene mutations in an ex vivo rat hepatocyte unscheduled deoxyribonucleic acid (DNA) synthesis (UDS) test. The study was in compliance with test method B.39 of European Commission directive 2000/32/EC. Three male rats (Crl: (WI) BR (SPF)) per dose (range-finding assay) or four male rats per dose per time point (main assay, except for the vehicle control group for 16-hour exposure, which used six rats) received clothianidin formulated in 0.5% (w/v) Cremophor at dose levels up to 5000 mg/kg bw by gavage. Animals were killed 4 or 16 hours after administration. Both net nuclear grain count values and fraction of cells in repair (nuclear grain count ≥ 5) were not elevated compared with controls. Clothianidin is not mutagenic under these experimental conditions (Brendler-Schwaab, 1999b).

Clothianidin (purity 96.4%; batch No. 30037120) was investigated for its potential to induce gene mutations in an ex vivo rat hepatocyte UDS test. The study was in compliance with test method

B.39 of European Commission directive 2000/32/EC and OECD Guideline No. 486 (Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*; 21 July 1997). Two rats (Wistar Han lbm:WIST (SPF)) of each sex per dose (range-finding assay) or four (main assay) male (starved) rats per dose per time point received clothianidin formulated in 0.5% (w/v) Cremophor at dose levels up to 2000 mg/kg bw by gavage. Animals were killed 2 or 16 hours after administration.

No dose level of the test item resulted in UDS induction in the hepatocytes of the treated animals compared with the concurrent vehicle controls. Neither the nuclear grains nor the resulting net grains were distinctly enhanced due to the in vivo treatment of the animals with the test compound for 2 hours or 16 hours, respectively. The fraction of cells in repair was unaffected.

Clothianidin does not induce UDS in vivo under these experimental conditions (Honarvar, 2003c).

Clothianidin (purity 99.8%; batch No. PF0000256K) was investigated for its potential to induce gene mutations in an ex vivo rat hepatocyte UDS test. The study was in compliance with test method B.39 of European Commission directive 2000/32/EC and OECD Guideline No. 486 (Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*; 21 July 1997). Two rats (Wistar Han lbm:WIST (SPF)) of each sex per dose (range-finding assay) or four (main assay) male (starved) rats per dose per time point received clothianidin formulated in 0.5% (w/v) Cremophor at dose levels up to 2000 mg/kg bw by gavage. Animals were killed 2 or 16 hours after administration.

No dose level of the test item resulted in UDS induction in the hepatocytes of the treated animals compared with the concurrent vehicle controls. Neither the nuclear grains nor the resulting net grains were distinctly enhanced due to the in vivo treatment of the animals with the test compound for 2 hours or 16 hours, respectively. The fraction of cells in repair was unaffected.

Clothianidin does not induce UDS in vivo under these experimental conditions (Honarvar, 2003d).

(c) Conclusion

Clothianidin (batch No. 30034708) was weakly positive at the limit dose of 5000 µg/plate in *S. typhimurium* strain TA1535 with metabolic activation in one bacterial reverse gene mutation assay (Thompson, 2000). However, in a further study by Herbold (1999b) of the same batch in the same strain, clothianidin was not mutagenic at doses up to 8000 µg/plate. Clothianidin was also consistently negative in the related, more sensitive strain, TA100. Plate incorporation and preincubation studies on three other batches of clothianidin at doses up to 5000 µg/plate demonstrate the absence of point mutagenic potential in all *S. typhimurium* strains used, including TA1535 (Otsuka, 1990a; Herbold, 1999a; Sokolowski, 2003). A clastogenic response occurred in vitro at the *TK* locus in mouse L5178Y cells (Durward, 2000a), both with and without metabolic activation. Batch Nos 30034708 and 30037120 also induced clastogenic responses in Chinese hamster lung cells in vitro (Wright, 2000) and in Chinese hamster V79 cells in vitro (Schulz, 2003a), respectively. However, in other in vitro mammalian tests with Chinese hamster V79 cells, clothianidin was not mutagenic at the *HPRT* locus (Brendler-Schwaab, 1999a; Poth, 2003a,b) and was not clastogenic (Schulz, 2003b). Clothianidin did not induce a genotoxic response in an in vitro DNA repair test (Otsuka, 1990b).

The fact that the in vitro clastogenicity was observed only at doses that induced greater than 50% cell loss and/or greater than 50% decrease in mitotic index suggests that this is most likely a high-cytotoxicity effect.

Clothianidin does not induce genotoxic responses in in vivo mouse micronucleus assays (Durward, 2000b; Honarvar, 2003a,b) or in vivo UDS assays (Brendler-Schwaab, 1999b; Honarvar, 2003c,d), indicating that clothianidin is not clastogenic and did not induce UDS in mammalian in vivo test systems.

Based on the weight of evidence, clothianidin is unlikely to be genotoxic in vivo.

2.5 Reproductive toxicity

(a) Multigeneration studies

Clothianidin (purity 95.2–96.0%; batch No. 30037120) was investigated in a one-generation dose range–finding study in rats. The study was partly in compliance with test method B.34 of European Commission directive 92/69/EEC and OECD Guideline No. 416 (Two-Generation Reproduction Toxicity Study; May 1983). Twenty randomly selected rats (Sprague-Dawley) of each sex per dose received clothianidin at a concentration of 0, 50, 100, 500 or 1000 ppm in the diet. Analytical verification revealed that dose levels were 86.3–88.8% of nominal values. Achieved test article intakes were 0, 2.9, 5.8, 29.1 and 58.9 mg/kg bw per day for males and 0, 3.4, 6.6, 34.2 and 68.6 mg/kg bw per day (premating period) and 0, 3.4, 6.4, 34.4 and 69.1 mg/kg bw per day (gestation period) for females. The test compound was administered to rats for about 8 weeks before mating, during gestation and through day 21 of lactation. F_1 litter size was not adjusted following delivery. The doses used were based upon the 90-day study in rats (Chambers, 1997a), in which the NOAEL was 1250 ppm.

Minimal decreases in body weight were observed in males at 500 ppm and in females at the top dose in week 1 of administration but were not considered toxicologically meaningful. There were no other indications of toxicity in parental animals. There were no abnormalities detected in the litters.

The NOAEL in this study was 1000 ppm, equal to 68.6 mg/kg bw per day, the highest dose tested (Astroff, 2000).

Clothianidin (purity 95.2–96.0%; batch No. 30037120) was investigated in a two-generation study of reproduction. Thirty randomly selected rats (Sprague-Dawley) of each sex per dose received clothianidin at a concentration of 0, 150, 500 or 2500 ppm in the diet. The study was partly in compliance with test method B.35 of European Commission directive 92/69/EEC. Accuracy, homogeneity and stability of the treated diet were confirmed by chemical analysis. Achieved test article intakes were 0, 10.2, 32.7 and 179.6 mg/kg bw per day for males and 0, 11.8, 37.9 and 212.9 mg/kg bw per day for females (calculations based on premating period for the first generation; similar intake during gestation). The test compound was administered throughout a 10-week premating phase, then throughout mating, gestation and lactation, for a total of about 16/20 weeks (F_0 males/F_0 females) or 20/24 weeks (F_1 males/F_1 females). The mating period was 2 weeks (1:1 mating ratio). Animals not delivering postmating were scheduled to be necropsied at day 24 and their cervical/uterine patency determined (via uterine horn flushing). F_1 litter size was adjusted following delivery to provide four males and four females per litter. Vaginal smears were examined daily in parental F_0 and F_1 females for 3 weeks prior to mating and in all females at termination, in order to determine estrous cyclicity (characterization: diestrus, presence of white blood cells; and estrus, cornification). For the F_1 pups selected for the next generation, preputial separation and vaginal patency were determined. A quantitative evaluation of the primordial ovarian follicle histology was scheduled in the F_1 generation on lactation day (LD) 2 if any ovarian or estrous cycle abnormality, female infertility or male germ cell abnormality was observed. In F_0/F_1 adults, one testis and caudal epididymis were processed for enumeration of spermatids and spermatozoids, respectively. Spermatozoid morphology and motility were examined on a distal vas deferens sample. Anogenital distance was measured in all F_2 pups at LD 0.

Significant body weight decreases were observed in both adult males and females of the F_0 and F_1 generations at the top dose. Relative feed consumption (g/kg bw per day) was generally increased (decreases were noted only during the first treatment week, indicating initial poor palatability). In both F_0 and F_1 animals, absolute and relative organ weight changes were in line with the observed body weight loss (Table 24).

There were no necropsy or histological findings showing a clear dose-related trend.

In both F_0 and F_1 animals, no difference in number and duration of estrous cycles between treated and control animals was observed on the basis of the smear analyses (Table 25).

Table 24. Two-generation reproductive toxicity of clothianidin in adult rats: body and organ weight data

	% relative to control							
	Dietary concentration (ppm)							
	0		150		500		2500	
	F_0	F_1	F_0	F_1	F_0	F_1	F_0	F_1
Body weight								
Males	—	—	—	—	—	—	↓8–10**	↓16–20**
Females								
- days 7–70[pm]	—	—	—	—	—	—	↓9–15**	↓16–19**
- days 0, 6, 13, 20[ge]	—	—	—	—	—	—	↓12–14**	↓13–16**
- days 0, 4, 7, 14, 21[la]	—	—	—	—	—	—	↓11–18**	↓13–15**
- day 14[la]	—	—	—	—	↓6.5*	—	—	—
Body weight gain								
Females (days 0, 6, 13, 20)[ge]	—	—	—	—	—	—	↓17**	↓8
Feed consumption								
Males	—	—	—	—	↓4* week 1[pm]	—	↓18** week 1[pm] ↑8* week 3;** weeks 4, 5, 8[pm]	↑16** weeks 1–10[pm]
Females	—	—	—	—	↑7.6* week 10[pm]	—	↓18** week 1[pm] ↑16** weeks 8–10[pm] ↑11* days 6–13[ge] ↑13* days 14–21[la]	↑21* weeks 1, 5–6[pm]; ** weeks 2–4, 7–10[pm] ↑11* days 6–13[ge] ↑13* days 7–21[la]
Organ weight								
Thymus								
- absolute (male)	—	—	—	—	—	—	↓16*	↓29*
- relative (male)	—	—	—	—	—	—	↓8	↓13*
- absolute (female)	—	—	—	—	—	—	↓35*	↓41*
- relative (female)	—	—	—	—	—	—	↓32*	↓32*
Ovary								
- absolute	—	—	—	—	—	—	↓19*	↓11
- relative	—	—	—	—	—	—	↓16*	—
Uterus								
- absolute	—	—	—	—	—	—	↓25*	↓21
- relative	—	—	—	—	—	—	↓20*	↓10

From Freshwater & Astroff (2000)
ge, gestation; la, lactation; pm, premating; * $P < 0.05$; ** $P < 0.01$ (parametric data: ANOVA + Dunnett's; non-parametric data: Kruskall-Wallis + Dunnett's or Mann-Whitney U-test [organ weight])

There were no relevant clinical observations in the pups.

At the top dose and in both sexes, a time-dependent decrease in body weight was observed in both generations. Body weight gain was impaired in the first-generation litters at 500 ppm and above and in both generations at the top dose.

A statistically significant increase in number of early stillborn deaths occurred in the 2500 ppm F_1 pups and in the 500 and 2500 ppm F_2 pups.

Table 25. Two-generation reproductive toxicity of clothianidin in adult rats: gross pathology and histopathology

	Dietary concentration (ppm)							
	0		150		500		2500	
	F_0	F_1	F_0	F_1	F_0	F_1	F_0	F_1
Developmental markers								
Preputial separation[a]	—	41.2	—	41.9	—	42.5**	—	47.9**
Body weight at preputial separation (g)	—	190	—	199	—	192	—	190
Vaginal opening[a]	—	32.4	—	32.2	—	32.1	—	34.7**
Body weight at vaginal opening (g)	—	104.6	—	104.4	—	98.8	—	90.6
Histopathology/cytopathology								
Sperm morphology								
- % normal	83.5	69.3	—	—	—	—	81.9	67.0
- % abnormal	15.9	30.0	—	—	—	—	16.0	29.0
- % detached	0.6	0.7	—	—	—	—	2.1	4.0
Total sperm count								
- epididymis	146.1	149.5	—	—	—	—	141.6	133.7
- testis	129.9	105.7	—	—	—	—	129.5	103.8
Sperm motility								
- % motile	82.9	81.7	—	—	82.6	79.0	79.2	73.4**
- % progressively motile	64.1	59.9	—	—	61.5	53.9	56.2**	46.1**
Estrous cycle								
- duration (days)	4.2	4.3	—	—	—	—	4.1	4.4
- number	3.3	3.4	—	—	—	—	3.7	3.4
Estrous stage (sacrifice)[b]								
n	*29*	*30*	*29*	*30*	*29*	*30*	*27*	*30*
- diestrus	18	19	19	18	25	16	24	25
- proestrus	2	0	0	3	1	0	1	1
- estrus	9	11	10	9	3	14	2	4
Ovarian follicle count (mean/ovary)								
- "non-anthral" follicles	12.17	—	—	—	—	—	—	12.22
- "anthral" follicles	3.94	—	—	—	—	—	—	4.53
- corpora lutea	3.71	—	—	—	—	—	—	3.24

From Freshwater & Astroff (2000)

* $P < 0.05$; ** $P < 0.01$ (parametric data: ANOVA + Dunnett's; non-parametric data: Kruskall-Wallis + Dunnett's or Mann-Whitney U-test [organ weight, histopathology]; non-parametric dichotomous data: χ^2 + Fisher Exact Test/Bonferroni adjustment)

[a] Expressed in days.

[b] Number of animals.

Litters evaluated for early stillborn deaths in the 2500 ppm F_1 and F_2 pups were not statistically elevated above controls. In light of the fact that the litter is generally accepted to be the appropriate unit for reproductive assessment and there was no effect on viable pup numbers (Table 26), the apparent increase in stillbirths was not considered to be toxicologically meaningful.

Thymus weight was decreased at 500 ppm (males) and above (males and females), and spleen weight was decreased at the top dose.

Single incidences of hydrocephalus and anophthalmia were reported at the top dose in F_1 pups. All other gross pathological findings were at low incidence and without a dose–response relationship.

Table 26. Two-generation reproductive toxicity of clothianidin in rats: pup data

	Dietary concentration (ppm)							
	0		150		500		2500	
	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
Litter data								
No. observed (day 21)	24	23	29	25	28	20	29	28
No. of pups missing[a]	2	1	2	4	1	4	4	6
No. of stillborn pups[a,b]								
- early[c]	0 (0)	1 (1)	3 (3)	3 (3)	3 (6)	5 (8*)	6 (9*)	4 (13*)
- late[d]	1 (1)	3 (3)	0 (0)	1 (1)	2 (3)	0 (0)	1 (1)	2 (2)
- total[a]	1 (1)	4 (4)	3 (3)	4 (4)	6 (9)	5 (8)	7 (10)	6 (15)
No. of viable pups[a]								
- day 0	14	14	14	14	14	13	13	12
- day 4	13	13	14	13	14	13	12	12
Birth index[e]	0.898	0.918	0.905	0.934	0.945	0.874	0.940	0.878
Live birth index[f]	1.00	0.998	0.993	0.991	0.984	0.974	0.979	0.965
Viability index[g]	0.992	0.980	0.994	0.981	0.987	0.988	0.924	0.970
Lactation index[h]	0.995	0.995	0.996	0.985	0.978	0.974	0.960	0.960
Body weight, male + female								
- day 0	—	—	—	—	—	—	↓8%*	↓3%
- day 4	—	—	—	—	—	—	↓16%**	↓9%
- day 7	—	—	—	—	—	—	↓18%**	↓13%**
- day 14	—	—	—	—	—	—	↓22%**	↓16%**
- day 21	—	—	—	—	—	—	↓26%**	↓21%**
Body weight gain, male + female	—	—	—	—	↓11%* (days 7–14)	—	↓29%** (days 0–21)	↓23%** (days 0–21)
Organ weights								
Thymus weight, male:								
- absolute	—	—	—	—	↓13%*	—	↓29%**	↓25%**
- relative	—	—	—	—	↓10%	—	↓5%	↓8%
Thymus weight, female:								
- absolute	—	—	—	—	↓10%	—	↓28%**	↓24%**
- relative	—	—	—	—	↓8%	—	↓7%	↓9%
Spleen weight, male:								
- absolute	—	—	—	—	—	—	↓30%**	↓31%**
- relative	—	—	—	—	—	—	↓7%	↓16%**
Spleen weight, female:								
- absolute	—	—	—	—	—	—	↓35%**	↓30%**
- relative	—	—	—	—	—	—	↓16%**	↓17%**

From Freshwater & Astroff (2000)

* $P < 0.05$; ** $P < 0.01$ (parametric data: ANOVA + Dunnett's; non-parametric data: Kruskall-Wallis + Dunnett's or Mann-Whitney U-test [organ weight, histopathology]; non-parametric dichotomous data: χ^2 + Fisher Exact Test/Bonferroni adjustment)

[a] Data expressed as litter incidence; early and late stillborn determined by observation of non-floating or floating lung.

[b] Litter (pups).

[c] Non-floating lung.

[d] Floating lung.

[e] Birth index = number of pups born/number of implantation sites.

[f] Live birth index = number of pups liveborn/total number of pups.

[g] Viability index = number of pups alive (day 4)/number of pups liveborn.

[h] Lactation index = number of pups alive (day 21)/number of pups post-culling (day 4).

In F_1 pups, preputial separation was delayed at 500 ppm and above. The notifier considered the delay of preputial separation at 500 ppm as part of the normal variation of the test laboratory (±5%). However, the trend showed a dose dependency. Body weights of all dose groups were comparable at the time of preputial separation. Vaginal opening was also retarded at the top dose (in parallel with a body weight decrease of −13%).

At the top dose, histopathological findings included a mammary gland cyst and chronic active inflammation (one male).

Total sperm count was only slightly affected at the top dose compared with controls; the differences did not reach statistical significance (Table 27). Also, sperm morphology revealed a subtle increase in the mean incidence of cells with detached spermatozoid at the top dose in both generations, but the differences between control and test groups were not statistically different (Table 28).

At the top dose, the proportion of motile sperm was slightly reduced (−10%) in the F_2 males, and progressive motility was reduced in both generations (−12% to −23%); there was no effect on motility at lower doses (Table 29).

The analysis of ovarian follicle count on serial sections revealed no remarkable differences between top-dose and control animals. Both number and duration of the complete estrous cycles were essentially unaltered in treated animals compared with controls. At termination, incidence of diestrus was slightly higher in F_1 females at 500 ppm and above and in F_1 and F_2 females at the top dose.

Clothianidin showed no toxicity to reproduction under these experimental conditions.

The NOAEL for both parental and offspring toxicity was 150 ppm, equal to 10.2 mg/kg bw per day, based on decreased body weight at 500 ppm, equal to 32.7 mg/kg bw per day, for parental animals and on decreased body weight and subsequent effect on preputial separation at 500 ppm, equal to 32.7 mg/kg bw per day, for offspring.

The NOAEL for reproductive toxicity was 2500 ppm, equal to 179.6 mg/kg bw per day, the highest dose tested (Freshwater & Astroff, 2000).

(b) Developmental toxicity

Rats

In a developmental toxicity range-finding study, eight successfully mated female rats (Sprague-Dawley Crl:CD®BR VAF/Plus®) per dose (mating ratio one male:one female) received clothianidin (purity 96%; batch No. 30034708) dissolved in a 0.5% aqueous solution of methylcellulose at a dose level of 0, 125, 250, 500 or 1000 mg/kg bw per day by gavage. The dose volume was in all cases 10 ml/kg bw. The protocol followed no guideline, as it was designed to set dose levels for a definitive study. Analytical verification revealed that dose levels were 93–101% of nominal values. Compound stability for 2 weeks in the vehicle at ambient temperature and compound homogeneity proved to be satisfactory. Negative control was obtained by treating with the vehicle. The data are not considered in the final evaluation.

At the top dose, all animals were found dead or killed moribund. At 250 mg/kg bw and above, clinical signs included localized alopecia, scant faeces and red perivaginal substance. At 500 mg/kg bw and above, piloerection, tremors, hypoactivity, emaciation, ptosis, coldness to touch, dehydration and chromorhinorrhoea were observed. At 1000 mg/kg bw, urine-stained abdomen and chromodacryorrhoea were noted. At necropsy, a dose-dependent incidence of small spleen was seen at 250 mg/kg bw and above. At all doses, body weights, body weight gains and feed consumption were decreased compared with the control animals. The decreases were time and dose related and started on day 1 of gavage. Spleen weights were decreased at all doses (confirming gross pathology) (Table 30).

Most fetal data indicate adverse effects at 250 mg/kg bw and above. Fetal external abnormalities were observed at that dose (n = 5) and included short snout (1), bilateral flexed forelimbs and hindlimbs (1), exencephaly (1), gastroschisis (abdominal wall fissure) (1), bilateral eye bulge

Table 27. Total sperm count

Generation	Group	*n*	Mean (standard error)	
			Epididymis	Testis
1	Control	30	146.1 (7.7)	129.9 (4.7)
	High	30	141.6 (8.1)	129.5 (3.9)
2	Control	30	149.5 (9.0)	105.7 (5.1)
	High	30	133.7 (11.4)	103.8 (5.5)

From Freshwater & Astroff (2000)

Table 28. Sperm morphology

Generation	Group	*n*[a]	Mean (standard error)[b]		
			% normal	% abnormal	% detached
1	Control	30	83.5 (1.1)	15.9 (1.1)	0.6 (0.1)
	High	30	81.9 (1.8)	16.0 (1.2)	2.1 (1.1)
2	Control	28	69.3 (2.0)	30.0 (2.0)	0.7 (0.2)
	High	29	67.0 (2.0)	29.0 (2.1)	4.0 (1.7)

From Freshwater & Astroff (2000)
[a] Animals with fewer than 100 sperm excluded.
[b] No statistically significant differences between groups at the 0.05 significance level.

Table 29. Sperm motility

Generation	Group	*n*	Mean (standard error)	
			% motile	% progressive
1	Control	30	82.9 (1.9)	64.1 (2.5)
	Low	28	82.6 (1.9)	66.2 (1.9)
	Mid	30	82.6 (1.6)	61.5 (2.2)
	High	29	79.2 (1.5)	56.2 (1.7)**
2	Control	29	81.7 (1.5)	59.9 (2.0)
	Low	29	82.6 (1.6)	61.7 (2.0)
	Mid	30	79.0 (1.9)	53.9 (2.2)
	High	29	73.4 (2.5)**	46.1 (2.7)**

From Freshwater & Astroff (2000)
** $P \leq 0.01$

depression (1) and short trunk (1). At 500 mg/kg bw (n = 2), findings were anasarca (body oedema) (2), short trunk (1), short tail (1) and umbilical hernia (1).

The MTD was considered to be 125 mg/kg bw; no NOAEL was observed (York, 1999a).

In a developmental toxicity study, 25 successfully mated female rats (Sprague-Dawley Crl:CD®BR VAF/Plus®) per dose (mating ratio one male:one female) received clothianidin (purity 95.2%; batch No. 30037120) dissolved in a 0.5% aqueous solution of methylcellulose at a dose level of 0, 10, 40 or 125 mg/kg bw per day by gavage on gestation days (GDs) 6–19. The dose volume was

Table 30. Teratogenicity range-finding study of clothianidin in rats

	Dose (mg/kg bw per day)				
	0	125	250	500	1000
Maternal data[a]					
Mortality					
- found dead	0	0	0	0	5[days 9, 10, 11]
- sacrificed	0	0	0	0	3[days 12, 14, 18]
Clinical signs	No	No	Yes	Yes	Yes
Feed consumption					
- g/day	—	↓18%	↓32%	↓49%	—
- g/kg bw per day	—	↓11%	↓21%	↓30%	—
Body weight					
- day 20	—	↓10%	↓19%	↓43%	—
- day 20[b]	—	↓12%	↓19%	↓31%	—
Body weight gain (g)					
- day 20	+125 ± 21	+88 ± 19	+52 ± 27	−49 ± 30	—
- day 20[b]	+46 ± 14	+12 ± 16	−9 ± 26	−53 ± 29	—
Absolute spleen weight	—	↓14%	↓14%	↓45%	—
Relative spleen weight	—	↓4%	—	↓6%	—
Gross pathology: small spleen incidence	0	0	1	6	7
Gravid uterine weight	—	↓7.3%	↓26%	↓95%	—
Caesarean section data[a]					
Number of pregnant females	8	7	6	8	7
No. of analysed litters	8	7	5[b]	8	7
No. of litters with ≥ 1 live fetuses	8	7	5[b]	2[c]	0
Corpora lutea (mean/ovary)	18.1	18.1	17.4	16.6	—
Implantations (litter incidence)	15.5	16.0	15.8[c]	14.1	—
Resorptions, early/late (litter incidence)	1.0 / 0.0	1.1 / 0.1	1.8 / 0.0	13.8 / 0.0	—
Live fetuses[d] (litter incidence)	14.5	14.7	14.0[c]	1.5[d]	—
Live male fetuses (%)[d]	54	53	51[c]	0[d]	—
Fetal weight (g),[d] male/female	3.53 / 3.46	3.22 / 3.21	2.66 / 2.49[c]	— / 1.04[d]	—
Fetal external anomalies[d]	—	—	Yes[c]	Yes[d]	—

From York (1999a)

[a] Maternal data are calculated for gestation days 6–20; caesarean section data are expressed in number of fetuses per litter.

[b] Corrected body weight = gestation body weight minus gravid uterine weight.

[c] Excludes one dam that gave birth to no live fetuses.

[d] Only dams with ≥ 1 live fetus taken into consideration. No statistical analysis reported.

in all cases 10 ml/kg bw. The protocol was in compliance with test method B.31 of European Commission directive 92/69/EEC and OECD Guideline No. 414 (Prenatal Developmental Toxicity Study; 12 May 1981). Analytical verification revealed that dose levels were 98–115% of nominal values. Compound stability for 2 weeks in the vehicle at ambient temperature and compound homogeneity proved to be satisfactory. Negative control was obtained by treating with the vehicle.

There were no deaths or relevant clinical signs. At the top dose, a slight decrease in body weight was observed from day 8. The body weight change was decreased at the top dose and was essentially attributable to effects during days 6–9. At 40 mg/kg bw per day, body weight gain was

impaired during days 6–9. No change of gravid uterine weight was observable when compared with the control dams.

Caesarean section observations revealed no remarkable differences between treated and control animals (Table 31). Fetal alterations occurred at similar incidences in litters of all dose groups, were comparable with recent historical controls or showed no consistency with dose (Table 32).

Clothianidin is not a developmental toxicant under these experimental conditions.

The maternal NOAEL was 10 mg/kg bw per day, based on decreased feed consumption and body weight effects at 40 mg/kg bw per day. The developmental and fetal NOAEL was 125 mg/kg bw per day, the highest dose tested (York, 1998a).

Rabbit

In a developmental toxicity range-finding study, five successfully mated female rabbits (New Zealand White, Hra:NZW(SPF)) per dose received clothianidin (purity 96%; batch No. 30034708) dissolved in a 0.5% aqueous solution of methylcellulose at a dose level of 0, 62.5, 125, 200 or 500 mg/kg bw per day by gavage on days 6–28 after mating. The dose volume was in all cases 5 ml/kg bw. Analytical verification revealed that dose levels were 93–102% of nominal values. Compound stability for 2 weeks in the vehicle at ambient temperature and compound homogeneity proved to be satisfactory. Negative control was obtained by treating with the vehicle.

All does at 125 mg/kg bw per day and above either died during the study or were killed moribund or due to abortion. At 62.5 mg/kg bw per day, one doe was terminated due to premature delivery. Two animals in the 125 mg/kg bw per day group died as a consequence of an intubation accident. At 62.5 and 125 mg/kg bw per day, dark urine was found. At 125 mg/kg bw per day and above, observations included a red substance in the cage pan (associated with litter resorption or moribundity) and scant/mucoid/no faeces. At 250 mg/kg bw per day and above, animals showed emaciation, hypoactivity and laboured breathing. At the top dose, additional signs included lacrimation, tilted head position, lost/impaired righting reflex and presence of red vaginal substance.

At 250 mg/kg bw per day and above, at necropsy, a trichobezoar (hairy conglomerate) was present in the stomach of a small number of rabbits, possibly as a consequence of decreased gastrointestinal activity. Dose-dependent decreases in body weight and feed consumption were observed at 125 mg/kg bw per day and above (Table 33). In the absence of statistical analysis, it was not possible to assess differences between the fetuses of the treated group (n = 28) and the control group (n = 48).

The MTD was considered to be 125 mg/kg bw per day; no NOAEL was identified (York, 1999b).

In a developmental toxicity study, 23 successfully mated female rabbits (New Zealand White, Hra:NZW(SPF)) per dose (mating ratio one male:one female) received clothianidin (purity 95.2%; batch No. 30037120) dissolved in a 0.5% aqueous solution of methylcellulose at a dose level of 0, 10, 25, 75 or 100 mg/kg bw per day by gavage on days 6–28 after mating. The dose volume was in all cases 10 ml/kg bw. The protocol was in compliance with test method B.31 of European Commission directive 92/69/EEC and OECD Guideline No. 414 (Prenatal Developmental Toxicity Study; 12 May 1981). Analytical verification revealed that dose levels were 96–103% of nominal values. Compound stability for 2 weeks in the vehicle at ambient temperature and compound homogeneity proved to be satisfactory. Negative control was obtained by treating with the vehicle.

Two animals were found dead at 75 and 100 mg/kg bw per day, and one animal was killed moribund at the high dose. Both decreased faecal output and orange/red urine were observed at 25 mg/kg bw per day and above. There were no relevant necropsy findings. Feed consumption was significantly decreased at 75 mg/kg bw per day and above, whereas uncorrected body weight (i.e. taking into account uterine weight) and body weight gain were affected only at the top dose. The reductions in uterine weight at 75 mg/kg bw per day and above were considered biologically significant (Table 34).

Table 31. Teratogenicity main study of clothianidin in rats

	Dose (mg/kg bw per day)			
	0	10	40	125
Maternal data				
Feed consumption:				
- days 6–9	—	—	↓9%*	↓47%**
- days 6–20	—	—	↓3.8%	↓17%**
Body weight:				
- day 20	—	—	—	↓5.3%**
- day 20[a]	—	—	—	↓6.8%**
Body weight gain:				
- days 6–9	—	—	↓42%**	↓152%**
- days 6–20	—	—	—	↓17%**
- days 6–20[a]	—	—	—	↓45%**
Fetal data				
Number of pregnant females	23	22	24	25
Corpora lutea (litter incidence)	15.9	15.8	15.9	16.0
Implantations (litter incidence)	13.6	14.3	14.2	14.3
Resorptions, early/late (litter incidence)	0.6 / 0.0	0.9 / 0.0	0.4 / 0.0	0.6 / 0.1
Live fetuses (litter incidence)	13.0	13.4	13.8	13.6
Live male fetuses (%)	49	49	52	48
Fetal weight (g), males/females	3.60 / 3.43	3.57 / 3.44	3.63 / 3.46	3.40 / 3.28
Fetal variations (fetal incidence % / litter incidence %)	4 / 34.8	3.1 / 40.9	3.6 / 37.5	4.7 / 40.0
Fetal malformations (fetal incidence % / litter incidence %)	0.7 / 8.7	0.3 / 4.5	0.3 / 4.2	0.3 / 4.0

From York (1998a)

* $P < 0.05$; ** $P < 0.01$ (continuous data: Dunnett's test [homogeneous variances]; Kruskall-Wallis/Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties]; discontinuous data: Kruskall-Wallis procedure)

[a] Corrected body weight = gestation body weight minus gravid uterine weight.

The test substance dose of 100 mg/kg bw per day tended to increase postimplantation loss, reduce fetal body weight and retard sternal ossification. These effects were considered related to administration of the test substance (Tables 35 and 36). Other alterations observed at 25 mg/kg bw per day and above (Table 36), including medially rotated hindlimbs, fused caudal vertebrae and absent hindpaw phalanges, were considered unrelated to administration of the test substance because 1) the fetal and/or litter incidences were within the ranges observed historically at the testing facility; 2) there was no dose-dependent pattern of effect; 3) the alteration was frequent in this rabbit strain; 4) the incidences did not significantly differ from the concurrent control group values; and/or 5) the alteration occurred in only one high dosage group litter. Other effects observed at the top dose, including small kidney, incompletely ossified sternal centra and absent hindpaw phalanges, were minimally but significantly increased on a litter incidence basis at the top dose.

The maternal NOAEL was 10 mg/kg bw per day, based on clinical signs at 25 mg/kg bw per day.

The developmental NOAEL was 75 mg/kg bw per day, based on increased resorption, reduced fetal body weight and retarded sternal ossification at 100 mg/kg bw per day (York, 1998b).

Table 32. Summary of fetal alterations in the main teratogenicity study of clothianidin in rats

Location	Parameter	Number of fetuses (litter incidence in %)[a]			
		Dose (mg/kg bw per day)			
		0	10	40	125
No. of litters evaluated		*23*	*22*	*24*	*25*
External	Umbilical hernia	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Oedema	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)
Visceral	Depressed eye bulge[m]	0 (0.0)	1 (4.5)	1 (4.2)	0 (0.0)
	Microphthalmia[m]	0 (0.0)	0 (0.0)	1 (4.2)	0 (0.0)
	Innominate artery absent[v]	0 (0.0)	0 (0.0)	1 (4.2)	1 (4.0)
	Umbilical artery descended to the left of the bladder	2 (8.7)	1 (4.5)	0 (0.0)	0 (0.0)
	Aortic arch dorsal to trachea/oesophagus[v]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
	Carotid (left) arises right of subclavian (right)[v]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
Skeletal	Small eye socket[m]	0 (0.0)	1 (4.5)	0 (0.0)	0 (0.0)
	Fused ribs[m]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
	Bifid centrum in thoracic vertebra[v]	1 (4.3)	0 (0.0)	1 (4.2)	2 (8.0)
	Cervical rib present at 7th cervical vertebra[v]	0 (0.0)	1 (4.5)	1 (4.2)	2 (8.0)
	Incompletely/not ossified sternal centra[v]	5 (21.7)	4 (18.2)	6 (25.0)	5 (20.0)
	Incompletely/not ossified pelvis[v]	1 (4.3)	3 (13.6)	3 (12.5)	2 (8.0)
	Short fibula and tibia in the hindlimbs	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)

From York (1998a)
m, malformation; v, variation
[a] Historical control litter incidence: microphthalmia = 0.59%, fused ribs = 0.46%, small eye socket/depressed eye bulge: not reported.

2.6 Special studies

(a) Neurotoxicity

Clothianidin is a neonicotinoid and has no structural relationship with organophosphate compounds. In repeated-dose toxicological investigations, the compound provoked no histopathological changes in the brain, and no changes in spinal cord or peripheral nerve pathology were reported. For these reasons, delayed neurotoxicity studies were not performed.

Studies to assess behavioural effects from acute and subchronic administration and effects on animals potentially exposed in utero or via the milk are described in detail below.

Acute neurotoxicity

In a preliminary range-finding study, five rats (Fischer 344 CDF (F-344)/BR) of each sex per dose received clothianidin (purity 95.2–96%) dispersed in 0.5% methylcellulose/0.4% Tween 80 in water by gavage at a dose level of 0, 250, 500 or 1000 mg/kg bw (dose volume 10 ml/kg bw). Deaths occurred from day 2 onwards at 500 mg/kg bw and above. Clinical signs were observed from 3 hours at 500 mg/kg bw and included tremors and decreased activity. Mortality incidence at 1000 mg/kg bw was 40% in the males and 100% in the females. Toxicity signs persisted for up to 72 hours following treatment. From these data, the peak of neurobehavioural effects was estimated to be 4 hours following treatment (Cain, Sheets & Stuart, 2000).

In the acute oral neurotoxicity study itself, 12 rats (Fischer 344 CDF (F-344)/BR) of each sex per dose received clothianidin (purity 95.2–96%; batch No. 30037120) dispersed in 0.5%

Table 33. Teratogenicity range-finding study of clothianidin in rabbits[a]

	Dose (mg/kg bw per day)				
	0	62.5	125	250	500
Maternal data					
Mortality					
- found dead	0	0	5[b] days 16, 18, 19, 24	5 days 13, 14, 15, 16, 17	4 days 14, 16
- sacrificed moribund	0	1 day 29	1 day 21	0	1 day 15
Clinical signs	No	Yes	Yes	Yes	Yes
Feed consumption	—	—	↓	↓	↓
Body weight					
- day 13	—	—	↓10%	↓17%	↓13%
- day 29	—	—	n.a.	n.a.	n.a.
Body weight gain	—	—	—	—	—
Gravid uterine weight	—	↓26%[c]	n.a.	n.a.	n.a.
Caesarean section data					
Number of pregnant females	5	5	5	5	5
Aborted/premature litters	0	1[d]	1[e]	0	0
Surviving litters	5	4	0	0	0
Corpora lutea (mean/ovary)	11.2	11.0	—	—	—
Implantations (litter incidence)	9.8	7.0	—	—	—
Resorptions, early/late (litter incidence)	0.2 / 0.2	0.0 / 0.0	—	—	—
Live fetuses (litter incidence)	9.6	7.0	—	—	—
Live male fetuses (%)	59.7	42.0	—	—	—
Fetal weight (g), males / females	36.97 / 36.37	40.66 / 38.25	—	—	—
Fetal external anomalies	—	—	—	—	—

From York (1999b)
n.a., not applicable
[a] Maternal data are calculated for gestation days 6–28; caesarean section data are expressed in number of fetuses per litter; no statistical analysis reported.
[b] Two of five deaths (days 16, 19) were attributed to intubation error.
[c] Excludes decedents, premature or abortive deliveries.
[d] Aborted.
[e] Premature.

methylcellulose/0.4% Tween 80 in water by gavage at a dose level of 0, 100, 200 or 400 mg/kg bw (dose volume 10 ml/kg bw). The protocol was in compliance with United States Environmental Protection Agency (USEPA) test method OPPTS 870.6200. Dose levels were 93.2–106% of nominal values. Compound stability for 1 week in the refrigerator and compound homogeneity were stated to be satisfactory. In addition to the clinical and mortality observations (daily) and body weight measurements (weekly), animals were tested for FOB end-points and for motor/locomotor activity on day −7, day 0 (hour 4), day 7 and day 14. FOB included the following observations: cage-side behaviour, behaviour during handling, open-field behaviour and reflex/physiological end-points. Motor/locomotor activity was studied by assessing rat behaviour in a figure-eight maze, equipped with infrared emitter/detector pairs. The movements were automatically sampled in 10-minute intervals (total test session: 90 minutes). Motor activity is defined as the number of beam interruptions per session, whereas locomotor activity is defined as the number of interruptions minus the consecutive counts for a given beam (until the next beam is encountered). All animals were subjected to gross

Table 34. Teratogenicity main study of clothianidin in rabbits: maternal data

	Dose (mg/kg bw per day)				
	0	10	25	75	100
No. of rabbits examined	*23*	*23*	*23*	*23*	*23*
Mortality					
- found dead	0	0	0	$2^{\text{days 25, 27}}$	$2^{\text{days 17, 20}}$
- sacrificed moribund	0	0	0	0	$1^{\text{day 19}}$
Clinical signs[a]					
- localized alopecia	$4^{(45)}$	$2^{(18)}$	$3^{(18)}$	$3^{(41)}$	$8^{(61)}$
- scant faeces	$1^{(1)}$	$3^{(4)}$	$4^{(10)}$	$10^{(40)}$**	$16^{(79)}$**
- no faeces	—	—	—	$1^{(1)}$	$11^{(22)}$**
- soft/liquid faeces	—	—	—	—	$1^{(5)}$
- orange urine	—	—	$2^{(4)}$	$9^{(38)}$**	$9^{(64)}$**
- red substance in pan	—	—	$1^{(1)}$	0	$4^{(8)}$**
- decreased motor activity	—	—	—	—	$1^{(1)}$
- loss of righting reflex	—	—	—	—	—
Feed consumption					
- g/day	—	—	—	↓17%*	↓37%**
- g/kg bw per day	—	—	—	↓15%*	↓34%**
Body weight					
- day 29	—	—	—	—	↓9%**
- day 29[b]	—	—	—	—	↓4%
Body weight gain					
- days 6–29	+0.32 ± 0.16	—	—	—	−0.02 ± 0.25**
- days 6–29[b]	−0.13 ± 0.21	—	—	—	−0.31 ± 0.17
Gravid uterine weight	—	—	—	↓11%	↓19%

From York (1998b)

* $P < 0.05$; ** $P < 0.01$ (continuous data: Dunnett's test [homogeneous variances]; Kruskall-Wallis/Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties]; discontinuous data: Kruskall-Wallis procedure)

[a] Number of rabbits with clinical sign $^{\text{(total number of observations)}}$.

[b] Corrected body weight = gestation body weight minus gravid uterine weight.

necropsy, and six rats of each sex per dose were chosen at random for perfusion and tissue collection. In control and top-dose animals, subsequent histopathological evaluation of selected tissues, including brain, spinal cord, dorsal root ganglia and peripheral nerve tissue (sciatic, tibial, sural), was performed. Sensitivity, reliability and validity of the test procedures have been established in previous studies with acrylamide, carbaryl and untreated rats (FOB) or with triadimefon, chlorpromazine and untreated rats (figure-eight maze). Rats selected for histopathology were euthanized by intraperitoneal pentobarbital injection (50 mg/kg bw), whereas all others were terminated by carbon dioxide asphyxiation.

There was no mortality. Clinical signs at the top dose in both males and females included tremors, decreased activity and ataxia. In 3 of 12 females, urine stain was observed, and 1 of 12 females exhibited red nasal and oral stain. Urine stain was also observed in 1 of 12 females in both the control and low-dose groups. These signs appeared on the day of treatment (day 0) and persisted up to day 1. There were no relevant effects on body weight. In general, all findings in the FOB and figure-eight maze were restricted to the day of treatment (Table 37). Most end-points revealed substantial effects at the top dose in both males and females (tremors, hypoactivity, decreased arousal, miosis following

Table 35. Teratogenicity main study of clothianidin in rabbits: caesarean section data

	Dose (mg/kg bw per day)				
	0	10	25	75	100
Number of surviving pregnant females	21	23	22	20	20
Aborted/premature litters	3[a]	0	0	1[a], 2[b]	6[a], 2[b]
Litters with > 3 live fetuses	0	0	2	0	1
Included in analysis	18	23	20	17	11
Corpora lutea (mean/ovary)	9.4	9.6	9.9	8.7	9.8
Implantations (litter incidence)	8.6	8.9	9.2	8.0	8.8
Resorptions, early/late (litter incidence):					
- number	2/3	0/3	2/5	0/4	10/5
- mean/litter	0.1/0.2	0.0/0.1	0.1/0.2	0.0/0.2	0.9/0.4
Live fetuses (litter incidence)	8.3	8.7	8.8	7.8	8.2
Live male fetuses (%)	51.8	47.2	50.2	48.8	39.6
Fetal weight male / female (g)	44.2 / 43.0	43.2 / 42.2	40.7 / 40.0	40.7 / 40.2	37.7 / 36.1**
% variations:					
- litter incidence	22.2	30.4	45.4	29.4	45.4
- fetal incidence	2.7	3.5	9.5**	4.5	8.2**
% malformations:					
- litter incidence	0.0	21.7	9.1	11.8	27.3
- fetal incidence	0.0	3.0**	1.7	1.5	5.9**
% litters with ≥ 1 variation	2.6	3.8	13.6	5.0	11.5
% litters with ≥ 1 malformation	0.0	2.9	1.5	1.6	9.0

From York (1998b)
* $P < 0.05$; ** $P < 0.01$
[a] Aborted.
[b] Premature.

light stimulus, decreased aerial righting response, hypothermia), with additionally gait incoordination and reduced approach response in the males. Biologically significant dose-related effects on arousal were detected at 100 mg/kg bw and above in the males and at 200 mg/kg bw and above in the females. The reduced activity observed in the FOB was confirmed by the findings in the figure-eight maze tests. In the top-dose males, no histological abnormality was reported. In the females of this dose group, the findings were as follows: minimal brain ventricle dilatation (1/6), left tibial nerve fibre degeneration (1/6), and right (1/6) and right + left (1/6) sciatic nerve fibre degeneration. In the female controls, incidences were as follows: right tibial nerve fibre degeneration (1/6), and right (1/6) and left (1/6) sciatic nerve myelin sheet vacuolization. All nerve lesions were graded minimal and of focal aspect, were mostly observed in either cross-section or longitudinal section, but not in both, and were devoid of concomitant inflammatory response. The oval/elongated shapes of some nerve fibres in the affected area were suggestive of a processing artefact rather than a compound-induced origin of the finding.

The neurotoxicity NOAEL was less than 100 mg/kg bw, based on reduced locomotor activity in males at this dose (Cain, Sheets & Stuart, 2000).

In the first follow-up acute neurotoxicity study, 10 male rats per dose received clothianidin at a dose of 0, 60 or 80 mg/kg bw. Analytical verification of doses revealed values ranging from 95%

Table 36. Summary of fetal alterations in the main teratogenicity study of clothianidin in rabbits

Location	Parameter	l/f	Number of fetuses (% incidence)[a]				
			Dose (mg/kg bw per day)				
			0	10	25	75	100
External	Medially rotated hindlimbs[v]	l	0	0	1 (4.5)	0	0
		f	0	0	3 (1.7)**	0	0
Visceral	Intermediate lung lobe absent[v]	l	0	0	0	3 (17.6)**	4 (36.4)**
		f	0	0	0	3 (2.2)	7 (8.2)**
	Small kidney[m]	l	0	0	0	0	1 (9.1)
		f	0	0	0	0	3 (3.5)**
Skeletal	Fused caudal vertebrae[m]	l	0	0	1 (4.5)	0	2 (18.2)**
		f	0	0	2 (1.1)	0	2 (2.4)**
	Incompletely ossified sternal centra[v]	l	0	0	0	0	2 (18.2)**
		f	0	0	0	0	2 (2.4)**
	Absent hindpaw phalanges[m]	l	0	0	0	0	2 (18.2)**
		f	0	0	0	0	2 (2.4)**

From York (1998b)

f, fetus; l, litter; m, malformation; v, variation; * $P < 0.05$; ** $P < 0.01$ (continuous data: Dunnett's test [homogeneous variances]; Kruskall-Wallis/Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties]; discontinuous data: Kruskall-Wallis procedure)

[a] Historical control incidences: 1) intermediate lung lobe absent: (i) litter incidence: n = 0–5, 0–27.8% (mean = 10.1%); (ii) fetal incidence: n = 0–7, 0–4.4% (mean = 1.54%); 2) small kidney: no reported data; 3) fused caudal vertebrae: (i) litter incidence: n = 0–1, 0–5.9% (mean = 1.4%); (ii) fetal incidence: n = 0–1, 0–1.9% (mean = 0.21%); 4) incompletely/not ossified sternebrae: (i) litter incidence: n = 0–10.5, 0–5.9% (mean = 1.8%); (ii) fetal incidence: n = 0–2, 0–1.2% (mean = 0.21%); 5) absent hindpaw phalanx: (i) litter incidence: n = 0–1, 0–5.9% (mean = 0.2%); (ii) fetal incidence: n = 0–1, 0–0.6% (mean = 0.02%).

to 105% of nominal concentrations, leading to actual doses of 0, 63 and 76 mg/kg bw. On the day of dosing, figure-eight maze activity was non-statistically significantly decreased in the treated animals at both dose levels, with, respectively, –40% and –24% (motor activity) and –40% and –26% (locomotor activity). However, as the two actual dose levels were quite similar, it was considered that the absence of a dose–response relationship was not easily interpretable, and a second follow-up study was designed with 60 mg/kg bw as the maximal dose.

There was no mortality, and there were no relevant clinical signs (Sheets & Gilmore, 2000).

A second follow-up study was conducted with 60 mg/kg bw as the maximal dose. In this study, 12 male rats (Fischer 344 CDF (F-344)/BR) per dose received clothianidin (purity 95.3–95.5%; batch No. 30037120) dispersed in 0.5% (w/w or volume per volume [v/v]) methylcellulose/0.4% Tween 80 in water by gavage at a dose level of 0, 20, 40 or 60 mg/kg bw (dose volume 10 ml/kg bw). The protocol was partly in compliance with USEPA test method OPPTS 870.6200. Dose levels were 89–101% of nominal values, and the dosing preparations were homogeneous. In addition to the clinical observations, animals were tested with a FOB and for motor/locomotor activity on day −7 and day 0 (hour 4). FOB and figure-eight maze observations were performed as described in the main study (Cain, Sheets & Stuart, 2000). The animals were not subjected to gross necropsy or histopathology. Rats were euthanized by carbon dioxide asphyxiation on day 2.

No statistically or biologically significant changes of any neurotoxicity parameter on the treatment day were detected in the treated animals when compared with the control group in the FOB and figure-eight maze. The neurotoxicity NOAEL was 60 mg/kg bw, and the neurotoxicity LOAEL was greater than 60 mg/kg bw, the highest dose tested (Sheets & Gilmore, 2000).

Table 37. Acute neurotoxicity study of clothianidin in rats: functional observational battery and figure-eight maze observations[a]

	Dose (mg/kg bw)							
	0		100		200		400	
	M	F	M	F	M	F	M	F
Functional observational battery[b]								
Home cage observations								
Tremors								
- score 1	—	—	—	—	—	—	7*	9*
- score 2	—	—	—	—	—	—	1	2
Decreased activity score 1	—	—	—	—	1	0	8*	11*
Open-field observations								
Posture								
- standing normally	—	—	—	—	—	—	7	7
- sitting or lying normally	—	—	—	—	—	—	5	5
Tremors								
- score 1	—	—	—	—	—	—	10*	6*
- score 2	—	—	—	—	—	—	1	5
Gait incoordination	—	—	—	—	—	—	1	0
Arousal								
- sluggish exploratory movements	1	0	3	0	4	2	9*	9*
- sluggish minimal movement	0	0	1	0	1	1	0	2
Reflex/physiological/manipulative observations								
Approach response: no reaction	—	—	—	—	—	—	1	0
Touch response: no reaction	—	—	—	—	—	—	0	2
Pupil response on light: pinpoint constriction	—	—	—	—	1	0	8*	9*
Aerial righting response								
- slightly uncoordinated	0	1	0	1	2	0	2	3
- landing on side	—	—	—	—	—	—	0	1
Body temperature (°C)	36.5	36.4	36.1	36.3	34.8*	35.1*	32.8*	32.7*
Figure-eight maze[c]								
Motor activity[d]	—	—	↓23% (1)*	—	↓59% (1,2)*	↓43% (1,2)*	↓81% (1,2)*	↓72% (1,2,3)*
Locomotor activity[d]	—	—	↓37% (1,2)*	—	↓62% (1,2)*	↓45% (1,2)*	↓88% (1,2)*	↓83% (1,2)*

From Cain, Sheets & Stuart (2000)

* $P < 0.05$ (ANOVA + Dunnett's *t*-test)

[a] Observations recorded on day 0.

[b] Incidences /12 animals (except body temperature); score 1, 2 = severity grade of observation.

[c] Per cent differences with concurrent control (number of movements during nine 10-minute intervals).

[d] Statistically significant differences observed during intervals 1, 2 or 3 out of 9 (10-minute intervals; total test session 90 minutes); comparison of measurements at day 0 with the pretreatment (day −7) values confirmed the historical control variability of ±20%.

Subchronic neurotoxicity

In a subchronic oral neurotoxicity study, 12 rats (Fischer 344 CDF (F-344)/BR) of each sex per dose were fed a diet containing clothianidin (purity 95.3–96%; batch No. 30037120) at a concentration in the diet of 0, 150, 1000 or 3000 ppm for 90 days. The protocol was in compliance with USEPA

test method OPPTS 870.6200. Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. Dose levels were 89.0–89.7% of nominal values. Compound stability was demonstrated for 1 week at ambient temperature and for 4 weeks in the refrigerator (6 °C), and compound homogeneity was satisfactory. Achieved doses (taking into account diet analysis results) were 0, 9.2, 60.0 and 177.0 mg/kg bw per day for males and 0, 10.6, 71.0 and 200.1 mg/kg bw per day for females. In addition to the clinical and mortality observations and body weight measurements, animals were examined with a FOB and motor/locomotor activity (figure-eight maze) on week −1, week 4, week 8 and week 13 (protocol as described previously; Cain, Sheets & Stuart, 2000). Prior to treatment and in week 12, animals were subjected to an ophthalmological examination in order to exclude animals with possible visual failure, precluding proper interpretation of neurotoxic end-points. All animals were subjected to gross necropsy, and six rats of each sex per dose were chosen at random for perfusion and tissue collection. In control and top-dose animals, subsequent histopathological evaluation of selected tissues was performed as described previously (Cain, Sheets & Stuart, 2000). The doses were selected taking into account the effects observed in a previous subchronic toxicity study (Wahle, 2000).

On day 4, one top-dose male was found dead. The animal showed decreased activity, ataxia and convulsions. This death (without explained cause) was probably incidental and unrelated to exposure to clothianidin because there were no other animals with any compound-related signs and there were no significant findings at necropsy.

There were no relevant clinical signs in the survivors. A slight body weight decrease (−5%) was observed at the top dose in males (weeks 11–13) and females (weeks 9–13). At the top dose, feed consumption was decreased in both males and females on most sampling times. The reduction was maximal during the first week of exposure, attaining about −17% (males) and −20% (females). There were no relevant findings in the FOB, figure-eight maze, gross necropsy or histopathology.

The no-observed-effect level (NOEL) was 1000 ppm, equal to 60 mg/kg bw per day, based on decreased feed consumption and decreased body weight.

There was no evidence of specific neurotoxicity. The neurotoxicity NOAEL was equal to 177 mg/kg bw per day, the highest dose tested (Sheets & Cain, 2000).

In a developmental neurotoxicity study, 25 randomly selected female rats (Crl:CD®(SD)IGS BR VAF/Plus®) per dose received clothianidin dissolved in corn oil (purity 95.5–95.9%; batch No. 30037120) at a dose level of 0, 150, 500 or 1750 ppm in the diet. The protocol was in compliance with USEPA test method OPPTS 870.6300 and with the draft OECD Guideline No. 426 (Developmental Neurotoxicity Study). Analytical verification (twice weekly from week 1 through week 8) revealed that dose levels were 90.7–96.3% of nominal values. Compound stability during 1 week in the diet at ambient temperature and during 4 weeks refrigerated as well as compound homogeneity had been determined in previously conducted studies and were claimed to be satisfactory (data not shown). Achieved test article intakes were 0, 12.9, 42.9 and 142.0 mg/kg bw per day (GDs 0–20) and 0, 27.3, 90.0 and 299.0 mg/kg bw per day (LDs 1–22). The mating period was 4 days (1:1 mating ratio). The males were not treated and were discarded after mating. The test compound was administered at F_0 during GDs 0–24 (if no delivery) or during GD 0 to postnatal day (PND) 22 (if delivery). F_1 animals were potentially exposed in utero, via the milk and via the feed during LDs 12–22.

In the pre-exposure period, clinical observations and body weight of F_0 animals were measured once per week. During the exposure period, the following were measured once per day: 1) autonomic dysfunction signs (lacrimation, salivation, palpebral closure, eye prominence, piloerection, urination, defecation), 2) abnormal appearance, posture, behaviour, movements; 3) feed consumption and body weight; 4) natural delivery observations (adverse signs during parturition, duration of gestation, litter size, day 1 pup viability); 5) gross necropsy of all F_0, and histopathology in case of gross lesions or when animal found death or killed moribund.

Until termination, all pups were checked (once or twice per day) for viability, clinical observations and general appearance. In the pre-weaning period, body weight was measured at PNDs 1, 5, 8, 12, 14, 18 and 22. In the post-weaning period, feed consumption was measured once per week as well as specific observations: *motor activity* was assessed as described previously; *sensory function* was tested by the auditory startle habituation test (peak response [gram] in the presence of noise stimulus, although without pre-pulse inhibition); and *learning and memory* were tested in the passive avoidance and water maze test. In particular, monitored end-points in passive avoidance and water maze were overall learning performance, short- and long-term retention and activity level/exploratory tendency in a novel environment.

At PND 5, litters were culled to five pups of each sex per litter. Per litter, one pup of each sex was randomly distributed to five subsets in order to perform different observations, neurotoxicity tests, and brain gravimetric or neuropathological examinations, including both morphometric measurements and thorough microscopic observations.

There were no relevant clinical signs in F_0 animals. During gestation and lactation, both feed consumption and maternal body weight were slightly decreased at the top dose, attaining statistical significance on some sampling times. Body weight change was affected only during gestation, with significant change on GDs 0–3. There were no relevant gross pathological or histopathological findings. The number of dams with stillborn pups was increased at 500 ppm and above (Table 38). However, the finding is of questionable toxicological significance in the absence of statistical significance and any dose–effect relationship.

There were no relevant findings in the litter parameters. The observed increase in number of pups found dead or presumably cannibalized at 150 ppm and the decreased lactation index at 150 and 500 ppm were considered unrelated to treatment because the findings were predominantly restricted to two litters (150 ppm) or one litter (500 ppm).

There were no relevant clinical signs. Pup body weights were altered from PND 14 to PND 22 (PND 14 corresponds with the approximate beginning of diet feeding). Changes in the lactation index at 150 and 500 ppm, when calculated on PND 12, were probably not induced by the compound, in the absence of a clear dose–effect relationship. On PND 22, no difference from controls was observed. At the top dose, reflex and physical development landmarks were unaltered when expressed as the average day postpartum where at least 50% of the pups had the developmental/reflex measure present.

Both passive avoidance and water maze performance were not altered by compound administration at any dose.

Average acoustic startle habituation (reactivity to auditory activity and habituation of responses with repeated presentation of stimuli) on PND 23 was significantly reduced in the top-dose females when compared with concurrent controls. The same trend was present in males, without attaining statistical significance. However, on PND 63, no significant difference from controls was observed at the top dose.

Motor activity was not affected at any dose on either PND 14 or PND 18. At the top dose of both males and females, reduced motor activity was observed for most sampling times on PND 22 (attaining statistical significance on the first sampling blocks) and for the first sampling blocks on PND 62. The effect was not statistically significant when average values of all sampling blocks were considered. However, the finding is considered toxicologically relevant, as the first sampling blocks cover the first exploratory movements of the animals (Table 39). Other spurious changes at intermediate doses were not considered to be of toxicological significance when the effect was unremarkable at the top dose.

There were no relevant findings in the gross pathology or brain weights. Detailed microscopic examinations of multiple coronal brain sections failed to indicate any effects of treatment on brain development (Table 40). There was also no evidence that any of the morphometric parameters measured on these same brains were affected by treatment, even though quantitative histometry on brain

Table 38. Developmental neurotoxicity study of clothianidin in rats: maternal data

	Dietary concentration (ppm)			
	0	150	500	1750
Feed consumption:				
- GDs 0–20	—	—	—	↓10%**
- LDs 1–22	—	—	—	↓5.6%**
Body weight:				
- GDs 0–20 (7*, 13*, 16–17*, 19*)	—	—	—	↓3–4%
- LDs 1–22 (1–3**, 6*, 7**, 13*, 14–15**, 19*)	—	—	—	↓4–8%
Body weight change				
- GDs 0–3	—	—	↓7.9%	↓63%**
- GDs 0–20	—	—	—	↓8.7%
No. of pregnant/delivering females	25	25	23	23
Duration of gestation (days)	22.6 ± 0.5	22.6 ± 0.5	22.6 ± 0.5	22.9 ± 0.3
Implantations[a]	14.6 ± 2.0	15.2 ± 2.0	15.4 ± 1.6	15.1 ± 1.8
Dams with stillborns: no. (%)	1 (4.0)	1 (4.0)	5 (21.7)	3 (13.0)

From Hoberman (2000)
GD, gestation day; LD, lactation day; * $P < 0.05$; ** $P < 0.01$ (continuous data: Dunnett's test [homogeneous variances]; Kruskall-Wallis/ Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties]; discontinuous data: Kruskall-Wallis procedure)
[a] On a per litter basis.

slices revealed significant increases of dentate gyrus (diagonal width) of the hippocampus and cerebellum thickness of about 10% and a concomitant decrease in germinal layer thickness of about 11% on PND 12 in the top-dose females. In the adult females at that dose, the dentate gyrus and cerebellum thickness were decreased by 5%, whereas caudate putamen was decreased by about 6%. However, the differences were not considered to be biologically significant and most likely reflect random differences between animals in the relative stage of brain development.

Neurotoxic effects from administration of clothianidin occurred only at doses that were developmentally toxic, as evidenced by reduced body weights, and did not persist into adulthood; therefore, clothianidin should not be considered a selective developmental neurotoxicant.

The parental systemic NOAEL was 500 ppm, equal to 42.9 mg/kg bw per day, based on body weight change at 1750 ppm.

The fetal systemic NOAEL was also 500 ppm, equal to 42.9 mg/kg bw per day, based on changes in pup weights at 1750 ppm.

The developmental neurotoxicity NOAEL was 1750 ppm, equal to 142 mg/kg bw per day, the highest dose tested

The NOAEL for reproductive toxicity was 1750 ppm, equal to 142 mg/kg bw per day, the highest dose tested (Hoberman, 2000).

(b) Immunotoxicity

In a study to examine the effect of clothianidin on the immune system, groups of rats received 0, 150, 500 or 3000 ppm of clothianidin in the diet for 28 days. The study was conducted according to the USEPA OPPTS guideline 870.7800. Consumed doses were 0, 13.8, 45.8 and 252.8 mg/kg bw per day for males and 0, 14.0, 46.2 and 253.0 mg/kg bw per day for females, respectively.

All rats survived to scheduled termination. No clinical observations related to test material administration occurred. Body weights and absolute feed consumption values were significantly reduced in male and female rats in the 3000 ppm exposure group for the entire study period (Table 41).

Table 39. Developmental neurotoxicity study of clothianidin in rats: pup data

	Dietary concentration (ppm)							
	0		150		500		1750	
Liveborn[a]	13.6 ± 2.2		14.2 ± 2.1		14.5 ± 1.8		14.0 ± 2.3	
Stillborn[a]	0.0 ± 0.2		0.0 ± 0.2		0.3 ± 0.5		0.1 ± 0.3	
Found dead (PND 9–12)[b]	0/239		18/248**		10/228*		0/220	
Viability index[b]	96.1		97.7		95.7		95.9	
Lactation index[a]:								
- PND 12	99.6		92.7**		94.8*		100.0	
- PND 22	100.0		97.4		99.4		97.5	
Pup weight[a]:	M	F	M	F	M	F	M	F
- PND 12	—	—	—	—	—	—	↓6.5%*	↓4%
- PND 14	—	—	—	—	—	↓6.8%*	↓13%**	↓13%**
- PND 18	—	—	—	—	—	↓7.1%**	↓16%**	↓16%**
- PND 22	—	—	—	—	—	↓5.7%*	↓15%**	↓15%**
- PND 23	—	—	—	—	—	↓4.3%	↓15%**	↓13%**
- PND 30	—	—	—	—	—	↓5.9%	↓8.5%**	↓5.8%*
- PND 37	—	—	—	—	—	↓3.5%	↓5.3%*	↓3%
Pup body weight gain:	M	F	M	F	M	F	M	F
- PNDs 5–22	—	—	—	—	—	—	↓18%**	↓18%**
Surface righting:								
- PND 3 (%)[c]	38.2 ± 14.9		25.4 ± 24.5*		25.3 ± 19.6*		20.1 ± 19.2**	
- t_{50}[d]	3.8 ± 2.0		3.8 ± 2.3		4.4 ± 2.1		3.5 ± 2.1	
Pinna unfolding:								
- PND 2 (%)[c]	7.7 ± 24.6		4.0 ± 20.0		0.0 ± 0.0		0.6 ± 2.8	
- PND 3 (%)[c]	60.5 ± 46.3		62.1 ± 39.7		40.9 ± 40.5		52.0 ± 39.6	
- t_{50}[d]	3.4 ± 0.8		3.4 ± 0.9		3.6 ± 0.6		3.5 ± 0.6	
Eye opening:								
- PND 12 (%)[c]	0.5 ± 2.3		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	
- PND 13 (%)[c]	6.0 ± 11.4		2.6 ± 7.9		1.2 ± 5.6		0.6 ± 2.9	
- PND 14 (%)[c]	25.3 ± 33.2		17.8 ± 29.8		17.4 ± 27.0		10.5 ± 20.9	
- t_{50}[d]	15.1 ± 0.7		15.3 ± 1.0		15.4 ± 0.8		15.4 ±0.8	
Acoustic startle:								
- t_{50}[d]	13.5 ± 1.0		13.4 ± 1.0		13.5 ± 0.6		13.4 ± 1.2	
Acoustic startle habituation:	M	F	M	F	M	F	M	F
- PND 23 (g)[e]	16 ± 9	21 ± 13	13 ± 6	18 ± 8	18 ± 13	16 ± 8	11 ± 4	11 ± 6**
- PND 63 (g)[e]	38 ± 31	30 ± 18	36 ± 33	26 ± 20	52 ± 38	21 ± 13	47 ± 23	30 ± 30
Motor activity								
Number of movements:								
- PND 22[f]	—	—	—	—	—	—	↓24% (1,2,3,4)*	↓10% (4*)
- PND 62[f]	—	—	—	—	—	—	↓2% (1,4)*	↓13% (4*)

Table 39 (continued)

	Dietary concentration (ppm)							
	0		150		500		1750	
Time spent in movement:								
- PND 22[f]	—	—	—	—	—	—	↓27% (1,3)*	↓19% (4*)
- PND 62[f]	—	—	—	—	—	—	—	—

From Hoberman (2000)

GD, gestation day; LD, lactation day; PND, postnatal day; * $P < 0.05$; ** $P < 0.01$ (continuous data: Dunnett's test [homogeneous variances]; Kruskall-Wallis/Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties]; discontinuous data: Kruskall-Wallis procedure)

[a] On a per litter basis.

[b] Pup incidence.

[c] Average % of pups meeting the criterion for the test on specified PND.

[d] t_{50} = average PND when ≥ 50% pups met the criterion.

[e] Response magnitude (recorded weight during startle in grams).

[f] % change of average (not statistically significant) value of 12 sampling blocks of 5 min; (1,..,..,4): number of blocks out of 12 sampling blocks where reduction attained statistical significance.

No changes related to clothianidin administration were observed at necropsy.

Absolute weights and the ratio of the spleen weight to the terminal body weight in the male and female rats did not differ significantly among the groups exposed to clothianidin.

Exposure to clothianidin did not cause any adverse effect on the immunoglobulin M (IgM) antibody-forming cell (AFC) response to the T cell–dependent antigen, sheep erythrocytes, in male and female rats when evaluated as both specific activity (AFC/10^6 spleen cells) and total spleen activity (AFC/spleen). The positive control, cyclophosphamide (CPS), produced the anticipated results, including a reduction in the immune response, body weight and body weight gain, feed consumption and spleen weights, as well as increased incidence of adverse clinical observations. The NOAEL for systemic toxicity was 500 ppm (45.8 mg/kg bw per day in male rats, 46.2 mg/kg bw per day in female rats) based on reduced body weight, body weight gains and feed consumption. The NOAEL for immunotoxicity was greater than 3000 ppm, the highest dose tested (Hoberman, 2004).

Clothianidin was tested for effects on developmental immunotoxicity in rats. Groups of 25 pregnant rats received test diets containing 0, 150, 500 or 2000 ppm clothianidin offered once daily on days 6 through 21 or 24 of presumed gestation. All surviving rats were terminated after completion of the 21-day postpartum period, and a gross necropsy was performed. The following parameters were evaluated: viability, consumed doses, clinical observations, maternal body weight and body weight changes, maternal behaviour, feed consumption values, necropsy observations, the number and distribution of corpora lutea, number of implantation sites, uterine contents, litter size and pup viability. Following weaning on PND 21, 202 male and 202 female rats (one pup of each sex per litter per assay, when possible) were selected for continuation on study. Of these rats, 190 male and 190 female rats were selected for immunological evaluation and assigned to two assays. One hundred and eighty rats were assigned to Assay 1: 20 rats of each sex in each of Groups I through IV and 10 rats of each sex in Group V (carrier and CPS). Two hundred rats were assigned to Assay 2: 20 rats of each sex in each of Groups I through IV and 10 rats of each sex in Groups VI (carrier and challenge) and VII (carrier, CPS and challenge).[1] The test substance in the diet or carrier was offered daily to these

[1] Note that group numbering was the same in Assay 1 and Assay 2. Hence, no rats were assigned to Groups VI and VII in Assay 1 or to Group V in Assay 2.

Table 40. Developmental neurotoxicity study of clothianidin in rats: female pup brain histometry data

	Thickness (μm, average of 10 brain slices)			
	Dietary concentration (ppm)			
	0	150	500	1750[a]
Dentate gyrus:				
- PND 12	919 ± 68	—	942 ± 70	1003* ± 81 (↑10%)
- PND 83	1562 ± 69	—	1506 ± 73	1483* ± 95 (↓5%)
Cerebellum:				
- PND 12	2856 ± 277	—	2946 ± 195	3149* ± 259 (↑10%)
- PND 83	4915 ± 267	—	4926 ± 115	4771 ± 255 (↓5%)
External germinal layer:				
- PND 12	37 ± 4.0	—	38 ± 4	33* ± 5 (↓11%)
Caudate putamen:				
- PND 83	3542 ± 218	—	3480 ± 174	3192* ± 156 (↓6%)

From Hoberman (2000)

* $P < 0.05$; ** $P < 0.01$ (Dunnett's test [homogeneous variances]; Kruskall-Wallis/Dunn's test [non-homogeneous variances, ≤ 75% ties] or Fisher's Exact test [> 75% ties])

[a] % change relative to controls in parentheses.

rats beginning on PND 21 through termination at exposures of 0 (carrier), 150, 500 or 2000 ppm in Groups I through IV, respectively.

Male and female rats assigned to Assay 1 (primary response) were administered 0.5 ml of sheep erythrocytes intravenously once 4 days before termination. Rats in Group V were administered CPS at 50 mg/kg bw via intraperitoneal injection for 4 consecutive days before termination. This injection was administered after the administration of the sheep erythrocytes. Male and female rats assigned to Assay 2 were administered *Candida albicans* formalin-fixed cells by subcutaneous injection in the right flank (0.2 ml) 8 days before challenge (except the "challenge-only" rats in Group VI). Rats in Group VII were administered CPS at 50 mg/kg bw via intraperitoneal injection for 4 consecutive days before challenge with *Candida albicans* chitosan antigen. Foot measurements were taken 3 days before euthanasia. All Assay 2 rats were then challenged by injection of the *Candida albicans* chitosan antigen into the foot pad (0.1 ml of 1.1 mg/ml). Subsequent foot measurements were taken approximately 24 and 48 hours after challenge. All rats assigned to Assay 1 were terminated following sensitization with the injection of sheep erythrocytes and/or administration of CPS. All surviving rats assigned to Assay 2 were terminated following the last foot measurement. The following parameters were evaluated: viability, consumed doses, clinical observations, autonomic behaviour, body weight and body weight changes, feed consumption values, thymus and spleen weights, immunological evaluations [spleen IgM AFC response, spleen cell number, delayed-type hypersensitivity response (footpad swelling)] and necropsy observations.

Terminal body weights in male and female rats were significantly reduced ($P \leq 0.01$) in the 2000 ppm exposure group. The absolute spleen and thymus weights for both male and female rats in the 2000 ppm exposure group were significantly reduced ($P \leq 0.01$), but the ratio of these weights to the terminal body weight did not differ significantly among the groups, indicating that the reduction in absolute weight was related to the terminal body weight. A significant increase ($P \leq 0.05$) in terminal body weights occurred in male rats in the 150 ppm exposure group. There were statistically significant decreases (24% and 22%) in spleen cell number following exposure to the high dose level (2000 ppm) of clothianidin for the male and female animals, respectively. This was not unexpected,

Table 41. Oral repeated-dose 28-day immunotoxicity study in rats: body weight and body weight changes

	Dietary concentration (ppm)							
	0		150		500		3000	
	M	F	M	F	M	F	M	F
Body weight (% of controls):								
- day 1	—	—	—	—	—	—	—	—
- day 8	—	—	—	—	—	—	−14.6**	−8.4**
- day 15	—	—	—	—	—	—	−12.8**	−9.9**
- day 22	—	—	—	—	—	—	−13.1**	−12.5**
- day 29	—	—	—	—	—	—	−12.8**	−12.6**
Body weight gain (% of controls):								
- days 1–8	—	—	—	—	—	—	−59.6**	−72.3**
- days 8–15	—	—	—	—	—	—	—	—
- days 15–22	—	—	—	—	—	—	—	−48.1**
- days 22–29	—	—	—	—	—	—	—	—
- days 1–29	—	—	—	—	—	—	−25.2**	−44.6**

From Hoberman (2004)
* $P < 0.05$; ** $P < 0.01$.

as the body and absolute spleen weights of these animals decreased as a result of the 2000 ppm dose of clothianidin. As expected, the positive control, CPS, produced statistically significant decreases in cell number when compared with the carrier group.

Exposure to clothianidin resulted in statistically significant increases in specific activity (AFC/10^6 spleen) (116%) and total spleen activity (AFC/spleen) (105%) in the low dose level (150 ppm) male animals. In addition, there were statistically significant increases in specific activity (143%) and total spleen activity (99%) in the high dose level (2000 ppm) female animals. These increases were due to the variability found in the Crl:CD (SD) rat strain. There were decreases in both specific and total spleen activities for the high dose level of clothianidin; however, these decreases did not reach the level of statistical significance. The positive control, CPS, produced statistically significant decreases in specific activity and total spleen activity when compared with the carrier animals for both the male and female animals.

There were no effects on the delayed-type hypersensitivity response assay at any of the dose levels of clothianidin at either the 24- or 48-hour time points for the male and female animals. At the 24-hour time point, which was the peak response, the positive control, CPS, produced suppression.

No deaths related to the test substance occurred. One F_0 generation female rat in the 2000 ppm exposure group was found dead on day 21 of lactation. This death was not considered related to the test substance because this was a single event.

All clinical observations during both the gestation and lactation periods were considered unrelated to the test substance. The number of rats with ptosis was significantly increased in the 2000 ppm exposure group compared with the control group value. Body weight gains in the 2000 ppm exposure group were significantly reduced for the gestation dosage period (calculated as GDs 6–20). Average body weights in the 2000 ppm exposure group were significantly reduced on GDs 9, 10, 13–16 and 18–20 compared with the control group values (Table 42).

Reflecting the weight reduction during gestation, average body weights in the 2000 ppm exposure group were significantly reduced during the lactation period. Body weight gains during

Table 42. Developmental immunotoxicity study in rats: maternal body weights and body weight change

	Dietary concentration (ppm)			
	0	150	500	2000
Number	25	25	25	25
Pregnant	24	21	25	25
Gestation				
Body weight (% of controls):				
- day 9	—	—	—	−2.9*
- day 10	—	—	—	−3.4*
- day 13	—	—	—	−3.7**
- day 14	—	—	—	−4.0**
- day 15	—	—	—	−3.6*
- day 16	—	—	—	−4.2*
- day 18	—	—	—	−4.5**
- day 19	—	—	—	−4.8**
- day 20	—	—	—	−5.6**
Body weight gain (% of controls):				
- days 0–6	—	—	—	—
- days 6–9	—	—	—	−71.1**
- days 9–12	—	—	—	—
- days 12–15	—	—	—	—
- days 15–18	—	—	—	—
- days 18–20	—	—	—	−24.6**
- days 6–20	—	—	—	−20.8**
- days 0–20	—	—	—	−14.3**
Lactation				
Body weight (% of controls):				
- day 1	—	—	—	−4.0*
- day 3	—	—	—	−8.5**
- day 6	—	—	—	−10.5**
- day 9	—	—	—	−10.1**
- day 13	—	—	—	−7.3**
- day 16	—	—	—	−7.0**
- day 21	—	—	—	−3.2*
Body weight gain (% of controls):				
- days 0–6	—	—	—	−273.9**
- days 6–9	—	—	—	−57.6**
- days 9–12	—	—	—	—
- days 12–15	—	—	—	181.8*
- days 15–18	—	—	—	—
- days 18–20	—	—	—	821.4*
- days 6–20	—	—	—	—

From Hoberman (2008)
* $P < 0.05$; ** $P < 0.01$

lactation were not affected by exposure to the test substance in the diet at concentrations as high as 2000 ppm.

Absolute and relative feed consumption values in the 2000 ppm exposure group were significantly reduced for the gestation dosage period (calculated as GDs 6–20).

Absolute and relative feed consumption values during the lactation period were significantly reduced in the 500 and 2000 ppm exposure groups for all intervals evaluated during lactation except the relative feed consumption value in the 500 ppm exposure group for LDs 6–9.

Pregnancy occurred in 24 (96.0%), 21 (84.0%), 25 (100%) and 25 (100%) rats in the 0 (carrier), 150, 500 and 2000 ppm exposure groups, respectively. The average pup weights per litter were significantly reduced in the 2000 ppm exposure group from PND 4 preculling until PND 21. All other parameters evaluated for natural delivery and litter observations were unaffected by administration of the test substance. No gross lesions occurred in the F_0 generation rats, and no clinical or necropsy observations in the F_1 generation pups were attributable to exposure.

All F_1 generation male and female rats survived to scheduled termination. No adverse clinical observations related to the test substance occurred. Body weights on day 1 postweaning (PND 22) were significantly reduced in the 500 and 2000 ppm exposed male rats and the 2000 ppm exposed female rats. In the male rats, body weights remained significantly reduced in both Assay 1 and Assay 2 in the 500 and 2000 ppm exposure groups through PND 36, and for female rats, body weights remained significantly reduced only in the 2000 ppm exposure group through PND 36. Terminal body weights were significantly reduced for male and female rats assigned to both Assay 1 and Assay 2 in the 2000 ppm exposure groups. Body weight gains were significantly reduced from PND 22 to PND 29 in both male and female rats in the 500 and 2000 ppm exposure groups. Body weight gains continued to be significantly reduced only in the 2000 ppm exposure group for male and female rats assigned to Assays 1 and 2 until termination, except for a non-statistically significant reduction for females in Assays 1 and 2 during the period from PND 36 to termination (Table 43).

Absolute feed consumption values were significantly reduced in the 500 ppm exposure group for male and female rats from PND 22 to PND 29 and for female rats in Assay 2 from PND 29 to PND 36 and from PND 22 to PND 36. In the 2000 ppm exposure group, absolute feed consumption values for male and female rats in both Assay 1 and Assay 2 were significantly reduced at all intervals evaluated, compared with the control group. Relative feed consumption values were significantly reduced only in the 2000 ppm exposure group for the male rats on PNDs 22–29 and significantly increased for the female rats assigned to Assay 1 from PND 29 to PND 36 and from PND 22 to PND 36.

No necropsy observations related to the test substance occurred in the F_1 generation rats.

Absolute, but not relative, thymus and spleen weights were significantly reduced for both male and female F_1 rats in the 2000 ppm group. These organ weight reductions are considered secondary to significant body weight and body weight gain reductions observed in this exposure group (Table 44).

The maternal NOAEL for general toxicity was 500 ppm, equal to 35 mg/kg bw per day during gestation and 68.3 mg/kg bw per day during lactation, based on reductions in body weight and feed consumption during gestation and lactation, reductions in body weight gains during gestation and from LD 0 to LD 6 and an increased incidence of ptosis.

The NOAEL for general toxicity in the F_1 generation male and female rats was 150 ppm (27.5 and 28.2 mg/kg bw per day for males in Assays 1 and 2 and 26.4 and 26.8 mg/kg bw per day for females in Assays 1 and 2, respectively). Significant reductions in body weight were present in male rats in the 500 and 2000 ppm groups and in female rats in the 2000 ppm group at weaning (PND 22). Reductions in body weight, body weight gain and feed consumption during the postweaning period persisted only in the 2000 ppm exposure group.

There were no immunologically relevant adverse effects on humoral immunity or cell-mediated immunity in male and female F_1 generation Crl:CD (SD) rats following exposure to clothianidin in

Table 43. Developmental immunotoxicity study: F_1 generation body weights and body weight changes

	Dietary concentration (ppm)							
	0		150		500		2000	
	M	F	M	F	M	F	M	F
n	*40*	*40*	*40*	*40*	*40*	*40*	*40*	*40*
Body weight (% of controls)								
- day 22	—	—	—	—	−3.8**	—	−28.2**	−28.1**
n	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Assay 1:								
- day 29	—	—	—	—	−7.5*	—	−26.3**	−29.3**
- day 36	—	—	—	—	−6.0*	—	−25.7**	−24.8**
- terminal body weight	—	—	5.9*	—	—	—	−25.6**	−20.6**
Assay 2:								
- day 29	—	—	—	—	−10.6*	−5.8*	−29.4**	−23.9**
- day 36	—	—	—	—	−8.1*	—	−26.4**	−20.6**
- terminal body weight	—	—	—	—	—	—	−25.5**	−18.5**
n	*40*	*40*	*40*	*40*	*40*	*40*	*40*	*40*
Body weight change (% of controls)								
- days 22–29	—	—	—	—	−10.1*	—	−27.8**	−24.9**
n	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Assay 1:								
- days 29–36	—	—	—	—	—	−11.3*	−24.8**	−15.1**
- day 36–termination	—	—	13.6*	—	—	—	−25.5**	—
- day 22–termination	—	—	6.0*	—	—	—	−25.3**	−17.1**
Assay 2:								
- days 29–36	—	—	—	—	—	—	−21.1**	−13.7**
- day 36–termination	—	—	17.8**	—	—	—	−23.0**	—
- day 22–termination	—	—	7.4	—	—	—	−24.1**	−14.6**

From Hoberman (2008)
* $P < 0.05$; ** $P < 0.01$

Table 44. Developmental immunotoxicity study: F_1 generation thymus and spleen weights

	Dietary concentration (ppm)							
	0		150		500		2000	
	M	F	M	F	M	F	M	F
n	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Terminal body weight (g)	202	161.2	214*	—	—	—	150**	128**
Spleen, absolute (g)	0.71	0.49	—	—	—	—	0.54**	0.38**
Spleen, relative (%)	0.355	0.304	—	—	—	—	—	—
Thymus, absolute (g)	0.72	0.57	—	—	—	—	0.57**	0.45**
Thymus, relative (%)	0.356	0.356	—	—	—	—	—	—

From Hoberman (2008)
* $P < 0.05$; ** $P < 0.01$

utero during gestation, via maternal milk and maternal feed during the postpartum period or via the diet during the postweaning period (Hoberman, 2008).

(c) Toxicity of metabolites

Both acute oral toxicity and mutagenicity (bacterial reverse gene mutation) studies were performed on TZNG, TZMU, TMG and MG (metabolites mostly found in all studied compartments), ATG-Ac (hen metabolite), ATMG-Pyr (goat metabolite) and MAI (environmental metabolite, generated by photolysis in natural water) (Table 45). On MNG, only a bacterial mutagenicity study, but no acute toxicity study, was reported. Additional studies on the environmentally relevant metabolite NTG, which was also a rat metabolite, included acute toxicity, subchronic toxicity, genotoxicity, and two-generation reproductive and developmental toxicity studies.

Acute oral toxicity studies on metabolites

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) per dose received TZNG (purity 98.6%; batch No. 89066524) dispersed in 5% w/v aqueous gum arabic by gavage at a dose level of 1125, 1350 or 1620 mg/kg bw (females) or 1450 mg/kg bw (males). Males were tested with only a single high dose, to check relative sensitivity to females. The dose volume was 10 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

In a preliminary range-finding study, two female rats were dosed at 1000, 1200, 1400, 1800 or 2000 mg/kg bw. Deaths occurred at 1200 mg/kg bw and above, on days 2–6. In the main study, mortality at 1350 mg/kg bw was one in five females (day 5), and at 1620 mg/kg bw, four of five females (days 3 and 4).

Clinical signs included lethargy, palpebral closure, arched gait and hunched posture, observed in all females and at all doses. Piloerection was present at 1350 mg/kg bw and above. By day 4, most surviving animals had recovered from these signs. At 1350 mg/kg bw, wasted appearance, stained snout and bradypnoea were detected in the animal that died on day 5. At the top dose, arched gait, ataxia, tremors, Straub tail (tail arched onto back) and hypothermia were restricted to a decedent.

Marked body weight losses were recorded from day 1 to day 8 at 1125 mg/kg bw and above, but not from day 8 to day 15.

Necropsy findings on decedents included small spleen and pale stomach (1350 mg/kg bw), dark/red lungs, distended/pale stomach and dark red areas/foci in stomach (1620 mg/kg bw) in females; and small (5/5) and soft (2/5) testes and distended colon (1/5) at 1450 mg/kg bw in males.

The LD_{50} for male rats was greater than 1450 mg/kg bw. The LD_{50} for female rats was 1481 mg/kg bw (Ruddock, 2000c).

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) of each sex per dose received TZMU (purity 99.3%; batch No. 89066326) dispersed in 5% w/v aqueous gum arabic by gavage at a dose level of 920, 1152, 1440, 1800 or 2250 mg/kg bw. The dose volume was 20 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

In a preliminary range-finding study, two rats of each sex were dosed at 200, 800, 1500, 4000 or 5000 mg/kg bw. Deaths occurred in both sexes at 1500 mg/kg bw and above, from hour 5 to day 2.

At all doses and in all animals, palpebral closure and lethargy were observed. Other commonly observed signs at 1152 mg/kg bw and above included proneness, ataxia, piloerection and hunched posture. At 1440 mg/kg bw, salivation, lacrimation and breathing irregularities were observed, as well as flaccidity and vasodilatation. Fatalities occurred at 1152 mg/kg bw and above on day 1 and day 2 (1152 mg/kg bw: 1/5 males, 3/5 females; 1440 mg/kg bw: 4/5 males, 4/5 females; 1800 mg/kg bw: 4/5 males, 3/5 females; 2250 mg/kg bw: 4/5 males, 5/5 females).

Table 45. Mammalian and environmental metabolites further assessed in toxicological studies[a]

Metabolite	Chemical name	Occurrence				
		R	G	H	P	E
TZNG	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine; thiazolylnitroguanidine	+	+	+	+	+
TZMU	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-methylurea; thiazolylmethylurea	+	+	−	+	+
TMG	(2-Chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-guanidine; thiazolmethyl-guanidine	+	+	+	+	+
MG	*N*-Methyl-*N*′-guanidine	+	−	−	+	+[b]
MNG	*N*-Methyl-*N*′-nitroguanidine	+	+	+	+	+
ATG-Ac	*N*′-[Amino(2-chlorothiazol-5-ylmethylamino)methylene] acetohydrazide	−	−	+	−	−
ATMG-Pyr	*N*′-[(2-Chlorothiazol-5-ylmethylamino)(methylamino)methylene]-2-oxo-propanohydrazide	−	+	−	−	−
MAI	3-Methylamino-1H-imidazo[1,5-c]imidazole	−	−	−	−	+
NTG or NG	Nitroguanidine	+	+	+	+	+

E, environmental; G, goat; H, hen; P, plant; R, rat

[a] Metabolites tested in acute oral toxicity (except for MNG) and bacterial mutagenicity studies.

[b] Photolytic breakdown product.

There were no relevant findings on body weight.

Necropsy findings at 1152 mg/kg bw and above were inflated/dark lungs, pale/mottled liver, yellow stomach mucosal surface and caecum impaction in decedents.

The LD_{50} for male rats was 1424 mg/kg bw. The LD_{50} for female rats was 1282 mg/kg bw (Ruddock, 1999a).

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) per dose received TMG (purity 96.0%; batch No. 30063517) dispersed in 5% w/v aqueous gum arabic by gavage at a dose level of 225, 650 or 1100 mg/kg bw (females) or 550 mg/kg bw (males). The dose volume was 20 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

In a preliminary range-finding study, two female rats per dose were administered the compound at 400, 570, 800, 1120 or 2000 mg/kg bw. Deaths occurred at 570 mg/kg bw and above, at 15 minutes to 3 hours after administration.

Lethargy, palpebral closure, tremors, piloerection and hunched posture were detected in the females at 225 mg/kg bw and above. At 650 mg/kg bw and above, animals exhibited proneness and twitching and occasionally breathing irregularities and ataxia.

Mortalities were observed from hour 1 and not later than day 2 (550 mg/kg bw: 3/5 males; 650 mg/kg bw: 4/5 females; 1100 mg/kg bw: 5/5 females).

There were no relevant findings on body weight.

Necropsy findings were dark/inflated lungs, dark liver and distension/reddening of the jejunum in decedents. Less commonly, lung and liver mottling and yellow jejunum were noted.

The LD_{50} for male rats was less than 550 mg/kg bw. The LD_{50} for female rats was 567 mg/kg bw (Ruddock, 1999c).

In an acute oral toxicity study with MG, five rats (Charles River CRL:CD®BR) of each sex per dose received MG·HCl (purity 98.8%; batch No. FHE05) dispersed in 5% w/v aqueous gum arabic by gavage at a dose level of 260, 355, 435, 530 or 650 mg/kg bw. The dose volume was 20 ml/kg bw.

The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC and OECD Guideline No. 401 (Acute Oral Toxicity; 24 February 1987).

In a preliminary range-finding study, two rats of each sex were dosed at 150, 500, 2000 or 5000 mg/kg bw.

Deaths occurred in both sexes at 500 mg/kg bw and above from 0.5 to 3 hours. Major clinical signs, although not at all doses, but treatment related, included lethargy, arched gait/posture, unkempt appearance, piloerection, tremor, ataxia, twitching, salivation and stained snout. Mortality occurred mostly on day 1, within 4 hours after administration (435 mg/kg bw: 3/5 females; 530 mg/kg bw: 1/5 males, 4/5 females; 650 mg/kg bw: 5/5 males, 5/5 females).

There were no relevant findings on body weight.

Necropsy findings included inflated/dark lung, distended/pale stomach and distension of the small intestine at 555 mg/kg bw and above in decedents. Other findings were renal pelvic dilatation, dark area of the spleen and enlarged mesenteric lymph nodes.

The LD_{50} for male rats was 550 mg/kg bw. The LD_{50} for female rats was 446 mg/kg bw (Ruddock, 1999b).

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) of each sex per dose received ATG-Ac (purity 94.4%[1]; batch No. 30068304) dispersed in corn oil by gavage at a single dose level of 2000 mg/kg bw. The dose volume was 10 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC.

In a preliminary range-finding study, two female rats per dose were dosed at 500, 1215 or 2000 mg/kg bw. No deaths occurred, either in the preliminary or in the main study.

In all males, tachypnoea was observed within 15 minutes up to 2 hours, whereas one in five males exhibited piloerection. The females showed no signs.

Body weight and necropsy were without relevant findings.

The LD_{50} for both male and female rats was greater than 2000 mg/kg bw (Ruddock, 2000a).

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) of each sex per dose received ATMG-Pyr[2] (purity 99%; batch No. 30070904) dispersed in 5% w/v aqueous gum arabic by gavage at a single dose level of 2000 mg/kg bw. The dose volume was 20 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC.

In a preliminary range-finding study, two female rats per dose were dosed at 500, 1215 or 2000 mg/kg bw.

No deaths occurred; no body weight changes, clinical signs or gross pathological changes were observed.

The LD_{50} for both male and female rats was greater than 2000 mg/kg bw (Ruddock, 2000a).

In an acute oral toxicity study, five rats (Charles River CRL:CD®BR) per dose received MAI (purity 96.0%; batch No. 30066002) dispersed in 5% w/v aqueous gum arabic by gavage at a dose level of 500, 650, 750 or 850 mg/kg bw (females) and 650 or 760 mg/kg bw (males). The dose volume was 20 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC.

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[1] Purity as stated on the analysis certificate. It was noted that degradation of the substance occurred in phosphate buffer within 3–15 minutes. When the HPLC analysis was conducted immediately after sample preparation, the compound was 97.8% pure.

[2] The compound was highly hygroscopic and apparently unstable, as storage under nitrogen was required.

In a preliminary range-finding study, two female rats per dose were administered the compound at 400, 600, 800 or 1000 mg/kg bw. Animals died or were killed on humane grounds at 800 mg/kg bw and above on day 2. Clinical signs were visible at 400 mg/kg bw and above, starting at 15 minutes after administration.

Lethargy and salivation were detected in all animals at all doses. Other common findings were lacrimation, palpebral closure, hunched/prone posture, piloerection, hypothermia and unkempt appearance. Additional findings at 750 mg/kg bw and above included stained snout, tremors, soft faeces, breathing disorders, stained tears/urine and ataxia. Most signs appeared on day 1 and were of various durations, some of them persisting until day 6. Mortality occurred from day 2 up to and including day 5 (650 mg/kg bw: 1/5 females; 750 mg/kg bw: 2/5 females; 760 mg/kg bw: 2/5 males; 850 mg/kg bw: 4/5 females).

Decreased body weight changes were observed in 1–2 rats per dose from the lowest dose to 750 mg/kg bw during the first observation week. Marked body weight loss was observed during the second week in the surviving rat at the top dose.

In the decedents, red/dark and inflated lungs, abnormal stomach contents/position, green surface in small intestine, gaseous distended and/or red jejunum, distended urinary bladder, renal pelvic dilatation and pale/mottled liver were observed. No abnormalities were detected in rats at termination.

The LD_{50} for male rats was not established precisely, but it was estimated to be between 850 and 2000 mg/kg bw. The LD_{50} for female rats was 758 mg/kg bw (Ruddock, 1999d).

In an acute oral toxicity study, seven rats (Sprague-Dawley) of each sex per dose received NTG (purity 99.6%; batch No. SOW 83H001-004) dispersed in methylcellulose/Tween 80 by gavage split into three injections given at hourly intervals (due to the viscosity of the compound) at a dose level of 5000 mg/kg bw. The dose volume was 10 ml/kg bw. The protocol was in compliance with test method B.1 of European Commission directive 92/69/EEC.

The predominant clinical signs associated with NTG administration were urinary excretion of a whitish precipitate (NTG) in the first 24 hours followed by excretion of a reddish urine for up to a week. NTG also affected the gastrointestinal tract, as it produced diarrhoea with perianal staining and irritation of the mucosa of the stomach and the small intestine.

Mortality was two of seven in the male group and two of six in the female group (one misdosed female was removed from the study).

The LD_{50} for both male and female rats was greater than 5000 mg/kg bw (Brown, Wheeler & Korte, 1988).

In an acute oral toxicity study taken from the open literature, five rats (Charles River F-344) of each sex received MNG (99.7%) dispersed in corn oil by gavage at a dose level of 1000 mg/kg bw. The dose volume was not reported. The protocol was not in compliance with test method B.1 of European Commission directive 92/69/EEC, and the study was not conducted according to GLP.

The LD_{50} (males and females) was in excess of 1000 mg/kg bw (Kinkead et al., 1993).

In summary (Table 46), some major (TZNG, > 5% of dose) or minor (TZMU, TMG, MG, < 5% of dose) rat metabolites are also detected in the hen, goat, plants and the environment. All of them have oral LD_{50} values less than 2000 mg/kg bw and should thus be considered intrinsically toxic by ingestion.

With clothianidin, clinical signs of transient neurological impairment were detected at the lowest dose tested (Cain, Sheets & Stuart, 2000; see also section 2.6(a)). Similar clinical signs were also observed with these metabolites, but at lower doses. Most clinical signs occurred, however, on the day

Table 46. Summary of oral acute toxicity of clothianidin metabolites in the rat

Metabolite	LD_{50} (mg/kg bw)		Purity (%)	Batch No.	Reference
	Males	Females			
TZNG	> 1450	1481	98.6	89066524	Ruddock (2000c)
TZMU	1424	1282	99.3	89066326	Ruddock (1999a)
TMG	< 550	567	96.0	30063517	Ruddock (1999c)
MG	550	446	98.8	FHE05	Ruddock (1999b)
ATG-Ac	> 2000	> 2000	94.4	30068304	Ruddock (2000a)
ATMG-Pyr	> 2000	> 2000	99.0	30070904	Ruddock (2000b)
MAI	> 850; < 2000	758	96.0	30066002	Ruddock (1999d)
NTG	> 5000	> 5000	99.6	SOW83H001-004	Brown, Wheeler & Korte (1988)
Clothianidin	> 1216, < 2000	> 523, < 1216	95.2	30037120	Sheets (2002a)

of administration. Other compounds, such as NTG (rat metabolite 1–4% of the administered dose), ATG-Ac (hen metabolite) and ATMG-Pyr (goat metabolite), were less toxic, with LD_{50} values greater than 2000 mg/kg bw.

Metabolite MG (CAS No. 471-29-4) may be considered a priori a potential plant residue of concern (known neurotoxicant, like most guanidino compounds), but it occurs only at very low levels. Thus, further testing on MG is not necessary.

Metabolite MNG was not tested for acute oral toxicity. However, as it was a rat metabolite accounting for about 13% of the dose, it is considered covered by the toxicological assessment of clothianidin.

Bacterial genotoxicity studies on metabolites

TZNG, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997).

Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to TZNG (purity 98.6%; batch No. 89066524) dissolved in DMSO. Final concentrations of TZNG were 0, 8, 40, 200, 1000 and 5000 µg/plate (test 1 ±S9 mix) and 0, 156.25, 312.5, 625, 1250, 2500 and 5000 (test 2 ±S9 mix). In test 2, 0.1 ml of bacteria was preincubated at 37 °C for 20 minutes in the presence of TZNG at the same final concentrations as in the treat-and-plate assay. In this test, the volume of test article was 0.05 ml rather than 0.1 ml, in an attempt to reduce possible solvent-induced toxicity, which may appear in the preincubation methodology.

In test 1, a decrease of bacterial background lawn was detected at the top dose in strain TA98 without S9. In test 2, a decrease of background lawn and/or decrease of spontaneous revertant number were observed in all strains except TA1535 in the presence and absence of S9. No reproducible increase of revertant numbers was observed in any strains, in either the presence or absence of S9.

TZNG is not mutagenic under these experimental conditions (Dawkes, 1999c).

TZMU, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The

study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997). Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to TZMU (purity 99.3%; batch No. 89066326) dissolved in DMSO, both in the presence and in the absence of S9 mix. Final concentrations of TZMU were 0, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate (test 2).

No bacteriotoxicity was detected in any strain at any dose. No TZMU treatment of any of the strains produced a statistically significant increase in revertant numbers in either the presence or absence of S9.

TZMU is not mutagenic under these experimental conditions (Dawkes, 1999a).

TMG, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997). Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to TMG (purity 96%; batch No. 30063517) dissolved in DMSO, both in the presence and in the absence of S9 mix. Final concentrations of TMG were 0, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate (test 2).

In test 2, thinning of the bacterial background lawn was observed at the top dose in the presence of S9 in all strains except TA1535 and TA102. No increase of revertant numbers was observed in any strain at any dose, either in the presence or in the absence of S9.

TMG is not mutagenic under these experimental conditions (Dawkes, 1999d).

MG, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997). Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to MG·HCl (purity 98.8%; batch No. FHE02) dissolved in purified water, both in the presence and in the absence of S9 mix. Final concentrations of MG were 0, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate (test 2).

Neither bacteriotoxicity nor increase of revertant numbers was detected in any strain at any dose, both in the presence and in the absence of S9.

MG is not mutagenic under these experimental conditions (Dawkes, 1999b).

MNG, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 2000/32/EC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997). Two tests were performed (plate incorporation [test 1] and preincubation [test 2]). Bacteria from strain *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) were exposed to

MNG (purity 97.3%; batch No. VE96026 Pt.17004/98) dissolved in DMSO at final concentrations of 0, 50, 158, 500, 1581 or 5000 µg/plate (all strains ±S9 mix) for 48 hours and in triplicate. In test 1 (plate incorporation test), 0.1 ml of an overnight culture of cells was exposed to the active ingredient and plated, whereas in test 2 (preincubation), the cells were preincubated at 37 °C for 20 minutes in the presence of the active ingredient.

No toxicity to any of the bacterial strains used was exhibited. In all experiments, MNG provoked no increase of revertant colonies compared with negative controls.

MNG is not mutagenic under these experimental conditions (Herbold, 2001).

ATG-Ac, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC. Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to ATG-Ac (purity 94.4%; batch No. 30068304) dissolved in DMSO, both in the presence and in the absence of S9 mix. Final concentrations of ATG-Ac were 0, 1.6, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate (test 2).

No bacteriotoxicity was detected in any strain at any dose. No ATG-Ac treatment of any of the strains produced an increase in revertant numbers sufficient to be considered as indicative of mutagenic activity in either the presence or absence of metabolic activation.

ATG-Ac is not mutagenic under these experimental conditions (Dawkes, 2000b).

ATMG-Pyr, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutation in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC. Two tests were performed (plate incorporation [test 1] and preincubation [test 2]). The experiment in test 1 on TA100 was conducted as a range-finding test. Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to ATMG-Pyr (purity 99.0%; batch No. 30070904) dissolved in DMSO, both in the presence and in the absence of S9 mix. Final concentrations of ATMG-Pyr were 0, 1.6, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate (test 2).

Neither bacteriotoxicity nor an increase of revertant numbers was detected in any strain at any dose, either in the presence or in the absence of S9.

ATMG-Pyr is not mutagenic under these experimental conditions (Dawkes, 2000a).

MAI, a metabolite of clothianidin, was investigated for its potential to induce mutations in an assay for gene mutations in *Salmonella* in the presence and absence of metabolic activation. The study was in compliance with test method B.14 of European Commission directive 92/69/EEC and OECD Guideline No. 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay; 21 July 1997). Two tests were performed (plate incorporation [test 1] and preincubation [test 2]; the experiment in test 1 on TA100 was conducted as a range-finding test). Overnight-grown cultures (0.1 ml) of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 were exposed to MAI (purity 99.9%; batch No. 30064522) dissolved in DMSO, both in the presence and in the absence of S9 mix. Final concentrations of MAI were 0, 8, 40, 200, 1000 and 5000 µg/plate (test 1) and 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate (test 2).

Background lawn thinning was observed in strain TA98 in the two tests at the top dose (±S9) and in test 2 at 2 mg/plate (−S9). No MAI treatment of any of the strains produced an increase in

revertant numbers sufficient to be considered as indicative of mutagenic activity in either the presence or absence of metabolic activation.

MAI is not mutagenic under these experimental conditions (Dawkes, 1999e).

In summary, the metabolites TZNG, TZMU, TMG, MG, MNG, ATG-Ac, ATGM-Pyr and MAI were adequately tested in the *S. typhimurium* reverse gene mutation assay, and none of them was shown to induce revertants above the spontaneous solvent control rate. These metabolites should be considered to be devoid of gene mutation potential.

Additional studies on the metabolite NTG

The environmentally relevant metabolite NTG (based upon estimated concentrations in the groundwater) was also a rat metabolite. In the rat metabolism study, it was demonstrated that NTG represented 2–4% of the dose. Therefore, taking into account the toxicity studies conducted using NTG, it can reasonably be assumed that the existing toxicity data fully cover the toxicity assessment of this compound.

The toxicological data demonstrate that the acute toxicity of NTG was low (> 5000 mg/kg bw per day; Brown, Wheeler & Korte, 1988) and that NTG has no primary irritating or sensitizing properties (Kinkead et al., 1993). Subchronic toxicity feeding studies (rats, mice) indicated that no adverse effects up to 1000 mg/kg bw per day have been observed. Various genotoxicity tests in vitro demonstrated that, overall, the compound was devoid of mutagenic activity (Krötlinger, 1992).

In a two-generation study, no maternal effects were observed at dietary concentrations up to and including 4000 ppm, and neither reprotoxic nor developmental parameters were affected in the litter up to and including the top dose (12 500 ppm) (Krötlinger, 1992). In a rat developmental study (gavage during GDs 6–15), both maternal and fetal toxicity were established at 316 mg/kg bw per day, and developmental toxicity was established at the same level, as skeletal growth retardation was observed at the top dose. No major malformations were observed (Krötlinger, 1992).

A study was conducted to examine the developmental toxicity of NTG in rabbits. The protocol was in compliance with test method B.31 of European Commission directive 92/69/EEC. Between 18 and 22 (top dose) inseminated rabbits (New Zealand White, mating ratio 1:2) per dose received NTG (purity 99%; batch Nos SOW84K010-A001 and SOW85F011-028) dissolved in 1% w/v carboxymethylcellulose by gavage at dose levels of 0, 100, 316 or 1000 mg/kg bw per day (dose volume 5 ml/kg bw) from GD 6 to GD 18 inclusive. The dosing suspensions were prepared once and kept refrigerated. Before daily dosing, refrigerated samples were warmed to attain room temperature by immersion in hot tap water for 30–60 minutes. Analytical verification and homogeneity of dosing formulations at the start of the study revealed that dose levels were acceptable when compared with nominal values. Stability was acceptable during 1 month after preparation. Negative control was obtained by treating with the vehicle.

During the period days 9–19 post-mating, six dams were found dead, and four moribund dams were euthanized. Feed consumption was decreased at the top dose during the period of treatment. Thereafter, consumption rebounded to control levels and was even greater than that of the control group. Gravid body weight was slightly lower than in controls in top-dose animals on days 18 and 29 after mating. However, when corrected for uterus weights, the difference from the control group was unremarkable on termination. As a consequence, body weight gain was negative at the top dose and slightly impaired at 316 mg/kg bw per day. Most signs (Table 47) were confined to the top-dose animals and included altered urine colour and consistency and low faeces production during the

Table 47. Oral teratogenicity study of NTG in rabbits (main study): maternal data

	Dose (mg/kg bw per day)			
	0	100	316	1000
Mortality no./18 (day of death):				
- found dead	0	0	0	3(11), 1(14), 1(15), 1(19)
- humanely terminated	0	0	0	1(9), 1(12), 2(19)
No. examined (gravid females)	13	15	15	18
Feed consumption:				
- days 6–18	—	—	—	↓39%
- days 18–29	—	—	—	↑
Body weight:				
- day 18	—	—	—	↓7.2%
- day 29	—	—	—	↓2.9%
- day 29 (corrected)	—	—	—	—
Body weight gain (kg):				
- days 6–18	+0.26	+0.23	+0.18	−0.10
- days 0–29 (corrected)	+0.21	+0.28	+0.28	+0.25
Clinical signs				
Thick, foamy urine:				
- days 6–18	2	5	7	16
- days 19–29	—	—	2	9
Red urine/red material in urine:				
- days 6–18	—	—	—	2
- days 19–29	—	—	—	1
Scant faeces:				
- days 6–18	1	1	0	9
- days 19–29	1	3	3	4
Red-stained paws:				
- days 6–18	—	—	—	2
Injured bloody toenails:				
- days 6–18	—	—	—	3
Mucus or rhinorrhoea:				
- days 6–18	—	—	—	2
Increased startle reflex:				
- days 6–18	—	—	—	3
Convulsions:				
- days 6–18	—	—	—	3
- days 19–29	—	—	—	1
Hypertonia:				
- days 6–18	—	—	—	5
- days 19–29	—	—	—	1
Hunched posture:				
- days 6–18	—	—	—	4

Table 47 (continued)

	Dose (mg/kg bw per day)			
	0	100	316	1000
Moved stiffly:				
- days 6–18	—	—	—	2
Disposition of animals				
Females inseminated	17	18	16	22
Dams died on test or humanely killed	0	0	0	10
Dams pregnancy not confirmed	0	0	0	2
Dams not pregnant	0	0	0	1
Dams pregnant	0	0	0	7
Dams examined on day 29				
Dams non-pregnant	4	3	1	1
Dams pregnant with live young	13	15	15	10
Dams pregnant with dead young	0	3	1	2
Dams pregnant with resorptions	3	13*	7	5*
Dams pregnant with only resorptions	0	0	0	1

From Coppes et al. (1988)
* $P < 0.05$ (Marascuilo's test of multiple comparison of proportions)

treatment period, signs that were also observed during the post-dosing period until termination. Other signs included injuries and stains on paws/toenails. Signs of neurological impairment were evident as cases of increased startle reflex, convulsions, increased muscle tightness and stiff movements, which were observed occasionally until study termination. In addition, occasional incidences of twitching, tremor, cyanosis, prostrate position, unconsciousness and salivation occurred, either during or after the treatment period. Other observed clinical signs were not dose related and considered incidental. No clear treatment-related effects were observed. The most frequent findings, occurring at comparable rates in all dose groups, including controls, were cysts on or dark red discoloured fallopian tubes.

The average numbers of corpora lutea and of implantations per litter were unaltered by the treatment (Table 48). The implantation rate (efficiency) was also not affected (i.e. no preimplantation loss was observed). However, the average number of resorptions as well as the resorption rate (post-implantation loss) were affected at all doses. Although dose dependency was not evident, statistically significant increases were observed at 100, 316 and 1000 mg/kg bw per day. The number of dead fetuses was also increased at 100 mg/kg bw per day and above, but again, no dose responsiveness was demonstrated, and the differences did not reach statistical significance. Fetal weights were about 14–17% lower at the top dose, compared with controls.

At the top dose, one individual exhibited a misplaced ureter and a cleft palate. Other findings at the top dose included a heart ventricle anomaly and an externally visible abdomen swelling. Other findings were observed at the low and/or intermediate dose, but not at the top dose. The absence of dose responsiveness of these findings, along with the singularity of the above-mentioned malformations, indicated that they were incidental. In contrast, the increased litter incidence of partially ossified sternebrae at 316 mg/kg bw per day and above was considered compound related. Further, absence of ossification in the olecranon, patellae and phalanges was observed at the top dose.

The maternal toxicity NOAEL was 100 mg/kg bw per day, and the maternal toxicity LOAEL was 316 mg/kg bw per day, based on decreased body weight (gain) and increased clinical signs.

The fetal toxicity NOAEL was 316 mg/kg bw per day, and the fetal toxicity LOAEL was 1000 mg/kg bw per day, based on decreased fetal weight.

Table 48. Oral teratogenicity study of NTG in rabbits: fetal data[a]

	Dose (mg/kg bw per day)			
	0	100	316	1000
Number of pregnant females	13	15	15	11
Corpora lutea	10.3 ± 2.0	11.2 ± 2.5	11.1 ± 1.7	11.0 ± 1.9
Implantations	9.5 ± 1.5	10.2 ± 1.8	9.3 ± 2.0	9.6 ± 2.0
Implantation efficiency[b] (%)	93.5 ± 9.9	92.9 ± 10.7	95.9 ± 18.3	90.1 ± 15.6
Resorptions[c]	0.2 ± 0.4	0.9 ± 0.5	0.5 ± 0.6	0.9 ± 1.4
Resorption rate[d] (%)	2.2 ± 4.1	9.3 ± 5.0*	6.6 ± 8.3*	9.7 ± 15.1*
Live fetuses	9.3 ± 1.3	9.0 ± 1.6	9.3 ± 3.0	8.7 ± 2.2
Live fetuses (%)	100.0 ± 0.0	97.5 ± 5.3	99.3 ± 2.8	98.2 ± 3.8
Dead fetuses	0.0 ± 0.0	0.3 ± 0.6	0.1 ± 0.3	0.2 ± 0.4
Dead fetuses (%)	0.0 ± 0.0	2.5 ± 5.3	0.7 ± 2.8	1.8 ± 3.8
Sex ratio (males/males + females)	0.424 ± 0.230	0.484 ± 0.202	0.441 ± 0.171	0.471 ± 0.136
Fetal weight (g):				
- males	42.8 ± 3.1	42.4 ± 5.3	43.6 ± 4.7	37.0 ± 7.0[#]
- females	43.4 ± 3.8	40.8 ± 5.7	41.3 ± 5.5	36.2 ± 6.7[#]
Fetal crown–rump length (cm):				
- males	10.5 ± 0.3	10.4 ± 0.4	10.6 ± 0.5	10.0 ± 0.7
- females	10.5 ± 0.4	10.3 ± 0.5	10.4 ± 0.5	9.9 ± 0.6
No. examined: fetuses (litters)	*121 (13)*	*135 (15)*	*131 (15)*	*87 (10)*
External/visceral examinations				
Swollen abdomen	—	—	—	1[e]
Ureter transversing midline	—	—	—	1[e]
Enlarged heart ventricle	—	—	—	1
Cleft palate[m]	—	—	—	1
Skeletal examinations				
Cleft palate[m]	—	—	—	1
Partially ossified sternebrae[v]	10 (5)	24 (8)	20 (10)	15 (8)
(% litter incidence)	(38%)	(53%)	(67%)	(80%)
Overall incidence				
Variations	46 (12)	64 (13)	61 (14)	52 (10)*

From Coppes et al. (1988)

m, malformation; v, variation; * $P < 0.05$ (Marascuilo's test of multiple comparison of proportions); [#] $P < 0.05$ (Newman-Keuls test)

[a] Values are group average litter data (± standard deviation), except when indicated otherwise.

[b] Implantation efficiency = (implantations/corpora lutea) × 100.

[c] Historical control (extracted from MARTA-MTA joint historical control data project, eight studies, 162 female Hra: (NZW)SPF, oral gavage studies (in-house historical control data were unavailable). Total resorptions/litter: mean 0.36, range 0–0.7.

[d] Resorption rate = (resorptions/implantations) × 100.

[e] Occurring in same fetus.

The developmental toxicity NOAEL was 100 mg/kg bw per day, and the developmental toxicity LOAEL was 316 mg/kg bw per day, based on increased skeletal variants (incomplete ossifications).

The apparently increased rate of resorptions per litter at 100 and 316 mg/kg bw per day was of dubious toxicological significance. As dose dependency was not evident and the incidences in the middle dose group (0.5) were within the range of what could be expected in control animals (up to 0.7), the relevance of the observation was questionable (Coppes et al., 1988).

Conclusion

In conclusion, metabolic activity in mammals and plants and hydrolytic activity in the environment result in the transformation of clothianidin to breakdown products that are relatively more toxic or of the same order of toxicity as the parent compound. As many of them are rat metabolites, occur at very low residue levels, are formed only under non-neutral acidity conditions or are environmentally not pertinent, the risk from exposure to these metabolites is considered very low, and further testing is not warranted.

(d) Pharmacological studies

In order to evaluate the pharmacological effects of clothianidin, a series of non-guideline studies were conducted according to GLP. Male CD (SD) SPF rats and male CD-1 (ICR) SPF mice were treated in vivo, and isolated ileum of male Hartley SPF guinea-pigs was treated in vitro, with clothianidin (purity 95.5%; batch No. 30037120). In the in vivo studies, the substance was dispersed in 0.5% (w/w or v/v) gum arabic solution and administered by single gavage at dose levels of 50, 100, 200 and 400 mg/kg bw (dose volume 10 ml/kg bw).

Both rats and mice were about 5 weeks of age (body weights: rats, 136–174 g; mice, 25–33 g), except for rats in the study of the circulatory system: 7 weeks of age (245–319 g). The doses were determined in previous range-finding tests in which three animals per dose were administered 1750, 2280, 2960, 3850 and 5000 mg/kg bw (rats) or 200, 280, 930, 550 and 770 mg/kg bw (mice) and observed during 1, 2, 4, 6, 8 and 12 hours and further daily for 1 week to monitor the appearance of any acute toxic signs and mortalities.

The study is accepted.

Methodologies

The study methodologies are described briefly as follows:

Mouse experiments:

(1) Effects on the central nervous system

(i) General physical condition and behaviour. Irwin's multiple observation method included alertness, passivity, stereotypy, grooming, vocalization, restlessness, irritability, reactivity, locomotor activity, response (touch, pain, startle), reflexes (Straub tail, pinna, corneal, ipsilateral flexor, pupil size), palpebral opening, exophthalmos, urination, salivation, lacrimation, writhing, piloerection, body temperature, skin colour, respiration, diarrhoea, tremors, twitching, convulsions, body position, gait (staggering, abnormal), muscle tone (limb, grip strength, body, abdominal), mortality. Observation periods after administration: 0.5, 1, 3, 6, 24 hours.

(ii) Anaesthetic effects. At 1 hour after administration, hexobarbital was injected intraperitoneally (80 mg/kg bw), and the animals were laid on an approximately 37 °C warming plate. Sleeping time, defined as the time between disappearance and recovery of the righting reflex, was recorded.

(2) Synergistic effects on convulsions (subthreshold stimulations)

(i) Electroshock stimulation. At 1 hour after administration, a current of 8.0 mA was applied via corneal electrodes (pulse duration 5 milliseconds; stimulation interval 10 milliseconds, stimulation period 0.6 milliseconds). Appearance of tonic flexor and extensor convulsions was monitored immediately after the current pulse.

(ii) Pentylenetetrazole stimulation. At 1 hour after administration, pentylenetetrazole was injected subcutaneously (55 mg/kg bw). Appearance of clonic and tonic extensor convulsions was monitored for 30 minutes after injection.

(3) *Effects on the gastrointestinal system.* At 1 hour after administration (subsequent to a fasting period of about 19 hours), charcoal (5% suspension in 5% gum arabic) was given by gavage (0.2 ml/animal). Animals were killed by cervical dislocation, and the gastrointestinal tract was isolated 30 minutes after charcoal gavage. Both length from pylorus to the farthest point reached by the charcoal (a) and total small intestine length (b) were measured. Transfer rate was expressed as a/b in %.

(4) *Effect on the skeletal muscles.* At 1, 3 and 6 hours after administration, animals were placed with their front paws on a horizontally stretched wire and observed as to whether they would grasp the wire with their hind paws within 10 seconds. Only those animals previously successful in grasping the wire within 5 seconds were selected for the experiment.

Rat experiments:

(1) *Effect on body temperature.* Before and 0.5, 1, 3 and 6 hours after administration, rectal temperature was registered with a digital thermometer.

(2) *Effect on the blood coagulation system.* At 1 hour after administration, blood was sampled from the inferior vena cava under ether anaesthesia. Trisodium citrate (3.2%) was added (1:9 v/v), plasma was separated by centrifugation (4 °C, 3000 revolutions per minute, 10 minutes) and both prothrombin time and activated thromboplastin time were determined using a fully automatic coagulometer.

(3) *Effects on the circulatory system.* Before and at 0.5, 1, 3 and 6 hours, systolic and mean blood pressure were determined plethysmographically by inserting the tail into the cuff of a non-intrusive automatic blood pressure meter. Heart beat was determined based on the pulse wave.

Guinea-pig experiments (in vitro):

Ileum from six male guinea-pigs (422–448 g, 6–8 weeks, killed by exsanguination under ether anaesthesia) was isolated and cut into strips (20–30 mm). Each strip was affixed to a Magnus bath containing 20 ml of Krebs solution (32 °C) and subjected to a static tensive load of approximately 0.5 g. The traction was measured by an isotonic transducer and recorded. Four strips per dose (under stabilized contraction condition) were exposed for 5 minutes to clothianidin, dissolved in DMSO, at final concentrations of 0 (solvent control), 10^{-6}, 10^{-5} and 10^{-4} mol/l, in the presence and absence of the contractile agonists acetylcholine (10^{-6} mol/l), histamine (3×10^{-6} mol/l) and barium (10^{-3} mol/l).

Range-finding study in rat

Mortality was observed at 3850 mg/kg bw (1/3 at day 4) and at the top dose (2/3 at 8 hours and day 6).

A decrease in spontaneous locomotor activity, prone posture and deep respiration were observed at each dose level. Additionally, deep respiration and tremors were observed at 2280 mg/kg bw and above, abnormal fur, prone posture and staggering gait at 2690 mg/kg bw and above, hunchback position at 3850 mg/kg bw and above, and convulsions and skin cyanosis at the top dose. The majority of the signs started on the day of administration, but some lasted until day 7 (last observation day). The maximal dose without fatalities was 3000 mg/kg bw.

Range-finding study in mouse

Mortality was observed at 550 mg/kg bw (1/3 at 4 hours) and at the top dose (2/3 at 1 hour and day 1).

A decrease in spontaneous locomotor activity, closed eyes and hypothermia were observed at each dose level. Additionally, staggering gait and tremors were observed at 390 mg/kg bw and above, hypothermia at 550 mg/kg bw and above, and closed eyes and skin cyanosis at the top dose. All signs

Table 49. General physical condition and behaviour after administration of clothianidin to female mice[a]

End-point	Dose (mg/kg bw)			
	50	100	200	400
Decrease in spontaneous locomotor activity	$3^{+;\ 0.5\ h}$	$3^{+,++;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h,\ 1\ day}$
Tremor	$1^{+;\ 0.5\ h}$	$1^{+;\ 0.5–1\ h}$	$3^{+,++;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h,\ 1\ day}$
Deep respiration	$1^{+;\ 1\ h}$	$3^{+;\ 0.5–3\ h}$	$2^{+;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h}$
Hypothermia	—	$3^{+;\ 1–3\ h}$	$3^{+;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h}$
Decrease in grooming	—	$3^{+;\ 0.5\ h}$	$3^{++;\ 0.5–1\ h}$	$3^{++;\ 0.5–6\ h}$
Mydriasis	—	$2^{+,++;\ 0.5–3\ h}$	$2^{+;\ 0.5–3\ h}$	$2^{+,++,+++;\ 0.5–6\ h}$
Decrease in reactivity	—	$1^{+;\ 0.5–3\ h}$	$3^{+;\ 0.5–3\ h}$	$3^{++;\ 0.5–6\ h}$
Prone position	—	$1^{+;\ 0.5–3\ h}$	$3^{+;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h}$
Staggering gait	—	$1^{+;\ 0.5\ h}$	$2^{+;\ 0.5–3\ h}$	$3^{+,++,+++;\ 0.5–6\ h,\ 1\ day}$
Decrease in body tone	—	$1^{+;\ 0.5–3\ h}$	$3^{+;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h}$
Decrease in abdominal muscle tone	—	$1^{+;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–3\ h}$	$3^{+,++;\ 0.5–6\ h}$
Decrease in touch response	—	—	$3^{+;\ 0.5–1\ h}$	$3^{+;\ 0.5–6\ h}$
Decrease in grip strength	—	—	$2^{+;\ 0.5\ h}$	$3^{+,++;\ 0.5–6\ h}$
Decreased limb tone	—	—	—	$3^{+,++;\ 1–6\ h}$
Inhibition of pinna reflex	—	—	—	$3^{+,++;\ 1–6\ h}$
Inhibition of ipsilateral flexor reflex	—	—	—	$1^{+++;\ 1\ h}$
Inhibition of corneal reflex	—	—	—	$1^{++;\ 1\ h}$
Straub tail	—	—	—	$1^{+;\ 1\ h}$
Skin cyanosis	—	—	—	$1^{++;\ 1\ h}$
Death	—	—	—	$13^{3\ h}$

From Unakami (2000)

[a] Observation conducted before and 0.5, 1, 3 and 6 hours and 1 day after dosing; control group (vehicle control): no observable effects. Values in table include number of animals with finding, degree of response ($^{+}$ slight, $^{++}$ moderate, $^{+++}$ severe) and duration of clinical sign (for all animals observed, i.e. n = 3/dose).

were remarkable on the day of administration and were resolved on day 2. Both onset and duration of the signs were correlated with the administered dose. The maximal dose without fatalities was 400 mg/kg bw.

General physical conditions and behaviour in mice

Effects were observed at 50 mg/kg bw and above (Table 49). In an additional separate experiment in which clothianidin was administered at 0, 12.5 and 25 mg/kg bw, no effects were observed.

Specific pharmacological effects in mice

The overall NOEL in these experiments was 12.5 mg/kg bw, based upon synergistic effects on convulsions at 25 mg/kg bw (Table 50).

Specific pharmacological effects in rats

From the results, it could be concluded that the NOEL was 100 mg/kg bw, when body temperature, heart rate and blood pressure data are considered. In an additional separate experiment in which clothianidin was administered at 0, 30 and 100 mg/kg bw, no statistically significant modification was detected at 100 mg/kg bw (Table 51).

Table 50. Specific pharmacological effects after administration of clothianidin to male mice

End-point	*n*	Dose (mg/kg bw)					
		0	6.25	12.5	25	75	225
Synergistic effects on convulsions[a]							
Electroshock[b]:							
- tonic flexor	10	1	3	2	8*	10*	10*
- tonic extensor	10	1	3	2	8*	10*	10*
Pentylenetetrazole:							
- clonic	10	0	n.a.	n.a.	2	0	0
- tonic extensor	10	2	n.a.	n.a.	0	0	0
Muscle tension suppression:							
- 1 h	8	0	n.a.	n.a.	0	0	4
- 3 h	8	0	n.a.	n.a.	0	0	4
- 6 h	8	0	n.a.	n.a.	0	0	2
Sleeping time[c]	8[d]	—	n.a.	n.a.	—	↑26%	↑63%*
Intestinal transfer rate[c]	8	—	n.a.	n.a.	—	↓60%*	↓78%*

From Unakami (2000)

n, number of mice/dose; n.a., dose level not tested; * $P < 0.05$ (chi-squared test and Dunnett's test)

[a] Chi-squared test.

[b] Results from two different experiments (trial 1 = dose 0, 6.25 and 12.5 mg/kg bw; trial 2 = dose 0, 25, 75 and 225 mg/kg bw; incidence equal in both controls).

[c] Dunnett's test.

[d] $n = 8$ mice/dose, except for the top-dose group, where $n = 6$ (two animals died after hexobarbital injection).

Effect on contraction agonists in guinea-pig ileum preparations

The effects of clothianidin on the contraction agonists acetylcholine, histamine and barium in isolated guinea-pig ileum preparations are illustrated in Table 52.

Conclusions

As was observed in the toxicological studies, the mouse was the most sensitive species for the effects of clothianidin. In the mouse, decrease of locomotor activity, tremors and deep respiration were observed at 50 mg/kg bw and above. At the higher doses, up to 400 mg/kg bw, clinical signs included hypothermia, mydriasis, abnormal gait/position and decreased muscle tone, indicating a nicotinic effect on the central nervous system.

The observed decreased limb tone at 400 mg/kg bw, decreased muscle strength at 225 mg/kg bw, decreased grip strength at 200 mg/kg bw and above and the reduced body tone and abdominal muscle tone at 100 mg/kg bw and above illustrate the ability of the compound to exert a suppression of the skeletal muscular system, with a NOEL for this effect at 50 mg/kg bw. The compound exerted an effect on the gastrointestinal system, as detected by charcoal transfer inhibition at 25 mg/kg bw and above. The NOEL for this effect is accordingly 12.5 mg/kg bw. This effect was also observed in vitro with guinea-pig ileum, where the contraction agonist barium was inhibited by about 12% by clothianidin. Sleeping time was lengthened at 75 mg/kg bw and above. At 225 mg/kg bw, the death of two of eight animals was considered the result of a synergistic action of clothianidin on the central respiratory centre depression by hexobarbital. At 25 mg/kg bw and above, both tonic flexor and tonic extensor convulsions were induced in the presence of subthreshold electroshock application. From these results, the NOEL for the central nervous effects of clothianidin is 12.5 mg/kg bw.

In the rat, the body temperature reduction at 300 mg/kg bw also indicates an effect on the central nervous sytem. The NOEL for this effect in the rat was 100 mg/kg bw. The NOEL for effects on the circulatory system was also 100 mg/kg bw (Unakami, 2000).

Table 51. Specific pharmacological effects after administration of clothianidin to male rats[a]

End-point	*n*	Dose (mg/kg bw)					
		0	30	100	300	1000	3000
Rectal temperature:							
- 0.5 h	6	—	—	—	↓1.3%	↓2.1%*	↓2.3%*
- 1 h	6	—	—	—	↓2.1%*	↓2.9%*	↓4.2%*
- 3 h	6	—	—	—	↓2.9%*	↓6.3%	↓10.3%
- 6 h	6	—	—	—	↓3.9%*	↓10.9%	↓17.0%
Heart rate:							
- 0.5 h	4	—	n.a.	—	↑12.3%*	↑6.5%	↑12.3%
- 1 h	4	—	n.a.	—	↑4.2%	↑4.4%	↑8.8%
- 3 h	4	—	n.a.	—	↑4.2%	↑6.4%	↑7.4%
- 6 h	4	—	n.a.	—	↑10.5%	↑10.0%	↑11.0%
Systolic blood pressure:							
- 0.5 h	4	—	n.a.	—	—	—	↓3.4%
- 1 h	4	—	n.a.	—	—	↓13%*	↓8.9%
- 3 h	4	—	n.a.	—	—	↓1.8%	↓4.5%
- 6 h	4	—	n.a.	—	↓1.8%	↓7.9%	↓11.4%
Mean blood pressure:							
- 0.5 h	4	—	n.a.	—	—	↓2.1%	↓4.2%
- 1 h	4	—	n.a.	—	—	↓15%*	↓10%
- 3 h	4	—	n.a.	—	—	↓5.4%	↓7.6%
- 6 h	4	—	n.a.	—	↓4.2%	↓15%*	↓12.5%
Prothrombin time, activated thromboplastin time	6	—	n.a.	n.a.	—	—	—

From Unakami (2000)

n, number of rats/dose; n.a., dose level not tested; * $P < 0.05$ (Dunnett's test trial 2)

[a] Results from two different experiments (trial 1 = dose 0, 30 and 100 mg/kg bw; trial 2 = dose 0, 300, 1000 and 3000 mg/kg bw). Results are given as % relative to control.

Table 52. Effect of clothianidin on contraction agonists in isolated guinea-pig ileum preparations

Concentration (mol/l)	% of contraction induced by each agonist (mean ± SD of four preparations/condition)		
	Acetylcholine (10^{-6} mol/l)	Histamine (3×10^{-6} mol/l)	Barium (10^{-3} mol/l)
0	101.6 ± 1.4	99.0 ± 2.0	101.9 ± 3.8
10^{-6}	101.1 ± 2.4	98.9 ± 1.8	98.1 ± 2.3
10^{-5}	104.4 ± 2.4	99.1 ± 1.2	99.2 ± 2.4
10^{-4}	98.8 ± 2.3	98.4 ± 1.7	89.5 ± 5.8*

From Unakami (2000)

SD, standard deviation; * $P < 0.05$ (Dunnett's test)

3. Observations in humans

No reports of adverse effects were identified during routine monitoring of production plant workers and among personnel involved in experimental biological testing or field trials. There is no evidence or information available to support any findings in relation to poisoning with clothianidin.

Comments

Biochemical aspects

Clothianidin was almost completely (90%) absorbed from the gastrointestinal tract within 24 hours following oral dosing of rats. The rate and extent of absorption were essentially independent of sex, dose or dose rate.

The compound was widely and homogeneously distributed throughout the tissues (time to maximum concentration = 1.5 hours), with a rapid decrease of residues to levels at or near the limit of quantification. There was no evidence of accumulation, although higher levels were detected in kidney and liver up to 4 hours post-dosing.

Within 24 hours, about 94–96% of the compound was excreted. Urinary excretion was the major elimination route, accounting for about 89–95%, with faecal elimination accounting for about 3–6%. The excretion profile over 72 hours after high-dose administration (elimination half-life = 1.9 hours) was almost identical to that after low-dose administration (elimination half-life = 1.2 hours), although the plasma concentration exhibited biphasic kinetics, suggesting moderate enterohepatic cycling.

Clothianidin metabolism was incomplete, with 56–74% of the dose being excreted unchanged over 72 hours. The main metabolic pathways were 1) oxidative demethylation and 2) cleavage of the nitrogen–carbon bond between the thiazolyl-methyl position and the nitroimino moiety. The main urinary metabolites recovered after low-dose testing were TZNG (7–11%), MNG (8–13%) and NTG (1–4%). In the faeces, MTCA (9%) and TMG (2%) were found. Other characterized metabolites were present at less than 2% of the dose.

Based upon the intended uses of clothianidin, representative metabolism studies in farm animals (goat, hen) were evaluated. It was demonstrated that the degradation pathways in farm animals were roughly comparable to those found in the rat (although absorption was probably somewhat lower) and that plant metabolism was less extensive. The major farm animal and plant metabolites (> 5% of the TRR) were also found in the rat, were structurally related to rat metabolites and/or were of lower toxicity. A notable exception was the plant metabolite MG, which is similar in toxicity to the parent compound, but which was observed only at low residue levels (maximally 0.25 ppm in sugar beet leaves at harvest).

Toxicological data

Clothianidin is of moderate acute oral toxicity, with an LD_{50} between 523 and 1216 mg/kg bw in rats and of 389 mg/kg bw in male mice. The dermal LD_{50} is greater than 2000 mg/kg bw, and the inhalation LC_{50} is greater than 5.54 mg/l (by gravimetry). Clothianidin is not irritating to the skin, is practically non-irritating to eyes and is not sensitizing to guinea-pig skin (maximization test).

In repeated-dose studies in mice, rats and dogs, no consistent toxicological profile was evident in any of the species at any of the dose ranges or study durations tested. Effects included lower body weights and body weight gains, decreased feed consumption and changes in some clinical chemistry parameters. In the rat, mild induction of hepatic CYP enzymes was observed in the 90-day feeding study. Hepatic induction was not assessed in either mouse or dog, although liver effects were also detected in dogs at a high dose.

In a 28-day feeding study in the mouse, atrophic changes in ovaries and uterus were reported at 2000 ppm (equal to 491 mg/kg bw per day). These changes in the reproductive system are considered to reflect the markedly reduced body weight gain.

Reports of increased ovary and uterus weights in a 90-day feeding study in rats at 1250 ppm (equal to 119 mg/kg bw per day for females) and above, accompanied by gross pathological and

histopathological findings (uterus fluid distension/uterus luminal dilatation), could not be confirmed in a second study. In the dog, the targets were the haematopoietic system and lymphoid organs (anaemia and leukopenia). The findings in the 30-day study were consistent with those found in the 90-day and 1-year study, with a peak effect around 5 weeks and a time-related adaptation at later times. The overall NOAEL for these effects was 1500 ppm (equal to 36.3 mg/kg bw per day).

The lowest relevant NOAEL for short-term studies was 500 ppm (equal to 27.9 mg/kg bw per day) from a 90-day study in the rat, on the basis of reduced body weight and body weight gain at 3000 ppm (equal to 202.0 mg/kg bw per day). A 90-day study in the mouse was considered unreliable due to deficiencies in the conduct of the study.

In an 18-month carcinogenicity study in mice with dietary concentrations of up to 2000 ppm, the onset of mortality in females occurred early in the study, and the overall mortality in females was increased. This was most likely due to exceedance of the MTD for a few months while the dose was adjusted. Body weight and body weight gain were reduced at 1250 ppm, and there was increased vocalization at this and the highest dose. There was an increase in hepatocellular hypertrophy at 1250 ppm and above. Fibromuscular hyperplasia of the cervix at 1250 ppm and above was observed, although such lesions are common in nulliparous ageing females. In males at 1250 ppm, there was increased incidence of myocardial degeneration. There was no statistically significant increase in the incidence of tumours of any site. The NOAEL was 350 ppm (equal to 47.2 mg/kg bw per day), based on body weight effects, clinical signs, and heart and cervical lesions at 1250 ppm.

Clothianidin was not carcinogenic in mice.

In a 24-month feeding study in rats with dietary concentrations up to 3000 ppm, feed consumption was reduced at 1500 ppm in males and at 500 ppm in females. Body weight and body weight gain were reduced in both sexes at 1500 ppm and above, mainly during the first year. Feed efficiency was unaffected. At the highest dose, there was clear histological evidence of local effects in the glandular stomach. The NOAEL for non-neoplastic effects in this study was 150 ppm (equal to 9.7 mg/kg bw per day), based on changes in terminal body weight and feed consumption at 500 ppm.

The incidence of hepatocellular carcinoma in male rats was slightly increased at 500 ppm (one at termination, two in unscheduled deaths) and at 3000 ppm (four in unscheduled deaths). As there was no relationship with dose or duration of treatment and as such tumours occur occasionally in untreated rats, it was concluded that these tumours were not compound related. Increases in the incidence of thyroid C-cell adenomas in the high-middle-dose and high-dose groups were not considered to be compound related, as there was no dose–response relationship in the incidence of adenomas plus carcinomas, and the combined incidence was not significantly increased in the top-dose group.

The Meeting concluded that clothianidin was not carcinogenic in rats.

The potential genotoxicity of clothianidin was tested in an adequate range of in vitro and in vivo studies. In general, clothianidin showed no evidence of mutagenicity. There was some evidence of clastogenicity in tests with mammalian cells in vitro at cytotoxic doses. Clothianidin was consistently negative in tests for genotoxicity in vivo.

The Meeting concluded that clothianidin was unlikely to be genotoxic in vivo.

On the basis of the absence of genotoxicity in vivo and the absence of carcinogenicity in the rat and the mouse, the Meeting concluded that clothianidin is unlikely to be carcinogenic in humans.

In a two-generation study of reproductive toxicity in rats at dietary concentrations up to 2500 ppm, both maternal and offspring toxicity were observed at 500 ppm and above, with decreased body weight (F_1, F_2) leading to lower body weight gains (F_1). Offspring toxicity was observed at the top dose and included delayed preputial separation and vaginal patency at clearly maternally toxic doses. The NOAEL for both parental and offspring toxicity was 150 ppm (equal to 10.2 mg/kg bw per day), based on decreased body weight at 500 ppm (equal to 32.7 mg/kg bw per day) for parental animals and on decreased body weight and subsequent effects on preputial separation at 500 ppm

(equal to 32.7 mg/kg bw per day) for offspring. The NOAEL for reproductive toxicity was 2500 ppm (equal to 179.6 mg/kg bw per day), the highest dose tested.

In rat developmental toxicity studies, the maternal NOAEL was 10 mg/kg bw per day, based on reductions in body weight gain and feed consumption. The NOAEL for developmental toxicity was 125 mg/kg bw per day, the highest dose tested.

In the rabbit, fetal and developmental toxicity occurred only at maternally toxic doses. The maternal NOAEL was 10 mg/kg bw per day, based on clinical signs (starting at gestation day 13) at 25 mg/kg bw per day, and the developmental NOAEL was 75 mg/kg bw per day, based on increased postimplantation loss, reduced fetal body weight and retarded sternal ossification at 100 mg/kg bw per day.

The Meeting concluded that clothianidin induced developmental toxicity only in the presence of maternal toxicity and that it was not teratogenic.

The acute neurotoxicity of clothianidin was investigated in three gavage studies in rats at doses up to 400 mg/kg bw. Clinical signs, including behavioural effects, were observed at the top dose on the day of treatment. Dose-dependent effects on arousal were observed at 100 mg/kg bw and above in males and at 200 mg/kg bw and above in females. There were no compound-related histopathological effects on neuronal tissue. The NOAEL for acute neurotoxicity was 60 mg/kg bw, on the basis of reduced locomotor activity in males at 100 mg/kg bw.

In a 13-week rat feeding study of neurotoxicity with dietary concentrations up to 3000 ppm, animals were assessed on weeks 4, 8 and 13 of clothianidin intake. No effects were observed on motor activity, learning or memory capacity. There were no histopathological changes in neuronal tissue. Thus, the NOAEL for neurotoxicity was 177 mg/kg bw per day, the highest dose tested.

A developmental neurotoxicity study was undertaken with clothianidin administered in the diet to rats at concentrations up to 1750 ppm (equal to 142 mg/kg bw per day during gestation and 299 mg/kg bw per day during lactation). The NOAEL for fetal and maternal toxicity was 42.9 mg/kg bw per day, based on changes in body weight at higher doses. At the top dose, subtle modification of acoustic startle habituation and motor activity observed in the pups immediately after weaning were considered secondary to nonspecific toxicity. No biologically significant effects on the central nervous system were observed histomorphometrically or histologically.

The Meeting concluded that clothianidin is not a developmental neurotoxicant. At relatively high doses, it can cause transient, acute neurobehavioural effects.

In a 28-day feeding study of the immunotoxicity of clothianidin in rats at doses up to 3000 ppm, body weights and feed consumption were significantly reduced in the high-dose group. Based on these changes, the NOAEL for systemic toxicity was 500 ppm (equal to 45.8 mg/kg bw per day). Clothianidin had no effect on the IgM AFC response to the T cell–dependent antigen (sheep erythrocytes). The NOAEL for immunotoxicity was 3000 ppm (equal to 252.8 mg/kg bw per day), the highest dose tested.

In a developmental immunotoxicity study in rats, pregnant animals were offered diets containing up to 2000 ppm clothianidin from day 6 of gestation. The maternal NOAEL for systemic toxicity was 500 ppm (equal to 35 mg/kg bw per day during gestation and 68.3 mg/kg bw per day during lactation), based on reductions in body weight and feed consumption and an increased incidence of ptosis at 3000 ppm. In the F_1 generation, the NOAEL for systemic toxicity was 150 ppm (equal to 26.4 mg/kg bw per day), based on reductions in body weight in males at weaning at 500 ppm. There were no immunologically relevant adverse effects on humoral immunity or cell-mediated immunity in male and female F_1 generation rats following exposure to clothianidin in the uterus during gestation, via maternal milk and maternal feed during the postpartum period or via the diet during the post-weaning period.

The Meeting concluded that clothianidin is not immunotoxic to adults or during development.

Some major (TZNG, > 5% of dose) and minor (TZMU, TMG, MG, < 5% of dose) metabolites of clothianidin in the rat are also detected in the hen, goat, plants and environment. They all have oral LD_{50} values less than 2000 mg/kg bw and should thus be considered intrinsically harmful by ingestion.

Other compounds, such as NTG (rat metabolite, 1–4% of the administered dose), ATG-Ac (hen metabolite) and ATMG-Pyr (goat metabolite), were less toxic, with LD_{50}s above 2000 mg/kg bw.

Metabolite MG may be considered a potential plant residue of concern (known neurotoxicant, like most guanidino compounds), but it is a rat metabolite and it occurred at very low residue levels in plant commodities used for animal feeding only. Thus, further testing on MG is not necessary.

Metabolite MNG was not tested for acute oral toxicity. However, as it was a rat metabolite accounting for about 13% of the dose, it was considered to be covered by the toxicological assessment of clothianidin.

Metabolites TZNG, TZMU, TMG, MG, MNG, ATG-Ac, ATGM-Pyr and MAI were tested in the *Salmonella typhimurium* reverse gene mutation assay, and all were negative.

In conclusion, metabolic activity in mammals and plants and hydrolytic activity in the environment result in the transformation of clothianidin to breakdown products that are relatively more toxic than or of the same order of toxicity as the parent compound. As many of these products are also rat metabolites, occur at very low residue levels and are not genotoxic, further testing is not warranted.

The Meeting concluded that the existing database on clothianidin was adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

An acceptable daily intake (ADI) of 0–0.1 mg/kg bw was established on the basis of the NOAEL in the chronic study in the rat of 9.7 mg/kg bw per day for decreased body weight and feed consumption. A safety factor of 100 was applied.

An acute reference dose (ARfD) of 0.6 mg/kg bw was established on the basis of the NOAEL of 60 mg/kg bw in the acute neurotoxicity study in the rat, based on reduced locomotor activity at 100 mg/kg bw. A safety factor of 100 was applied.

The Meeting considered that the effects seen in mice at 50 mg/kg bw per day in pharmacological studies were marginal and transient (less than 0.5–1 hour) at this dose level, whereas at the next dose level, 100 mg/kg bw per day, several effects were evident simultaneously in the same animals for longer times (3 hours).

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	350 ppm, equal to 47.2 mg/kg bw per day	1250 ppm, equal to 171.4 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 251.9 mg/kg bw per day[b]	—

Species	Study	Effect	NOAEL	LOAEL
Rat	Ninety-day studies of toxicity[a]	Toxicity[c]	500 ppm, equal to 27.9 mg/kg bw per day	1250 ppm, equal to 96.0 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[a]	Toxicity	150 ppm, equal to 9.7 mg/kg bw per day	500 ppm, equal to 32.5 mg/kg bw per day
		Carcinogenicity	3000 ppm, equal to 157 mg/kg bw per day[b]	—
	Two-generation study of reproductive toxicity[a]	Parental toxicity	150 ppm, equal to 10.2 mg/kg bw per day	500 ppm, equal to 32.7 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 10.2 mg/kg bw per day	500 ppm, equal to 32.7 mg/kg bw per day
		Reproductive toxicity	2500 ppm, equal to 179.6 mg/kg bw per day[b]	—
	Developmental toxicity study[d]	Maternal toxicity	10 mg/kg bw per day	40 mg/kg bw per day
		Embryo and fetal toxicity	125 mg/kg bw per day[b]	—
	Acute neurotoxicity study[d]	Neurotoxicity[c]	60 mg/kg bw	100 mg/kg bw
	Developmental neurotoxicity study[a]	Maternal toxicity	500 ppm, equal to 42.9 mg/kg bw per day	1750 ppm, equal to 142 mg/kg bw per day
		Offspring toxicity	500 ppm, equal to 42.9 mg/kg bw per day	1750 ppm, equal to 142 mg/kg bw per day
		Developmental neurotoxicity	1750 ppm, equal to 142 mg/kg bw per day[b]	—
	Immunotoxicity study[a]	General toxicity	500 ppm, equal to 45.8 mg/kg bw per day	3000 ppm, equal to 252.8 mg/kg bw per day
		Immunotoxicity	3000 ppm, equal to 252.8 mg/kg bw per day[b]	—
	Developmental immunotoxicity study[a]	Maternal toxicity	500 ppm, equal to 35–68.3 mg/kg bw per day	2000 ppm, equal to 120.6–249.7 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 26.4 mg/kg bw per day	500 ppm, equal to 88.9 mg/kg bw per day
		Developmental immunotoxicity	2000 ppm, equal to 337.7 mg/kg bw per day[b]	—
Rabbit	Developmental toxicity study[d]	Maternal toxicity	10 mg/kg bw per day	25 mg/kg bw per day
		Embryo and fetal toxicity	75 mg/kg bw per day	100 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity[a,e]	Toxicity	1500 ppm, equal to 36.3 mg/kg bw per day	2000 ppm, equal to 46.4 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Values for NOAEL and LOAEL taken from two different studies.
[d] Gavage administration.
[e] Two or more studies combined.

Estimate of acceptable daily intake for humans

0–0.1 mg/kg bw

Estimate of acute reference dose

0.6 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to clothianidin

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; 90% within 24 h
Distribution	Wide; highest concentrations in kidney and liver
Potential for accumulation	None
Rate and extent of excretion	Largely complete within 72 h
Metabolism in animals	Moderately metabolized; excreted unchanged at 56–74% at 72 h; main pathway was oxidative demethylation and cleavage of the nitrogen–carbon bond between the thiazolyl-methyl position and the nitroimino moiety
Toxicologically significant compounds in animals, plants and the environment	Parent compound and animal metabolites TZNG, MNG, NTG, MTCA and TMG; main plant metabolite is MG
Acute toxicity	
Rat, LD_{50}, oral	523–1216 mg/kg bw
Mouse, LD_{50}, oral	389 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.54 mg/l (4.5 h, nose-only exposure)
Rabbit, dermal irritation	Non-irritating
Rabbit, eye irritation	Practically non-irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson and Kligman test)
Short-term studies of toxicity	
Target/critical effect	Decreased body weights and body weight gain, decreased kidney weights, decreased feed consumption, clinical chemistry changes
Lowest relevant oral NOAEL	27.9 mg/kg bw per day (90-day study in rats)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased feed consumption and body weights
Lowest relevant NOAEL	9.7 mg/kg bw per day (rat carcinogenicity study)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Clothianidin unlikely to be genotoxic in vivo; metabolites not genotoxic (Ames test)
Reproductive toxicity	
Reproduction target/critical effect	None
Lowest relevant reproductive NOAEL	179.6 mg/kg bw per day (highest dose tested)

Developmental target/critical effect	Decreased fetal weight, increased postimplantation loss, decreased sternal ossification centres
Lowest relevant developmental NOAEL	75 mg/kg bw per day (rabbit)

Neurotoxicity/delayed neurotoxicity

Acute neurotoxicity target/critical effect	Decreased locomotor activity
Lowest relevant acute neurotoxic NOAEL	60 mg/kg bw per day
Short-term neurotoxicity target/critical effect	Decreased body weight and feed consumption
Lowest relevant subchronic neurotoxic NOAEL	60 mg/kg bw per day
Developmental neurotoxicity target/critical effect	No biologically significant effects
Lowest relevant developmental neurotoxic NOAEL	142 mg/kg bw per day (highest dose tested)

Other toxicological studies

Twenty-eight-day immunotoxicity	No effects on the immune system
Developmental immunotoxicity	No effects on the immune system

Medical data

	No data

Summary

	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw	Rat, 2-year study	100
ARfD	0.6 mg/kg bw	Rat, acute neurotoxicity study	100

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CYPROCONAZOLE

First draft prepared by
P.V. Shah[1], Midori Yoshida[2] and Maria Tasheva[3]

[1] Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, United States of America (USA)
[2] Division of Pathology, National Institute of Health Sciences, Tokyo, Japan
[3] National Service for Plant Protection, Sofia, Bulgaria

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Explanation

Cyproconazole is the International Organization for Standardization (ISO)–approved name for (2*RS*, 3*RS*, 2*SR*, 3*SR*)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)-butan-2-ol (International Union of Pure and Applied Chemistry [IUPAC]), for which the Chemical Abstracts Service (CAS) No. is 94361-06-5. The cyproconazole structure exists in four stereoisomeric forms as two diastereoisomeric pairs of enantiomers. Cyproconazole is a 1:1 mixture of the two diastereomeric pairs, each of which is a 1:1 mixture of the enantiomers (i.e. all four stereoisomers are present in similar amounts).

Cyproconazole is a broad-spectrum triazole fungicide. It acts by inhibiting sterol biosynthesis in fungi (demethylation inhibitor). Cyproconazole has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All pivotal studies with cyproconazole were certified as complying with good laboratory practice (GLP) unless otherwise stated.

Evaluation for acceptable daily intake

Unless otherwise stated, studies evaluated in this monograph were performed by GLP-certified laboratories and complied with the relevant Organisation for Economic Co-operation and Development (OECD) and/or United States Environmental Protection Agency (USEPA) test guidelines.

1. Biochemical aspects

1.1 Absorption, distribution and excretion

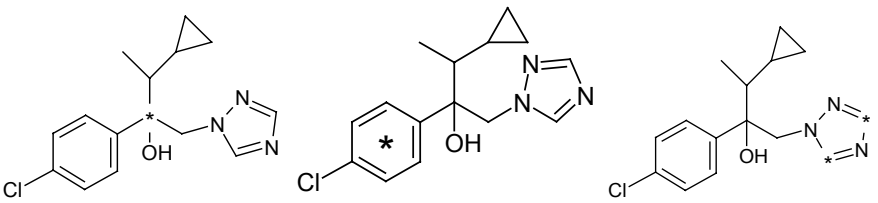
Rats

The absorption, distribution and elimination of cyproconazole were studied after oral dosing of cyproconazole radiolabelled with ^{14}C, as shown in Figure 1.

The absorption, distribution and metabolism of cyproconazole labelled with ^{14}C [α-(4-chlorophenyl)-α-(1-cyclopropyl-ethyl)-1H-1,2,4-triazole-1 (α-^{14}C) ethanol] (Figure 1) were studied in Kfm: WIST Wistar rats. Cyproconazole (both carbon labelled and non-radioactive) was dissolved in dimethyl sulfoxide (DMSO), and the radiochemical purity of the carbon-labelled cyproconazole was greater than 97%. Cyproconazole was administered either by gastric intubation or by injection into the femoral vein (0.3–0.5 ml volume). The specific activity of cyproconazole was 0.72 MBq/mg. The radioactive dose ranged from 5.7 to 9.5 MBq/kg body weight (bw) except for bile duct–cannulated animals (0.93–1.2 MBq/kg bw). Pretreatment in the multidose experiment was with unlabelled compound. The ratio of diastereomers was 1:1. The study design and sampling times are described in Table 1.

No significant $^{14}CO_2$ was detected in exhaled air in the pilot study with a 10 mg/kg bw single gavage dose. More than 85% of the administered dose was excreted into urine and faeces within 168 hours after dosing. Female rats excreted more of the administered dose via urine (35–42%) than did male rats (28–33%). The faecal elimination in female rats was less (43–54%) than that of male rats (59–69%). The bile duct–cannulated rats excreted approximately 76% and 60% of the administered dose in the bile in male and female rats, respectively (Table 2). Absorption was somewhat delayed in the high-dose animals but remained linear in the dose range. After a single oral low dose, the maximal blood levels were reached after 3 and 9 hours in males and females, respectively (Table 3). After the high oral dose administration, the maximum blood levels were reached later than in the low-dose

Figure 1. Position of the radiolabel on cyproconazole used in pharmacokinetic studies in rats



(α-^{14}C) ethanol label U-phenyl ring label Triazole label

* position of label

Table 1. Study design and sampling details of cyproconazole absorption, distribution and metabolism in Wistar rats

Experiment	Dose (mg/kg bw)	Route	No. of animals	Comments
Group 1	10	Intravenous	5 males/females	Sampling of urine was after 4, 7 and 24 h and at daily intervals thereafter. Faeces were collected at daily intervals. About 50 mg of blood was taken from the tail vein at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 24, 48, 72, 96, 120, 144 and 168 h. At 168 h post-dosing, 2 rats/sex were subjected to tissue distribution analysis by whole-body autoradiography and 3 rats/sex by liquid scintillation counting (organs).
Group 2	10	Oral gavage	5 males/females	Same as Group 1
Group 3	10	Oral gavage (bile duct–cannulated)	5 males/females	Urine, faeces and bile were collected at intervals of 0–24, 24–48, 48–72 and 72–144 h. Radioactivity in carcass was determined after 144 h.
Group 4	10 for 14 days	Oral gavage	6 males/females	Same as Group 1
Group 5	130	Oral gavage	5 males/females	Same as Group 1

study (at about 30–40 hours), indicating a lower absorption rate. The maximal blood radioactivity levels amounted to 2.2 and 2.5 µg/ml for a single 10 mg/kg bw dose in males and females, respectively. After pretreatment, these values were slightly lower. However, because of the similar urinary excretion in these experiments, pretreatment is considered not to influence the extent of absorption. Blood levels in females increased roughly linearly between the dose levels of 10 and 130 mg/kg bw. In males of the high-dose group, the somewhat lower maximum concentration in blood (C_{max}) was probably due to the delayed absorption. The radioactivity is eliminated from the blood with a half-life of about 25–30 hours, with no significant differences between sexes and dosing regimes (Table 3). Most tissues were found to contain some traces of radioactivity at the end of the study (168 hours); excretion was almost complete at this time. Approximately 75.5% (males) and 60% (females) of the dose were excreted in the bile by 144 hours. At least 90% of the biliary excretion occurred within the first 24 hours. About 20% of the radioactivity excreted by the bile was reabsorbed in the gastrointestinal tract. The urinary excretion accounted for 30% and 40% of the administered dose in males and females, respectively, which may reflect a reduction in the absorption of cyproconazole by males, as levels of radioactivity were found to be the same in the bile.

Tissue residues at study termination (168 hours; Table 4) were highest in liver and adrenals (mainly cortex), followed by the renal fat, kidney and spleen (after intravenous application only). In all experiments, the tissue concentrations were slightly higher in females than in males. There was no

Table 2. Cumulative excretion of radioactivity in rat urine, faeces and bile

Time interval (h)	% of administered dose									
	10 mg/kg bw p.o.		130 mg/kg bw p.o.		10 mg/kg bw p.o. bile cannulated		10 mg/kg bw p.o. multiple dose		10 mg/kg bw i.v.	
	M	F	M	F	M	F	M	F	M	F
Urine										
0–24	9.0	14.5	2.4	2.2	3.4	5.5	13.3	15.3	13.7	12.1
0–48	20.6	33.6	9.0	13.5	9.2	25.3	21.7	29.0	25.9	25.8
0–72	24.4	37.3	20.4	30.2	9.4	26.2	25.6	32.7	29.3	34.0
0–144	27.7	40.7	28.0	41.4	9.5	26.8	27.6	34.7	32.6	38.2
0–168	27.8	41.0	28.5	41.7	—	—	27.8	34.9	32.9	38.4
Faeces										
0–24	12.4	18.7	9.3	1.6	—	—	31.6	17.8	16.6	6.7
0–48	36.4	38.3	24.5	9.5	—	—	48.7	36.5	49.1	25.8
0–72	46.3	47.4	42.4	29.1	—	—	55.1	44.6	57.5	40.7
0–144	57.8	53.4	59.8	42.5	4.5	4.8	59.6	48.8	67.6	50.5
0–168	58.6	53.9	60.3	43.0	—	—	60.1	49.2	68.5	50.8
Bile										
0–24	—	—	—	—	73.9	53.8	—	—	—	—
0–48	—	—	—	—	74.7	57.4	—	—	—	—
0–72	—	—	—	—	75.3	59.0	—	—	—	—
0–144	—	—	—	—	75.5	59.6	—	—	—	—
Carcass	—	—	—	—	0.6	1.0	—	—	—	—
Total	86.5	94.9	88.8	84.8	90.0	92.1	88.0	84.1	101.3	89.2

From Schweitzer (1987a)
F, female; i.v., intravenous; M, male; p.o., per os (by mouth)

Table 3. Blood level parameters in rat

Parameter	10 mg/kg bw p.o.		130 mg/kg bw p.o.		10 mg/kg bw p.o. multiple dose		10 mg/kg bw i.v.	
	M	F	M	F	M	F	M	F
C_{max} (µg/ml)	2.2	2.5	13.4	23.6	1.7	1.2	4.1	2.9
T_{max} (h)	3	9	43	36	5	5	n.a.	n.a.
AUC[a] (µg·h/ml)	6.0	8.5	5.7	9.6	3.7	4.0	8.6	11.1
$t_{½}$ elimination (h)	29	31	25	30	30	33	26	29

From Schweitzer (1987a); Völlmin & Karapally (1992)
AUC, area under the concentration–time curve; F, female; M, male; n.a., not applicable; $t_{½}$, half-life; T_{max}, time to C_{max}
[a] Blood concentrations used for AUC calculation were corrected for dose.

unusual retention of compound-derived materials in the rats, and multiple dosing did not influence the distribution patterns.

In conclusion, absorption of cyproconazole was almost complete, independent of sex and dose regime. It was almost completely excreted in the bile and urine in 168 hours. After 7 days, residues in organs and tissues were very low (predominantly in liver and adrenal cortex via oral route), and there was no significant accumulation in the body (Schweitzer, 1987a).

Table 4. Residues in rat tissues 168 hours after dosing

Tissue	Residues (mg/kg bw equivalents of cyproconazole)							
	10 mg/kg bw p.o.		130 mg/kg bw p.o.		10 mg/kg bw p.o. multiple dose		10 mg/kg bw i.v.	
	M	F	M	F	M	F	M	F
Blood	0.05	0.06	0.39	0.52	0.04	0.03	0.07	0.10
Plasma	0.04	0.04	0.39	0.65	0.02	0.02	0.05	0.07
Brain	0.01	0.02	0.13	0.26	0.01	0.01	0.02	0.04
Testicles	0.02	n.a.	0.13	n.a.	0.01	n.a.	0.01	n.a.
Epididymis	0.02	n.a.	0.26	n.a.	0.01	n.a.	0.02	n.a.
Uterus	n.a.	0.04	n.a.	0.91	n.a.	0.03	n.a.	0.07
Ovaries	n.a.	0.10	n.a.	0.65	n.a.	0.05	n.a.	0.46
Muscle	0.01	0.02	0.13	1.04	0.01	0.01	0.03	0.04
Skin	0.05	0.06	0.78	4.55	0.03	0.08	0.05	0.12
Renal fat	0.19	0.26	2.73	4.42	0.07	0.10	0.33	0.51
Heart	0.02	0.04	0.26	0.52	0.02	0.02	0.04	0.09
Thymus	0.01	0.02	0.13	0.26	0.02	0.01	0.05	0.10
Pancreas	0.03	0.04	0.26	0.52	0.03	0.03	0.06	0.13
Thyroid	0.02	0.08	0.26	0.91	0.02	0.02	0.05	0.10
Bone marrow	0.02	0.02	0.13	0.39	0.01	0.02	0.15	0.13
Lymph nodes	0.02	0.03	0.26	0.52	0.02	0.03	0.05	0.08
Salivary glands	0.02	0.04	0.26	0.39	0.02	0.02	0.04	0.07
Spleen	0.02	0.04	0.26	0.52	0.02	0.02	0.55	0.50
Adrenal	0.32	0.54	3.25	5.33	0.17	0.21	0.64	0.96
Kidney	0.15	0.14	1.82	1.82	0.10	0.07	0.23	0.27
Lung	0.05	0.05	0.52	0.65	0.03	0.05	0.17	0.32
Liver	0.59	0.52	5.46	6.50	0.36	0.32	0.92	1.22

From Schweitzer (1987a)
F, female; i.v., intravenous; M, male; n.a., not applicable; p.o., per os (by mouth)

In a whole-body autoradiography study, ^{14}C-labelled cyproconazole [α-(4-chlorophenyl)-α-(1-cyclopropyl-ethyl)-1H-1,2,4-triazole-1 (α-^{14}C) ethanol] was administered to 10 female Kfm: WIST Wistar rats as a single dose of 10 mg/kg bw by gavage intubation in DMSO. Two rats were sacrificed at 3, 6, 24, 48 and 96 hours after dosing. Animals were immediately deep-frozen at −75 °C and embedded in a 2% semiliquid gel of sodium carboxymethylcellulose (CMC). Sagittal slices (40 µm thick) were prepared and dehydrated. Tissue concentrations of radioactivity were determined directly from the autoradiograms.

At all time intervals, most tissue levels were higher than that of blood, pointing to a large volume of distribution. The maximal value amounted to about 20 mg equivalent of cyproconazole per kilogram of tissue in the liver and adrenal. The maximal tissue levels reached at 6 hours post-dosing were in good agreement with the half-life of absorption estimated in the female rats (3.3 hours). The elimination of radioactivity from most tissues occurred monophasically with a half-life of 11 hours, except in liver (21 hours), blood (22 hours) and adrenal. In the adrenal, the elimination was rapid initially until 24 hours and then significantly slower, with a terminal half-life of 47 hours. At 96 hours, radioactivity in most tissues was below the limit of detection, except for liver, adrenal and kidney; the limit of detection was 100 µg equivalent of cyproconazole per kilogram of tissue (Schweitzer, 1987b).

In a separate rat metabolism study, [phenyl-U-^{14}C]cyproconazole (radiochemical purity 95.9%) in 2:2:1 polyethylene glycol 200:ethanol:water volume per volume (v/v) was administered to female HanBrl: WIST (SPF) rats by gavage at a nominal dose level of 0.5 mg/kg bw. Animals were dosed with the radioactive test substance daily for up to 14 days, and groups of four rats were killed 1, 7, 14 or 20 days after the first administration. The distribution of radioactivity between tissues was determined in all subgroups. Radioactivity in the urine, faeces and blood was measured, and metabolites in the urine and faeces were identified in the subgroup of rats killed 20 days after the first administration.

Recovery of [phenyl-U-^{14}C]cyproconazole was 97.1% of the total administered dose 7 days following 14 repeated doses (Table 5). An excretion steady state was reached 4 days after the first dose, with the majority of the total administered dose (96.5%) recovered in the faeces (56.3%) and urine (40.2%). The cage wash accounted for 0.19%, whereas the carcass/tissues retained less than 0.42% of the total administered dose. Three days after the final dosing, radioactivity was almost completely excreted. Blood kinetics showed increasing residue values with ongoing administrations, peaking at approximately 0.075 mg cyproconazole equivalents per kilogram within 8 days after the start of dosing. The highest blood levels were found 10 days after the start of dosing (0.08 mg cyproconazole equivalents per kilogram). After administration of the final dose, blood residues declined rapidly, with a depletion half-life of approximately 40 hours.

The distribution of radioactivity in tissues, blood and plasma is presented in Table 6. The levels of radioactivity reached a plateau by 7 days after the start of dosing and decreased rapidly once dosing ceased. The calculated half-life for the depletion of radioactivity (assuming monophasic first-order kinetics) from the tissues ranged from 1 to 3 days. The greater persistence and longer half-life of radioactivity in blood compared with plasma indicated some partitioning of radioactivity into the red blood cells. The highest concentrations of radioactivity were observed in the liver (1.37 mg cyproconazole equivalents per kilogram), adrenals (0.93 mg/kg), lungs (0.56 mg/kg), fat (0.49 mg/kg), kidneys (0.25 mg/kg), pancreas (0.22 mg/kg) and ovaries (0.16 mg/kg) 7 days after the start of dosing. All other tissues had concentrations of radioactivity that were comparable to or below the concentrations in blood over the time course.

1.2 Metabolism

The quantitative distribution of urinary metabolite fractions is presented in Table 7, and the nomenclature and structure of cyproconazole and its metabolites are presented in Appendix 1. Urine was found to contain at least 21 metabolite fractions by two-dimensional thin-layer chromatography (TLC). Individual fractions were detected in similar proportions on days 0–1, 6–7 and 13–14. The combined urinary metabolite fractions accounted for 20.8–41.0% of the daily administered doses. More than 92.5% of the radioactivity was extractable from faeces, accounting for 25.6–56.3% of the administered daily dose (Table 7). Faeces were found to contain at least 13 metabolite fractions by two-dimensional TLC. The combined faecal metabolite fractions accounted for 23.7–52.5% of the administered daily dose, whereas non-extractable radioactivity accounted for 1.9–3.8% of the daily dose. Two of the fractions (fractions 9 and 10) were identified as unmetabolized parent, and fraction 6 was identified as NOA 421153 (a diol metabolite of cyproconazole).

In summary, the orally administered test substance was rapidly absorbed from the gastrointestinal tract into the systemic circulation and was almost completely excreted within 3 days after the last dosing. Four days after the start of dosing, the amount of daily excretion remained nearly constant during the dosing period. The major part of the daily administered dose was excreted with the faeces, and a somewhat smaller part was excreted via the kidneys. The serial blood samples, taken at daily intervals during the whole experimental period, reached a plateau residue level at about 0.075 mg cyproconazole equivalents per kilogram within 8 days after the start of dosing. After the

Table 5. Mean recovery of radioactivity in excreta, tissues and carcass of female rats following 14 consecutive daily oral doses of [phenyl-U-^{14}C]cyproconazole[a]

Matrix	Time interval (days)	% of the administered dose
Urine	0–14	36.82 ± 1.73
	0–20	40.17 ± 1.93
Faeces	0–14	52.57 ± 2.74
	0–20	56.29 ± 2.48
Cage wash	0–20	0.19 ± 0.10
Total excretion	0–20	96.65 ± 1.47
Tissues	0–20	0.06 ± 0.02
Carcass	0–20	0.36 ± 0.10
Total residues	0–20	0.42 ± 0.12
Total recovery[b]	0–20	97.07 ± 1.37

From Hassler (2003)

[a] Data were obtained from Tables 5 and 6 on pages 37 and 38 of the study report; data are the mean of four rats.

[b] Defined as the sum of urine and faeces (0–20 days), cage wash, tissues and carcass.

Table 6. Tissue concentrations of radioactivity during and following 14 daily oral doses of [^{14}C] cyproconazole (0.5 mg/kg bw) to female rats[a]

Tissue	Concentration (mg cyproconazole equivalents/kg)				Half-life (h)
Days after start of dosing	1	7	14	20	—
Days after last dose	1	1	1	7	—
Adrenals	0.6461	0.9347	0.8639	0.0314	30
Blood	0.0288	0.0631	0.0687	0.0152	66
Brain	0.0544	0.0846	0.0864	0.0016	25
Fat	0.3298	0.4895	0.5078	0.0122	27
Kidneys	0.1403	0.2546	0.2615	0.0327	48
Liver	0.6591	1.3698	1.2526	0.0883	38
Lungs	0.3475	0.5618	0.5537	0.0185	29
Muscle	0.0366	0.0584	0.0599	0.0018	28
Ovaries	0.0959	0.1567	0.1531	0.0060	31
Pancreas	0.1093	0.2239	0.1901	0.0040	26
Plasma	0.0371	0.0658	0.0669	0.0028	31

From Hassler (2003)

[a] Data extracted from page 27 of the study report. Average of four rats per interval.

last of 14 consecutive daily doses, the blood residues declined rapidly, reaching half the maximum concentration within 3 days. The tissue residues determined at four different time points during and after the dosing period showed a profile similar to that observed in serial blood. The metabolite patterns of urine and faeces, investigated at three different time intervals during the dosing period, were essentially identical for all analysed time intervals. About 13% of the daily dose was excreted as unchanged parent via the faeces. Besides parent, the metabolite NOA 421153 was assigned to the faecal metabolite pattern, accounting for about 6% of the daily dose. There was no evidence for an accumulation of cyproconazole and/or related residues in tissues and organs (Hassler, 2003).

Table 7. Distribution of metabolite fractions in urine and faeces after single and multiple oral dose administration of [^{14}C]cyproconazole to female rats

Metabolite fraction	% of daily dose			Metabolite fraction	% of daily dose		
	Urine 0–1 day	Urine 6–7 days	Urine 13–14 days		Faeces 0–1 day	Faeces 6–7 days	Faeces 13–14 days
Fraction 1	0.9	1.8	1.3	Fraction 1	2.3	5.7	6.8
Fraction 2	0.8	1.8	1.3	Fraction 2	0.6	1.1	1.2
Fraction 3	2.4	4.3	3.1	Fraction 3	0.1	0.8	1.4
Fraction 4	1.2	3.3	3.0	Fraction 4	0.1	0.8	0.7
Fraction 5	1.2	1.9	1.4	Fraction 5	3.1	7.0	7.1
Fraction 6	0.2	0.4	0.4	Fraction 6 (NOA 421153)	2.2	6.2	6.9
Fraction 7	0.9	2.1	1.7	Fraction 7	2.2	3.3	3.4
Fraction 8	1.2	3.6	2.4	Fraction 8	0.3	1.1	1.3
Fraction 9	0.2	0.4	0.4	Fraction 9 (SAN 619 F)	1.1	1.1	1.1
Fraction 10	0.2	0.7	0.6	Fraction 10 (SAN 619 F)	8.4	12.8	12.3
Fraction 11	0.1	0.2	0.1	Fraction 11	2.4	7.0	7.5
Fraction 12	2.0	4.3	4.0	Fraction 12	0.5	1.3	1.4
Fraction 13	2.3	3.7	2.9	Fraction 13	0.4	1.2	1.3
Fraction 14	4.5	6.0	6.0	Non-extractable	1.9	3.6	3.8
Fraction 15	0.3	1.2	4.8				
Fraction 16	1.3	1.8	2.9				
Fraction 17	0.2	0.5	3.3				
Fraction 18	0.4	0.5	0.6				
Fraction 19	0.1	0.2	0.2				
Fraction 20	0.1	0.2	0.2				
Fraction 21	0.2	0.7	0.4				
Total	**20.8**	**39.8**	**41.0**	**Total**	**25.6**	**53.1**	**56.3**

From Hassler (2003)

The excreta (urine, faeces and the bile) analysed were derived from an earlier toxicokinetic study in the rat (Schweitzer, 1987a). Urine and faecal samples from the single oral low dose (10 mg/kg bw), single oral high dose (130 mg/kg bw) and repeated-dose (0- to 168-hour interval) experiments as well as bile from the single oral low dose study (0- to 144-hour interval) were collected and stored for analyses. The faecal samples of male and female rats from 0 to 168 hours were pooled. The faeces, bile and urine were successfully processed, and metabolites were extracted (methanol, acetone, dichloromethane) and evaporated. The extractable (organic and aqueous phase) radioactivity and non-extractable radioactivity were determined. An enzyme hydrolysis (β-glucuronidase, arylsulfatase) was also performed. The faecal samples were extracted by a suitable solvent, and separation of the metabolites was achieved mainly by preparative TLC. The extracts were concentrated and applied on other TLC plates for further separation by means of other solvent systems. For final separation and purification, the samples were subjected to reversed-phase high-performance liquid chromatography (HPLC). Information about the structure of the metabolites was obtained by nuclear magnetic resonance (NMR) spectroscopy. The structure was then determined by fast atom bombardment mass spectra and confirmed by electron impact mass spectra. Qualitative and quantitative compositions of metabolites in the various extracts (urine, faeces, bile) were determined by comparative TLC with reference standards. The radioactivity of the TLC scrapings was measured by liquid scintillation

counting. The radioactivity of the extractable and non-extractable faeces (after combustion) was determined by liquid scintillation counting.

Cyproconazole was extensively metabolized in the rat. In the excreta, 35 metabolites and the parent compound were detected. Approximately 10% of the unchanged cyproconazole was primarily in the faeces, and a trace amount (< 1%) was in the urine. Among the metabolites identified in the urine and faeces, 13 were more prevalent, and all other metabolites were in very low quantity (≤ 1%). Among the more prominent fractions in urine were NOA 421152 (M3 and M4), NOA 408616, NOA 421154 (M18) and NOA 452669 (M30/33) (Table 8). In faeces, NOA 421152 (M3 and M4) and NOA 421153 (M14) were the major metabolites besides parent (Table 9). Further metabolites at significant amounts were NOA 421152 (M4), NOA 421153 (M9), NOA 452154, NOA 451353, NOA 421154 (M18) and NOA 452668. The cyproconazole diastereomer A (= M1) appeared to be metabolized to a greater extent than the diastereomer B (= M2), which is corroborated by the lower amount of A (0.6–2.1%) excreted by the rat, compared with that of B (5.3–12.0%). This difference was also observed in slightly higher amounts of metabolites related to diastereomer A than to diastereomer B. The metabolic pathway does not appear to depend on the dosage or sex of the animals.

The metabolic pathway of cyproconazole in rats involved the following reactions:

- oxidative elimination of the triazole ring;
- hydroxylation of the carbon bearing the methyl group;
- oxidation of the methyl group to the carbinol and further to the carboxylic acid;
- reductive elimination of the carbon bearing the methyl group, yielding a benzyl alcohol, which is further oxidized to the corresponding ketone.

In summary, cyproconazole was extensively metabolized in rats, independent of the dose or sex of the animals. Diastereomer A (= M1) was transformed more extensively than diastereomer B (= M2) (Karapally, Völlmin & Spielmann, 1987a).

In the above-described metabolite identification study in rats by Karapally, Völlmin & Spielmann (1987a), diastereomer A of cyproconazole seemed to be more extensively metabolized than diastereomer B, as indicated by diastereomers A and B (M1 and M2, respectively) found in the excreta. Therefore, a separate study was conducted to confirm the differences in the metabolism of diastereomers A and B. In this study, two groups of six female Wistar rats received a single oral gavage dose of cyproconazole diastereomer A or B, each at 130 mg/kg bw. The radiochemical purity of ^{14}C-labelled cyproconazole diastereomer A and B was greater than 91% and 98%, respectively. DMSO was used as the vehicle for administration. Urine and faeces were collected at 24-hour intervals for 4 days following administration. Faecal samples were extracted with methanol. All faecal extracts from each of group A and group B were pooled and enzymatically hydrolysed (β-glucuronidase, arylsulfatase). All urine samples from each of group A and group B were pooled and then subjected to enzymatic hydrolysis (β-glucuronidase, arylsulfatase). A two-dimensional TLC system was used to determine the composition of metabolites in the various extracts. The radioactive spots were detected by photographic techniques. Quantification of the metabolites was performed by scraping radioactive spots from the plates, and the radioactivity was determined by liquid scintillation counting.

The excretion rates of both cyproconazole diastereomers A and B were found to be in the same range (Table 10). Animals administered diastereomer A excreted less test substance via the urine than did animals administered diastereomer B. In faeces, diastereomer A (= M1) was metabolized to a greater extent than diastereomer B (= M2). The amount of unchanged diastereomer A (3.4%) was approximately 5-fold less than the amount of unchanged diastereomer B (15.2%). This was further corroborated by the amounts of metabolites derived from diastereomer A compared with those derived from diastereomer B (Table 11).

Table 8. Composition of the total excreted (0–168 hours) radioactivity in rat urine (free and conjugated)

Metabolite No.	% of administered dose					
	Male			Female		
	Dose (mg/kg bw)					
	10	130	10	10	130	10
	Single	Single	Multiple	Single	Single	Multiple
SAN 619 A (M1)	< 0.1	< 0.1	0.1	0.1	< 0.1	< 0.1
SAN 619 B (M2)	< 0.1	0.1	0.1	0.3	0.3	0.3
NOA 421152 (M3)	0.8	1.0	1.9	4.4	4.9	4.1
NOA 421152 (M4)	0.2	0.5	0.3	1.4	2.1	1.0
M5	< 0.1	< 0.1	0.1	0.2	0.1	0.2
M6 and/or M7	0.2	—	0.3	0.4	0.4	0.3
M8	—	—	—	—	—	—
NOA 421153 (M9)	< 0.1	< 0.1	< 0.1	0.1	0.2	0.1
NOA 452154 (M10)	0.2	—	< 0.1	1.0	0.1	0.1
NOA 421154 (M11)	0.3	0.2	0.6	0.7	0.3	0.9
M12	0.3	0.5	0.8	0.7	0.6	0.7
NOA 451353 (M13)	0.3	< 0.1	0.2	0.2	< 0.1	0.2
NOA 421153 (M14)	0.7	0.7	0.8	1.6	1.4	1.2
NOA 408616 (M15)	0.6	1.6	0.7	1.0	2.1	0.5
CGA 123420 (M123420)	0.2	0.3	0.4	0.3	0.7	0.6
M17/35	0.1	0.1	0.1	0.2	0.2	0.1
NOA 421154 (M18)	0.3	—	0.2	0.9	0.4	2.0
M19	—	—	—	—	—	—
NOA 452668 (M20)	0.9	1.0	1.6	0.6	0.4	0.9
NOA 405870 (M21)	—	—	—	0.7	0.3	—
NOA 405872 (M22–M26/36)	1.6	1.8	0.8	1.9	1.7	1.2
M27	0.4	0.4	0.1	0.2	< 0.1	—
M28	—	—	—	—	—	—
M29	< 0.1	< 0.1	—	—	< 0.1	—
NOA 452669 (M30/33)	0.2	0.8	0.2	2.3	4.6	0.8
M31	—	—	—	0.2	0.2	—
M32		< 0.1	—	< 0.1	—	—
M34	—	—	—	—	< 0.1	—
M37	0.8	0.2	0.4	1.0	0.3	0.4
Sum of unknowns	—	< 0.1	—	0.3	—	—
Polar fraction	19.3	18.7	17.6	20.0	19.9	18.8
Non-extractable	—	—	—	—	—	—
Total	**27.8**	**28.4**	**27.8**	**41.0**	**41.7**	**34.9**

From Karapally, Völlmin & Spielmann (1987a)

Table 9. Composition of the total excreted (0–168 hours) radioactivity in rat faeces (free and conjugated)

Metabolite No.	% of administered dose					
	Male			Female		
	Dose (mg/kg bw)					
	10	130	10	10	130	10
	Single	Single	Multiple	Single	Single	Multiple
SAN 619 A (M1)	0.7	0.6	0.6	2.0	0.7	0.8
SAN 619 B (M2)	10.8	10.0	11.5	7.1	5.0	7.1
NOA 421152 (M3)	12.8	13.2	10.8	7.5	7.8	6.0
NOA 421152 (M4)	1.1	2.4	0.9	1.2	1.8	0.7
M5	0.1	0.4	< 0.1	0.3	0.2	< 0.1
M6 and/or M7	0.5	1.1	1.6	0.9	0.6	0.6
M8	—	—	0.9	0.3	0.3	0.6
NOA 421153 (M9)	2.7	3.7	2.1	0.8	1.3	1.2
NOA 452154 (M10)	2.0	0.4	2.0	0.8	0.2	0.9
NOA 421154 (M11)	0.8	0.9	1.3	0.9	0.3	0.6
M12	—	—	—	—	—	—
NOA 451353 (M13)	1.8	0.9	1.5	2.6	0.4	0.5
NOA 421153 (M14)	4.5	6.9	5.4	8.1	4.2	5.5
NOA 408616 (M15)	0.6	1.1	0.7	0.4	0.7	0.5
CGA 123420 (M123420)	0.1	0.5	< 0.1	0.2	1.2	0.3
M17/35	—	—	—	—	—	—
NOA 421154 (M18)	1.2	0.8	1.6	3.9	1.0	5.5
M19	—	0.4	< 0.1	—	—	—
NOA 452668 (M20)	5.1	2.7	5.1	1.1	0.8	2.6
NOA 405870 (M21)	0.1	0.2	—	0.2	0.2	—
NOA 405872 (M22–M26/36)	0.4	0.3	1.3	0.3	0.5	1.3
M27	—	—	—	—	—	—
M28	—	—	—	—	—	—
M29	—	—	—	—	—	—
NOA 452669 (M30/33)	0.3	0.3	—	< 0.1	0.5	0.2
M31	—	—	—	—	—	—
M32	< 0.1	< 0.1	< 0.1	< 0.1	0.2	—
M34	—	—	—	—	0.2	—
M37	—	—	—	—	0.3	< 0.1
Sum of unknowns	—	—	—	—	—	—
Polar fraction	7.7	7.8	6.7	8.4	8.9	9.2
Non-extractable	5.0	5.8	5.5	6.8	5.6	4.8
Total	**58.6**	**60.2**	**60.1**	**53.9**	**43.0**	**49.2**

From Karapally, Völlmin & Spielmann (1987a)

Table 10. Cumulative excretion of radioactivity in urine and faeces of rat[a]

Time period (h)	% of administered dose (mean ± standard deviation)	
	Diastereomer A	Diastereomer B
Urine		
0–24	2.5 ± 1.3	3.3 ± 1.4
0–48	14.5 ± 1.5	12.4 ± 3.2
0–72	29.8 ± 3.3	33.2 ± 7.0
0–96	39.0 ± 2.9	50.7 ± 2.6
Faeces		
0–24	—	2.3 ± 3.0
0–48	2.6 ± 2.2	6.8 ± 6.5
0–72	22.0 ± 16.0	9.3 ± 7.7
0–96	33.8 ± 13.5	28.5 ± 1.6
Total excretion		
0–96	72.8 ± 13.8	79.2 ± 4.1

From Karapally, Völlmin & Spielmann (1987b)
[a] Data extracted from Table 2 of the study report.

Eighteen metabolites from diastereomer A and 21 metabolites from diastereomer B were detected in the excreta (Table 12). At least five metabolites were formed from both diastereomers, indicating that no diastereomeric pair exists for these metabolites and that therefore at most one chiral centre is left in their chemical structures. (This was later proved not to be the case for NOA 405870 [M21] [Völlmin & Karapally, 1992].) The following metabolites are found to be diastereomeric pairs: NOA 421152 (M3/4), NOA 421153 (M9/14), NOA 421154 (M11/18) and NOA 452669 (M30/33).

In summary, the route of excretion and its temporal course were about in the same range for both diastereomers. Based on the amount of parent material found in the excreta, diastereomer A was more extensively biotransformed than diastereomer B. Based on the metabolite profiles and the structure of the metabolites, the course of metabolism was similar for both diastereomers. There were a number of differences with respect to the absence or presence of individual metabolites, depending on the individual diastereomer; however, these were at such low levels that they are not considered to be significant (Karapally, Völlmin & Spielmann, 1987b).

A separate metabolism study in Wistar rats was conducted to verify the presence or absence of two major metabolites (M21/M21a and M36), which were found in the milk and in the urine of lactating goats. Two groups of Han Ibm: Wistar rats (three of each sex per dose) were administered cyproconazole labelled with ^{14}C [α-(4-chlorophenyl)-α-(1-cyclopropyl-ethyl)-1H-1,2,4-triazole-1 (α-^{14}C) ethanol] via single oral gavage dose at 10 or 130 mg/kg bw. The radiochemical purity was greater than 98%. The animals were housed individually in metabolism cages for 7 days. The urine and faeces of each animal were collected separately at 7, 24, 48, 72, 96, 120 and 144 hours post-dosing. Urine samples were pooled separately for male and female rats and subjected to extensive extraction, portioning and enzymatic hydrolysis. Metabolites were isolated by TLC and identified by NMR spectroscopy at 360 MHz, with the structures verified by fast atom bombardment mass spectra. Final verification was by the comparison of the spectroscopic data with those of synthesized standards.

As a result of the low recovery of radioactivity, no further analysis was performed for the low-dose group. From the urine of the high-dose male rats, a mixture of the diastereomers NOA 405870

Table 11. Composition of the total excreted (0–96 hours) radioactivity in rat (free and conjugated)

Metabolite No.	% of excreted radioactivity					
	Diastereomer A			Diastereomer B		
	Urine	Faeces	Total	Urine	Faeces	Total
SAN 619 A (M1)	1.5	1.9	3.4	—	—	—
SAN 619 B (M2)	—	—	—	4.7	10.5	15.2
NOA 421152 (M3)	13.3	18.8	32.1	—	—	—
NOA 421152 (M4)	—	—	—	11.4	1.9	13.3
M5	0.2	—	0.2	—	—	—
M6 and/or M7	0.5	0.5	1.0	0.7	0.3	1.0
M8	—	0.8	0.8	—	—	—
NOA 421153 (M9)	—	—	—	1.0	0.9	1.9
NOA 452154 (M10)	—	—	—	0.6	< 0.1	0.6
NOA 421154 (M11)	—	—	—	1.2	< 0.1	1.2
M12	—	—	—	1.0	< 0.1	1.0
NOA 451353 (M13)	—	—	—	0.4	< 0.1	0.4
NOA 421153 (M14)	4.4	8.5	12.9	—	—	—
NOA 408616 (M15)	2.8	—	2.8	5.7	0.1	5.8
CGA 123420 (M16)	0.8	< 0.1	0.8	4.6	< 0.1	4.6
M17	0.3	< 0.1	0.3	0.2	< 0.1	0.2
NOA 421154 (M18)	0.7	1.3	2.0	—	—	—
M19	—	—	—	0.3	0.1	0.3
NOA 452668 (M20)	0.1	0.4	0.5	—	—	—
NOA 405870 (M21)	0.2	—	0.2	0.8	< 0.1	0.8
M22	—	—	—	0.4	0.1	0.5
M23	—	—	—	0.2	—	0.2
M24	—	—	—	0.4	—	0.4
M25	0.2	—	0.2	—	—	—
M26	—	—	—	0.1	< 0.1	0.1
M27	0.2	—	0.2	0.2	—	0.2
M28	< 0.1	< 0.1	< 0.1	—	—	—
M29	< 0.1	< 0.1	< 0.1	—	—	—
NOA 452669 (M30/33)	5.4	0.6	6.0	4.6	< 0.1	4.6
M31	—	—	—	0.1	—	0.1
M32	0.2	—	0.2	—	—	—
M34	0.1	0.3	0.4	—	—	—
M35	—	—	—	—	< 0.1	< 0.1
NOA 405872 (M36)	—	—	—	—	0.1	0.1
Sum of unknowns	0.4	—	0.4	0.2	0.3	0.5
Conjugates	—	0.4	0.4	—	0.9	0.9
Polar fraction + non-extractable	23.4	11.1	34.5	23.9	21.5	45.4
Total	**55.0**	**45.0**	**100.0**	**62.7**	**37.3**	**100.0**

From Karapally, Völlmin & Spielmann (1987b)

Table 12. Composition of the total excreted (0–96 hours) radioactivity in rat (free and conjugated)

Metabolite No.	% of excreted radioactivity					
	Diastereomer A			Diastereomer B		
	Urine	Faeces	Total	Urine	Faeces	Total
SAN 619 A (M1)	1.5	1.9	3.4	—	—	—
SAN 619 B (M2)	—	—	—	4.7	10.5	15.2
NOA 421152 (M3)	13.3	18.8	32.1	—	—	—
NOA 421152 (M4)	—	—	—	11.4	1.9	13.3
M5	0.2	—	0.2	—	—	—
M6 and/or M7	0.5	0.5	1.0	0.7	0.3	1.0
M8	—	0.8	0.8	—	—	—
NOA 421153 (M9)	—	—	—	1.0	0.9	1.9
NOA 452154 (M10)	—	—	—	0.6	< 0.1	0.6
NOA 421154 (M11)	—	—	—	1.2	< 0.1	1.2
M12	—	—	—	1.0	< 0.1	1.0
NOA 451353 (M13)	—	—	—	0.4	< 0.1	0.4
NOA 421153 (M14)	4.4	8.5	12.9	—	—	—
NOA 408616 (M15)	2.8	—	2.8	5.7	0.1	5.8
CGA 123420 (M16)	0.8	< 0.1	0.8	4.6	< 0.1	4.6
M17	0.3	< 0.1	0.3	0.2	< 0.1	0.2
NOA 421154 (M18)	0.7	1.3	2.0	—	—	—
M19	—	—	—	0.3	0.1	0.3
NOA 452668 (M20)	0.1	0.4	0.5	—	—	—
NOA 405870 (M21)	0.2	—	0.2	0.8	< 0.1	0.8
M22	—	—	—	0.4	0.1	0.5
M23	—	—	—	0.2	—	0.2
M24	—	—	—	0.4	—	0.4
M25	0.2	—	0.2	—	—	—
M26	—	—	—	0.1	< 0.1	0.1
M27	0.2	—	0.2	0.2	—	0.2
M28	< 0.1	< 0.1	< 0.1	—	—	—
M29	< 0.1	< 0.1	< 0.1	—	—	—
NOA 452669 (M30/33)	5.4	0.6	6.0	4.6	< 0.1	4.6
M31	—	—	—	0.1	—	0.1
M32	0.2	—	0.2	—	—	—
M34	0.1	0.3	0.4	—	—	—
M35	—	—	—	—	< 0.1	< 0.1
NOA 405872 (M36)	—	—	—	—	0.1	0.1
Sum of unknowns	0.4	—	0.4	0.2	0.3	0.5
Conjugates	—	0.4	0.4	—	0.9	0.9
Polar fraction + non-extractable	23.4	11.1	34.5	23.9	21.5	45.4
Total	**55.0**	**45.0**	**100.0**	**62.7**	**37.3**	**100.0**

From Karapally, Völlmin & Spielmann (1987b)

(M21/M21a) was isolated. Based on the NMR spectrum, the ratio of isolated NOA 405870 (M21 to M21a) was 10:1. The free extractable amounts of these metabolites accounted for 0.06% and 0.04% of the administered dose in urine of males and females, respectively. NOA 405872 (M36) was not detected in the urine of male and female rats in this study (Völlmin & Karapally, 1992).

A separate metabolism study was conducted to determine the presence of NOA 405872 (M36) in the excreta of rats. This metabolite was detected in the milk and excreta of lactating goats. Two groups of Han Wistar rats (three males and three females per group) were administered an oral dose of [^{14}C-triazole]cyproconazole (purity > 98%) at about 5 mg/kg bw and 130 mg/kg bw. Urine and faeces were collected separately daily for 4 days. Approximately 20% of the urine from male and female rats of the same group was pooled for extraction. The urine samples were acidified and fractionated by solid-phase extraction. The eluted methanol fractions were reduced to a small volume for TLC analysis. For both groups, equal fractions of the faeces of each animal (20% for group A and 10% for group B) were pooled and homogenized. The faecal samples were extracted several times with methanol, and the extracts were pooled and concentrated to a small volume for TLC analysis. Isolation and purification of cyproconazole metabolite NOA 405872 were done by a series of TLC runs in different solvent systems. Identification was confirmed by two-dimensional TLC with a reference standard, by HPLC co-chromatography and by mass spectroscopy.

NOA 405872 (M36) accounted for 0.056% of rat urinary radiocarbon in the low-dose group (0.022% of the applied dose) and 0.037% in the high-dose group (0.019% of the applied dose). In faeces, NOA 405872 (M36) accounted for 0.014% of the recovered radiocarbon in the low-dose group (0.006% of the applied dose) and 0.011% in the high-dose group (0.003% of the applied dose).

In this study, the presence of NOA 405872 (M36) in the urine and faeces of rats was confirmed (Briswalter & Yu, 1994).

An in vitro metabolism study was performed in order to compare the formation of metabolites of cyproconazole during incubation with subcellular liver fractions of the rat and mouse. Liver samples from Han Wistar rats and CD-1 mice treated with cyproconazole were taken from a previously conducted study. Two rats and two mice were used from the high-dose groups after 4 weeks of treatment and from the control groups. High-dose rats were treated with 1400 ppm (107 mg/kg bw per day) and high-dose mice with 200 ppm (28 mg/kg bw per day). Livers were frozen by immersion in liquid nitrogen and kept frozen at about −70 °C. Subcellular fractions (microsomes and cytosol) were prepared by tissue homogenization and centrifugation at 9000 × *g* and at 105 000 × *g*, respectively. In vitro incubations were performed for 5 or 120 minutes at 37 °C using subcellular fractions (microsomes about 3.0–3.2 mg protein per millilitre and cytosol about 2.3–2.5 mg protein per millilitre) in Tris HCl, about 35 MBq/ml [^{14}C]cyproconazole and one equivalent of "cold" cyproconazole. The following cofactors were added to the assay: reduced nicotinamide adenine dinucleotide phosphate (NADPH) (3 mmol/l), flavin adenine dinucleotide (50 µmol/l), pyridoxal phosphate (20 µmol/l) and glutathione (GSH) (1 mmol/l). All incubations were terminated with ice-cold methanol. Analysis was performed using an HPLC system with ultraviolet (UV) diode array and/or radiochemical detector. The non-radioactive standards were detected from their UV signal at 288 nm. In order to reduce interference from endogenous compounds and cofactors, backflush methodology was used.

Cyproconazole was metabolized in vitro by rat liver fractions from control and pretreated animals after 120 minutes of incubation at a rate of 6.5% and 20.5%, respectively (Table 13). In contrast to rats, control mice exhibited a much slower rate of metabolism in vitro, approximately 0.8% after 120 minutes. In the presence of pretreated mouse liver fractions, the in vitro metabolism of cyproconazole was increased to about 5.6% after 120 minutes.

Table 13. In vitro metabolic rate of cyproconazole

Species	Metabolic rate (%)		Fold induction by pretreatment
	Control	4-week pretreated	
Rat	6.5	20.5	3.2
Mouse	0.8	5.6	7.2

From Dorobek & Müller (1995)

A spectrum of about 20–30 different metabolites was observed during the in vitro incubations. The metabolites NOA 421154 (M11 and M18), NOA 421153 (M9 and M14) and NOA 421152 (M4) could be identified from control and pretreated rats. Metabolites CGA 123420 and NOA 408616, however, were found only in very small quantities in pretreated rats only. The metabolic pattern was identical in control and pretreated liver fractions, at least with regard to the relevant peaks/metabolites and differing only in the rate of metabolism. The dominant metabolites in both control and pretreated liver fractions were the diastereomers NOA 421153 (M9/M14), which together amounted to about 50% of all the metabolites. Although the metabolic rate of cyproconazole in the presence of mouse liver fractions was lower than that of the rat, similar spectra of metabolites were found. The metabolites NOA 421154 (M11), NOA 421153 (M14), NOA 421154 (M18), NOA 421152 and NOA 408616 could be identified in control mouse incubations. The metabolite NOA 421153 (M9) was not formed, whereas the peak area of metabolite NOA 421152 (M4) was not increased, compared with the corresponding blank value. During incubations in the presence of pretreated mouse liver fractions, a similar pattern of metabolites was detected, compared with control liver. Furthermore, the metabolites M9 and M4 could be identified in at least one animal. In contrast to the rat metabolite profile, the mouse metabolite profile showed no one predominating metabolite. Also noted was the fact that the diastereomer pairs NOA 421252 (M3/M4), NOA 421153 (M9/M14) and NOA 421154 (M11/M18) occurred at similar levels.

The results of this study demonstrate that the in vitro clearance capacity of control and pretreated rat liver for cyproconazole is about 8- and 4-fold higher, respectively, compared with the mouse liver. Although metabolism in mouse liver fractions occurred at a lower level, the increase between control and pretreated liver was twice that of the rat. The data clearly show the inducibility of the respective enzyme systems in both species. The rat liver in vitro metabolism of cyproconazole follows mainly one route (i.e. to metabolites NOA 421153 [M9/M14]), whereas the mouse liver formed the metabolites NOA 421152 (M3/M4), NOA 421153 (M9/M14) and NOA 421154 (M11/M18) to a similar extent. The patterns of the major metabolites found in the incubations with rat liver fractions are essentially in agreement with the metabolism described for the rat in vivo. The comparison indicates some quantitative and qualitative differences in the in vitro metabolism of cyproconazole in rat and mouse liver (Dorobek & Müller, 1995).

The proposed metabolic pathway of cyproconazole in rats is shown in Figure 2.

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity of cyproconazole is summarized in Table 14.

Figure 2. Proposed metabolic pathway of cyproconazole in rats (modified from Völlmin & Karapally, 1992)

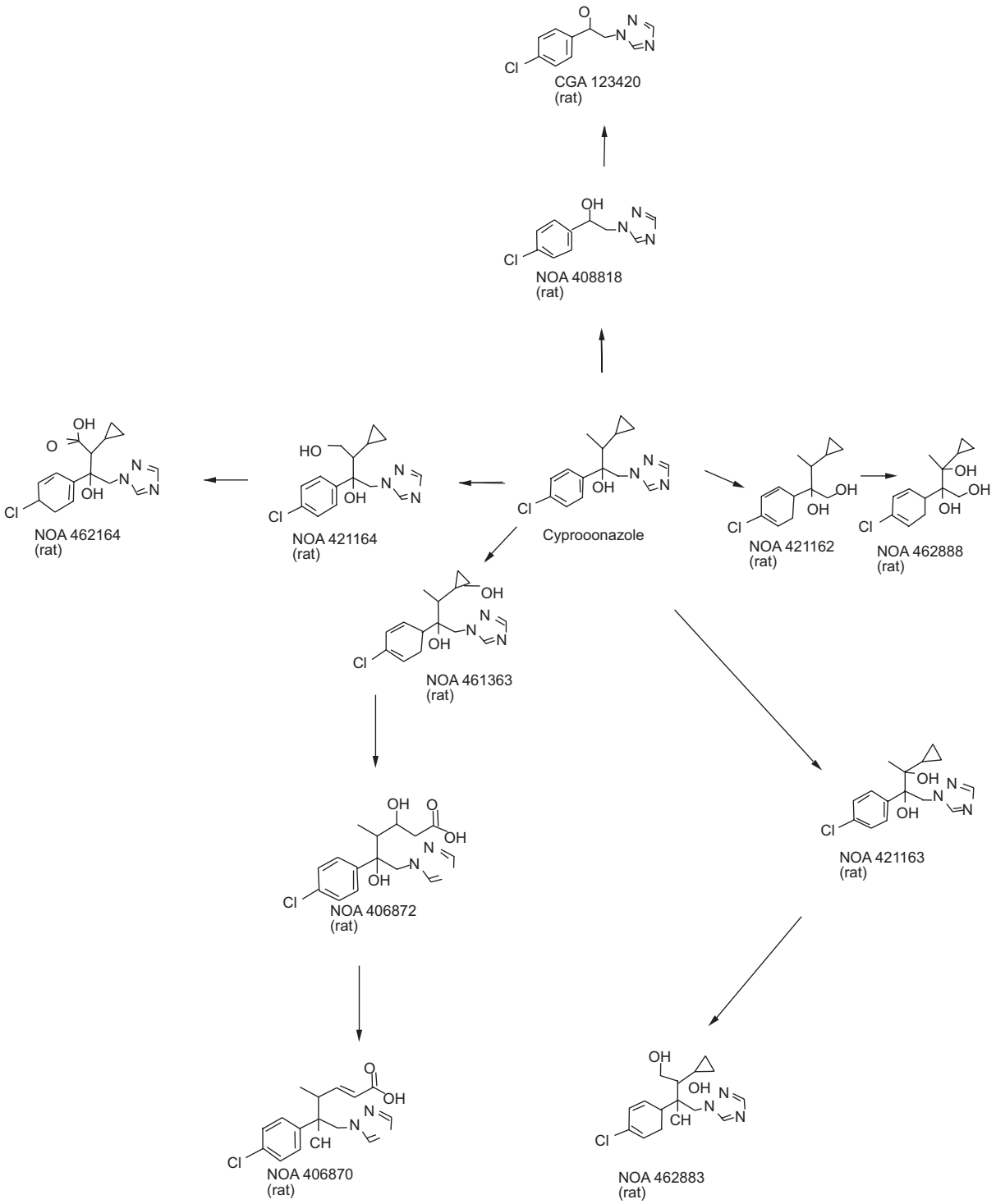


Table 14. Acute toxicity of cyproconazole

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Reference
Mouse	NMRI	M, F	Oral	M: 200 F: 218	—	Hamburger et al. (1984b)
	CD-1	M	Oral	M: 270	—	Hamburger (1987)
Rat	Han Wistar	M, F	Oral	M: 1115 F: 1342 Combined 1290	—	Hamburger et al. (1984a); Hamburger (1988)
	Sprague-Dawley	F	Oral	F: 350	—	Durando (2005a)
Rabbit	New Zealand White	F	Oral	F: 460	—	Hamburger, Gerber & Klotzsche (1985a)
Rat	Han Wistar	M, F	Dermal	M and F > 2000	—	Hamburger et al. (1984c)
	Sprague-Dawley	M, F	Dermal	M and F > 2000	—	Durando (2005b)
Rabbit	New Zealand White	M, F	Dermal	M and F > 2000	—	Hamburger & Klotzsche (1985)
Rat	Han Wistar	M, F	Inhalation (nose only)	—	M and F > 5.645	Ullmann (1985)
	Han Wistar	M, F	Inhalation (nose only)	—	M and F > 2.03	Durando (2005c)
Rabbit	New Zealand White	M	Dermal irritation	Non-irritating	—	Hamburger, Gerber & Klotzsche (1985b)
	New Zealand White	M, F	Dermal irritation	Slightly irritating	—	Durando (2005d)
Rabbit	New Zealand White	M	Ocular irritation	Non-irritating	—	Hamburger, Gerber & Klotzsche (1985c)
	New Zealand White	M, F	Ocular irritation	Mildly irritating	—	Durando (2005e)
Guinea-pig	Ibm: GOHI; SPF	F	Dermal sensitization (maximization test)	Non-sensitizing	—	Arcelin (1992)
	Dunkin-Hartley	M, F	Dermal sensitization (Buehler test)	Non-sensitizing	—	Durando (2005f)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male

(a) Oral administration

Mice

Groups of male and female young adult NMRI mice (five of each sex per dose) were given cyproconazole (purity 94.4%) as a single dose of 0, 125, 160, 250, 320, 400, 500, 640 or 800 mg/kg bw by gavage in DMSO. A preliminary study was done in order to establish a dosing regimen testing dose levels from 125 to 4000 mg/kg bw. Animals were treated orally by gavage at a dose volume of 10 ml/kg bw. Animals were observed for 1 hour following treatment and at hourly intervals on day 1 and twice daily thereafter. Body weights were recorded on days 1, 7 and 14. Necropsy was performed on all animals.

The most common symptoms were signs of daze (males and females), weakness (males and females) and ataxia (females only) at all dose levels. Less common were exophthalmus and lacrimation, also found to occur at all dose levels in both males and females. At higher dose levels, animals showed signs of decreasing respiratory rate and laboured breathing. First onset of symptoms occurred by 9–10 minutes after dosing in the 800 mg/kg bw dose group. The longest duration of symptoms was 96 hours in the 320 mg/kg bw male group and 110 hours in the 500 mg/kg bw female group. Recovery was complete in all groups by 120 hours. There were no differences in terminal body weights between the control and treated groups. There were no significant treatment-related effects noted at necropsy in surviving and decedent animals. The oral median lethal doses (LD_{50}) for cyproconazole in mice were 200 mg/kg bw for males and 218 mg/kg bw for females (Hamburger et al., 1984b).

Groups of male young adult CD-1 mice (five per dose) were given cyproconazole (purity 96.2%) as a single dose of 0, 100, 160, 250 or 400 mg/kg bw by gavage in deionized water. Animals were treated orally by gavage at a dose volume of 10 ml/kg bw. Animals were observed for 1 hour following treatment and at hourly intervals for the first 7 hours on day 1. On the following 12 days, observations were done twice a day. Body weights were recorded on days 1, 7 and 14. Necropsy was performed on all animals.

Earliest onset of lethality occurred 39 hours after dosing in the 400 mg/kg bw group. The most common symptoms were weakness, dizziness, decreased movement, flaccidity, ataxia, and laboured and decreased respiration. The first onset of symptoms was noted after 41 minutes post-dosing in the 400 mg/kg bw group. The longest duration of symptoms lasted 72 hours in the 250 mg/kg bw group. Recovery was complete in the survivors of all groups by 96 hours. Gross pathology at necropsy did not reveal any particular findings in any organ or tissue, except for the livers of dead animals, which were partly autolytic. Histopathology revealed no clear signs of liver toxicity except for cytoplasmic vacuolation found in two animals of the 160 and 250 mg/kg bw groups, respectively. The oral LD_{50} for cyproconazole in male mice was 270 mg/kg bw (Hamburger, 1987).

Rats

Groups of male and female young adult Han Wistar rats (five of each sex per dose) were given cyproconazole (purity 95.7%) as a single dose of 200–6400 mg/kg bw for males and 200–8000 mg/kg bw for females by gavage in DMSO. Animals were observed for mortality and clinical signs at 1-hour intervals for the first day and twice daily thereafter for 13 days. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

No treatment-related effects on body weight were observed, except for reduced body weights in the 500 mg/kg bw females in the first 7 days. The most common symptoms were signs of daze (males and females), weakness (males and females) and ataxia (females only) at all dose levels. Less common were exophthalmus and lacrimation, also found to occur at all dose levels in both males and females. At higher dose levels, animals showed signs of decreasing respiratory rate and laboured breathing. First onset of symptoms occurred within 1 hour after dosing in all male groups and at 500 mg/kg bw and higher in females. Recovery of survivors occurred within 48–96 hours at lower doses and by 12 days at higher doses. Gross pathology at necropsy did not show any particular findings in any organ or tissue in decedents or in surviving animals. The oral LD_{50}s of cyproconazole in rats were 1115 mg/kg bw for males and 1342 mg/kg bw for females (Hamburger et al., 1984a). The combined oral LD_{50} for both sexes was 1290 mg/kg bw (Hamburger, 1988).

In a second study, fasted female young adult Sprague-Dawley rats (1–3 animals per dose) were given cyproconazole (purity 98.2%) as a single dose ranging from 110 to 1100 mg/kg bw by gavage in 1% weight per weight (w/w) solution of CMC in distilled water using the up-and-down procedure. Animals were observed for mortality and clinical signs within the first several hours after dosing and

at least once daily thereafter for up to 14 days. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

No treatment-related effects on body weight were observed. No treatment-related effects were observed in females (two) treated at 110 mg/kg bw. Two animals at 350 mg/kg bw and one animal at 1100 mg/kg bw died following signs of toxicity (including hypoactivity, abnormal posture and reduced faecal volume). The necropsy of the rats in the 110 mg/kg bw treatment group was unremarkable. Gross necropsy of the decedents in the 350 and 1100 mg/kg bw cyproconazole treatment groups revealed discoloration of the intestines. The acute oral LD_{50} of cyproconazole was estimated to be 350 mg/kg bw in female rats, with a 95% confidence interval of 58.05–1430 mg/kg bw (Durando, 2005a).

Rabbits

Groups of fasted female New Zealand White rabbits (five animals per dose; two animals at the top dose) were given cyproconazole (purity 95.6%) as a single dose (0, 320, 500, 640 or 800 mg/kg bw) by gavage in 4% w/w solution of CMC in distilled water. Animals were observed for mortality and clinical signs at hourly intervals on the first day after dosing and twice daily thereafter for up to 14 days. Body weights were recorded on days 1, 7 and 14. A gross necropsy was performed on all animals.

The most common clinical signs were weakness, daze, prone position (on side), decreased movement, flaccidity, ataxia, and laboured and decreased respiration. The first onset of symptoms occurred 3 hours after dosing in the 640 mg/kg bw group. The longest duration of symptoms was 384 hours in the 500 mg/kg bw group. Recovery was complete in survivors of all groups by 408 hours. All surviving animals from the low dose had gained body weight at the termination of the study. All other animals lost body weight. Gross pathology did not show any particular findings in any organ or tissue at necropsy. The acute oral LD_{50} of cyproconazole was estimated to be 460 mg/kg bw in female rabbits (Hamburger, Gerber & Klotzsche, 1985a).

(b) Dermal application

Rats

Five male and five female young adult Han Wistar rats were exposed dermally to cyproconazole (purity 95.7%) at 2000 mg/kg bw applied to a shaved dorsal area representing about 10% of the body surface at a dose volume of 4 ml/kg bw (of a 50% solution in DMSO). The treated area was covered by porous gauze and attached by tape. The treated site was rinsed with water and soap 24 hours after the treatment. Teflon collars were placed around the neck of the animals to prevent ingestion of the test material. Animals were observed for mortality and clinical signs at hourly intervals on the first day after dosing and twice daily thereafter for up to 14 days. Body weights were recorded on days 1, 7 and 14. A gross necropsy was performed on all animals.

No mortality occurred following dermal application of cyproconazole at a dose of 2000 mg/kg bw for 24 hours. Body weight gain was slightly retarded during the first week in males, whereas all females lost weight. Body weight development of both sexes recovered at least partly during the second week after treatment. No clinical signs of toxicity were observed in the study, except that all animals showed signs of weakness for 1–2 days after treatment. Necropsy revealed no abnormal changes. The dermal LD_{50} of cyproconazole in rats was greater than 2000 mg/kg bw for males and females (Hamburger et al., 1984c).

In another acute dermal toxicity study, young adult Sprague-Dawley rats (five males and five females) were dermally exposed to cyproconazole (purity 98.2%) as a dry paste (70% w/w mixture in distilled water) for a single 24-hour application to approximately 10% of the body surface area at

a limit dose of 2000 mg/kg bw. All animals were observed for mortality, signs of gross toxicity and behavioural changes during the first several hours after application and at least once daily for 14 days after dosing. Body weights were recorded on days 1, 7 and 14 (termination) following dosing.

No treatment-related effects were seen on mortality, clinical signs, body weights, irritation or gross necropsy findings. The acute dermal LD_{50} of cyproconazole technical was greater than 2000 mg/kg bw in male and female rats (Durando, 2005b).

Rabbits

Five male and five female young adult New Zealand White rabbits were exposed dermally to cyproconazole (purity 95.6%) at 2000 mg/kg bw applied to approximately 10% of the (shaved) intact body surface area, moistened with a 0.9% sodium chloride solution at 3 ml/kg bw. The test substance was maintained in contact with the skin for 24 hours using a porous gauze and attached by tape at the edges. The rabbits were observed for 14 days. Animals were observed for mortality and clinical signs first after 2 hours from dosing and then every hour on the first day and twice daily thereafter for 14 days. Body weights were recorded on days 1, 2, 3, 4, 6, 8, 10 and 14. A gross necropsy was performed on all animals.

No treatment-related effects were seen on mortality, clinical signs, irritation or gross necropsy findings. Body weight gain was reduced in males up to day 3 post-dosing, whereas females lost weight over the course of the first 4 days. Body weight for both sexes partially recovered during the remainder of the study. The dermal LD_{50} of cyproconazole in rabbits was greater than 2000 mg/kg bw for males and females (Hamburger & Klotzsche, 1985).

(c) Exposure by inhalation

Rats

Groups of five male and five female young adult Han Wistar rats were exposed by nose only to cyproconazole (purity 95.0%) for 4 hours at a concentration of 2606 or 5645 mg/m^3. Rats were observed for 14 days. The actual concentration of cyproconazole in the chamber was determined gravimetrically. The animals were observed for clinical signs of toxicity 4 times during the first day and daily thereafter. Individual body weights were recorded at day 1 (pretest), day 8 and day 15 of the test. A necropsy was performed on all animals. The following histopathology was done on the high-dose animals: nasal cavity, lungs with mainstream bronchi, liver, kidneys, adrenal glands and all gross lesions. For the low-dose animals, only gross lesions were examined.

The measured concentrations were within the acceptable range (2492–2710 mg/m^3 and 5490–5802 mg/m^3 at the low and high doses, respectively). The mean particle size distribution observed was approximately 41% and 61% in the range of 1–5 µm for the low and high doses, respectively.

No animals died during the study. Slight sedation, dyspnoea and ruffled fur were observed in all animals 4 hours post-dosing. All rats had recovered completely 24 hours after initiation of exposure. Body weight development was not affected by treatment. No treatment-related macroscopic or microscopic findings were observed. Under the study conditions utilized, it can be concluded that the inhalation LC_{50} of cyproconazole at 4 hours in rats was higher than 5645 mg/m^3 air (5.65 mg/l air) (Ullmann, 1985).

In another acute inhalation study, groups of five male and five female young adult Sprague-Dawley rats were exposed by nose only to cyproconazole (purity 98.7%) for 4 hours at a concentration of 2.03 mg/l. Chamber concentration and particle size were determined periodically. Following exposure, the animals were retained without treatment for 14 days. All animals were observed for mortality, signs of gross toxicity and behavioural changes at least once daily for 14 days after dosing or until death occurred. Body weights were recorded prior to administration and again on days 7 and

14 (termination) following dosing or after death. At the end of the scheduled period, the animals were killed and subjected to a gross postmortem examination.

Atmospheres generated had mean aerodynamic particle sizes of 3.0–3.1 μm, with a geometric standard deviation of 1.90.

There were no treatment-related effects on mortality, clinical signs, body weights or necropsy findings. Under the study conditions utilized, it can be concluded that the inhalation LC_{50} of cyproconazole at 4 hours in male and female rats was greater than 2.03 mg/l (Durando, 2005c).

(d) Dermal irritation

In a study of primary dermal irritation, three male young adult New Zealand White rabbits were dermally exposed to 1.0 ml of cyproconazole (purity 94.4%) applied to a 6 cm^2 gauze pad covered with plastic. The test material was in contact with the skin for 4 hours. The adjacent skin area served as the control. Dermal irritation was scored according to the "Method of testing primary irritant substances" (Code of Federal Regulations Title 16, Section 1500.41) after 30 minutes and then daily for 3 days.

No irritation was observed on any rabbits following application of cyproconazole. Under the conditions of this study, it is concluded that cyproconazole is non-irritating to the skin of rabbits (Hamburger, Gerber & Klotzsche, 1985b).

In another study of primary dermal irritation, one female and two male young adult New Zealand White rabbits were dermally exposed to 0.5 g of cyproconazole (purity 98.2%) as a dry paste (70% w/w mixture in distilled water) for a single 4-hour application to one 6 cm^2 intact site on each animal. Dermal irritation was scored according to the Draize method after 60 minutes and then daily for 3 days.

One hour after patch removal, very slight erythema was noted for all three treated dose sites. All animals were free from dermal irritation within 24 hours. Under the conditions of this study, it is concluded that cyproconazole is slightly irritating to the skin of rabbits (Durando, 2005d).

(e) Ocular irritation

In a study of primary eye irritation, 100 mg of cyproconazole (purity 94.4%) was instilled into the conjunctival sac of one eye of each of three male New Zealand White rabbits. The eyes were not washed. Irritation was scored by the method of Draize at 0.5 hour and 1, 2 and 3 days after exposure.

Slight redness (grade 1) of the conjunctivae was noted 0.5 hour after treatment. No other findings were noted thereafter. Under the conditions of this study, it is concluded that cyproconazole is non-irritating to the eyes of rabbits (Hamburger, Gerber & Klotzsche, 1985c).

In another study of primary eye irritation, 0.1 ml (0.06 g) of cyproconazole (purity 98.2%) was instilled into the conjunctival sac of one eye of each of three New Zealand White rabbits (one male and two females). The eyes were not washed. Irritation was scored by the method of Draize at 1 hour and 1, 2 and 3 days after exposure.

Within 1 hour after test substance instillation, "positive" iritis and conjunctivitis were noted for all three treated eyes. The mean severity of irritation scores were 15.0, 4.0, 1.3 and 0.0 at 1, 24, 48 and 72 hours post-instillation. The overall incidence and severity of irritation decreased with time. Under the conditions of this study, it is concluded that cyproconazole is mildly irritating to the eyes of rabbits (Durando, 2005e).

(f) Dermal sensitization

In a study of dermal sensitization with cyproconazole (purity 95.6%), young female Ibm: GOHI (Himalayan spotted) SPF guinea-pigs were tested using the maximization method of Magnusson &

Kligman. For the main study, 10 female guinea-pigs were assigned to a control group, and 20 to the treatment group. In this study, the test concentrations chosen were 5% for intradermal induction and 25% for epidermal induction and challenge. DMSO was used as the vehicle. Skin reactions at the challenge sites were observed at 24 and 48 hours after challenge applications and graded according to the Draize scoring scale. Body weights were recorded at the start and end of the test. A positive control group with 2-mercaptobenzothiazol was included for a sensitivity check of the guinea-pig strain.

Epidermal induction caused grade 1 erythema in 8 of 20 animals and in 2 of 20 animals after 24 and 48 hours, respectively. No positive reactions were evident at the application site in any of the animals following 24 and 48 hours after challenge application. No toxic symptoms were evident in the guinea-pigs of the control or test group. Under the study conditions utilized, cyproconazole is considered as non-sensitizing in this maximization test (Arcelin, 1992).

In another study of dermal sensitization with cyproconazole (purity 98.2%), male and female young adult Dunkin-Hartley guinea-pigs were tested using the method of Buehler. Ten animals were used in the controls, and 20 animals in the test group. For the main study, the concentration of the test substance was 75% (w/w) in a 1% w/w solution of CMC for three weekly inductions and the challenge exposures. Results of the pilot study determined that a 75% test substance concentration was the highest concentration that caused no skin irritation. Once each week for 3 weeks, 0.4 g of the test substance in vehicle formulation was applied to the left side of each animal using an occlusive 25 mm Hill Top Chamber. The chambers were applied to the test sites on the left shoulder and secured by a bandage. After 6 hours, the bandage and chambers were removed, and the test sites were cleaned. Approximately 24 and 48 hours after each induction application, skin reactions were scored using the Draize method. Similarly, the challenge phase was conducted 27 days after the first induction dose. Body weights were recorded prior to the initial induction and again the day after the challenge application.

Following challenge of previously induced guinea-pigs with a 75% w/w mixture of cyproconazole technical in 1% w/w aqueous CMC, very faint erythema (0.5) was noted for 5 of 20 test sites 24 hours after challenge. Irritation cleared from all affected sites by 48 hours. Following challenge of naive controls with a 75% w/w mixture of cyproconazole technical in 1% w/w aqueous CMC, very faint erythema (0.5) was noted for 3 of 10 naive control sites 24 hours after challenge application. Irritation cleared from all affected sites by 48 hours. The positive response observed in the historical positive control validation study with α-hexylcinnamaldehyde technical validates the test system used in this study. There were no treatment-related effects on body weight during the study. Under the study conditions utilized, it is concluded that cyproconazole is not a skin sensitizer in guinea-pigs as determined by the Buehler method (Durando, 2005f).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In an oral study of toxicity in mice, cyproconazole (purity 95.6%) was administered to 10 CD-1 (Swiss origin) mice of each sex per dose at dietary concentrations of 0, 5, 15, 300 or 600 ppm (equivalent to 0, 0.7, 2.2, 43.8 or 88.7 mg/kg bw per day for males and 0, 1.0, 3.2, 70.2 or 128.2 mg/kg bw per day for females, respectively) for 13 weeks. The test concentrations were confirmed analytically; almost all mean measured concentrations of the test diets were within ±15% of nominal. Mortality and clinical signs were checked twice daily (once daily on holidays and weekends). Body weights and feed consumption were recorded weekly, including day 1. All animals were subjected to a detailed

gross pathological examination. Organs were collected, weighed and prepared for histopathology in accordance with OECD guidelines.

The study authors stated that there were no treatment-related clinical signs. Clinical signs were not summarized in tables or reported for individual animals. There was no treatment-related mortality. One high-dose male was killed in a handling accident during week 4; all of the other animals survived until terminal sacrifice. At 600 ppm, feed consumption was significantly decreased in females during week 1 (18%; $P < 0.01$), and overall average weekly feed consumption was decreased by 10% (not significant). Feed consumption was decreased, but not significantly, in 600 ppm males throughout the study. Body weight gain in treated male mice was reduced in the two highest dose groups throughout the entire treatment period, resulting in terminal body weights that were 9.5% and 11% below those of controls for the 300 and the 600 ppm groups, respectively (Table 15). In female mice, body weight gain was below control values at the high dose. Terminal body weight of this group was 7.4% less than that of the control group. A less marked effect was also seen in 300 ppm females. Males and females in the 600 ppm group had mean body weight losses during the first week of treatment, whereas controls gained weight (males: −1.2 g vs +1.0 g; females: −0.2 g vs +0.7 g for controls). No growth or very little growth was observed after week 8 in either sex administered the 300 or 600 ppm diets. Increases in feed conversion ratios (feed consumed per unit weight gain) were observed in males and females, but not in a dose-related manner.

Absolute liver weights in the 300 and 600 ppm males and females were increased by 46% and 79%, respectively, for males and by 63% and 100%, respectively, for females. Relative (to body weight) liver weights were significantly increased by 63% and 104%, respectively, for males and by 73% and 124%, respectively, for females. Relative spleen weights were statistically significantly increased in males at 300 ppm (0.32 vs 0.22 in controls) and 600 ppm (0.33 vs 0.22 in controls) (an increase of about 48–50% compared with controls). Gross findings included enlarged liver in 2 of 10 males at 600 ppm (0/10 controls) and accentuated lobular pattern in the liver in 5 of 10 males at 600 ppm (2/10 controls) and 9 of 10 females at 600 ppm (1/10 controls). Histopathological changes included single-cell hepatocyte necrosis in 2 of 10 males and 3 of 10 females at 300 ppm and in 5 of 10 males and 7 of 10 females at 600 ppm; periacinar hepatocytic vacuolation in 4 of 10 males and 10 of 10 females at 600 ppm; periacinar hepatocytic eosinophilia in 7 of 10 males at 300 ppm and 8 of 10 males at 600 ppm; and centriacinar hepatocytic eosinophilia in 6 of 10 males and 8 of 10 females at 600 ppm. None of these lesions were observed in male or female controls. The changes in the liver were considered to be an adaptive response of the body and not adverse.

The lowest-observed-adverse-effect level (LOAEL) for cyproconazole in CD-1 mice was 300 ppm, equal to 43.8 mg/kg bw per day, based on decreased body weight gain in both sexes and increased relative weight of spleen in males. The no-observed-adverse-effect level (NOAEL) was 15 ppm, equal to 2.2 mg/kg bw per day (Warren, Skinner & Karapally, 1987).

Rats

In a 28-day dose range–finding study of toxicity, groups of 16 male and 16 female Kfm: WIST rats of Han Wistar origin were given diets containing cyproconazole (purity 95.7%) at a dietary concentration of 0, 10, 30, 100, 300 or 1000 ppm (equivalent to 0, 0.8, 2.3, 8.1, 25.3 or 96.2 mg/kg bw per day for males and 0, 0.9, 2.9, 9.8, 31.5 or 127.6 mg/kg bw per day for females, respectively) for 28 days. Diets were analysed for homogeneity and stability of the test substance prior to study initiation and at weeks 2 and 4 of the study. Mortality and clinical signs (including neurological, oral and behavioural inspection) were checked daily. Body weights and feed consumption were recorded weekly, including day 1. Laboratory investigations were carried out on eight animals of each sex per group at the end of week 2 and at the end of the treatment period (animals investigated in week 2 were not investigated in week 4). All animals were necropsied, and selected organs were weighed. A complete macroscopic and microscopic examination was performed.

Table 15. Body weight gain of male and female mice treated with cyproconazole for 90 days

Sex	Treatment	Body weight gain (g)[a]	
		Weeks 0–4	Weeks 0–13
Males	Control	3.0	6.8
	5 ppm	3.7 (+23%)	7.1 (+4%)
	15 ppm	3.3 (+10%)	5.9 (−13%)
	300 ppm	1.6 (−47%)	3.9 (−43%)
	600 ppm	1.4 (−53%)	2.17 (−68%)
Females	Control	2.2	3.7
	5 ppm	2.3 (+5%)	3.8 (+3%)
	15 ppm	2.0 (−9%)	3.0 (−19%)
	300 ppm	1.3 (−41%)	2.9 (−22%)
	600 ppm	0.3 (−86%)	2.4 (−35%)

From Warren, Skinner & Karapally (1987)
[a] % difference from control shown in parentheses.

No mortality was observed in the study. No clinical signs of toxicity were observed in the study. Body weights were decreased in high-dose males and females throughout the study. Body weights were also decreased in males and females in the 300 ppm group during weeks 1 and 2; however, they were comparable to respective control values at study termination. Body weight gain was also decreased in high-dose males and females. Feed consumption was not altered in males. The only differences from controls were noted in high-dose females, among which feed consumption increased during weeks 2 (16.7%), 3 (28.7%) and 4 (47.8%). All of these increases were statistically significant, but they were not consistent with the body weights. A statistically significant increase in the mean corpuscular haemoglobin concentration (MCHC) was seen in high-dose males at week 2 but not at week 4. Sporadic changes in haemoglobin, haematocrit, mean corpuscular volume (MCV), white blood cell counts, lymphocytes and platelets were seen, but the changes were small and of doubtful toxicological significance. Elevated levels of blood urea nitrogen were statistically significant in high-dose males at both intervals (22–28% increase) and in high-dose females (25% increase) at 2 weeks. Cholesterol was increased in high-dose females (77%), and an increased trend was evident in males. Increased alanine aminotransferase (ALT) activity was observed in high-dose males (74% at 2 weeks and 49% at 4 weeks) and high-dose females at 4 weeks (27%). Lactate dehydrogenase (LDH) activity was increased in males and females in the 300 and 1000 ppm groups at 2 and 4 weeks, which was statistically significant. Bilirubin was decreased in males at 300 and 1000 ppm at week 4 and in corresponding females at both intervals, but only the change in high-dose females at week 4 was statistically significant. It is evident from these data that compound-induced clinical chemistry changes occurred in the 300 and 1000 ppm dose groups. The mean values indicate that there was a trend towards increased calcium oxalate in dosed males and increased amorphous urate in high-dose males and females; however, an examination of the individual data showed that these increases were influenced by sporadic high values and/or abnormally low control values. Therefore, these changes do not appear to be compound related.

Absolute liver weight in males was increased in a dose-related manner at 100 ppm and above, reaching statistical significance at the high dose (41.3%). Relative liver weight was significantly increased at 300 and 1000 ppm (16% and 62%, respectively). In females, absolute liver weight was increased in a dose-dependent manner at 100 ppm and above and reached statistical significance in the two highest dose groups (18% and 35%, respectively). The same holds true for the relative liver weight, which reached significance at 300 and 1000 ppm (15% and 45%, respectively). Tubular

calcification was observed in the kidneys of females at comparable rates in all groups; no relationship to treatment was apparent. The only obvious treatment-related effects were observed in liver; increased incidences of vacuoles, attributed to reversible storage, were present in males and females at 300 ppm and in males at 1000 ppm. Hepatocytomegaly, predominantly centrilobular, was noted in high-dose males and females. Focal mononuclear hepatitis was present in the same treatment groups, but the incidence was not dose related. The liver toxicity seen in this study (increased liver weights, liver-related clinical chemistry parameters and histopathological findings) was considered to be an adaptive response of the body and not adverse.

Based on the results of this study, the NOAEL was 100 ppm, equal to 8.1 mg/kg bw per day, based on reduced body weight gain in females, changes in clinical chemistry, organ weight changes and histopathological findings in the liver seen at the LOAEL of 300 ppm, equal to 25.3 mg/kg bw per day (Skinner et al., 1985a).

In an oral study of toxicity in rats, cyproconazole (purity 95.7%) was administered for 13 weeks in the diet to 15 Kfm: WIST rats of Han Wistar origin of each sex per dose in the diet at a dose level of 0, 20, 80 or 320 ppm (equal to 0, 1.5, 6.4 or 23.8 mg/kg bw per day for males and 0, 1.9, 7.0 or 31.1 mg/kg bw per day for females, respectively). An additional 15 animals of each sex in the control and high-dose groups were observed for a 4-week recovery following a 13-week dietary exposure to cyproconazole. Diets were analysed for homogeneity and stability of the test substance prior to study initiation and at monthly intervals during the study. Mortality and clinical signs (including neurological, oral and behavioural inspection) were checked daily. Body weights and feed consumption were recorded weekly, including on day 1. Laboratory investigations (haematology, chemistry and urinalysis) were carried out on 10 animals of each sex per dose at weeks 4, 8 and 13 and for recovery animals at weeks 14 and 18. At study termination, all animals were subjected to a detailed gross pathological examination. Organs were collected, weighed and prepared for histopathology.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable (85–101% of the nominal).

There were no mortalities in the study. The only clinical symptom noted in dosed animals was piloerection, which was observed in males only (8/15 at the middle dose and 14/30 at the high dose). In males, mean body weight was not affected during the first 8 weeks of treatment (Table 16). A slight reduction in body weight gain was noted from week 8 until the end of treatment in high-dose males (−10%; including recovery animals). Body weight gain during recovery was comparable between control and high-dose males. The body weight of high-dose females was slightly below that of the control group at the end of treatment (−3.5%; including recovery animals). Body weight gain was reduced in high-dose females by 14% during the last 5 weeks of treatment. Body weight gain during recovery was comparable between control and high-dose females. The two lower dose groups were not affected.

Feed consumption was not affected by treatment except for a slight reduction in week 1 for the high-dose males of the recovery group but not for the main group. Creatinine levels were increased in 20 and 320 ppm dose group females ($P < 0.01$) at all intervals during the treatment period and in 20 and 320 ppm dose group males at weeks 8 ($P < 0.01$) and 13 ($P < 0.01$ and $P < 0.05$ for 20 and 320 ppm dose groups, respectively). Calcium levels were depressed in 20 and 320 ppm dose group females at week 4 ($P < 0.05$), in 20 ppm males ($P < 0.01$) and females ($P < 0.05$) at week 8 and in 20 ppm ($P < 0.01$) and 320 ppm males ($P < 0.05$) and 80 ppm ($P < 0.05$) and 320 ppm ($P < 0.01$) females at week 13. A slight increase in creatinine level was also observed in high-dose males of the recovery group at week 14, but not at week 18. Calcium levels were not affected in animals of the recovery group both at 14 and at 18 weeks. Sodium levels were significantly higher than those of the controls at week 13 among all males and females receiving 320 ppm. However, at week 4, sodium levels among males receiving 80 or 320 ppm appeared lower than those of the controls. High-dose animals of the recovery

Table 16. Body weight gain of control and high-dose animals for selected periods in the first 90-day toxicity study in rats with cyproconazole

Sex	Treatment	Body weight gain (g)[a]				
		Weeks 0–4	Weeks 4–8	Weeks 8–13	Weeks 0–13	Weeks 14–17
Males	Control	129	64	49.5	242.5	22
	320 ppm	127.5 (−1.2%)	61 (−4.7%)	44.5 (−10.1%)	233 (−3.9%)	20 (−9.1%)
Females	Control	51	24.5	21.5	97	10
	320 ppm	48.5 (−4.9%)	24.5 (−0%)	18.5 (−14.0%)	91.5 (−5.7%)	10 (−0%)

From Skinner et al. (1985b)
[a] % difference from control shown in parentheses.

group of both sexes showed increased sodium levels at week 14, but not at week 18. The changes in these clinical parameters are inconsistent from sampling point to sampling point and show no dose–response relationship. Values for rats receiving 20 ppm often appeared more disturbed than those for rats receiving 80 ppm. This absence of a dose-related pattern argues against these changes being a finding of toxicological significance. However, the disappearance of these changes following a withdrawal period of 4 weeks may be taken to indicate a substance-related change. A slight elevation of aspartate aminotransferase (AST) values among females receiving 80 or 320 ppm was noted at week 13. However, the magnitude of the effect did not reach a level of toxicological significance. Blood urea values were increased in high-dose males and females of the recovery group only. This effect was found both at week 14 and at week 18. However, owing to the absence of any effect in the main study group at any time point, the significance of a constant difference in urea levels among withdrawal group rats during recovery was obscure. No treatment-related effects on the urinalysis parameters were observed.

No treatment-related lesions were observed during macroscopic examination. Although not always statistically significant, increases in liver weights (absolute and relative to body weights) were seen in 80 and 320 ppm males and 320 ppm females. Elevated liver weights were not observed in animals examined after the recovery period. Vacuolated hepatocytes with single large or several small predominantly centrilobular vacuoles were present in 6 of 15 males treated at 320 ppm. This represents reversible lipid storage and is considered to be treatment related. Five males and four females treated at 320 ppm showed a distinct lobular pattern, probably due to enlarged activated cytoplasm. This is secondary to increased functional demands and is reversible. There were no treatment-related liver changes in male or female recovery rats. The liver toxicity seen in this study (increased liver weights, liver-related clinical chemistry parameters and histopathological findings) was considered to be an adaptive response of the body and not adverse. No treatment-related changes were observed in any other tissue or organ.

The NOAEL was 80 ppm, equal to 6.4 mg/kg bw per day. The LOAEL was 320 ppm, equal to 23.8 mg/kg bw per day, based on slight impairment of body weight gain and increased creatinine levels. The study authors set a no-observed-effect level (NOEL) at 20 ppm, equal to 1.5 mg/kg bw per day (Skinner et al., 1985b).

In a second oral study of toxicity in rats, cyproconazole (purity 95.5%) was administered for 13 weeks to 15 Hanlbm: WIST (SPF) Wistar rats of each sex per dose in the diet at a dose level of 0, 20, 350, 700 or 1400 ppm (equivalent to 0, 1.4, 24.7, 52.8 and 106.8 mg/kg bw per day in males and 0, 1.6, 29.6, 57.3 and 118.1 mg/kg bw per day in females, respectively). Five rats of each sex per dose were subjected to neuropathological examination and also evaluated in the functional observational battery (FOB) and for the assessment of motor activity. Diets were prepared at monthly intervals and stored at room temperature. Stability, homogeneity and dietary concentrations were

confirmed analytically. All rats were examined twice daily for signs of toxicity and mortality. Body weight, water consumption and feed consumption were measured weekly. Ophthalmoscopic examinations were performed on all animals prior to initiation of treatment and on all controls and 1400 ppm animals on day 86. Urinalysis parameters were evaluated in all animals at the end of treatment. FOBs were performed at pretest and at weeks 4, 9 and 13 at about the same time each day and were always conducted before the assessment of motor activity. Animals were observed in the home cage, during handling and in an open field. Observations covered the functional domains of central nervous system activity, central nervous system excitation and sensorimotor, autonomic and physiological functions. Neurological examinations were performed, including sensorimotor functions (approach, touch, vision, audition, pain, vestibular), autonomic functions (pupillary reflex, body temperature) and sensorimotor coordination (grip strength, landing footsplay). At termination, blood was taken for haematological and clinical chemistry analysis. Animals were then subjected to a gross necropsy, and organ weights were obtained for selected organs. Histopathological examination of selected tissues was conducted on all animals. Animals scheduled for neuropathology were deeply anaesthetized with pentobarbital (supplemented with heparin) and sacrificed by in situ perfusion with neutral buffered formalin (4%). Appropriate samples of organs and tissues were collected as outlined in the guidelines (OECD guideline 408; USEPA Federal Insecticide, Fungicide, and Rodenticide Act guideline 82-1). Processing and microscopic examination of these tissues were done for control and high-dose animals only.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable. The prepared samples were stable at room temperature.

No treatment-related effect was observed on survival, clinical signs, the FOB, feed consumption ratios, water consumption, the eyes, gross pathology or neuropathology. A minimal, transient (week 4) decrease in mean rectal temperature in high-dose animals was considered likely to be related to exposure to the test item, but was not toxicologically significant. Body weights were statistically significantly decreased throughout treatment in the 700 ppm males (10–14%) and 1400 ppm males (19–28%) and females (10–18%). Cumulative body weight gains (Table 17) were statistically significantly decreased throughout the study in the 700 ppm males (15–35%) and 1400 ppm males (28–69%) and females (17–76%). Cumulative body weight gain was most severely curtailed during the first week of treatment and progressively became more like that of controls throughout the study. Body weight was decreased in the 700 ppm females at week 1 (8%), and body weight gain was decreased by 31% during the first week. This transient response was not considered adverse. The body weights and body weight gains of the other treated groups were similar to those of the controls.

At and above 350 ppm, absolute and relative (to body weight) liver weights were increased ($P < 0.05$) by 13–55% in both sexes. Increased incidences of liver fatty change in males and liver hypertrophy in both sexes were observed. Increased incidences of thyroid gland follicular hypertrophy were noted in both sexes. Pituitary gland distal lobe hypertrophy was observed in the males. Additionally, relative (to body weight) spleen weights were decreased ($P < 0.05$) in the females by 14–25%, compared with controls. Additional effects were observed on the liver. Increased ($P < 0.001$) γ-glutamyl transpeptidase (GGT) was observed in the 1400 ppm males and in the females at and above 700 ppm. Additional differences ($P < 0.01$) noted at 1400 ppm included increased ALT and AST activities in the males, decreased triglyceride levels in the males and increased cholesterol and globulin levels in the females. Increased ($P < 0.05$) leukocytes were observed in the groups at and above 700 ppm. Several types of leukocytes were increased in number in the 700 and/or 1400 ppm groups, including neutrophils, lymphocytes, monocytes and large unstained cells. Urinary leukocyte levels were increased ($P < 0.05$) in the 1400 ppm males. In addition to the increase in relative spleen weight, spleen congestion was also observed in the females at and above 700 ppm. Additionally, a decreased incidence of extramedullary haematopoiesis in the spleen was noted in the 1400 ppm

Table 17. Decrease in cumulative body weight gain in the second 90-day toxicity study in rats with cyproconazole

Sex	Treatment	Weeks 0–4		Weeks 4–8		Weeks 8–13		Weeks 0–13	
		Body weight gain (g)	% difference from control	Body weight gain (g)	% difference from control	Body weight gain (g)	% difference from control	Body weight gain (g)	% difference from control
Males	Control	157.0	—	76.7	—	42.2	—	275.9	—
	20 ppm	153.1	−2.5	81.4	6.1	43.3	2.6	277.8	0.7
	350 ppm	143.2	−8.8	83.6	9.0	44.8	6.2	271.5	−1.6
	700 ppm	117.2	−25.4	71.2	−7.2	46.9	11.1	235.3	−14.7
	1400 ppm	88.9	−43.4	70.4	−8.2	40.7	−3.6	200.0	−27.5
Females	Control	79.9	—	28.2	—	15.9	—	124.0	—
	20 ppm	78.8	−1.4	31.7	12.4	18.1	13.8	128.6	3.7
	350 ppm	69.3	−13.3	31.5	11.7	19.2	20.8	120.0	−3.2
	700 ppm	69.8	−12.6	28.5	1.1	16.8	5.7	115.1	−7.2
	1400 ppm	59.7	−25.3	25.8	−8.5	17.9	12.6	103.3	−16.7

From Gerspach (1999)

group. Increased incidences of the following microscopic lesions were also observed: 1) fatty change in the adrenal cortex in males at and above 700 ppm; 2) single-cell necrosis in the adrenal gland cortex in females at and above 700 ppm; 3) ceroid deposition in the adrenal gland cortex in females at and above 700 ppm; and 4) renal haemosiderosis in the 1400 ppm males and in females at and above 700 ppm. The liver toxicity seen in this study (increased liver weights, liver-related clinical chemistry parameters and histopathological findings) was considered to be an adaptive response of the body and not adverse.

The NOAEL was 20 ppm, equivalent to 1.4 mg/kg bw per day. The LOAEL was 350 ppm, equivalent to 24.7 mg/kg bw per day, based on decreased body weight gains (Gerspach, 1999).

When the results of the two dietary studies of toxicity were combined, an overall NOAEL of 80 ppm, equal to 6.4 mg/kg bw per day, was established, based on decreased body weight gains seen at the LOAEL of 320 ppm, equal to 23.8 mg/kg bw per day and above.

Dogs

In a 90-day study of toxicity, groups of four male and four female Beagle dogs were given diets containing cyproconazole (purity 95.6%) at a concentration of 0, 20, 100 or 500 ppm (equal to 0, 0.8, 4.0 or 18.2 mg/kg bw per day for males and 0, 0.7, 3.3 or 19.2 mg/kg bw per day for females, respectively) for 13 weeks. The dogs were inspected twice per day for mortality, moribundity and clinical signs. Eyes of the controls and 500 ppm dose groups were examined prior to dosing and at termination. Body weights were recorded weekly. Feed consumption was measured twice weekly and calculated on a weekly basis. Blood for measurement of haematological and clinical chemistry parameters was collected from all dogs before the test and after 4, 8 and 13 weeks of treatment. Urinalysis was conducted 2 weeks prior to the initiation of the study and at weeks 4, 8 and 13. At the end of the study, a complete gross postmortem examination was done. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

The prepared diets were stored at 3–5 °C. Stability of the test material was analysed prior to commencement of feeding and at monthly intervals thereafter. The mean concentrations of cyproconazole in the diets (weeks 1, 4, 8 and 13) were within acceptable limits (±15% of nominal).

All dogs survived until the scheduled terminal sacrifice. The investigators indicated that "slack muscle tone" was observed in all animals at 500 ppm. This change was first observed in week 1, and all dogs were affected after 2 weeks of treatment. This effect persisted until the end of the study in three of four males and two of four females. No other relevant clinical changes were noted. No lesions were reported during ophthalmoscopic examination. Body weight gain was inhibited in males and females at 500 ppm. Mean terminal body weights of dogs in this group were 11% and 9% for males and females, respectively, relative to corresponding control weights. Body weight gain was more pronounced in male dogs of the 500 ppm dose group in the first 6 weeks of the study (−64% of the controls) (Table 18). In 500 ppm dose males, feed consumption was slightly lower throughout the study: 19% lower during the first 6 weeks and 11% lower during the last 7 weeks, in comparison with controls.

Increased platelet counts were observed in 500 ppm dose males and females only. These counts exceeded control as well as baseline values at all post-treatment intervals. Haematological analysis revealed a reduction in red blood cell parameters (haemoglobin, haematocrit and red blood cells) at the study initiation. This reduction progressed to a greater than 7% reduction by week 13 in males of the 500 ppm dose group. Similarly, females of the 500 ppm dose group showed a reduction in red blood cell parameters, but not as great as those observed in 500 ppm males. Changes in clinical chemistry parameters observed in 500 ppm dose dogs that appeared to be treatment related included decreases in levels of bilirubin, total cholesterol, high-density lipoprotein (HDL; HDL cholesterol), triglycerides, total protein and albumin and increases in alkaline phosphatase and GGT activities. Other fluctuations noted in 500 ppm dose animals that did not appear to be related to treatment because they were not dose related or were comparable to baseline values included increases in creatinine, ALT, creatine phosphokinase, glutamate dehydrogenase, cortisol and testosterone levels, as well as decreases in calcium and inorganic phosphorus levels.

Although not always statistically significant, the noted treatment-related changes were observed in both sexes at the 4-, 8- and 13-week intervals, with the exception of decreased cholesterol (total and HDL) levels, observed at weeks 8 and 13 (only intervals for measurement), and increased GGT activity at week 13 (measured at weeks −1, 0 and 13). There were no noted changes in the investigated urinary parameters indicative of a compound-related effect. Absolute and relative liver weights of males and females of the 500 ppm dose group and males of the 100 ppm group were higher than those of the controls; statistical significance was achieved among 500 ppm animals only. Absolute and relative brain weights of 500 ppm females were slightly increased compared with control values. This difference was minimal in degree and, in the absence of a similar effect among male dogs, is not considered to represent an effect of toxicological significance. Macroscopic examination did not reveal treatment-related changes. The only microscopic changes that appeared to be related to treatment were observed in the liver of males and females of the 100 and 500 ppm dose groups. Hepatocytomegaly occurred at a frequency of two of four for both males and females at 100 ppm and to a mild or moderate degree in four of four males and females at 500 ppm. Also, degeneration of single hepatocytes and cytoplasmic inclusions were each observed at a rate of one of four at the 500 ppm dose in both males and females. Other noted changes occurred sporadically or were observed at comparable frequencies between groups and could not conclusively be associated with treatment.

In summary, treatment at 500 ppm caused retardation of body weight gain and well-defined hepatotoxicity. At 500 ppm, there were clear changes in clinical chemistry parameters (levels of bilirubin, cholesterol, albumin, etc.) as well as a significant increase in the number of platelets. These changes in clinical chemistry parameters can possibly be associated with interference with the adrenocorticosteroid synthesis pathway, as similarly observed in a previous 3-month feeding study in the rat (Gerspach, 1999). However, histological examination revealed no changes in the adrenal gland. A number of these changes have also been associated with toxic effects on the liver and, due to their slight recovery over the study progression, indicate a reversible response. Furthermore, histological

Table 18. Body weight gain of male and female dogs in a 90-day toxicity study with cyproconazole

Sex	Treatment	Body weight gain (kg)[a]	
		Weeks 0–6	Weeks 0–13
Males	Control	1.4	2.1
	20 ppm	1.4 (0%)	2.4 (+14%)
	100 ppm	1.6 (+14%)	2.4 (+14%)
	500 ppm	0.5 (−64%)	1.4 (−33%)
Females	Control	0.7	1.2
	20 ppm	1.0 (+43%)	1.9 (+58%)
	100 ppm	0.7 (0%)	1.3 (+8%)
	500 ppm	0.3 (−57%)	0.5 (−58%)

From Warren et al. (1986)
[a] % difference from control shown in parentheses.

analysis of dogs receiving 100 ppm over a 52-week treatment period (Warren et al., 1988a) revealed similar and less severe histopathological findings in one male dog only, further indicating that this is an adaptive response. The increased liver weight and histological analysis demonstrate that at doses above 100 ppm, this adaptive response degenerates into a toxic effect with the manifestation of increased hepatomegaly and single-cell death, which are consistent with initiation of liver damage due to exposure to cyproconazole.

The NOAEL was 100 ppm, equal to 3.3 mg/kg bw per day, based on decreased body weight gain and increased alkaline phosphatase activity seen at the LOAEL of 500 ppm, equal to 18.2 mg/kg bw per day. The study authors set a NOEL of 20 ppm, equal to 0.7 mg/kg bw per day (Warren et al., 1986).

In a 1-year study of oral toxicity, groups of four male and four female Beagle dogs were given diets (dietary admixture) containing cyproconazole (purity 95.6%) at a concentration of 0, 30, 100 or 350 ppm (equal to 0, 1.0, 3.2 or 12.1 mg/kg bw per day for males and 0, 1.0, 3.2 or 12.6 mg/kg bw per day for females, respectively) for 53 weeks. The dogs were inspected twice per day for mortality, moribundity and clinical signs, except for weekends and holidays (checked once per day). Eyes of the control and 350 ppm dose groups were examined prior to dosing and at termination. Body weight and feed consumption were recorded weekly. Blood for measurement of haematological and clinical chemistry parameters was collected from all dogs before the test (−2 weeks and −1 week) and during treatment at 3, 6 and 9 months as well as at termination. Urinalysis was conducted 2 weeks prior to the initiation of the study and at 3, 6 and 9 months during treatment and at termination. At the end of the study, a complete gross postmortem examination was done. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically. Samples of livers from all dogs were analysed for cytochrome P450 (CYP) content, GSH content, glutathione *S*-transferase (GST) activity and *p*-aminophenol hydroxylase activity.

The prepared diets were stored in a refrigerator at 4 °C. Stability and homogeneity of the diets were analysed. Analysis of the diets provided indicates that the test substance was homogeneously distributed in the diets and the measured concentrations were within the acceptable range of target concentrations.

There were no deaths or unscheduled sacrifices during the study. Subdued behaviour (during weeks 4–8) and lower body weight gain (during the first 4 weeks) were observed in one high-dose

female. Feed consumption also tended to be low in this animal up to week 10. Thereafter, this animal displayed behaviour comparable that of the other dogs. One 100 ppm female displayed subdued behaviour, with body weight loss and low feed consumption during week 2. One high-dose female failed to come into heat during the study, and terminal histological examination revealed immature ovaries. Body weight gain for the high-dose males was decreased during the first few weeks of the study; the decrease was greatest at week 9 (25% of the controls) and became comparable to that of controls thereafter (Table 19). Males in the 100 ppm dose group showed slightly increased body weights. Over the study, treated males had higher feed consumption compared with controls. There were no effects of treatment on feed consumption except that a few individuals showed slightly diminished values at the beginning of the treatment.

There were no treatment-related ocular changes in either sex. Increased platelet counts were observed in the high-dose animals throughout the study. Marginal changes in the platelets were also observed in the males at 100 ppm at various sampling points; however, there was no clear effect of the treatment. No effects on prothrombin time were observed in the 100 ppm and 350 ppm dose group animals at week 48, even though the platelet counts were increased. A tendency to depressed levels of bilirubin (never achieved statistical significance), cholesterol (statistically significant in males and females at weeks 13 and 38, in females at week 51), total protein (statistically significant in males and females at week 13 and in males at weeks 25 and 38) and albumin (statistically significant in both sexes at all time points) was noted at 350 ppm. The minimal depression of bilirubin levels in males receiving 100 ppm at week 25 is considered to be of no biological relevance, as the bilirubin level in males receiving the high dose on this occasion was comparable to that of controls. Triglyceride levels were lower in females receiving 350 ppm throughout the study (statistically significant at weeks 13 and 38). In males of this group, a depression in triglyceride levels was seen only at week 13 (not significant). Triglyceride levels of males in the middle-dose group were marginally depressed at week 13. Calcium levels among males and females of the high-dose group were lower than those of controls; statistical significance was achieved in males and females at week 13 and in males at week 25. Alkaline phosphatase values in males and females of the high-dose group were markedly higher than those of the controls throughout the study, achieving statistical significance at week 13 (females only) and at weeks 25, 38 and 51 (males and females). The reduced calcium levels were not accompanied by any bone abnormalities; therefore, they were not considered to be toxicologically relevant. Alkaline phosphatase levels were non-significantly increased in females (from the start of treatment) of the middle-dose group. Glutamate dehydrogenase activities were also slightly elevated in high-dose males and females, achieving statistical significance at one occasion only (in males only, at week 25). ALT activities of only one male dog of the high-dose group appeared to be elevated throughout the study, but no other dog of this group appeared affected until week 51, when the mean for this group became significantly higher than that of the controls. There were sporadic statistically significant alterations in a few urinary parameters compared with control values, but none could be attributed to the treatment. The pH of the high-dose males was more alkaline than the control value at weeks 12, 25 and 38, but statistical significance was not attained. The CYP content in the liver showed a statistically significant increase at 100 ppm in female dogs and at 350 ppm in both sexes. In contrast, the results obtained from measurements of *p*-aminophenol hydroxylase activities (marker enzyme for cytochrome P448 induction) indicate no induction at any dose level. Therefore, cyproconazole is considered to be a CYP inducer and is not associated with cytochrome P448. Analysis of GSH levels displayed a reduction across all dose levels, without a dose–response relationship, significant at 30 and 100 ppm in males and at 100 ppm in females, in comparison with control values.

The livers of two males of the high-dose group appeared enlarged, with pronounced lobular patterning. No other macroscopic findings considered to be treatment related were observed. Absolute and relative liver weights of male dogs showed a dose-related increase; however, only those of animals treated at 350 ppm were statistically significantly increased. A minor, non-significant effect

Table 19. Body weight gain of male and female dogs treated with cyproconazole for 1 year

Sex	Treatment	Body weight gain (kg)[a]	
		Weeks 0–9	Weeks 0–52
Males	Control	1.9	3.4
	30 ppm	1.9 (0%)	5.3 (+56%)
	100 ppm	2.1 (+11%)	4.5 (+32%)
	350 ppm	1.4 (−26%)	3.7 (+9%)
Females	Control	1.0	2.3
	30 ppm	0.8 (−20%)	2.0 (−13%)
	100 ppm	1.5 (+50%)	3.2 (+39%)
	350 ppm	0.8 (−20%)	1.8 (−22%)

From Warren et al. (1988a)
[a] % difference from control shown in parentheses.

on liver weights of females was seen. Minimally increased relative weights were observed for kidney and pituitary of high-dose dogs, without achieving statistical significance. However, there was no clear treatment-related trend with respect to the kidney and pituitary, and, in the absence of corroborative evidence, these apparent findings are considered to be of dubious toxicological significance. The liver was shown to be the target organ. The most prominent findings were laminar eosinophilic intrahepatocytic bodies, present in all male and two female dogs treated with 350 ppm and in one male of the 100 ppm group. These bodies are thought to be associated with hypertrophy of the endoplasmic reticulum, which is an adaptive change allowing increased metabolism of xenobiotics. The presence of large masses of endoplasmic reticulum will result in increased liver size and weight. Canalicular bile plugs were found in two males of the 350 ppm group. The liver toxicity seen in this study (increased liver weights, liver-related clinical chemistry parameters and histopathological findings) was considered to be an adaptive response of the body and not adverse. An increase in intrahepatocytic pigment was also apparently related to treatment in male dogs (one at 100 ppm and three at 350 ppm), but not in females. These findings may reflect a slight degree of intrahepatic cholestasis, which is supported by the increased alkaline phosphatase activity. Occasional occurrence of changes in other tissues, which are considered not to be treatment related, included immaturity of the ovaries in one female of the 350 ppm group, degeneration of the testicular germinal epithelium in one male of the 100 ppm group, glomerular lipoidosis of kidneys in one male of the 100 ppm group, unilateral renal aplasia with compensatory hypertrophy of the centrolateral kidney in one female of the 100 ppm group, perivascular mixed inflammatory cells in the cerebrum and midbrain of one male of the 350 ppm group and an aggregation of subpendymal plate cells in the midbrain of one male of the 100 ppm group. Other recorded lesions were those commonly found in Beagle dogs at this age.

The NOAEL is 100 ppm (equal to 3.2 mg/kg bw per day), based on decreased body weight gain in male dogs seen at the LOAEL of 350 ppm (equal to 12.1 mg/kg bw per day). The study authors set a NOEL of 30 ppm (equal to 1.0 mg/kg bw per day) (Warren et al., 1988a).

(b) Dermal application

Rats

In a repeated-dose dermal toxicity study, groups of 10 Hanlbm: WIST (SPF) rats of each sex per dose received a dermal application of cyproconazole (purity 95.5%) in an aqueous suspension of 1% CMC and 0.1% Tween 80 at doses of 0, 10, 100 or 1000 mg/kg bw per day, 6 hours per day, 5 days per week, for the first 3 weeks and every day thereafter. Approximately 10% of the total body

surface area on the back of each rat was clipped free of hair prior to study initiation and as necessary thereafter. The appropriate volume (5 ml/kg bw) of cyproconazole suspension was applied to the clipped dorsal area and held in contact with the skin with gauze patches. Patches were covered with aluminium foil and fastened with adhesive tape. After 6 hours, the dressings were removed, and the treated sites were rinsed with lukewarm water. Dosing solutions were analysed for concentration, homogeneity and stability of the test substance. Animals were examined for mortality and signs of toxicity twice daily. Detailed clinical examinations (in the home cage, in a standard arena and during handling) were performed pretest and once weekly thereafter. Animals were weighed daily, and body weights were recorded weekly. Water consumption was measured weekly. Feed consumption was measured pretest and weekly thereafter. Ophthalmoscopic examination was performed on all rats before the start of dosing and prior to termination (control and high-dose groups). Urinalysis was not conducted. At termination of the dosing period, haematological and clinical chemistry determinations were performed, and each animal was examined externally and internally for macroscopic changes. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

The concentrations in the dosing solutions were within the acceptable range. The dosing solutions were homogeneously distributed and were stable at room temperature.

There were no treatment-related deaths, clinical signs of toxicity or clinical signs of irritation and no biologically significant treatment-related effects on body weight, body weight gain, feed consumption, motor activity or ophthalmoscopic examination. Males and females in the 1000 mg/kg bw per day group had increases of 36% in haemoglobin distribution width, suggesting anisochromia of red blood cells. Prothrombin time was also increased in males and females in the 1000 mg/kg bw per day group by 29% and 32%, respectively, compared with controls. In addition, males and females in the 1000 mg/kg bw per day group also had increases in monocyte counts of 54% and 34%, respectively, compared with controls. An increase of 88% in large unstained cell counts was noted in 1000 mg/kg bw per day females when compared with controls. Plasma globulin levels were significantly increased ($P \leq 0.01$) in males in the 1000 mg/kg bw per day group by 9% and in females in the 100 and 1000 mg/kg bw per day groups by 8% and 16%, respectively. The albumin to globulin ratio was significantly decreased ($P \leq 0.01$) in males and females in the 1000 mg/kg bw per day group by 13 and 11, respectively. Protein levels were significantly increased ($P \leq 0.05$ or $P \leq 0.01$) in females in the 100 and 1000 mg/kg bw per day groups by 5% and 9%, respectively. Cholesterol levels were significantly increased ($P \leq 0.01$) in females in the 100 and 1000 mg/kg bw per day groups by 43% and 39%, respectively. Minor effects were noted in several electrolytes in males and females in the 100 and 1000 mg/kg bw per day groups. There was a significant increase in one hepatic enzyme (ALT) and a marginal increase in a second hepatic enzyme (AST) in males in the 1000 mg/kg bw per day group. No treatment-related effects were observed on organ weights except for increased absolute and relative liver weights in high-dose males (19–25%) and females (26–28%). No treatment-related macroscopic findings were reported, except for the enlargement of the liver observed in one male and one female in the 1000 mg/kg bw per day group. There was an increased incidence and/or severity of acanthosis at the skin application sites in males and females in the 100 and 1000 mg/kg bw per day groups. There was an increased incidence of centrilobular hepatocellular hypertrophy in males in the 100 and 1000 mg/kg bw per day groups and in females in the 1000 mg/kg bw per day group. An increased incidence of haemosiderosis of the spleen was observed in males in the 1000 mg/kg bw per day group and in females in the 100 and 1000 mg/kg bw per day groups. An increased incidence of hypertrophy of the thyroid follicular epithelium was observed in males and females in the 100 and 1000 mg/kg bw per day groups. The study author suggested that the hypertrophy of hepatocytes and thyroid follicular epithelium as well as the higher protein levels and increased enzyme activities are indicative of adaptive metabolic processes and therefore are not considered as adverse. However, there appears to be a mild anaemia present, with additional evidence provided by the deposition of

haemosiderin in the spleen. This would not constitute an adaptive metabolic process. In addition, the effects on the liver appear to be more extensive than just an adaptive response when there are effects on protein metabolism and the formation of increased cholesterol.

The LOAEL was 1000 mg/kg bw per day, based on the range of effects observed (haematology, clinical chemistry and histopathology) at 1000 mg/kg bw per day. The NOAEL (systemic) was 100 mg/kg bw per day (Sommer, 2000).

(c) Exposure by inhalation

Rats

In a 16-day study of toxicity after repeated doses by inhalation, groups of five male and five female KFM-Han Wistar rats were exposed to cyproconazole (purity 96.2%) via the nose only for 6 hours per day, 5 days per week, for a total of 12 exposures. The nominal dose levels were 0, 0.01, 0.1 and 1.0 mg/l. Mean air flow in the exposure chambers was 1.4 l/min per animal. Recovery duration after the last exposure was 15 days. Aerosol concentration was determined gravimetrically using glass fibre filters once pretest and daily thereafter during exposure periods. Particle size distribution was determined at least once daily in the high dose and in one of the lower doses using a stage cascade impactor. Oxygen concentration, humidity and temperature in the exposure chamber were measured daily. The animals were observed twice daily for mortality and once for clinical signs of toxicity. Feed consumption was recorded weekly, and individual body weights were recorded twice during the acclimatization period (9 days) and weekly thereafter. Blood samples for haematology and clinical chemistry were collected from all animals of the control and low-dose groups and from three males and three females of the mid-dose group on day 17 and from two males and two females of the mid-dose group following the 15-day recovery period. At termination, each animal was examined externally and internally for macroscopic changes. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

Gravimetric determination of the aerosol concentrations revealed means of 0.017 mg/l (low dose), 0.099 mg/l (middle dose) and 1.026 mg/l (high dose). Particle size determinations showed comparable distributions over time and dose. As a mean of all determinations, 90% of the particles were found to be smaller than 3.0 μm.

All animals in the high-dose group either died or were sacrificed by day 6. All high-dose animals exhibited slight hunched posture, laboured respiration, ruffled fur and weakness. In mid-dose animals, slight squealing, howling and crying were noted in one male and three females. The same symptoms were observed in one male and two females of the low-dose group. Feed consumption was comparable in the treated and control groups. No effects of treatment on body weights of treated males and females were noted. No treatment-related effects were observed on haematological parameters, except for slightly dose-related increases in white blood cell counts in male and female rats. Bilirubin levels were slightly, but statistically significantly, reduced in male animals of the low- and mid-dose groups. LDH and alkaline phosphatase activities were slightly lower in mid-dose males (not statistically significant). In female animals, effects were seen only in the middle-dose group; these effects were related to liver toxicity—slight to moderate increases in AST and ALT levels and high LDH activity. None of these effects were statistically significant. After recovery, they all returned to normal levels. In male animals, there was a statistically significant increase in absolute liver and kidney weights in the low- and mid-dose groups and in relative liver and kidney weights in the mid-dose group. In female animals, there was only a slight increase in relative liver weight in the mid-dose group. Recovery data indicate the reversibility of these effects.

No macroscopic findings were recorded. Treatment-related findings were recorded in the respiratory tract, liver and spleen. In the high-dose rats, slight squamous metaplasia of nasal epithelium was recorded in two females. Slight or moderate hepatocellular hypertrophy occurred in four males

and two females. Minimal to marked fatty change of liver cells was recorded in five males and four females. Minimal or slight vacuolation of macrophages in the splenic pulp was recorded in two males and two females, and slight to moderate depletion of lymphocytes was evident in the spleen of four females. Hepatocellular hypertrophy was also recorded in two males and one female of the mid-dose group and one male of the low-dose group. At the end of the recovery period, hepatocellular hypertrophy was not recorded in two males and one female of the high-dose group. Spleen weights were not recorded in this study; however, histopathological examination revealed effects at the high dose and haemosiderosis in the females at the middle dose. Haemosiderosis has previously been noted in other subchronic studies with cyproconazole and is considered treatment related.

As the weak effects observed at the lowest concentration represent the kind of effects from which the animals recovered completely at the higher concentration of 0.1 mg/l, this nominal concentration of 0.01 mg/l, corresponding to an achieved concentration of 0.017 mg/l, is considered to represent the no-observed-adverse-effect concentration (NOAEC) of the study (Bernstein et al., 1987). On a body weight basis, this concentration corresponds to 4.9 mg/kg bw per day (6 hours of exposure per day; 0.8 litres of air per kilogram body weight per minute).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity, groups of 50 male and 50 female CD-1 mice, including two control groups, were given diets containing cyproconazole (purity 95.6%) at dose levels of 0, 5, 15, 100 and 200 ppm, equal to 0, 0.69, 1.84, 13.17 and 27.85 mg/kg bw per day for males and 0, 1.03, 2.56, 17.65 and 36.30 mg/kg bw per day for females, for 81 and 88 weeks, respectively. An additional 10 animals of each sex in the 0 and 200 ppm groups were sacrificed at week 13 as satellite groups. The initial age was 6 weeks. Premix and final diets were analysed for homogeneity and stability of the test substance at least every 2 months. The mice were housed individually. Mortality and gross signs of ill-health or reaction to treatment were checked twice a day. Body weight and feed intake were measured weekly throughout the entire study period. After 52 and 78 weeks and (for females) prior to termination, blood smears were prepared from all surviving animals following superficial venesection of the tail. Differential white blood cell counts were performed only on the smears of all animals from the 0 and 200 ppm dose groups. All animals were subjected to a detailed gross pathological examination. At termination, organ weights were obtained for adrenals, brain, heart, kidneys, liver, gonads, pituitary and spleen. Histopathological examination of selected tissues was conducted on all animals in the control and high-dose groups.

Values for test compound were generally in agreement with nominal values. The percentage deviation from nominal values, determined at various intervals throughout the treatment period, were −20% to +36%, −13% to +27%, −16% to +23% and −22% to +6% at dose levels of 5, 15, 100 and 200 ppm, respectively. Homogeneity and stability results were not provided in the study report. It was not considered that these variations were sufficient in degree or frequency to affect the interpretation of results of the study.

Mortality among mice in both sexes at 100 or 200 ppm appeared less than that among the controls or among mice receiving 5 or 15 ppm (Table 20).

No treatment-related symptoms were observed. The majority of symptoms were signs of ill-health preceding death. Serology samples showed the presence of antibodies to mouse hepatitis virus, *Mycoplasma pulmonis* and Sendai virus. The presence of antibodies, however, did not correlate with an adverse effect on the study due to these pathogens. Body weight developments in both sexes at 100 or 200 ppm were retarded. Feed consumption was not affected by treatment. No treatment-related differences were revealed in blood smears in both sexes at 0 and 200 ppm at different time points. At

Table 20. Mortality in a carcinogenicity study in mice

Period	Number of decedents/total number of animals[a]					
	0 ppm (control 1)	5 ppm	15 ppm	100 ppm	200 ppm	0 ppm (control 2)
Males						
Week 0–termination	31/50 (62%)	30/50 (60%)	25/50 (50%)	21/50 (42%)	14/50 (28%)	34/50 (68%)
Females						
Week 0–termination	33/50 (66%)	26/50 (52%)	30/50 (60%)	14/50 (28%)	23/50 (46%)	27/50 (54%)

From Warren et al. (1989)
[a] % mortality shown in parentheses.

termination, relative liver weights of animals of both sexes that received 100 or 200 ppm were significantly increased: in males by 22% and 42% at 100 and 200 ppm, respectively, and in females by 29% and 59% at 100 and 200 ppm, respectively. The analysis of the effect of the presence of liver nodules on the liver weights indicated that the increase in liver weight was not a consequence of the masses. Absolute and relative liver weights of the satellite groups (200 ppm) terminated after 13 weeks of treatment were significantly increased in males and females. There was a significant increase in the incidence of hepatic accentuated lobular pattern and of hepatic masses at 15, 100 and 200 ppm in males and at 100 and 200 ppm in females compared with each of the two control groups. The hepatic masses corresponded to the treatment-related increases in hepatic adenomas and carcinomas in both sexes. The females at 200 ppm also had an increased incidence of areas of hepatic change and enlargement. The males at 200 ppm also had a small increase in the incidence of granular liver. In the males at 100 and 200 ppm, there were reduced incidences of gastrointestinal tracts with abnormal contents in all regions examined apart from the stomach, which was unaffected. This treatment-related effect was not seen in females. Renal findings that may relate to treatment were restricted to a reduction in the incidence of granular kidneys in the 200 ppm females and to an apparent reduction in size of the kidneys in the 200 ppm male group. Skin ulceration was increased in the 200 ppm female group. In the males at 100 and 200 ppm, oedema of the subcutis was reduced in incidence. In the 100 ppm female group, the same effect was observed. The incidence of flaccid testes corresponding to an increased testicular germinal epithelial deficit was increased in males at 200 ppm only. The major treatment-related non-neoplastic changes are shown in Table 21.

In the liver, the major toxic changes (single-cell necrosis, diffuse hypertrophy, centriacinar hepatocytic vacuolation) were increased at 100 and 200 ppm in both sexes, although the male mice were more severely affected. The incidence of focal hepatocytic hyperplasia was increased at 200 ppm in females. Several non-neoplastic changes in male mice were increased: optic nerve gliosis (200 ppm), skin ulceration (200 ppm) and cellulitis (100 and 200 ppm), epididymal aspermia (100 and 200 ppm) and testicular germinal epithelial deficit (100 and 200 ppm). In females, aortic arteritis (200 ppm) and lymphoid hyperplasia in the mesenteric lymph modes (200 ppm) were increased. The lack of dose dependencies in the incidences of testicular lesions and aspermia in the epididymis and lack of testicular toxicity in the 90-day study of mice conducted at doses up to 88.7 mg/kg bw (Warren, Skinner & Karapally, 1987) indicated that cyproconazole has no potential for testicular toxicity. In the satellite group (13 weeks of treatment), the incidences of periacinar hepatocytic hypertrophy in both males and females, periacinar hepatocytic vacuolation in males and non-zonal hepatocytic fat vacuolation in females were significantly increased by the treatment. Treatment-related effects on incidences of neoplastic changes are shown in Table 22.

The incidences of hepatocytic adenoma in males at 100 and 200 ppm and in females at 200 ppm and of hepatocytic carcinoma in males at 15 and 100 ppm and in females at 200 ppm were significantly increased for all animals. The incidence of hepatocytic carcinoma in males at

Table 21. Non-neoplastic findings in a carcinogenicity study in mice[a]

	Incidence (number observed out of total evaluated)											
	Males						Females					
	0 ppm (control 1)	5 ppm	15 ppm	100 ppm	200 ppm	0 ppm (control 2)	0 ppm (control 1)	5 ppm	15 ppm	100 ppm	200 ppm	0 ppm (control 2)
Liver												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Focal inflammation	1	1	4	5*	8**	1	1	5	9	5	4	6
Single-cell necrosis	0	2	3	14***	25***	2	0	3*	2	4*	9***	0
Diffuse hepatocytic hypertrophy	4	4	6	26***	36***	10	5	6	6	7	20***	8
Periacinar hepatocytic hypertrophy	1	2	5*	4*	2	0	0	1	0	0	3*	0
Centriacinar hepatocytic vacuolation	0	0	0	1	3*	0	0	0	0	4*	3*	0
Periacinar hepatocytic vacuolation	0	4*	3	1	1	1	0	0	1	17***	6**	1
Focal hepatocytic hyperplasia	1	0	2	2	2	1	0	0	1	0	5**	0
Testes												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	—	—	—	—	—	—
Germinal epithelial deficit	22	31	29	34**	33*	23	—	—	—	—	—	—
Epididymides												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	—	—	—	—	—	—
Aspermia	10	20	15	26**	21*	15	—	—	—	—	—	—
Optic nerve												
Total evaluated	*45*	*45*	*44*	*45*	*46*	*41*	—	—	—	—	—	—
Gliosis	0	2	2	2	3*	0	—	—	—	—	—	—
Skin												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Ulceration	4	5	4	6	9*	2	1	4	3	5	6	3
Cellulitis	4	5	6	8*	8*	1	1	4	3	5	6	3
Heart												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Aortic arteritis	0	0	0	0	0	1	1	0	0	2	4*	0
Mesenteric lymph nodes												
Total evaluated	*48*	*45*	*49*	*49*	*47*	*50*	*47*	*49*	*46*	*50*	*50*	*50*
Lymphoid hyperplasia	0	0	2	2	0	1	3	2	1	2	13*	7

From Warren et al. (1989)
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with pooled controls 1 + 2)
[a] Data extracted from Table 5 of the study report.

15 ppm was increased. To confirm the significance of the increase, an analysis of trends in association between incidences of all neoplastic tumours and dosage of cyproconazole was performed (Table 23).

Table 22. Neoplastic changes in a carcinogenicity study in mice

	Incidence (number observed out of total evaluated)											
	Males						Females					
	0 ppm (control 1)	5 ppm	15 ppm	100 ppm	200 ppm	0 ppm (control 2)	0 ppm (control 1)	5 ppm	15 ppm	100 ppm	200 ppm	0 ppm (control 2)
All animals												
Total evaluated	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Hepatocytic adenoma	3	4	5	12**	12**	3	0	0	0	2	6**	0
Hepatocytic carcinoma	0	0	3*	3*	1	0	0	0	0	0	7***	0
Animals killed or dying during the treatment period												
Total evaluated	*31*	*30*	*25*	*21*	*14*	*34*	*33*	*26*	*30*	*14*	*23*	*27*
Hepatocytic adenoma	1	2	1	0	2	0	0	0	0	0	3*	0
Hepatocytic carcinoma	0	0	2	2	1	0	0	0	0	0	5**	0
Animals killed after 82 weeks of the treatment period												
Total evaluated	*19*	*20*	*25*	*29*	*36*	*16*	*17*	*24*	*20*	*36*	*27*	*23*
Hepatocytic adenoma	2	2	4	12*	10	3	0	0	0	2	3	0
Hepatocytic carcinoma	0	0	1	1	0	0	0	0	0	0	2	0

From Warren et al. (1989)
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with pooled controls 1 + 2)
[a] Data extracted from Tables 5, 8 and 10 of the study report.

Table 23. Combined liver adenoma and carcinoma in a carcinogenicity study in mice

	Males					Females				
	Dietary concentration (ppm)									
	0	5	15	100	200	0	5	15	100	200
No. of animals	100	50	50	50	50	100	50	50	50	50
No. of affected animals	6	4	8	15	13	0	0	0	2	13
Statistical significance (not adjusted for age)	###	n/s	*	***	***	###	n/s	n/s	n/s	***
Age-adjusted analysis: statistical significance	##	n/s	n/s	**	n/s	###	n/s	n/s	n/s	***

From Warren et al. (1989)
##, significant trend over affected groups, $P < 0.01$; ###, significant trend over affected groups, $P < 0.001$; n/s, not significant pairwise comparison, $P > 0.05$; *, significant pairwise comparison, $P < 0.05$; **, significant pairwise comparison, $P < 0.05$; ***, significant pairwise comparison, $P < 0.001$

The analysis of the liver tumours indicated a positive association at doses of 15, 100 or 200 ppm in males and 200 ppm in females (Table 23). However, there is an uneven distribution of survival in this study (see Table 20). Better than expected survival was found in males at 15, 100 and 200 ppm and in females given 100 ppm. Animals with longer survival have a greater risk of developing tumours. Thus, males with the greater lifespan bore the most liver tumours. The Peto analysis (age-adjusted analysis for fatal, incidental and total tumours) sets out to assess whether the greater lifespan was the

only cause of the greater incidences or whether there was an effect of treatment (Table 23). The results in Table 20 indicate an effect of difference of survival; that is, there was a treatment-related effect in the combination of fatal and incidental tumours in males at 100 ppm and in females at 200 ppm. In addition, in female mice of all three categories (fatal, incidental and combined), the effect was present at the highest dose level only. In male mice, the effect (combined) of the 15 ppm group was no longer significant with Peto analysis ($P = 0.188$), and that of the 200 ppm group fell just below the level of significance ($P = 0.052$). In male mice of the 100 ppm group, the effect was significant in the combined and incidental tumour categories, but not in the fatal tumour group. Although there were a number of neoplasms present in animals of the control and treated groups, their incidences, except liver tumours, were not statistically significant, and they were considered not to be treatment related.

The LOAEL in this study was 100 ppm, equal to 13.2 mg/kg bw per day, based on reduced body weight gains, the toxic changes in the liver and the increase in incidence of adenoma and adenocarcinoma in the liver. The NOAEL in this study was 15 ppm, equal to 1.84 mg/kg bw per day (Warren et al., 1989).

Mode of action (MOA) studies for liver tumour development were conducted (see section 2.6 and Appendix 2).

Rats

In a combined long-term study of toxicity and carcinogenicity, groups of 70 male and 70 female Han Wistar (Kfm: WIST) rats were given diets containing cyproconazole (purity 95.6%) at dose levels of 0, 20, 50 or 350 ppm, equal to 0, 1.01, 2.22 and 15.59 mg/kg bw per day for males and 0, 1.24, 2.73 and 21.76 mg/kg bw per day for females, for 118–119 weeks and 121–122 weeks, respectively. Premix and final diets were analysed for homogeneity and stability of the test substance prior to study initiation and at monthly intervals during the study. Mortality was checked twice a day. Detailed examination of skin, fur, eyes, mucous membranes, and respiratory and circulatory activity and palpation of masses (if indicated) were conducted biweekly. Body weight and feed consumption were determined weekly up to 13 weeks of treatment, then for weeks 15, 17, 19 and 21, then again weekly. Ophthalmological examination was performed during weeks 98 and 99 on all surviving rats of the control and 350 ppm groups. Haematological, clinical chemistry and urinalysis examinations were carried out on 10 rats of each sex per group at weeks 14, 26, 52, 78 and 105 and at treatment termination. Ten rats of each sex per group were sacrificed after each of 52 and 78 weeks of treatment. For the rats killed at week 52, samples of the liver were frozen and analysed for glycogen content, glucose-6-phosphatase activity and fructose-1,6-diphosphatase activity. The remaining surviving animals were sacrificed at treatment termination. A gross necropsy was conducted on all animals. At interim and terminal sacrifices, organ weights were obtained for adrenals, brain, heart, kidneys, liver, gonads, pituitary (termination only) and spleen from all animals. Histopathological examination of selected tissues was conducted on all animals.

The analysed concentrations of test material in the diets were reported as generally acceptable and within 10% of nominal concentrations. All dietary concentrations were higher (22–50%) than nominal concentrations at 28 weeks. Deviations from nominal were greater than 15% at seven, six and two intervals of analysis at nominal levels of 20, 50 and 350 ppm, respectively. Mean concentrations for the entire study, however, were within 5% of the nominal at all dose levels. Homogeneity and stability were not provided in the study report. Dietary concentrations at 50 ppm and lower doses sometimes deviated over 30% during the first 8 months, which raises concern as to the exact dose delivered to the animals.

A slightly lower number of decedents was noted among rats of the high-dose group (350 ppm) by week 79. However, at termination, the mortality of the males was 72%, 68%, 68% and 56%, and the mortality of the females was 64%, 62%, 58% and 66%, at 0, 20, 50 and 350 ppm, respectively. There were no treatment-related symptoms during the study. The behaviour of control and treated rats

remained similar. Mean body weights of both sexes at 350 ppm were significantly depressed throughout the study. The cumulative body weight gains were below control levels, varying by 5–10% and 10–21% in males and females, respectively. Body weight and body weight gain of both sexes at 20 or 50 ppm were not affected by treatment with cyproconazole. Feed consumption was increased in high-dose females during the first 13 weeks by 7% compared with the control group. Part of this increase may be due to food scattering. No effect was seen in males. No treatment-related findings were observed in ophthalmological examination. No treatment-related changes in haematological parameters were observed except for a significant decrease in lymphocytes and a significant increase in neutrophils in males at 350 ppm at week 14. These changes were considered to be of no toxicological relevance, because of lack of time and dose dependency and no corresponding findings among females. At the clinical chemistry examination, a decrease in total bilirubin level and increase in GGT activity in both sexes at 350 ppm were observed at many time points, although the changes were not statistically significant. The levels of bilirubin and GGT in the rat are normally very low and close to the limit of detection by the methodology employed. The significance of these findings is therefore questionable. Significant increases in total protein and globulin levels in females, a higher trend of cholesterol levels in females and increases in AST and ALT values in males were observed at 350 ppm during the late portion of the study, although the magnitudes of these changes were small. A marginal but consistent increase in urea levels in females at 350 ppm was noted. Corticosteroid levels at week 52 were unaffected. No treatment-related changes in urinalysis were observed. Analysis of livers for glycogen content, glucose-6-phosphatase activity and fructose-1,6-diphosphatase activity revealed no clear treatment-related effects. A marked reduction in body weight (varying from 15% to 20%) in females at 350 ppm at all scheduled sacrifices affected the analysis of organ weights to a small extent. Relative liver and kidney weights were significantly increased in females at 350 ppm at all or most time points. There were no treatment-related gross findings. Histopathologically, hepatocellular fatty change was found in males at 350 ppm, with increased incidence and severity at all time points. An increased incidence of hepatocellular hypertrophy was seen in females at 350 ppm at week 78 (Table 24).

Numerous other non-neoplastic lesions were diagnosed in various organs and tissues. The type, incidence and severity of these lesions were considered to be similar in both treated and control rats. A number of neoplasms were diagnosed in rats of all dose groups. The type, incidence and malignancy of these neoplasms were considered to be similar in both treated and control rats.

The LOAEL was 350 ppm (equal to 15.59 mg/kg bw per day), based on the body weight depression in both sexes. The NOAEL was 50 ppm (equal to 2.22 mg/kg bw per day). Under the conditions of this study, cyproconazole was not carcinogenic to rats (Warren et al., 1988b).

2.4 Genotoxicity

Cyprocobazole was negative for mutagenicity in various in vivo and in vitro genotoxicity assays (Table 25).

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a two-generation study of reproductive toxicity, groups of 26 male and 26 female KFM Wistar rats were given diets containing cyproconazole (purity 95.6%) at a concentration of 0, 4, 20 or 120 ppm (equal to 0, 0.28, 1.39 and 8.29 mg/kg bw per day for males and 0, 0.33, 1.67 and 9.88 mg/kg bw per day for females, respectively). Treatment started after a 14-day acclimation period and 10 weeks before mating and continued for the dams through weaning of offspring at 21 days postpartum. Treatment

Table 24. Selected non-neoplastic findings in the liver in a 2-year rat study

Findings	Incidence							
	Males				Females			
	Control	20 ppm	50 ppm	350 ppm	Control	20 ppm	50 ppm	350 ppm
Hepatocellular fatty change								
Terminal sacrifice, including intercurrent deaths	23/50	19/49	29/50	38/50	23/50	15/50	15/50	10/50
Interim sacrifice week 52	4/10	5/10	7/10	10/10	1/10	—	—	1/10
Interim sacrifice week 78	3/10	1/10	4/10	10/10	—	—	—	—
Hepatocellular hypertrophy								
Terminal sacrifice, including intercurrent deaths	—	—	1/50	1/50	—	—	—	—
Interim sacrifice week 52	—	—	—	—	—	—	—	—
Interim sacrifice week 78	—	—	—	—	—	—	—	4/10

From Warren et al. (1988b)

for males was continued for approximately 3 weeks after termination of the mating period. On day 4 postpartum, litters were culled to four males and four females per litter, when more than four fetuses of each sex were in the group. Randomly, 26 males and females of the F_1 generation (one male and one female per litter if possible) were treated at the same dose levels as the F_0 parents from the time of weaning. These treated rats were then mated at 15 weeks of age. Mating of siblings was avoided. Dosing continued through the weaning of F_2 offspring. Males of both generations were weighed weekly throughout the study. Dams were weighed weekly during premating periods and on days 0, 7, 14 and 20 of pregnancy. Dams and offspring were weighed on days 0, 7, 14 and 21 postpartum. All animals were examined daily for mortality and clinical signs, and females were observed 3 times a day from day 20 of gestation for signs of parturition. Feed consumption was monitored at the same time as body weight was recorded, except during the mating period, when both males and females had access to the same feeder. Parturition was observed and described. Litter size, sex distribution and malformations of pups were recorded. Sacrificed parental animals were necropsied. Organs or tissues with major macroscopic abnormalities were removed and processed for histopathology. The liver of each animal killed at scheduled termination was weighed and investigated histopathologically. In addition, the following tissues were prepared for histopathological investigations: vagina, cervix, uterus, ovaries, testes, epididymides, seminal vesicles, prostate, coagulating gland and pituitary gland.

Prepared diets were analysed for stability, homogeneity and concentrations before the start of the treatment and every second month thereafter. Prepared diets were stable for 14 days at room temperature.

No treatment-related clinical signs were noted in either the F_0 or F_1 parental generation, and no parental animals died during treatment. The only clinical signs noted in F_0 animals were in a single high-dose female that failed to deliver and showed signs of distress and increased respiration. Clinical signs in the F_1 generation were minor and did not appear to be treatment related. No treatment-related mortality occurred in the parental animals of both generations. Body weight and feed consumption were comparable between treated groups and controls during the entire study period. A marginal/slight increase in relative liver weight was seen in F_0 males (5.5% greater than controls) and females (4.4% greater than controls), attaining statistical significance in males only ($P > 0.05$). Slight liver fatty change was noted at increased incidence in high-dose F_0 males (Table 26). This observation is of questionable significance, as no toxicity was seen in F_0 females or F_1 males and females. No other treatment-related changes were observed. Indices reflecting mating success were comparable between

Table 25. Results of studies of genotoxicity with cyproconazole

End-point	Test system	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Reverse mutation (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538	1.0–5000 µg/plate ± metabolic activation in DMSO	95.6	Negative	Hoorn (1986)
HGPRT gene mutation	Chinese hamster ovary cell line V79	20–200 µg/ml ± metabolic activation in DMSO	94.4	Negative	Miltenburger (1985b)
Mitotic non-disjunction assay (aneuploidy)	*Saccharomyces cerevisiae*	10–550 µg/ml ± metabolic activation in DMSO	Not specified	Negative	Hoorn (1985)
Chromosomal aberration	Chinese hamster ovary cells	100–200 µg/ml without and 100–250 µg/ml with metabolic activation in DMSO	95.6	Weakly positive	Enninga (1988)
				Negative (re-evaluation)	McEnaney (1992)
	Chinese hamster ovary cells	60–500 µg/ml without and 15–200 µg/ml with metabolic activation in DMSO	95.6	Negative	Murli (1990)
	Chinese hamster ovary cells	25–800 µg/ml ± metabolic activation in DMSO	100	Negative	Saigo (1995)
UDS (DNA repair)	Rat primary hepatocytes	0.15–15 µg/ml ± metabolic activation in DMSO	96.2	Negative	Curren (1988)
Morphological transformation assay	Syrian hamster embryo cells	20–200 µg/ml ± metabolic activation in DMSO	94.4	Negative	Miltenburger (1985a)
In vivo					
Micronucleus formation	Swiss random mice (5 males and 5 females per group)	0, 16.7, 55.7, 167 mg/kg bw (single oral dose in DMSO)	Not specified	Negative	Taalman (1985)
Cytogenetic assay (chromosomal aberrations)	ICO:CDI (CRL) mice (5 males and 5 females per group)	0, 50, 100, 200 mg/kg bw in arachis oil	95.5	Negative	Ogorek (1999)
Dominant lethal assay	Sprague-Dawley rats (20 males per group)	0, 20, 40, 80 mg/kg bw in corn oil	95.6	Negative	Putman (1991)

DNA, deoxyribonucleic acid; UDS, unscheduled DNA synthesis

treated groups and controls. Several females in all groups of both generations failed to become pregnant; however, there were no treatment-related differences between treated and control groups. The fertility indices in the F_0 and F_1 females were comparable between treated and control groups. Numbers of implantation sites per dam did not significantly differ among groups. The mean pregnancy length in the low-dose F_0 and F_1 females was comparable to that of their respective controls. An increased gestation length was observed in some mid- and high-dose F_0 females. A single mid-dose animal delivered a single pup on day 24 of gestation. A single female of the high-dose F_0 group failed to deliver and was not included in the calculations of gestation length. However, this female had only a single implantation site and no fetuses at termination. In the F_0 generation, the females delivered on day 22 or 23; however, the distribution between those 2 days varied among groups. Delivery on day 22 occurred for 66% of the controls and 50% of the dams in the 20 and 120 ppm groups, indicating a slight increase in the length of pregnancy in some animals of these dose groups, which may have been related to treatment. However, there was no such increase in the F_1 generation dams.

Table 26. Incidence of liver fatty changes in F_0 males in a two-generation reproductive toxicity study in rats

Liver fatty change	Incidence (number out of total number evaluated)			
	Controls	4 ppm	20 ppm	120 ppm
No. of tissues evaluated	*26*	*26*	*26*	*26*
Total	10	12	12	19*
Minimal	4	3	2	4
Slight	6	8	9	14
Moderate	—	1	1	1

From Eschbach et al. (1987)
* $P < 0.05$

No treatment-related malformations were evident. Treatment did not have an apparent effect on sex distribution in either F_1 or F_2 pups. No treatment-related clinical signs were noted in F_1 or F_2 pups. No treatment-related effects on body weights were observed at any of the investigated postnatal intervals in either F_1 or F_2 pups. The mean number of implantation sites was decreased in the high-dose group (−7.2% compared with controls); however, the value was within the historical control range. A decreased litter size at birth was seen in this group (−12% compared with controls). Neither parameter was affected in the F_1 generation. Among pups in the F_1 generation, there was a dose-related increase in prenatal/perinatal mortality in the mid- and high-dose groups (13.6% and 16.3%, respectively). The prenatal/perinatal mortality, 10.7%, was also considered relatively high in the control group by the author. As shown in Table 27, there was a corresponding slight increase in postnatal mortality (days 0–21 postpartum) in the mid- and high-dose groups (6.6% and 8.1%). Postnatal days 0–4 were most affected. However, the apparent increases at 20 ppm in prenatal/perinatal and postnatal mortality in the F_1 litters result from one single F_0 dam that lost 100% of its pups (12/13 prenatal/perinatal losses, the single surviving pup dying in the first 4 days). As there was no effect in the F_1 generation, this event was unlikely to be treatment related. In the F_2 litters, there was a slight increase in prenatal/perinatal mortality at the high dose only. There was a slightly higher postnatal mortality during days 0–4 in the F_2 high-dose group only (7.6% greater than controls). Pup weights were not affected in either F_0 or F_1 generations.

Administration of cyproconazole in the diet of rats in this two-generation reproductive toxicity study produced minimal signs of parental toxicity in F_0 males at the highest dose tested. A slight (statistically significant) increase in relative liver weight was associated with a marginally increased incidence of liver fatty change. This observation is of questionable significance, as no toxicity was seen in F_0 females or F_1 males and females. In addition, the 90-day rat studies (Skinner et al., 1985b; Gerspach, 1999) indicated that the NOAEL for liver effects was in excess of 80 ppm (6.4 mg/kg bw per day). An increase in gestation length in some high-dose F_0 females may have been related to treatment, but was not observed in the F_1 generation. In the F_0 generation, there was a slight, statistically non-significant decrease in the number of implantations in the high-dose group. In addition, there was a dose-related increase in prenatal/perinatal mortality in the high-dose groups in the F_0 and F_1 generations (16.3% and 12.6%, respectively). There was a corresponding slight increase in postnatal mortality (days 0–21 postpartum) in the high-dose group of the F_0 and F_1 generations (8.1% and 7.6%, respectively). Treatment with cyproconazole had no effects at 4 ppm.

The NOAEL for parental systemic toxicity was 20 ppm (equal to 1.39 mg/kg bw per day), based on slight fatty changes (vacuolated hepatocytes) seen at the LOAEL of 120 ppm (equal to 8.29 mg/kg bw per day). The NOAEL for reproductive and offspring toxicity was equal to or greater than 120 ppm (equal to or greater than 8.29 mg/kg bw per day), the highest dose tested. The LOAEL for reproductive and offspring toxicity was not established. The study authors set a NOEL of 4 ppm

Table 27. Litter data (calculated from litter values) in the two-generation reproductive toxicity study in rats

Parameter	Generation	0 ppm	4 ppm	20 ppm	120 ppm
Mean live pups/dam:					
- Day 0	F_1	11.2	11.5	10.8	9.8↓
- Day 4[a]		7.8	7.7	7.5	7.2↓
- Day 21		7.8	7.7	7.4	7.1↓
- Day 0	F_2	11.0	11.7	12.0	10.9↓
- Day 4[a]		7.7	7.7	7.8	7.2↓
- Day 21		7.7	7.6	7.7	7.1↓
Prenatal and perinatal loss (%)	F_1	10.7	7.8	13.6↑	16.3↑
	F_2	11.3	7.3	5.6	12.6↑
Mean postnatal loss (%):					
- Days 0–4	F_1	0.3	1.6	5.6	7.6↑
- Days 0–21		0.3	1.6	6.6	8.1↑
- Days 0–4	F_2	2.2	4.2	1.4	5.8↑
- Days 0–21		2.2	5.9	2.9	7.6↑
Sex ratio at birth (males/females)	F_1	0.49	0.47	0.49	0.49
	F_2	0.48	0.50	0.49	0.55
Mean pup weight at birth (g):					
- Male	F_1	5.9	5.8	5.9	5.9
- Female		5.6	5.5	5.6	5.7
- Male	F_2	5.8	6.0	5.8	6.0
- Female		5.5	5.8	5.4	5.7
Mean pup weight at day 21 postpartum (g):					
- Male	F_1	45.8	45.4	46.4	44.2
- Female		44.1	44.6	44.4	43.5
- Male	F_2	46.2	49.4	49.0	47.1
- Female		44.9	47.6	46.9	45.5

From Eschbach et al. (1987)
[a] After culling.

(equal to 0.33 mg/kg bw per day) (Eschbach et al., 1987). However, the original report did not consider the fact that apparently increased litter mortality in the 20 ppm group was considerably affected by the total loss of one single dam (Doubovetzky, 1998).

(b) Developmental toxicity

Rats

In a non-GLP range-finding study of developmental toxicity, groups of five pregnant KFM-Han Wistar rats were given cyproconazole (purity 95.6%) at a dose of 0, 7.5, 30, 75 or 120 mg/kg bw per day by gavage in 4% CMC in distilled water from day 6 to day 15 of gestation, inclusive. The dosing solutions were prepared daily. All rats were observed twice daily for clinical signs of toxicity, mortality and moribundity. Maternal body weights were recorded on day 0 until day 21 postpartum. Feed consumption was recorded on days 6, 11, 16 and 21 postcoitum. On day 21 of gestation, all surviving dams were sacrificed and subjected to gross necropsy. Fetuses were removed by caesarean section. Examinations at sacrifice comprised uterine weight, number and positions of implantations, number of corpora lutea in each ovary, individual fetal weights, percentage preimplantation loss, percentage postimplantation loss, and early and late intrauterine deaths. All fetuses were weighed,

Table 28. Maternal body weight gain in a range-finding teratogenicity study in rats[a]

Gestation period	Maternal body weight gain (g)[b]				
	Dose (mg/kg bw per day)				
	0	7.5	30	75	120
Days 0–6	23	22	23	22	20
Days 6–11	17	12 (−29%)	12 (−29%)	10 (−41%)	3 (−82%)
Days 11–16	23	26 (+13%)	21 (−9%)	15 (−55%)	18 (−22%)
Days 6–16	40	38 (−5%)	33 (−18%)	25 (−38%)	21 (−48%)
Days 16–21	46	56 (+22%)	47 (+2%)	31 (−33%)	33 (−28%)
Days 6–21	86	94 (+9%)	80 (−7%)	56 (−35%)	54 (−37%)

From Becker (1985a)

[a] No statistical evaluation of the data was performed.

[b] % difference from control shown in parentheses.

sexed and examined for external malformations/variations. The uteri of all females found to be not pregnant at necropsy were placed in an aqueous solution of ammonium sulfide to accentuate possible haemorrhagic areas of implantation sites.

No deaths occurred and no symptoms or clinical signs were observed in any group. No abnormal findings were noted at necropsy in any females. A reduction in body weight gain was apparent in the high-dose animals (2/5 with live fetuses) in the early days of treatment (Table 28). Weight gain was otherwise similar to that of controls in other groups. Mean body weights were lower from 30 mg/kg bw per day upwards, but this was due to the greatly reduced number of fetuses. Feed consumption was reduced in the 75 and 120 mg/kg bw per day groups throughout treatment, amounting to 10% less than controls at 75 mg/kg bw per day and 16.7% less at 120 mg/kg bw per day.

Reproduction parameters were not affected at 7.5 mg/kg bw per day. At 30 mg/kg bw per day and above, increased numbers of early resorptions (24–54% of implants) and reduced fetal body weights were observed. Two of five dams at 75 mg/kg bw per day and three of five dams at 120 mg/kg bw per day were found to have lost all implantations by resorption. The following malformations and/or anomalies were found upon external investigations:

- Control — no malformations or anomalies
- 7.5 mg/kg bw per day — no malformations or anomalies
- 30 mg/kg bw per day — 1 palatoschisis (in 47 fetuses)
- 75 mg/kg bw per day — 1 palatoschisis (in 16 fetuses)
- 120 mg/kg bw per day — 10 of 11 fetuses with palatoschisis (2 litters)

The NOAEL for maternal toxicity was 30 mg/kg bw per day. The LOAEL for maternal toxicity was 75 mg/kg bw per day, based on decreased body weight gain and feed consumption. The NOAEL for developmental toxicity was 7.5 mg/kg bw per day, based on a dose-related increase in early resorption at 30 mg/kg bw per day and above and a dose-related increase in palatoschisis (cleft palate) seen at the LOAEL of 30 mg/kg bw per day and above. The study author concluded that the NOAEL was 7.5 mg/kg bw per day (Becker, 1985a).

In the main study of developmental toxicity, groups of 25 pregnant KFM-Han Wistar rats were given cyproconazole (purity 95.6%) at a dose of 0, 6, 12, 24 or 48 mg/kg bw per day by gavage in 4% CMC in distilled water (10 ml/kg bw volume) from day 6 to day 15 of gestation, inclusive. The

Table 29. Maternal body weight gain in main teratogenicity study in rats

Gestation period	Maternal body weight gain (g)[a]				
	Dose (mg/kg bw per day)				
	0	6	12	24	48
Days 0–6	18	18	17	18	17
Days 6–11	17	16 (−6%)	14 (−18%)**	12 (−29%)**	11 (−35%)**
Days 11–16	27	26 (−4%)	25 (−7%)	23 (−15%)**	22 (−19%)**
Days 6–16	44	42 (−5%)	39 (−11%)	35 (−20%)**	33 (−25%)**
Days 16–21	48	51 (+6%)	53 (+10%)	43 (−10%)**	42 (−12%)**
Days 6–21	92	93 (+1%)	92 (0%)	78 (−15%)**	75 (−18%)**

From Becker (1985b)
** $P \leq 0.01$
[a] % difference from control shown in parentheses.

dosing solutions were prepared daily. The concentration, stability and homogeneity of the dosing solution were determined once during the treatment period. All rats were observed twice daily for clinical signs of toxicity, mortality and moribundity. Maternal body weights were recorded on day 0 until day 21 postpartum. Feed consumption was recorded on days 6, 11, 16 and 21 postcoitum. On day 21 of gestation, all surviving dams were sacrificed and subjected to gross necropsy. The fetuses were removed from the uterus by caesarean section, sexed, weighed, examined for gross external abnormalities and fixed either for viscera and brain investigations according to Wilson (1/3 fetuses) or for skeletal examination (2/3 fetuses). The uteri and contents of all pregnant females were weighed on the day of scheduled sacrifice and used to determine the corrected body weight gain. The uteri of all females found to be not pregnant at necropsy were placed in an aqueous solution of ammonium sulfide to accentuate possible haemorrhagic areas of implantation sites.

No deaths occurred and no symptoms or clinical signs were observed in any group of this study that could be considered treatment related. Similarly, no treatment-related findings were noted macroscopically at necropsy in any females. Evidence of maternal toxicity included inhibited body weight gain (18%) during treatment (gestation days 6–11) at dose levels of 12 mg/kg bw per day and above (Table 29). Body weight gain of the 12 mg/kg bw per day group beyond day 11 was similar to that of the control group. Decreased body weight and feed consumption among females were seen in the 24 and 48 mg/kg bw per day dose groups. These differences in maternal body weights could have been influenced by treatment-related intrauterine effects (e.g. increased number of resorptions, decreased fetal weight). Net body weight change (= body weight gain from day 6 to day 21 minus uterine weight) showed no difference among groups.

Evidence of fetal toxicity was apparent from observed dose-related increases in the number of litters with supernumerary ribs at doses of 24 and 48 mg/kg bw per day. The increase in supernumerary ribs at 6 (5.6%) and 12 (8.2%) mg/kg bw per day was within the historical control range (8.3% of the historical control value). At 24 and 48 mg/kg bw per day, a significantly increased postimplantation loss was noted, which was dose related. The total losses consisted mainly of early resorptions and, to a lesser extent, late resorptions; these amounted to 22.2% and 30.6% of the implantations at 24 and 48 mg/kg bw per day, respectively. Preimplantation loss was not affected by treatment. The mean fetal body weights in the 24 and 48 mg/kg bw per day groups were significantly reduced (−8.3%) compared with that of the control. External investigations revealed the presence of one runt in each of the 6, 12, 24 and 48 mg/kg bw per day groups. This finding was considered to be incidental and not related to cyproconazole treatment. At the highest dose, one fetus was noted with a hydrocephalus, and two fetuses, one of which was a runt, had a palatoschisis (also reported as cleft

Table 30. Fetal abnormalities observed in main teratogenicity study in rats

Parameter	Dose (mg/kg bw per day)				
	0	6	12	24	48
External examinations					
No. of fetuses examined	248	248	271	217	177
No. of malformations (no. of litters affected)	0 (0)	1 (1)	1 (1)	1 (1)	3 (3)
Visceral examinations					
No. of fetuses examined	78	79	91	71	60
No. of malformations (no. of litters affected)	0 (0)	0 (0)	0 (0)	1 (1)	3 (3)
Total no. of palatoschises (no. of litters affected)	—	—	—	—	2 (2)
Total no. of hydrocephalus (no. of litters affected)	—	—	—	1 (1)	2 (2)
Skeletal examinations					
No. of fetuses examined	170	169	180	146	117
No. of anomalies (no. of litters affected)	5 (4)	4 (4)	10 (7)	14 (8)	6 (6)

From Becker (1985b)

palate) (Table 30). At 24 mg/kg bw per day, a hydrocephalus internus was noted during the visceral investigations. In the 24 and 48 mg/kg bw per day groups, the incidence of incompletely ossified phalangeal nuclei and calcanea with still absent ossification was increased compared with that of the control. These findings were considered to be the result of the reduced mean body weights of fetuses and not a specific effect of treatment on skeletal development.

The maternal toxicity NOAEL was 6 mg/kg bw per day, based on body weight gain reduction during early treatment at the LOAEL of 12 mg/kg bw per day. The developmental NOAEL was 12 mg/kg bw per day, based on reduced fetal body weight, increased postimplantation loss, increases in supernumerary ribs and increased fetal malformations seen at the LOAEL of 24 mg/kg bw per day and above. The study author considered the NOEL to be 12 mg/kg bw per day (Becker, 1985b); however, the study author did not consider the decrease in body weight gain at 12 mg/kg bw per day to be a treatment-related effect (Eschbach, 1989).

In a non-GLP published study of developmental toxicity, groups of 20 pregnant Wistar rats were given cyproconazole (unknown purity) at a dose of 0, 20, 50 or 75 mg/kg bw per day by gavage in aqueous solution of CMC (10 ml/kg bw volume) from day 6 to day 16 of gestation, inclusive. Groups of five pregnant females were treated at a dose of 100 mg/kg bw per day. Other procedures were similar to those of the standard guideline developmental toxicity study in rats.

Treatment of pregnant rats revealed maternal toxicity at all investigated dose levels as manifested by reduced body weight gain early during treatment. The depressed body weights during the early treatment period (6–11 days) were 37%, 37% and 63% below the control values at 20, 50 and 75 mg/kg bw per day, respectively. The full biological and toxicological relevance of this effect cannot be assessed due to the lack of individual data on weight gains. Fetotoxicity in the form of increased fetal resorptions and reduced body weight and size was statistically significant at 50 mg/kg bw per day and above. Fetotoxicity was also evident in the low-dose group in terms of reduced mean fetal weights. A treatment- and dose-related increase in serious developmental effects, including cleft palate, internal hydrocephali and hydronephrosis of the ureter as well as delayed ossification, was seen at all doses. The effects were apparent at 20 mg/kg bw per day, at lower incidence and/or lower degree of severity. No NOEL could be established in this study, either for maternal or for embryo/fetotoxicity (Machera, 1995).

Rabbits

In a study of developmental toxicity, groups of 16 pregnant hybrid Chinchilla rabbits were given cyproconazole (purity 95.6%) at a dose of 0, 2, 10 or 50 mg/kg bw per day by gavage in 4% CMC in distilled water (dose volume 4 ml/kg bw) on days 6–18 of gestation, inclusive. The dosing solutions were prepared daily. Concentration, stability and homogeneity of cyproconazole in the vehicle were determined once during the treatment period. Body weight was recorded daily from day 0 until day 28 postcoitum, and feed consumption was recorded on days 6, 11, 15, 19, 24 and 28 postcoitum. Mortality, clinical signs and symptoms were recorded at least twice daily. All females were killed on day 28 postcoitum by cervical dislocation, and fetuses were removed by caesarean section. Postmortem examinations included gross macroscopic inspection of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The fetuses were removed from the uterus, sexed, weighed, examined for gross external abnormalities and prepared for internal examinations. Fetuses were dissected carefully to investigate body cavities and organs. Skin was removed, and crania were examined for ossification. Heads were fixed and cross-sectioned to examine the cephalic viscera. Trunks were then cleared in a potassium hydroxide solution, and skeletons were stained for examination. The uteri and contents of all pregnant females were weighed on the day of scheduled sacrifice and used to determine the corrected body weight gain. The uteri of all females found to be not pregnant at necropsy were placed in an aqueous solution of ammonium sulfide to accentuate possible haemorrhagic areas of implantation sites.

The measured concentrations of cyproconazole ranged from 73.3% to 93.3% and from 48.0% to 80.0% of nominal concentrations immediately after preparation and 90 minutes after preparation, respectively. The test material concentrations in the dosage suspensions for the 10 and 50 mg/kg bw per day groups were not within acceptable limits (±15% of nominal concentration) immediately after preparation. Ninety minutes after preparation, none of the dosage suspension/test material concentrations were within acceptable limits. Triplicate analyses of each dosage suspension also showed that these mixtures were not homogeneous. In the face of such inconsistency, it is difficult to have confidence in the exposure levels reported for the study.

No deaths occurred and no symptoms or clinical signs that could be considered treatment related were observed in any group of this study. Similarly, no treatment-related findings were noted macroscopically at necropsy in any females. Body weight development of dams of the low- and middle-dose groups did not differ from that of the control group (Table 31). High-dose animals showed a mean loss in body weight between the first and second days of treatment of 105 g. They returned to their day 6 body weight by day 12 only. Day 6 to day 11 body weight gain was therefore 127% below control. Animals partly recovered from this, resulting in an overall body weight gain during the entire treatment period that was 38% below control. Mean corrected body weight gains (= body weight gain from day 6 to day 28 minus uterus weight) were similar in all groups, indicating that maternal changes in body weight gain could be due to increased resorptions. Feed consumption was decreased (−26.9%, $P < 0.05$) in the high-dose group during the initial phase of the treatment period (days 6–11). Noted differences between treated and control groups at other intervals were not remarkable. Reproduction parameters were not affected in the 2 mg/kg bw per day group (Table 32). A significantly increased postimplantation loss was recorded at 50 mg/kg bw per day. An apparently treatment- and dose-related increase in implantation loss was also seen at 10 mg/kg bw per day. The total losses consisted mainly of early resorptions and late resorptions; these amounted to 7% and 16.4% of the implantations at 10 and 50 mg/kg bw per day, respectively. The mean fetal body weights were not affected.

Evidence of developmental toxicity included hydrocephalus internus, observed in one fetus at each dose level, and one agenesia of the diaphragm in the control, one partial agenesia of the diaphragm at 10 mg/kg bw per day and one agenesia of the left kidney and ureter at the high dose. Slight microphthalmia was found in four fetuses of one litter in the 10 mg/kg bw per day dose group.

Table 31. Differences in body weight gain of dams over the treatment period in the developmental toxicity study in Chinchilla rabbits with cyproconazole

Group (mg/kg bw per day)	Body weight change (g)[a]								Corrected body weight gain (%)[b]
	Days postcoitum								
	0–6	6–11	11–15	15–19	6–19	19–24	24–28	6–28	
1 (0)	152 (+4.7%)	45 (+1.3%)	85 (+2.5%)	48 (+1.4%)	178 (+1.4%)	60 (+1.7%)	34 (+0.9%)	272 (+8.0%)	−4.9
2 (2)	125 (+4.1%)	55 (+1.7%)	75 (+2.3%)	71 (+2.1%)	201 (+6.3%)	68 (+20%)	40 (+1.2%)	309 (+9.6%)	−4.5
3 (10)	160 (+5.2%)	71 (+2.2%)	69 (+2.1%)	59 (+1.7%)	199 (+6.2%)	63 (+1.8%)	37 (+1.1%)	299 (+9.2%)	−3.7
4 (50)	187 (+6.2%)	−12 (−0.4%)	50 (+1.6%)	73 (+2.2%)	111 (+3.4%)	66 (+2.0%)	33 (+1.0%)	210 (+6.5%)	−4.9

From Becker (1986)

[a] % change shown in parentheses.

[b] Corrected body weight gain as a percentage of weight on day 6.

The incidence of hydrocephalus internus was 0.85, 0.83 and 0.93 for the low-, mid- and high-dose fetuses and 0.08 for the historical control incidence. Hydrocephaly was also seen at two dose levels in a developmental toxicity study in rats (Eschbach, 1989) and also in the published study in rats with this test material (Machera, 1995); however, this anomaly did not occur in the concurrent controls of either study. In another developmental toxicity study in New Zealand White rabbits, hydrocephaly was not seen (Müller, 1991).

The maternal NOAEL was 10 mg/kg bw per day, and the LOAEL was 50 mg/kg bw per day, based on decreased body weight gains and feed consumption. The developmental toxicity NOAEL was 10 mg/kg bw per day, based on increases in postimplantation loss seen at the LOAEL of 50 mg/kg bw per day, the highest dose tested. The study author set a NOAEL for maternal toxicity at 10 mg/kg bw per day and concluded that there was no embryotoxicity or teratogenicity at doses up to and including 50 mg/kg bw per day (Becker, 1986).

In a second study of developmental toxicity, groups of 18 pregnant New Zealand White rabbits were given cyproconazole (purity 94.8%) at a dose of 0, 2, 10 or 50 mg/kg bw per day by gavage in 1% CMC in distilled water (dose volume 5 ml/kg bw) on days 6–18 of gestation, inclusive. The dosing solutions were prepared weekly as a suspension in vehicle. The concentration and homogeneity of cyproconazole in the vehicle were determined during the first and last weeks of the treatment. It was stated in the study report that the stability was determined and proven during the range-finding study. All animals were examined twice daily for morbidity and mortality. Body weight was recorded on days 0, 6, 9, 12, 15, 19, 24 and 28 postcoitum. Feed consumption was recorded for days 0–2, 2–4, 4–6, 6–9, 9–12, 12–15, 15–17, 17–19, 19–21, 21–24, 24–26 and 26–28 postcoitum. On day 28 postcoitum, surviving female rabbits were killed, dissected and examined macroscopically for pathological changes. Animals found dead and females showing signs of abortion were also necropsied and examined for pathological changes. Ovaries and uteri were removed and examined. Uterine contents, position of fetuses in the uterus and number of corpora lutea were recorded. Fetuses were killed by intrapleural injection of sodium pentobarbital, sexed, weighed, examined for gross external abnormalities and prepared for internal examinations. Fetuses were microdissected to investigate body cavities and organs. Heads were removed from approximately half of the fetuses, fixed and examined for visceral abnormalities by modified Wilson's technique. In the remaining fetuses, examination of the brain was performed by a mid-coronal cut. The carcass of all fetuses was examined for skeletal abnormalities after

Table 32. Summary of reproduction data in the developmental toxicity study in Chinchilla rabbits with cyproconazole

Parameter	Dose (mg/kg bw per day)			
	0	2	10	50
Number of mated females	16	16	16	16
Number of non-pregnant females	1	1	—	—
Number of females aborted	—	1	—	—
Number of females with live fetuses at necropsy (= numbers used for calculations)	15	14	16	16
Number of corpora lutea:				
- Per group	133	124	137	138
- Mean per dam	8.9	8.9	8.6	8.6
Number of implantations:				
- Per group	126	119	129	128
- Mean per dam	8.4	8.5	8.1	8.0
Preimplantation loss:				
- Per group	7	5	8	10
- Mean per dam	0.5	0.4	0.5	0.6
Live fetuses:				
- Per group	123	118	120	107
- Mean per dam	8.2	8.4	7.5	6.7
- % of implantations	97.6	99.2	93.0	83.6
- % males	53.7	46.6	50.8	48.6
Dead fetuses	0	0	0	0
Early resorptions:				
- Per group	3	0	7	10
- Mean per dam	0.2	0.0	0.4	0.6
- % of implantations	2.4	0.0	5.4	7.8
Late resorptions:				
- Per group	0	1	2	11*
- Mean per dam	0.0	0.1	0.1	0.7
- % of implantations	0.0	0.8	1.6	8.6
Total resorptions:				
- Per group (= total postimplantation losses)	3	1	9	21*
- Mean per dam	0.2	0.1	0.6	1.3
- % of implantations	2.4	0.8	7.0	16.4
Mean weight of live fetuses	35.1	35.4	36.8	34.2

From Becker (1986)
* $P \leq 0.05$

Alizarin staining. The uteri of apparently non-pregnant females at necropsy were placed in an aqueous solution of ammonium sulfide to accentuate possible haemorrhagic areas of implantation sites.

The data provided show that the test material was suspended homogeneously in the CMC, and the concentrations attained were close to the nominal concentrations (89–105% for homogeneity and 91–94% for concentration measurements). Stability data were not provided in the study report.

There were two deaths, one in the mid-dose group (14 days postcoitum) and one in the high-dose group (22 days postcoitum), which were apparently incidental. Low water consumption was observed in all groups, but the incidence was greatest at the high dose level. Two animals aborted in

Table 33. Maternal body weight gain in a teratogenicity study in New Zealand White rabbits

Gestation period	Maternal body weight gain (g)[a]			
	Dose (mg/kg bw per day)			
	0	2	10	50
Days 0–6	357	292	319	279
Days 6–9	71	71 (0%)	55 (−23%)	−37** (−152%)
Days 9–12	102	58 (−43%)	69 (−32%)	121 (+18%)
Days 12–15	72	80 (+12%)	83 (+16%)	47 (−34%)
Days 15–19	105	72 (−32%)	64 (−39%)	91 (−14%)
Days 6–19	350	281 (−20%)	268 (−23%)	222 (−37%)
Days 19–28	193	253 (+31%)	223 (+15%)	270 (+40%)

From Müller (1991)
** $P \leq 0.01$
[a] % difference from control shown in parentheses.

both the low-dose and high-dose groups (on postcoitum days 27 and 20 at the low dose and both on day 19 at the high dose). The author stated that as there was no dose–response relationship and as similar incidences of abortions have been observed in this strain of rabbit at this testing facility, as reported in the historical control data submitted in the final report, the abortions in this study are considered incidental. There was 100% intrauterine death at necropsy in three low-dose, one mid-dose and one high-dose female. There was no dose–response relationship observed, and similar incidences are reported in the historical control data. No other findings were reported. In high-dose animals, a group mean body weight loss was observed from day 6 to day 9 postcoitum (Table 33). Beyond day 9, no significant effects on body weight development were seen. The high-dose group displayed a statistically significant decrease in mean daily feed consumption compared with the control value from days 6 to 9 postcoitum (and from days 6 to 19 postcoitum; $P < 0.05$ not attained). Necropsy of females did not reveal any treatment-related findings. The highest number of non-pregnant animals occurred in the high-dose group.

There was a dose-related decrease in the number of implantations compared with the control group when comparing all does or only those with live fetuses at necropsy. Although all dose levels displayed an increased per cent postimplantation loss (when all does were considered), the increase was inversely related to dose. Number of fetuses per doe, fetal sex distribution and mean fetal weight were similar in all groups. Fetal malformations and variations were observed in all three dose groups and in the control group (Table 34). In the control group, three fetuses from three litters showed external or skeletal malformations: kinked tail, fused ribs or malpositioned digits. In the low-dose group (2 mg/kg bw per day), one fetus was viscerally malformed, showing dysplasia of the retina. In the mid-dose group (10 mg/kg bw per day), six fetuses (one of them dead) from three litters showed external/visceral and skeletal malformations. Some of these malformations concerned ribs and vertebrae, the position of hindlimbs, the tail and the abdominal wall. The outcome of this group was dominated by one litter. Four of the six affected fetuses were from this single litter (positions 1–4 in right horn of uterus). One of these four was found dead at necropsy, and two others had extremely low body weights (21.5 and 22.8 g compared with the group mean of 40.8 g). These three fetuses were found with omphalocele, umbilical hernia and arthrogryposis. The remaining three affected fetuses of this group were found with skeletal malformations only. In addition to the one dead fetus, there were four resorptions. In the high-dose group (50 mg/kg bw per day), 15 fetuses from seven litters were malformed. These malformations were found in sternebrae and ribs, vertebral column, hindlimbs, tail and kidneys. In one of these fetuses, a general oedema was found. Owing to the types and

Table 34. Fetal abnormalities in a teratogenicity study in New Zealand White rabbits[a]

Parameter	Dose (mg/kg bw per day)			
	0	2	10	50
No. of fetuses examined externally (no. of dead fetuses)	110 (1)	90	75 (2)	60 (1)
No. of fetuses examined viscerally	110	90	73	59
No. of fetuses examined skeletally	110	90	73	59
Malformations				
No. of fetuses with external/visceral malformations (no. of litters affected)	2 (2)	1 (1)	2 (1)	7 (5)
No. of fetuses with skeletal malformations (no. of litters affected)	1 (1)	0 (0)	3 (3)	13 (7)
Total no. of fetuses with malformations:				
- No. of fetuses (no. of litters affected)	3 (3)	1 (1)	5 (3)	15 (7*)
- Average % malformed fetuses	2.7	1.1	6.1	25
- % of litters affected	18.8	9.1	21.4	70
Variations				
No. of fetuses with external/visceral variations (no. of litters affected)	3 (2)	3 (1)	3 (2)	2 (2)
No. of fetuses with skeletal variations (no. of litters affected)	110 (16)	88 (11)	72 (14)	58 (10)

From Müller (1991)
* $P \leq 0.05$
[a] Data extracted from Table 11 in the study report, page 56.

incidences of malformations, and when compared with the concurrent and historical control data, a treatment-related effect is concluded for the high-dose group. A significant incidence of malrotated hindlimbs was recorded in the high-dose animals. There was one incidence of this malformation in the 10 mg/kg bw per day group also. This malformation was not seen in the historical control data presented, and association with treatment cannot be excluded at present.

The maternal NOAEL was 10 mg/kg bw per day and the LOAEL was 50 mg/kg bw per day, based on decreased body weight gain and feed consumption. The developmental toxicity NOAEL was 10 mg/kg bw per day and the LOAEL was 50 mg/kg bw per day, based on the increased incidence of malformed fetuses and litters with malformed fetuses, mainly affecting sternebrae, ribs, vertebral column, hindlimbs and tail. The study author indicated that there was no developmental toxicity at 10 mg/kg bw per day (Müller, 1991).

2.6 Special studies

(a) Acute neurotoxicity

No studies were submitted.

(b) Short-term study of neurotoxicity

No studies were submitted.

(c) Studies on metabolites

Metabolites M21/M21a and M36 (also named NOA 405870 and NOA 405872, respectively) were found in the milk and in the urine of lactating goats. They were found in the rat in minor

amounts only (0.02–0.06% of applied dose in urine). Therefore, the toxicological profile of these metabolites was investigated. The IUPAC name for metabolite M21/M21A is 5-(4-chlorophenyl)-5-hydroxy-4-methyl-6-(1H-1,2,4-triazol-1-yl)-2-hexanoic acid. The IUPAC name for metabolite M36 is 5-(4-chlorophenyl)-3,5-dihydroxy-4-methyl-6-[1,2,4]triazol-1-yl-hexanoic acid.

(i) Acute oral toxicity study of metabolite NOA 405870 (M21/M21a)

Groups of male and female young adult HanIBM: Wistar rats (five of each sex per dose) were given cyproconazole metabolite M21/M21a (purity 99.8%) as a single gavage dose of 2000 mg/kg bw. The test substance was administered as a solution in polyethylene glycol 400 (dose volume of 10 ml/kg bw). Animals were observed for mortality and clinical signs at 1-hour intervals for the first day and twice daily thereafter during weekdays and once on weekends. Body weights were recorded on days −7, 0, 1–3, 7 and 14. A gross necropsy was performed on all animals.

No mortality was observed during the study. No treatment-related effects on body weight were observed. Symptoms were unspecific, including piloerection, reduced activity, slowed and laboured breathing and hunched posture. All animals were free of symptoms by day 5. No treatment-related abnormal findings were noted at the necropsy. The acute oral LD_{50} of cyproconazole metabolite M21/M21a was greater than 2000 mg/kg bw for male and female rats (Warren, Müller & Carpy, 1992a).

(ii) Genotoxicity of metabolite NOA 405870 (M21/M21a)

In a microbial reverse gene mutation study, *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 or TA1537 were exposed to cyproconazole metabolite M21/M21a (purity 100%) at doses of 8–5000 µg/plate with or without S9 activation (initial trial) and 312.5–5000 µg/plate with or without S9 activation (confirmatory trial). The S9 fraction was derived from Aroclor 1254–induced Sprague-Dawley male rat livers, and the test material was delivered to the test system in DMSO.

Slight toxicity was noted in the first experiment at the highest concentration with strains TA98 and TA1537 (without S9) and TA102 (with S9). A narrower concentration range was selected for all strains in the second experiment. In this experiment, slight toxicity was seen at the highest concentration with strain TA98 (without S9) and TA1537 (with and without S9). Metabolite NOA 405870 (M21/M21a) did not reveal an increased incidence of revertant colonies in any of the experiments conducted. The positive controls induced the expected mutagenic responses in the appropriate tester strain. There was, however, no evidence that the cyproconazole metabolite M21/M21a induced a mutagenic effect under any test conditions (Clare, 1992a) (see Table 35 below).

(iii) Acute oral toxicity study of metabolite NOA 405872 (M36)

Mice

Groups of male and female young adult Crl:CD1 (ICR) BR mice (five of each sex per dose) were given cyproconazole metabolite M36 (purity 98.0%) as a single gavage dose of 2000 mg/kg bw. The test substance was administered as a solution in polyethylene glycol 400 (dose volume of 10 ml/kg bw). Animals were observed 4 times during the day of treatment and once daily for the following 14 days. Body weights were recorded on days 1 (pre-administration), 4, 8 and 15. A gross necropsy was performed on all animals.

One female animal died spontaneously on test day 7. There were no clinical signs and no findings at necropsy with this animal. Therefore, this death was considered not to be treatment related. No other death occurred during the 14-day observation period after treatment with cyproconazole metabolite M36. No clinical signs were observed. No treatment-related effects on body weight were observed. The acute oral LD_{50} of cyproconazole metabolite M36 was greater than 2000 mg/kg bw for male and female mice (Pfister, 1995).

Rats

Groups of male and female young adult HanIBM: Wistar rats (five of each sex per dose) were given cyproconazole metabolite M36 (purity 97.0%) as a single gavage dose of 2000 mg/kg bw. The test substance was administered as a solution in polyethylene glycol 200 (dose volume of 10 ml/kg bw). Animals were observed for mortality and clinical signs at 1-hour intervals for the first day and twice daily thereafter during weekdays and once on weekends. Body weights were recorded on days −7, 0, 1–3, 7 and 14. A gross necropsy was performed on all animals.

No animals died during the 14-day observation period after treatment with cyproconazole metabolite M36. Symptoms were unspecific and mainly limited to piloerection. All animals were free of symptoms by day 8. No treatment-related abnormal findings were noted at the necropsy. The acute oral LD_{50} of cyproconazole metabolite M36 was greater than 2000 mg/kg bw for male and female rats (Warren, Müller & Carpy, 1992b).

(iv) Short-term toxicity study of metabolite NOA 405872 (M36)

Rats

In a 28-day study of toxicity, groups of five male and five female HanIbm: WIST Wistar rats were given diets containing cyproconazole metabolite M36 (purity 98%) at a dietary concentration of 0, 1500, 5000 or 20 000 ppm (equal to 0, 155, 527 and 2772 mg/kg bw per day for males and 0, 176, 528 and 2126 mg/kg bw per day for females, respectively). Diets were prepared weekly. All rats were observed for mortality and signs of toxicity twice a day (once on weekends), and each was subjected to a detailed check for signs of reaction to treatment once a week, which included palpation. Body weights were recorded on the day before commencement of treatment and weekly thereafter. Mean feed consumption was determined weekly. Haematological and clinical chemistry investigations were carried out on all animals after 4 weeks of treatment at necropsy. Urinalysis was performed after 2 weeks of treatment. Eye examinations were not performed. At study termination, animals were sacrificed by carbon dioxide asphyxiation. All animals were subjected to a detailed gross pathological examination. Organs were collected, weighed and prepared for histopathology.

Test diets were analysed prior to study initiation for homogeneity of mixing and stability of test material in diet as part of another study (Study No. 553R; a 5-week study), and the results were provided in the study report (Warren et al., 1995a).

There were no deaths in either sex at the low- and mid-dose levels and no apparent signs of toxicity. At the high dose, two rats of each sex died (one female after 6 days of treatment, one male after 12 [13, according to the pathology report] days, one female after 17 days and one male after 23 days). The deaths were sudden with no prior evidence of a toxic effect, and none of the rats that died was observed in a moribund state. Because two of the dead rats were partially cannibalized when found, the remaining high-dose rats were individually housed for the remainder of the study (from day 18). Piloerection and emaciation were observed most frequently in the high-dose group in both sexes. In the results section of the report text, it is stated that minimal degrees of hunched posture and lethargy, recorded only in high-dose rats, were noted in logbook notes of the Study Director and veterinarian; however, the incidence was not provided. Body weight gain in high-dose males and females was reduced in the first week of treatment. Subsequently, these animals lost body weight. The two other dose groups were not affected. There was no statistically significant effect on feed consumption, although slightly reduced values were determined for high-dose females towards the end of the study. At week 4, there was no apparent effect on haematology in either sex at any dose level, although differences were noted. There were several differences noted in clinical chemistry parameters among the groups in both sexes that are consistent with kidney and/or liver toxicity, as well as starvation/malnutrition, but as the group sizes are small (2–5), a definitive statement regarding the differences is not possible. The increase in alkaline phosphatase activity observed in both sexes at the high dose

suggests liver effects, but a decrease might be expected with respect to the losses in body weight at this dose level. There was a decrease in urinary protein in the high-dose males (43% of control value), which the authors attributed to reduced protein secretion from underdeveloped accessory sexual glands, secondary to the marked reduction in growth of these rats. Additionally, it was stated that the slightly more dilute urine in these high-dose males may indicate an impaired ability of the kidneys to concentrate the urine. There was no effect on urine volume in the high-dose males, and females did not display a similar change. There were no apparent effects observed in either sex in the mid- and low-dose groups. Macroscopic lesions were observed in the high-dose group only and included small seminal vesicles in males, small uteri in females and filamentous inclusions in the testes of two of five males. Because of cannibalization of the two females that died, examination of these rats was limited. Six of the 10 high-dose rats displayed a small thymus, and no thymus was found in one high-dose rat. These findings are most likely attributable to the decreased growth observed in these rats. Two high-dose females displayed pallor of the liver, suggesting this as a target organ. The observed differences in organ weights, which were mainly decreases at the high dose in both sexes, may be attributed to decreased growth/malnutrition. Treatment-related effects (hepatocellular vacuolation) were observed in the liver in both sexes at the middle and high doses and in the kidneys (vacuolation of the proximal tubule cells) in both sexes at the high dose. The vacuolation in both organs corresponds to the microvesicular lipid deposits found in sections stained for fat with Sudan III and was of a minimal to slight severity. Other changes observed at the high dose were attributable to the decreased growth (emaciation) of these rats (atrophy/hypoplasia/retarded maturation of the uterus, mammary gland, ovaries, vagina, testes, prostate and seminal vesicles; hypocellular bone marrow; diffuse cortical lymphoid depletion of the thymus; lymphoid depletion of the spleen; degranulation of secretory ducts of salivary glands; and atrophy of adipose tissue).

The NOAEL was 1500 ppm (equal to 155 mg/kg bw per day), based on increased relative liver weights in females and hepatocyte vacuolation in both sexes at the LOAEL of 5000 ppm (equal to 527 mg/kg bw per day) (Warren et al., 1995a).

(v) Genotoxicity of metabolite NOA 405872 (M36)

In three tests (in vitro Ames test and mouse lymphoma cell assay plus in vivo mouse micronucleus assay), NOA 405872 showed no evidence of any mutagenic potential (Table 35). Although NOA 405872 showed apparent clastogenic potential in one in vitro system (Chinese hamster ovary cells), this finding was not confirmed in vivo and therefore not considered to be of relevance for humans.

(d) Weight of evidence for the mode of action for cyproconazole in mice

To clarify the MOA for mouse liver tumours, several studies including new data were submitted.

(i) 14-day dietary study for the evaluation of liver effects in three strains of mice

A 14-day dietary study was conducted to give sufficient data to allow a mouse strain (C57BL/6J or C3H/HeNClrBR) to be selected for subsequent constitutive androstane receptor (CAR)-knockout mouse experiments, based on a liver response including adenomas and carcinomas in CD-1 mice treated with cyproconazole (Milburn, 2006c). An additional goal of the study was to help elucidate what range of key events may be operative in the MOA for liver tumours in cyproconazole-treated mice. The three strains of male mice (CD-1, C57BL/6J and C3H/HeNClrBR) were administered 0, 200 and 450 ppm cyproconazole or 850 ppm phenobarbital (PB) in diet for 14 days. Five male mice per group at each time point were sacrificed after 2, 7 and 14 days of treatment. Additionally, five male mice per strain/treatment/time point were implanted with an osmotic pump containing bromodeoxyuridine (BrdU) 3 days before termination to clarify cell proliferating activity in the liver.

In all mouse strains, liver weights were clearly and time-dependently increased following treatment with PB and cyproconazole at both dose levels (Table 36).

Table 35. Results of studies of genotoxicity with cyproconazole metabolites

End-point	Test system	Concentration or dose	Purity (%)	Result	Reference
NOA 405872 (M36)					
Reverse mutation (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	1st mutagenicity test: 8–5000 µg/plate ± metabolic activation in DMSO 2nd mutagenicity test: 312.5–5000 µg/plate ± metabolic activation in DMSO	97.0	Negative	Clare (1992b)
Mouse lymphoma (gene mutation or clastogenicity)	L5178Y $TK^{+/-}$ mouse lymphoma cells	3400 µg/ml ± metabolic activation in DMSO	95.0	Negative	Wollny (2000)
Chromosomal aberration	Chinese hamster ovary V79 cells	28.1–3600 µg/ml ± metabolic activation in DMSO	95.0	Positive	Czich (2001)
Mouse bone marrow micronucleus test	CD-1 mice	5 male mice at 24 h and 5 male mice at 48 h, single gavage dose of 2000 mg/kg bw in DMSO	95.0	Negative	Fox (2001)
NOA 405870 (M21/M21a)					
Reverse mutation (Ames test)	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	1st mutagenicity test: 8–5000 µg/plate ± metabolic activation in DMSO 2nd mutagenicity test: 312.5–5000 µg/plate ± metabolic activation in DMSO	100	Negative	Clare (1992a)

Table 36. Intergroup comparison of liver weight adjusted for body weight in cyproconazole- and phenobarbital-treated mice for 14 days

Mouse strain / day	Dietary concentration (ppm), test substance			
	0 (control)	200, cyproconazole	450, cyproconazole	850, PB
CD-1:				
- Day 3	2.34	2.49	2.73**	2.81**
- Day 8	2.12	2.93**	3.44**	2.80**
- Day 15	2.20	2.90**	3.71**	2.95**
C57BL/6J:				
- Day 3	1.24	1.44*	1.54 NS	1.55*
- Day 8	1.12	1.51**	2.03**	1.54**
- Day 15	1.17	1.49**	1.82*	1.57**
C3H/HeNClrBR:				
- Day 3	1.37	1.60**	1.67**	1.70**
- Day 8	1.39	1.88**	2.20**	1.95**
- Day 15	1.55	2.00**	2.46**	2.11**

From Milburn (2006c)

[a] Statistically significant difference from control group mean: *$P < 0.05$; ** $P < 0.01$ (Student's *t*-test, two-sided); NS, not significantly different compared with control value

This increase in liver weight was consistent with the centrilobular (PB) or centrilobular/pan-lobular (cyproconazole) liver hypertrophy and the proliferation of smooth endoplasmic reticulum membranes observed with the same animals. Slight single-cell necrosis in the liver and hepatocyte

vacuolation (lipid content) were increased with PB and cyproconazole in three strains with time dependency, although slight differences were detected. In blood biochemical examination, plasma cholesterol (PB only), HDL or low-density lipoprotein (LDL) were decreased in C57BL/6J and C3H/HeNClrBR strains (Table 37).

The cell proliferation and apoptosis in the liver of all three strains and expression of selected genes in the liver known to play a role in regulation of cell cycle control, cell proliferation and apoptosis or specific CYP isoenzymes (Daujat, Neel & Piette, 2001; De Smaele et al., 2001; Hino et al., 2002; Columbano et al., 2005; Ellinger-Ziegelbauer et al., 2005; Huang et al., 2005; Iida et al., 2005) were measured in C3H/HeNClrBR mice, genetic background strains of CAR-null mice, by means of real-time polymerase chain reaction (PCR). Treatment with PB and cyproconazole resulted in an early stimulation of hepatocellular proliferation in all three strains, although this was not statistically significant in C57BL/6J mice treated with PB. The recording of apoptosis via terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick end labelling (TUNEL) staining in all strains was decreased, but statistical significance was limited. Liver expressions of *Mdm2*, *Gadd45β* and *Cyp2b10* were increased and of *Tsc22* were decreased. There was no clear indication for *Rb1* gene expression (Table 38).

In immunoblotting analysis, cyproconazole and PB induced CYP2B protein expression in liver microsomes of three strains, the induction corresponding to the increased expression of *Cyp2b* gene. These data demonstrate that cyproconazole is a strong CYP2B inducer mediated by CAR as well as PB in the CD-1, C57BL/6J and C3H/HeNClrBR mouse liver (Milburn, 2006c).

Based on the results of this study (Milburn, 2006c), either the C3H/HeNClrBR or the C57BL/6J strain of mice was sufficiently similar to the CD-1 strain. Information from this study was utilized to determine the strain (C3H) for subsequent studies with CAR-null mice, as well as the time of maximal effect with PB treatment (3 days) or cyproconazole treatment (7 days) for certain parameters.

(ii) Exposure to cyproconazole in wild-type and CAR-null C3H male mice for 7 days

Male C3H wild-type and CAR-null mice (6–8 weeks of age) were treated with cyproconazole at 200 or 450 ppm in diet for 7 days (Milburn, 2006a). Mice (3–5 per group) were killed on day 8. The study was conducted in two separate experimental batches.

Plasma cholesterol level was decreased in wild-type mice, and triglyceride levels were decreased in both genotypes (wild-type and CAR-null mice). Plasma alkaline phosphatase activity was slightly increased in both types of mice. An increase in ALT activity in wild-type mice but not in CAR-null mice was correlated with the liver histopathological findings, such as single-cell necrosis or inflammatory cell infiltration, observed in wild-type mice only. Absolute and adjusted (for body weight) liver weights were increased at 200 and 450 ppm in wild-type mice (Table 39).

In CAR-null mice, absolute liver weights were not different from control, but liver weights adjusted for body weight were slightly, but statistically significantly, increased at 450 ppm. Histopathologically, centrilobular or panlobular hepatocellular hypertrophy was observed in wild-type mice at 200 and 450 ppm but not in CAR-null mice. Although minimal to slight vacuolation in hepatocytes was detected in treated groups in wild-type mice only, Oil Red O stain demonstrated that treatment with cyproconazole increased positive hepatocyte lipid content moderately in wild-type mice and minimally to slightly in CAR-null mice. Cyproconazole increased Ki67 labelling indices of hepatocytes at both doses in wild-type mice; labelling indices were slightly increased at 450 ppm in CAR-null mice. Expression of *Gadd45β* gene in the liver was increased in wild-type mice at 200 and 450 ppm and in CAR-null mice only at 450 ppm (Table 40). Treatment-related *Cyp2b10* gene expression was over 100 times greater in wild-type mice at 200 and 450 ppm, but several times greater at

Table 37. Liver micropathology in cyproconazole- and phenobarbital-treated mice for 14 days

	CD-1				C57BL/6J				C3H/HeNClrBR			
	Cyproconazole			PB	Cyproconazole			PB	Cyproconazole			PB
	Dietary concentration (ppm)											
	0	200	450	850	0	200	450	850	0	200	450	850
Day 3												
Single-cell necrosis[a]:												
- Minimal	0	4	1	3	0	1	1	4	0	1	1	1
- Slight	0	0	0	1	0	0	0	0	0	0	0	0
Hepatocyte hypertrophy, slight grade[a,b]	0	0	4	5	0	0	2	4	0	0	0	0
Hepatocyte vacuolation, moderate grade[a]	0	0	0	2	0	0	0	0	0	0	0	0
Hepatocyte lipid content (Oil Red O), grade[c]:												
- Minimal	2	1	0	1	1	1	1	0	0	1	0	0
- Slight	2	1	0	2	0	1	3	0	1	0	2	2
- Moderate	1	2	2	2	0	2	1	0	0	3	3	3
- Marked	0	1	3	0	0	0	0	0	0	0	0	0
Day 8												
Single-cell necrosis[a]:												
- Minimal	0	3	2	4	0	3	4	2	0	1	9	0
- Slight	0	0	7	1	0	0	5	0	0	0	1	0
Hepatocyte hypertrophy, slight grade[a,b]	0	7	10	10	0	9	9	10	0	5	10	10
Hepatocyte vacuolation[a]:												
- Minimal	0	0	4	1	0	0	0	0	0	0	0	0
- Slight	0	0	2	0	0	0	0	0	0	0	0	0
Hepatocyte lipid content (Oil Red O), grade[c]:												
- Minimal	3	0	0	1	0	0	0	1	0	0	0	3
- Slight	1	1	0	0	0	2	2	0	1	1	0	2
- Moderate	0	1	0	1	0	3	1	0	0	1	4	0
- Marked	0	3	5	1	0	0	1	1	0	2	1	0
Day 15												
Single-cell necrosis[a]:												
- Minimal	0	4	7	5	0	6	8	0	0	0	2	1
- Slight	0	0	0	0	0	0	1	0	0	0	0	0
Hepatocyte hypertrophy, slight grade[a,b]	0	10	10	10	0	9	10	10	0	10	10	10
Hepatocyte vacuolation[a]:												
- Minimal	0	1	6	0	0	0	0	0	0	0	0	0
- Slight	0	0	1	0	0	0	0	0	0	0	0	0
Hepatocyte lipid content (Oil Red O), grade[c]:												
- Minimal	4	0	0	1	1	2	1	1	1	0	0	0
- Slight	0	0	0	0	0	0	0	0	0	2	1	0
- Moderate	1	2	1	2	0	1	2	0	0	3	4	0
- Marked	0	3	2	0	0	2	2	0	0	0	0	0

From Milburn (2006c)

[a] Data from 10 animals per group; results for main study animals (5) and BrdU animals (5) combined.

[b] Hypertrophy was described as centrilobular in PB-treated mice and centrilobular/panlobular in cyproconazole-treated mice.

[c] Data from five animals per group; analysis of BrdU animals only.

Table 38. Quantitative real-time PCR analysis of Cyp2b10, Mdm2, Gadd45β, Tsc22 and Rb1 gene expression in the C3H/HeNClrBR mouse liver

Termination day	Treatment	Dietary concentration (ppm)	Fold change in gene expression (after normalization to *Hprt*)				
			Cyp2b10	*Mdm2*	*Gadd45β*	*Tsc22*	*Rb1*
3	Control	0	1	1	1	1	1
	Cyproconazole	200	47**	1.12	4.7*	0.61	1.04
		450	98**	1.67**	36.5**	0.29**	1.05
	PB	850	61	1.21**	6.8*	0.51	1.14**
8	Control	0	1	1	1	1	1
	Cyproconazole	200	167**	0.97	5.7**	0.81	1.06
		450	292**	1.39*	30.1**	0.40**	1.01
	PB	850	143**	1.16	16.9**	0.68	1.08
15	Control	0	1	1	1	1	1
	Cyproconazole	200	280**	1.04	8.2**	0.77	0.92
		450	446**	1.36**	27.7**	0.41**	0.81
	PB	850	212**	1.01	21.4**	0.61	0.82

From Milburn (2006c)
* $P < 0.05$; ** $P < 0.01$. Analysis of variance was performed on control values. Fold changes in gene expression are derived from the levels of the control group.

Table 39. Liver weights in wild-type and CAR-null mice treated with cyproconazole

Dietary concentration (ppm)	Liver weights (g)			
	Wild type		CAR null	
	Control	Cyproconazole	Control	Cyproconazole
Absolute liver weights				
200	1.39	1.80**	1.47	1.58
450	1.41	1.90**	1.53	1.53
Adjusted (for body weight) liver weights				
200	1.38	1.80**	1.46	1.59
450	1.29	2.02**	1.43	1.61*

From Milburn (2006a)
Statistically significant difference from control group mean: * $P < 0.05$; ** $P < 0.01$ (Student's *t*-test, two-sided)

both doses in CAR-null mice. *Mdm2* gene expression was not altered by cyproconazole in wild-type or null mice.

Cyproconazole caused a strong increase in antibody for rat CYP2B by immunoblotting in wild-type mice, but not in CAR-null mice. In wild-type mice, but not in CAR-null mice, a moderately increased activity of 7-benzyloxyresorufin *O*-debenzylase (BROD) at both dose levels and a weakly increased activity of 7-pentoxyresorufin *O*-dependentylase (PROD) at 200 ppm were observed. Both isoenzymes are known to be induced by PB (Honkakoski et al., 1992; Jarukamjorn et al., 1999). At both 200 and 450 ppm, coumarin 7-hydroxylase activity was strongly increased by 849% and 1135% in wild-type mice and by 472% and 458% in CAR-null mice, compared with relevant control values, respectively. This activity is catalysed by CYP isoenzyme CYP2A5 (Lavery et al., 1999) and is known to be inducible in the mouse liver by barbiturates (Honkakoski et al., 1992).

Table 40. Quantitative real-time PCR analysis of gene expression in mice given cyproconazole

Strain	Dietary concentration (ppm)	Fold change in gene expression (after normalization to *Hprt*)		
		Cyp2b10	*Mdm2*	*Gadd45*β
C3H wild type	0	1.00	1.00	1.00
	200	147.03**	0.93	2.46*
	450	294.07*	1.00	6.06**
CAR null	0	1.00	1.00	1.00
	200	2.46**	0.93	1.00
	450	4.00*	1.23	4.29*

From Milburn (2006a)

* $P < 0.05$; ** $P < 0.01$. Analysis of variance was performed on control values. Fold changes in gene expression are derived from the levels of the control group.

In conclusion, administration of cyproconazole for 7 days to wild-type and CAR-null mice at dietary concentrations at 200 and 450 ppm was associated with major differences in the qualitative and quantitative response of the liver. Wild-type mice responded with a strong pleiotropic response of the liver, which comprised increased size, hepatocellular hypertrophy, stimulatory effects on hepatocyte cell cycle, induction of CYP and, particularly at the high dose, slight liver damage. CAR-null mice were much less affected, if at all. The differences in response of liver weights, histopathology and gene expression between wild-type and CAR-null mice are largely similar to those observed with the model enzyme inducer PB (Milburn, 2006a). The slight but significant increase in liver weight of CAR-null mice might indicate other pathways of liver hypertrophy, including pregnane X receptor (PXR). The toxicological significance of the changes in vacuoles in hepatocytes using CAR-null mice remained undetermined, because nuclear receptor signalling, including CAR, regulates lipid metabolism (Ory, 2004; Yamamoto et al., 2004; Rezen et al., 2009).

(iii) Phenobarbital: Exposure of wild-type and CAR-null C3H male mice via the oral (dietary) route for 3 and 7 days

To investigate the effects of PB on liver histopathology and related biochemical events in wild-type mice (C3H) and CAR-null mice, male mice were treated with PB admixed in the diet at 850 ppm for 3 or 7 days (Milburn, 2006b). Clinical observations, body weights and feed consumption were monitored throughout the study. At termination, clinical chemistry was performed, liver weights were recorded and liver samples were taken for microscopic examination. The Ki67 labelling index was determined to evaluate hepatocyte proliferation. Liver microsomes were prepared for CYP enzyme activity assays, sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis. In addition, messenger ribonucleic acid (mRNA) was isolated from frozen liver samples, and expression of selected genes was analysed by quantitative real-time PCR. Plasma cholesterol level was slightly reduced in wild-type mice for 3- and 7-day treatments, but the reduction in CAR-null mice was slight for the 3-day treatment only. Three-day treatment with PB slightly increased plasma ALT and AST activities in wild-type mice but did not affect these enzymes in the CAR-null animals. After 7 days of treatment, the inexplicably high values and the large variability in animals, including controls, prevented interpretation of ALT or AST levels. Absolute and adjusted (for body weight) liver weights were increased in wild-type mice after PB treatment for 3 and 7 days, but adjusted (for body weight) liver weights were not increased in CAR-null mice (Table 41). The increased liver weights correlated with centrilobular hypertrophy of hepatocytes in wild-type mice but not in CAR-null mice. Oil Red O staining for hepatocyte lipid demonstrated increased severity in wild-type mice after treatment for 3 days, but a marginal tendency in wild-type mice at the 7-day treatment and in CAR-null mice at both time points. PB treatment increased Ki67

Table 41. Liver weights adjusted for body weight in wild-type and CAR-null mice administered phenobarbital

Treatment	Adjusted liver weight (g)			
	Wild-type mice		CAR-null mice	
	Dietary PB concentration (ppm)			
	0	850	0	850
3 days	1.35	1.85**	1.46	1.39*
7 days	1.44	1.98**	1.53	1.56

From Milburn (2006b)
Statistically significant difference from control group mean: * $P < 0.05$; ** $P < 0.01$ (Student's *t*-test, two-sided)

Table 42. Liver cell proliferation results: Ki67 labelling index

Treatment	Ki67 labelling index (mean ± standard deviation)			
	Wild-type mice		CAR-null mice	
	Dietary PB concentration (ppm)			
	0	850	0	850
3 days	0.58 ± 0.21	4.94** ± 0.92	0.28 ± 0.09	0.10** ± 0.04
7 days	0.68 ± 0.20	2.08** ± 0.73	0.37 ± 0.23	0.48 ± 0.28

From Milburn (2006b)
Statistically significant difference from control group mean: ** $P < 0.01$ (Student's *t*-test, two-sided)

labelling indices of hepatocytes (Brown & Gatter, 1990) in wild-type mice, but not in CAR-null mice (Table 42).

Gene expression of *Mdm2* and *Gadd45β*, which are associated with the regulation of proliferation and/or apoptosis in the rodent liver and inducible by treatment with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene or PB in a CAR-dependent manner (Columbano et al., 2005; Huang et al., 2005), was analysed. Although no or minimal increase of *Mdm2* expression in wild-type and CAR-null mice after the treatment was observed, expression of the *Gadd45β* gene was increased in wild-type mice and less increased in CAR-null mice (Table 43).

For liver biochemical parameters, PROD activities and CYP2B immunoblotting were greatly increased by the treatment at 3 and 7 days in wild-type mice, but there was no effect in CAR-null mice. BROD activities were increased only in wild-type mice at 3 days by treatment. PB increased coumarin 7-hydroxylase activities in wild-type mice treated for 7 days only. These data confirm the known strong potency of PB as an inducer of murine hepatic CYP isoenzymes of gene subfamily *Cyp2b* (Honkakoski et al., 1992; Nerurkar et al., 1993a,b; Jarukamjorn et al., 1999; Lavery et al., 1999; Wei et al., 2000, 2002; Maglich et al., 2002; Ueda et al., 2002; Columbano et al., 2005; Huang et al., 2005). This study also demonstrated that responses by the liver to PB treatment for 3 or 7 days in wild-type mice were different from those in CAR-null mice (Milburn, 2006b).

(iv) Four-week liver cell proliferation study in rats and mice (with serial sacrifices)

Male Wistar rats and CD-1 mice at 12 weeks of age were treated with cyproconazole for 0, 1, 2, 3, 4, 7, 14, 21 and 28 days at 0, 20, 350 or 1400 ppm (rats) or 0, 15, 100 or 200 ppm (mice) (Warren et al., 1995b). Five animals per dose group were sacrificed after the different time periods given above.

Table 43. Quantitative real-time PCR analysis of gene expression in mice given phenobarbital

Strain	Dietary PB concentration (ppm)	Fold change in gene expression (after normalization to *Hprt*)					
		Cyp2b10		*Mdm2*		*Gadd45*β	
		Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Wild type	0	1.00	1.00	1.00	1.00	1.00	1.00
	850	238.86**	194.01**	1.07	1.07	6.96**	4.92**
CAR null	0	1.00	1.00	1.00	1.00	1.00	1.00
	850	0.71	5.28*	1.23*	1.23	2.00*	2.14**

From Milburn (2006b)

* $P < 0.05$; ** $P < 0.01$. Analysis of variance was performed on control values. Fold changes in gene expression are derived from the levels of the control group.

Rats at 350 and 1400 ppm exhibited decreased body weight gain. From day 4, a dose-related increase in liver weight was apparent, affecting the mid- and high-dose animals of both species. Histopathology showed time- and dose-related centrilobular hepatocyte hypertrophy and hepatocyte vacuolation in the mid- and high-dose rats and all treated mice. Various combinations of hepatocyte vacuolation were also found in both rats and mice from all treated groups. In the rat, the mean labelling index of BrdU for the high-dose group showed a distinct peak on day 7 (not statistically significant) and a rapid decrease by day 14 (Figure 3). No significant differences were detected at 20 or 350 ppm. In mice, the mean labelling index of the high-dose group showed a distinct peak on days 2 and 3 with a subsequent decrease by day 14. For the 15 and 100 ppm groups, there were statistically significant increases, compared with control, for day 3 only. Generally, the increase in cell proliferation in the mouse study correlated with the onset of the hepatocyte hypertrophy.

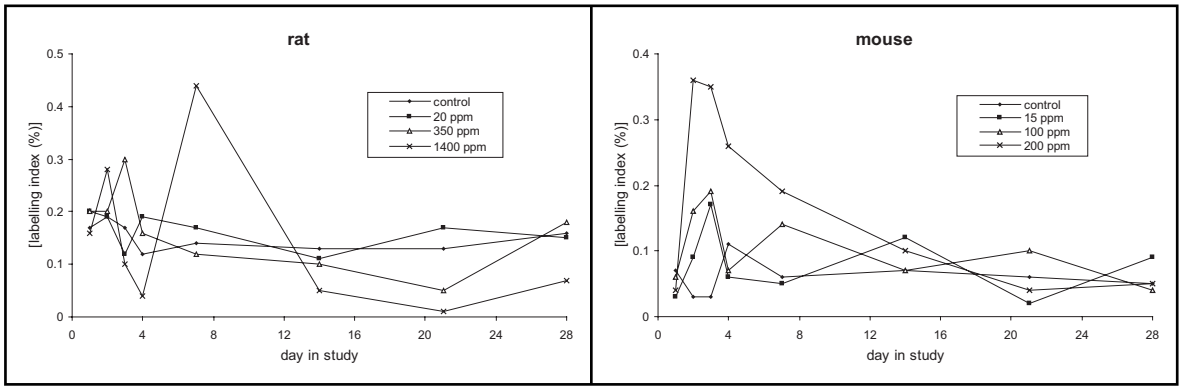
In conclusion, liver cell proliferation showed distinct species differences. Although the rat hepatocytes did not increase cell proliferation after the treatment, the mouse hepatocytes showed distinct increases in cell proliferation at an early phase after the treatment. This early increase in cell proliferation most likely represented a rapid response to a stimulus.

(v) Comparative histopathological evaluation of the effects of cyproconazole and propiconazole on the liver of male mice

Propiconazole (CGA 64250), another member of the group of triazole fungicides, showed a pattern of effects similar to that of cyproconazole, including liver weights, induction of xenobiotic metabolizing enzymes, hepatocyte proliferation and induction of liver tumours in mice. The histopathological re-evaluation was intended to compare the histopathological effects of subchronic treatment with cyproconazole and propiconazole on the liver of male mice (Weber, 1999). In the study on propiconazole, groups of five male mice were each treated with 0, 850 or 2500 ppm and sacrificed after 1, 2, 3, 4, 7, 14, 28 and 60 days. In addition, this study included PB-treated groups (850 ppm, five male mice each).

Effects on liver weight in male mice were comparable in the 850 ppm propiconazole group and the 200 ppm cyproconazole group. A slightly more pronounced effect was seen with PB at 850 ppm. The strongest effect on liver weight was found with propiconazole at 2500 ppm. Hypertrophic hepatocytes were mainly found in centrilobular and midzonal lobular compartments in all treated groups except the low dose of cyproconazole, being more prominent in propiconazole and PB groups. Minimal hepatocellular necrosis was observed in cyproconazole- or propiconazole-treated animals. PB induced minimal to moderate hepatocellular necrosis. Although a clear relationship to treatment could not be established in mice treated with cyproconazole, propiconazole (at both dose levels) and PB induced an increase in mitotic activity mainly around day 2. Centrilobular hepatocyte vacuolation was mainly observed after the treatment with cyproconazole and propiconazole, but not PB.

Figure 3. Liver cell proliferation study: hepatocellular proliferation in male rats and mice



In conclusion, there were no significant qualitative differences between these two triazoles, and quantitative differences mostly reflected the different degrees of induced hepatomegaly. The overall histopathological picture obtained with the two triazoles resembles that of PB (Warren et al., 1995b).

(vi) Liver enzyme induction study in rat and mouse

Examination of liver enzyme induction profiles in rats and mice following cyproconazole or PB treatment for up to 21 days was conducted. The liver samples were obtained from Han-Wistar male rats and CD-1 male mice treated with cyproconazole, contained in the diet, for 4 and 21 days at dose levels of 1400 and 200 ppm, respectively, and from the corresponding controls (Warren et al., 1995b). Using fractions extracted from the samples of livers from rats and mice (Warren et al., 1995b), the following parameters and enzyme activities were investigated: total CYP content, NADPH–CYP reductase (NCPR), ethoxyresorufin *O*-deethylase (EROD, a CYP1A-related activity), PROD (a CYP2B-related activity), total GSH content, GST and uridine diphosphate-glucuronosyltransferase (UDPGT) (Dorobek & Müller, 1995). The results of this study show that cyproconazole is a liver enzyme inducer (Table 44). Although qualitatively the effects were very similar in the two species, the effects were more prominent in rats than in mice. To confirm, the lower PROD induction in mice was further investigated and discussed in the second enzyme induction study (Trendelenburg, 2001). In the second study, CD-1 mice (Crl:CD®-1 (ICR) BR; five of each sex per group; about 6 weeks old) were treated on 14 consecutive days at dose levels of 0, 50, 100 or 200 ppm cyproconazole and 850 ppm PB. Liver enzyme induction was analysed using immunoblotting of microsomal CYP contents and enzyme activities in the subcellular liver fraction of mice (Table 45) (Trendelenburg, 2001).

Liver enzyme induction similar to that observed in the first study was observed in the second study.

In conclusion, the inducing profile was qualitatively very similar to that observed in both sexes with 850 ppm PB, which was fed as a reference compound in this study. Thus, cyproconazole is a strong PB-type inducer of xenobiotic metabolizing enzymes in the mouse liver.

(vii) 13-week dose range–finding feeding study in CD-1 mice

CD-1 mice initially aged 6 weeks (10 animals of each sex per group) were treated for 13 weeks at a dose level of 0, 5, 15, 300 or 600 ppm in a dose range–finding study (Warren, Skinner & Karapally, 1987).

Body weight depression in males and liver enlargement or histopathological liver changes in both sexes were observed at 300 or 600 ppm, indicating that the two highest doses are excessive for a long-term study. Both 5 and 15 ppm showed no treatment-related effects. The dose of 15 ppm is

Table 44. Liver enzyme induction (first study): effects of cyproconazole on liver enzymes and parameters of male rats and mice

Parameter	% of control			
	Rats		Mice	
	4 days	21 days	4 days	21 days
Total CYP	453**	444**	296**	283**
EROD	305**	247**	176	155
PROD	9754**	10 653**	424*	323**
NCPR	140	126*	270**	306**
UDPGT	97	180*	87	76
GST	139	260**	133*	140**
GSH	152*	94	111	108

From Dorobek & Müller (1995)
* $P < 0.05$; ** $P < 0.01$

therefore the NOAEL in this study, corresponding to 2.2 mg/kg bw per day in males and 3.2 mg/kg bw per day in females. As it should be recognized that the spacing between the NOAEL and the LOAEL (300 ppm) was very large, the "real" NOAEL for this study is expected to be somewhere in between these two dose levels (Warren, Skinner & Karapally, 1987).

3. Observations in humans

Syngenta, formerly Sandoz Agro Ltd, has monitored occupational exposure to cyproconazole. A report by the company physician summarizes investigations of the health conditions of Syngenta employees exposed to cyproconazole in the production, formulation and packaging plant and in research laboratories (covering the period 1988–1994). No adverse effects were reported (Hertner & Schulze-Rosario, 2001).

Questionnaires have been filled in by Syngenta field trial personnel. The reported cases give no evidence for a sensitizing potential of cyproconazole in humans. However, a few individuals may occasionally experience local skin reactions when exposed to formulations by direct contact. No cases of serious poisoning with cyproconazole or cyproconazole-containing formulations have been reported to Syngenta. The few publications on occupational exposure, reviewed by Hertner & Schulze-Rosario (2001), do not report on significant adverse findings correlated with cyproconazole exposure.

Comments

Biochemical aspects

In a toxicokinetic study, male and female rats were given cyproconazole uniformly labelled with ^{14}C either in the phenyl ring or at the α-carbon position as a single dose at 10 or 130 mg/kg bw or as 14 repeated doses of 10 mg/kg bw per day followed by a single oral dose of radioactive cyproconazole at 10 mg/kg bw. Cyproconazole was rapidly and extensively (86%) absorbed from the gastrointestinal tract and rapidly excreted from the body in urine and faeces (85% of the administered dose) within 168 hours. The majority of excretion occurred in the first 48 hours. The bile duct–cannulated rats excreted approximately 76% and 60% of the administered dose in the bile in males

Table 45. Liver enzyme induction in mice (second study)

Parameter	% of control							
	Male				Female			
	Cyproconazole			PB	Cyproconazole			PB
	50 ppm	100 ppm	200 ppm	850 ppm	50 ppm	100 ppm	200 ppm	850 ppm
Enzyme activities in subcellular liver fractions of mice								
MROD	n.s.	168	n.s.	315	160	159	157	380
EROD	n.s.	n.s.	n.s.	214	252	275	260	593
PROD[a]	257	343	404	2448	372	316	295	3147
BROD[a]	520	694	825	4431	1040	1003	942	7750
COH	839	778	1248	969	628	737	1149	623
LA 11-OH	n.s.	n.s.	n.s.	299	203	162	146	592
LA 12-OH	n.s.	n.s.	n.s.	n.s.	192	157	151	n.s.
mEH	n.s.	n.s.	197	182	n.s.	214	412	394
UDPGT	n.s.	139	n.s.	154	129	138	n.s.	196
GST	123	131	180	185	n.s.	184	408	354
Immunoblot analysis of microsomal CYP contents								
CYP1A isoenzymes	141	184	186	222	137	147	149	188
CYP2B isoenzymes Band 1	741	845	1239	1931	1701	2749	3151	3537
CYP2B isoenzymes Band 2	322	295	422	490	791	930	832	716
CYP3A isoenzymes	264	338	387	352	298	569	712	507
CYP4A isoenzymes	97	82	95	96	122	76	128	41

From Trendelenburg (2001)

COH, coumarin 7-hydroxylase; LA 11-OH, lauric acid 11-hydroxylase; LA 12-OH, lauric acid 12-hydroxylase; mEH, microsomal epoxide hydrolase; MROD, 7-methoxyresorufin *O*-demethylase; n.s., value not significantly different from control at $P = 0.05$ (Dunnett's test)

[a] An inhibitory factor present in microsomes from cyproconazole-treated animals strongly disturbed the accurate determination of this activity.

and females, respectively. Approximately 5% of the administered dose was recovered in the faeces in the cannulated rats. The absorption, distribution and excretion of cyproconazole were similar in rats administered repeated doses and single low and high doses. No significant radioactivity was detected in the exhaled air following a single oral dose of 10 mg/kg bw. Less than 0.42% of the administered dose was found in the carcass and tissues at 168 hours following 14 repeated doses. Tissue residues were highest in liver and adrenals (mainly cortex), followed by fat and kidney. There was no evidence of bioaccumulation in any tissues in rats. Cyproconazole was extensively metabolized, with a greater number of metabolites identified in the urine compared with the faeces. The metabolic profile revealed 35 metabolites, ranging from less than 0.1% to 4.9% and from less than 0.1% to 13.2% of the administered dose in the urine and faeces, respectively. Approximately 11% of the parent compound was detected in the faeces, and less than 0.4% in the urine. The predominant metabolic reactions of cyproconazole in the rat were 1) oxidative elimination of the triazole ring, 2) hydroxylation of the carbon bearing the methyl group, 3) oxidation of the methyl group to the carbinol and further to the carboxylic acid and 4) reductive elimination of the carbon bearing the methyl group, yielding a benzyl alcohol, which is further oxidized to the corresponding ketone.

Toxicological data

Cyproconazole has moderate acute toxicity when administered by the oral route to mice (LD_{50} of 200 mg/kg bw) and female rats (LD_{50} of 350 mg/kg bw). The LD_{50} in rats and rabbits treated dermally was greater than 2000 mg/kg bw. The LC_{50} in rats treated by inhalation (nose only) was greater than 5.6 mg/l. Cyproconazole was slightly irritating to the eyes and skin of rabbits. Cyproconazole was not a skin sensitizer in guinea-pigs as determined by the Magnusson & Kligman (maximization) test and the Buehler test.

The liver was the target organ for cyproconazole in short-term toxicity studies in mice, rats and dogs. Disturbances in lipid metabolism were also observed in all species studied. The NOAEL in a 90-day study of toxicity in mice was 15 ppm (equal to 2.2 mg/kg bw per day), based on decreased body weight gain in both sexes and increased relative spleen weights in males seen at 300 ppm (equal to 43.8 mg/kg bw per day).

The NOAEL in a 28-day study of toxicity in rats was 100 ppm (equal to 8.1 mg/kg bw per day), based on reduced body weight gain in females, changes in clinical chemistry, organ weight changes and histopathological findings in the liver seen at 300 ppm (equal to 25.3 mg/kg bw per day). Two 90-day dietary toxicity studies in rats were available, with a combined NOAEL of 80 ppm (equal to 6.4 mg/kg bw per day), based on reduced body weight gain seen at 320 ppm (equal to 23.8 mg/kg bw per day).

The NOAEL in the 90-day and 1-year toxicity studies in dogs was 100 ppm (equal to 3.2 mg/kg bw per day), based on retarded body weight gain seen at 350 ppm (equal to 12.1 mg/kg bw per day) and above.

The carcinogenic potential of cyproconazole was studied in mice and rats. In mice, the major changes following administration of cyproconazole occurred in the liver. There were a number of toxic effects (focal hepatocytic inflammation, single-cell hepatocytic necrosis and diffuse hepatocytic hypertrophy) at the two highest dose levels (100 and 200 ppm) in both sexes. The male mice were more severely affected than the females. The non-liver findings in mice were not considered treatment related. A treatment-related increase in the incidence of combined adenomas and carcinomas was found in males and females at 200 ppm and in males at 100 ppm. The NOAEL in this study was 15 ppm (equal to 1.8 mg/kg bw per day), and the LOAEL was 100 ppm (equal to 13.2 mg/kg bw per day).

To clarify the MOA of mouse liver tumours, mechanistic studies were conducted in which the liver effects of cyproconazole and PB in various strains of mice were compared. The results of these studies indicated that cyproconazole as well as PB produced similar effects in a dose-related manner in mice. Studies using CAR-null and wild-type mice treated with cyproconazole or PB for up to 7 days clearly indicated early gene expression changes in CAR regulation (*Cyp2b10, Gadd45β*), biochemical changes (induction of CYP2B-dependent enzyme activities), hypertrophy, fat vacuolation and increased single-cell necrosis in the liver, indicating that these effects were a consequence of CAR activation by cyproconazole as well as PB. Based on these mechanistic studies, the Meeting concluded that development of liver tumours in mice administered cyproconazole depends upon CAR activation.

In the 2-year toxicity and carcinogenicity study in rats, the NOAEL was 50 ppm (equal to 2.2 mg/kg bw per day), on the basis of body weight depression at 350 ppm (equal to 15.6 mg/kg bw per day). There was no evidence of treatment-related tumorigenesis in the rat.

Cyproconazole gave a negative response in an adequate range of in vitro and in vivo genotoxicity tests.

The Meeting concluded that cyproconazole was unlikely to be genotoxic.

On the basis of the absence of genotoxicity, the absence of carcinogenicity in rats and no carcinogenicity in mice by an MOA relevant to humans, the Meeting concluded that cyproconazole is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproductive toxicity in rats, reproductive parameters were not affected at the highest dose tested (120 ppm, equal to 8.3 mg/kg bw per day). The NOAEL for parental systemic toxicity was 20 ppm (equal to 1.4 mg/kg bw per day), based on a significant increase in relative liver weight and an increased incidence of slight fatty changes (vacuolated hepatocytes) in the liver at 120 ppm (equal to 8.3 mg/kg bw per day). These effects in the liver cannot be unequivocally attributed to the activation of CAR. Toxicity was less pronounced in the F_0 females and was not seen in F_1 males and females. The NOAEL for reproductive and offspring toxicity was 120 ppm (equal to 8.3 mg/kg bw per day), the highest dose tested.

There are three developmental toxicity studies in rats: the main study, a non-GLP range-finding study and a published study. Cyproconazole caused significantly diminished body weight gain during the early phase of treatment (days 6–11) as well as reduced feed consumption in all three studies. In the dose range of 20–30 mg/kg bw per day, body weight gain was 29–37% below that of control groups. Major fetal malformations in these studies were cleft palate (also reported as palatoschisis) and internal hydrocephalus. These malformations occurred at dose levels of 20 mg/kg bw per day and above. The NOAEL for maternal toxicity in the main developmental study was 6 mg/kg bw per day, based on reduced maternal body weight gain during gestation days 6–11 and decreased feed consumption seen at 12 mg/kg bw per day. The developmental NOAEL in the main study in rats was 12 mg/kg bw per day, based on decreased fetal body weights, increased postimplantation loss, increases in supernumerary ribs and increased fetal malformations (e.g. cleft palate) seen at 24 mg/kg bw per day.

There are two developmental toxicity studies available in rabbits. In the first study, treatment of pregnant Chinchilla rabbits with cyproconazole resulted in maternal body weight loss and reduced feed consumption early during treatment at the highest dose level of 50 mg/kg bw per day. A slightly increased number of postimplantation losses were also noted in this group. No treatment-related fetal abnormalities were observed. The NOAEL for maternal and developmental toxicity in Chinchilla rabbits was 10 mg/kg bw per day. In the second study, New Zealand White rabbits were treated at the same dose levels. As in the previous study, the high dose resulted in maternal toxicity in the form of body weight loss and reduced feed consumption early during treatment. The incidence of postimplantation losses was not affected. In contrast to the first study, the second one revealed an increased number of fetal malformations, mainly affecting sternebrae, ribs, vertebral column, hindlimbs and tail. The NOAEL for maternal and developmental toxicity was 10 mg/kg bw per day.

The Meeting concluded that cyproconazole can cause developmental toxicity, including malformations, but only at doses that are maternally toxic.

In a 90-day dietary study of toxicity in rats, five rats of each sex per dose were subjected to neuropathological examination and were also evaluated in the FOB and for the assessment of motor activity. No effects on FOB parameters, motor activity or neuropathology were observed at doses up to 1400 ppm (equal to 106.8 mg/kg bw per day).

No adverse effects due to occupational exposure to cyproconazole were reported in workers working in the production, formulation and packaging plant or in research laboratories.

The Meeting concluded that the existing database on cyproconazole was adequate to characterize the potential risk to fetuses, infants and children.

Toxicological data on metabolites

Several toxicological studies were conducted on cyproconazole metabolites M21/M21a and M36 (also named NOA 405870 and NOA 405872, respectively). The IUPAC name for metabolite M21/M21a is 5-(4-chlorophenyl)-5-hydroxy-4-methyl-6-(1H-1,2,4-triazol-1-yl)-2-hexanoic acid. The IUPAC name for metabolite M36 is 5-(4-chlorophenyl)-3,5-dihydroxy-4-methyl-6-[1,2,4]triazol-1-yl-hexanoic acid. These metabolites were found in the rat in minor amounts only (0.02–0.06% of applied dose in urine). They were found in the milk and in the urine of lactating goats. Therefore, the toxicological profile of these metabolites was investigated.

The acute oral LD_{50} for metabolite M21/M21a (NOA 405870) was greater than 2000 mg/kg bw. The metabolite was negative for mutagenicity in a bacterial reverse mutation assay (Ames test). For metabolite M36 (NOA 405872), an oral LD_{50} value of greater than 2000 mg/kg bw was observed in mice and rats. Based on the results from three genotoxicity studies, it is concluded that M36 is unlikely to be genotoxic in vivo. A 28-day feeding study in rats resulted in deaths at the test limit dose of 20 000 ppm and significant reductions in body weight at 5000 ppm and above. The NOAEL in this study was 1500 ppm (equal to 155 mg/kg bw per day), based on reduced body weights seen at 5000 ppm (equal to 527 mg/kg bw per day).

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.02 mg/kg bw on the basis of the overall NOAEL of 2.2 mg/kg bw per day from the 2-year study of toxicity and carcinogenicity and the multigeneration reproduction study in rats based on reduced body weight gain and liver toxicity seen at higher doses. A safety factor of 100 was applied. This ADI was supported by the NOAEL of 15 ppm (equal to 2.2 mg/kg bw per day) observed in a 90-day toxicity study in mice on the basis of reduced body weight gain observed at 300 ppm (equal to 43.8 mg/kg bw per day).

The Meeting established an acute reference dose (ARfD) of 0.06 mg/kg bw on the basis of a maternal toxicity NOAEL of 6 mg/kg bw per day in studies of developmental toxicity in rats, based on body weight loss during the early treatment period (gestation days 6–11) and reduced feed consumption seen at 12 mg/kg bw per day. The ARfD is protective of developmental toxicity seen at a slightly higher dose in rabbits.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mice	Ninety-day study of toxicity[a]	Toxicity	15 ppm, equal to 2.2 mg/kg bw per day	300 ppm, equal to 43.8 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 2.2 mg/kg bw per day	350 ppm, equal to 15.6 mg/kg bw per day[b]
		Carcinogenicity	350 ppm, equal to 15.6 mg/kg bw per day[b]	—
	Multigeneration study of reproductive toxicity[a]	Parental toxicity	20 ppm, equal to 1.4 mg/kg bw per day	120 ppm, equal to 8.3 mg/kg bw per day[b]
		Reproductive toxicity	120 ppm, equal to 8.3 mg/kg bw per day[b]	—
		Offspring toxicity	120 ppm, equal to 8.3 mg/kg bw per day[b]	—
	Developmental toxicity study[c,d]	Maternal toxicity	6 mg/kg bw per day	12 mg/kg bw per day
		Embryo and fetal toxicity	12 mg/kg bw per day	24 mg/kg bw per day
Rabbit	Developmental toxicity study[c,d]	Maternal toxicity	10 mg/kg bw per day	50 mg/kg bw per day[b]
		Embryo and fetal toxicity	10 mg/kg bw per day	50 mg/kg bw per day[b]
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	100 ppm, equal to 3.2 mg/kg bw per day	350 ppm, equal to 12.1 mg/kg bw per day[b]

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Two or more studies combined.

Estimate of acceptable daily intake for humans

0–0.02 mg/kg bw

Estimate of acute reference dose

0.06 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to cyproconazole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly absorbed, > 86% within 144 h
Dermal absorption	Not available
Distribution	Widely distributed in tissues; highest residues in liver, adrenal, fat and kidney
Potential for accumulation	None
Rate and extent of excretion	Rapid and extensive
Metabolism in animals	Extensively metabolized (35 metabolites identified)
Toxicologically significant compounds (animals, plants and environment)	Cyproconazole and 1,2,4-triazole

Acute toxicity	
Rat, LD_{50}, oral	350 mg/kg bw (rats)
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.6 mg/l (4 h exposure, nose only)
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Slightly irritating
Guinea-pig, dermal sensitization	Not a sensitizer (Magnusson & Kligman test, Buehler test)

Short-term studies of toxicity	
Target/critical effect	Reduced body weights in mice and rats
Lowest relevant oral NOAEL	2.2 mg/kg bw per day (90-day study of toxicity in mice)
Lowest relevant dermal NOAEL	100 mg/kg bw per day (rats)
Lowest relevant inhalation NOAEC	0.017 mg/l, equal to 4.9 mg/kg bw per day (16-day inhalation study in rats)

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver
Lowest relevant NOAEL	2.2 mg/kg bw per day (2-year carcinogenicity study in rats)
Carcinogenicity	Not carcinogenic in rats; carcinogenic in mice by MOA not relevant to humans

Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	None
Lowest relevant reproductive NOAEL	8.3 mg/kg bw per day (rats; highest dose tested)
Developmental target/critical effect	Developmental toxicity, including teratogenicity, only at maternally toxic dose in rats and rabbits
Lowest relevant developmental NOAEL	10 mg/kg bw per day (rats and rabbits)
Neurotoxicity/delayed neurotoxicity	
Subchronic neurotoxicity	Not neurotoxic (90-day study in rats)
Mechanistic data	
	Mechanistic studies supporting CAR-mediated liver toxicity and tumours in mice
Medical data	
	No adverse effects reported

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	2-year study of toxicity and carcinogenicity and multigeneration study of reproductive toxicity in rats	100
ARfD	0.06 mg/kg bw	Developmental toxicity studies in rats	100

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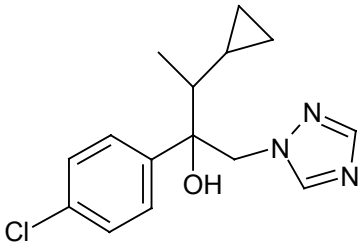
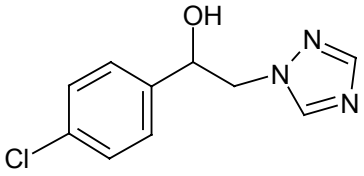
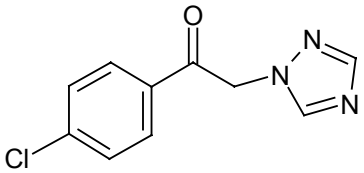
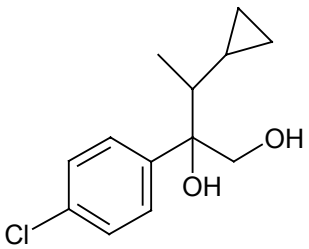
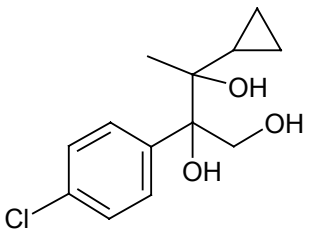
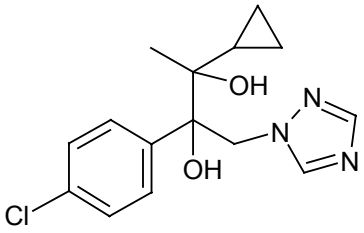
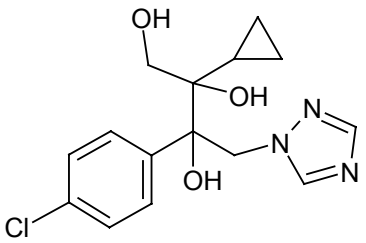
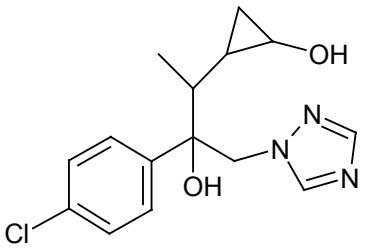
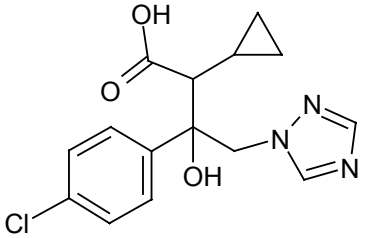
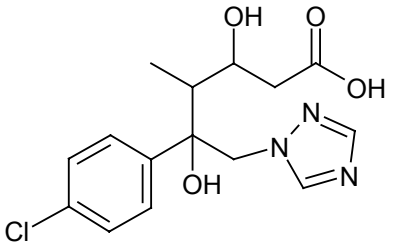
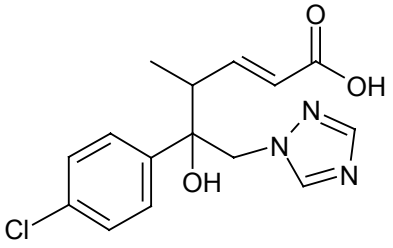
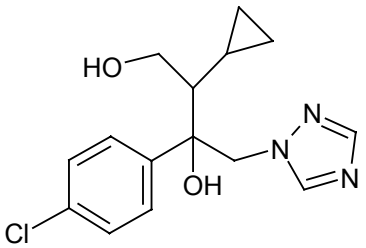
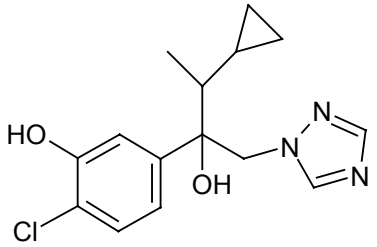
Appendix 1: Metabolites formed in the rat from cyproconazole

Code number (alphabetical order)	Designation in reports	IUPAC name	Structure
Cyproconazole SAN 619 F CGA 221949	M1/M2	2-(4-chlorophenyl)-3-cyclopropyl-1-[1,2,4]triazol-1-yl-butan-2-ol	
NOA 408616	M15	1-(4-chlorophenyl)-2-[1,2,4]triazol-1-yl-ethanol	
CGA 123420	M16	1-(4-chlorophenyl)-2-(1H-1,2,4-triaz-ol-1-yl)-ethanone	
NOA 421152	M3/M4	2-(4-chlorophenyl)-3-cyclopropyl-1,2-butanediol	
NOA 452669	M30/M33	2-(4-chlorophenyl)-3-cyclopropyl-butane-1,2,3-triol	
NOA 421153	M9/M14	2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)-2,3-butanediol	

Code number (alphabetical order)	Designation in reports	IUPAC name	Structure
NOA 452668	M20	3-(4-chlorophenyl)-2-cyclopropyl-4-[1,2,4]triazol-1-yl-butane-1,2,3-triol	
NOA 451353	M13	2-[2-(4-chlorophenyl)-2-hydroxy-1-methyl-3-[1,2,4]triazol-1-yl-propyl]-cyclopropanol	
NOA 452154	M10	3-(4-chlorophenyl)-2-cyclopropyl-3-hydroxy-4-[1,2,4]triazol-1-yl-butyric acid	
NOA405872	M36	5-(4-chlorophenyl)-3,5-dihydroxy-4-methyl-6-[1,2,4]triazol-1-yl-hexanoic acid	
NOA 405870	M21/M21a	5-(4-chlorophenyl)-5-hydroxy-4-methyl-6-(1H-1,2,4-triazol-1-yl)-2-hexanoic acid	
NOA 421154	M11/M18	3-(4-chlorophenyl)-2-cyclopropyl-4-(1H-1,2,4-triazol-1-yl)-1,3-butanediol	

Code number (alphabetical order)	Designation in reports	IUPAC name	Structure
NOA 410714	M31/M38	2-chloro-5-(2-cyclopropyl-1-hydroxy-1-[1,2,4]triazol-1-ylmethyl-propyl)-phenol	

Appendix 2: Application of the IPCS conceptual framework for cancer risk assessment

A2.1 Introduction

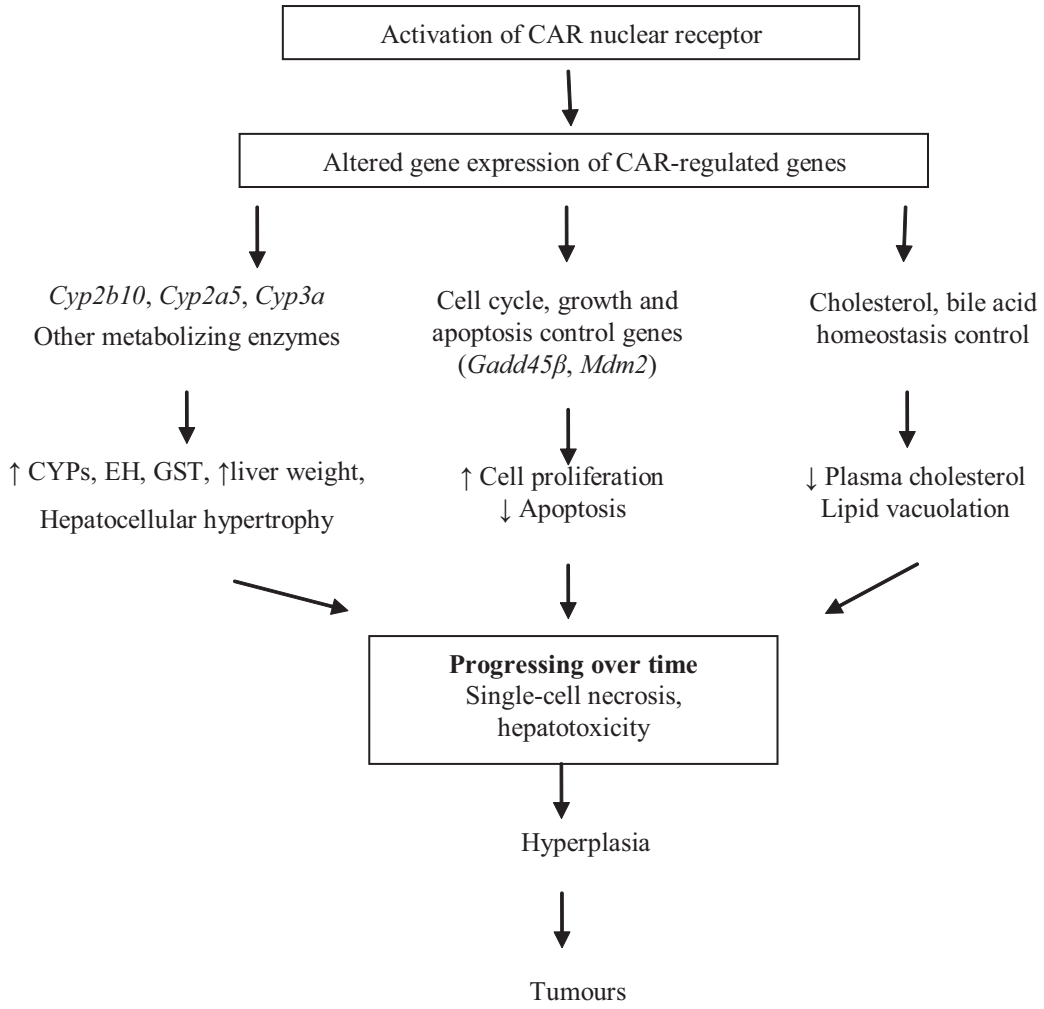
The data relating to the postulated MOA below are summarized in section 2.6(d). The analytical approach applied to the postulated MOA is based on the International Programme on Chemical Safety (IPCS) conceptual framework for evaluating an MOA for chemical carcinogens (Sonich-Mullin et al., 2001; Boobis et al., 2006; Holsapple et al., 2006). Administration of cyproconazole was associated with a statistically significant increase in hepatocellular tumours in male and female CD-1 mice. In male mice, there were statistically significant positive trends for adenomas and for combined tumours. Males also had statistically significant pairwise increases in carcinomas at the three highest doses (15, 100 and 200 ppm), adenomas at the 100 ppm level and combined tumours at the two highest doses (100 and 200 ppm). In female mice, there were also statistically significant positive trends for adenomas, carcinomas and combined tumours. All three parameters were statistically significant by pairwise comparison for females at the high dose (200 ppm).

In rats, no treatment-related tumour findings were observed in either sex at doses up to and including 350 ppm. An evaluation of the weight of evidence to support a non-mutagenic MOA for liver tumours in mice is summarized below.

A2.2 Summary description of the postulated mode of action

Development of liver tumours in mice administered cyproconazole orally is initiated by activation of CAR in liver hepatocytes. Following alteration of CAR-regulated genes, induction of CYP2B-dependent enzymes, hepatocyte hypertrophy and cell proliferation, suppression of apoptosis and perturbed liver function (e.g. decreased plasma cholesterol) are early events. This is a non-genotoxic mitogenic MOA. Over time, progression of hepatotoxicity, including single-cell necrosis associated with increased cell replication and suppression of normal apoptotic processes, might be associated with clonal expansion of potentially mutated cells, which leads to preneoplastic foci and development of liver tumours. Activation of CAR is considered to be the required initiating event leading to the cascade of key events that might result in the induction of liver tumours in cyproconazole-treated mice. No evidence of carcinogenicity of cyproconazole in CAR-null mice has been provided. Figure 4 outlines the key events in the postulated MOA for cyproconazole-induced liver tumours.

Figure 4. Key events in the mode of action for liver tumours in mice



A2.3 Key events in the mode of action

1. **Activation of CAR.** Activation of CAR is considered a critical initiating event in the MOA for liver tumorigenesis by cyproconazole. This was demonstrated through the use of CAR-null mice (CAR −/−) and the wild-type C3H mice (CAR +/+), where a pleiotropic liver response was reported only in the wild-type mice administered cyproconazole, but little or no response was reported in the CAR-null mice. A study investigating the liver effects of cyproconazole and PB in CD-1 mice, the outbred strain used in the cancer bioassay, and in C57BL/6J (C57) and C3H/HeNClrBR (C3H) inbred strains, for which CAR-null mice are available, indicated that cyproconazole and PB produced similar effects in a dose-related manner in CD-1, C57 and C3H mice (Milburn, 2006a,b,c).
2. **Altered expression of CAR-responsive genes.** CAR-responsive expression of genes associated with cell cycle control (*Mdm2*), cell proliferation (*Mdm2*, *Gadd45β*), apoptosis (*Tsc22*) and xenobiotic metabolism (*Cyp2b10*) was measured by real-time PCR in C3H wild-type and CAR-null mice. Marked increases in *Gadd45β* and *Cyp2b10* mRNA levels were observed in the liver of C3H mice treated with 200 and 450 ppm cyproconazole following 2, 7 and 14 days of treatment. Slight increases in *Mdm2* expression were seen at 450 ppm at all time points analysed (days 2,

7 and 14), whereas dose-dependent decreases in *Tsc22* (a pro-apoptotic gene) expression were observed following 2, 7 and 14 days of treatment. A comparison of gene expression in C3H wild-type and CAR-null mice showed markedly lower induction of *Cyp2b10* mRNA in CAR-null mice, supporting the role of CAR in the regulation of *Cyp2b10* expression. Expression levels of *Mdm2* and *Gadd45β* in cyproconazole-treated CAR-null mice were similar to or slightly lower than the levels observed in the wild-type mice. A similar expression pattern was observed in the PB-treated (positive control) mice (Milburn, 2006a,b).

3. **Cellular proliferation (mitogenic response).** The temporal sequence of events where there is an initial burst of cellular proliferation followed by a decline approaching background levels is a hallmark feature demonstrating a mitogenic MOA. There was a statistically significant increase in cell proliferation (as measured by BrdU labelling) after 3 days of treatment with up to 200 ppm cyproconazole in C3H mice. Cell proliferation returns to control levels by day 14 following continuous treatment with cyproconazole. To further investigate the role of CAR in the proliferative response, liver cells from C3H wild-type and CAR-null mice treated with 200 or 450 ppm cyproconazole for 7 days were measured by positive cells to Ki67 antibody. Statistically significant increases in cell proliferation (Ki67 labelling) were observed in the liver of wild-type mice after 7 days of treatment, whereas CAR-null mice showed a proliferative response similar to that of the vehicle control at 200 ppm and a reduced proliferative response (relative to wild-type mice) at 450 ppm (Warren et al., 1995,b; Milburn, 2006c).

4. **Induction of metabolizing enzymes.** CAR is known to transcriptionally activate specific xenobiotic metabolism genes, including *Cyp2b* and *Cyp3a*. To evaluate CYP2B protein levels in treated mice, western blot analysis was performed on liver extracts isolated from wild-type and CAR-null mice treated with 200 or 450 ppm cyproconazole for 7 days. A strong induction (relative to controls) of CYP2B protein levels was observed in wild-type mice at 200 and 450 ppm cyproconazole. In contrast, no increase in CYP2B protein was observed in the CAR-null mice treated with cyproconazole. CYP activity (specifically, CYP2B10 activity) was assessed by measuring PROD and BROD activities in wild-type and CAR-null mice treated with cyproconazole. Increased BROD activity was observed in wild-type mice fed 200 or 450 ppm cyproconazole for 7 days. Elevated PROD activity was seen in wild-type mice at 200 ppm, but not at 450 ppm. No increase in BROD or PROD activity was observed in the CAR-null mice at any dose (Milburn, 2006b,c).

5. **Changes in liver histopathology.** Hepatocyte hypertrophy, vacuolation and single-cell necrosis were observed as early as 7 days in wild-type mice fed 200 or 450 ppm cyproconazole. No evidence of hepatocyte hypertrophy, vacuolation or necrosis was seen in the CAR-null mice fed cyproconazole for 7 days. Increased incidences of hepatocellular hypertrophy and vacuolation and single-cell necrosis were also observed in male CD-1 mice at 28 days and male and female CD-1 mice following treatment with cyproconazole for 13 weeks (300 and 600 ppm) and 18 months (100 and 200 ppm) (Warren, Skinner & Karapally, 1987; Warren et al., 1989; Milburn, 2006b).

6. **Increased liver weight.** A statistically significant increase in liver weight (adjusted for terminal weight) was observed in male C3W wild-type mice, but not in male CAR-null mice, fed 850 ppm cyproconazole for 3 days. Similar results were seen in C3H wild-type and CAR-null mice following 7 days of treatment with cyproconazole at 200 or 450 ppm with the exception of a slight increase in relative (but not absolute) liver weight in CAR-null mice at 450 ppm. Increased liver weights were also observed in cyproconazole-treated male and female mice at 13 weeks (300 and 600 ppm) and 18 months (100 and 200 ppm) (Warren, Skinner & Karapally, 1987; Warren et al., 1989; Milburn, 2006b).

7. **Liver tumours.** Administration of cyproconazole was associated with an increased incidence of hepatocellular tumours at 100 and 200 ppm in male CD-1 mice and 200 ppm in female CD-1 mice (Warren et al., 1989).

A2.4 Concordance of dose–response relationships

Nearly all of the key events in the MOA were observed in mice at the tumorigenic doses of 100 and 200 ppm. Very few of the key events occurred at the NOAEL in the 18-month mouse study (15 ppm), and those that did occur appeared to be transient in nature. In studies of up to 28 days' duration, hypertrophy and vacuolation in the livers of mice were observed at 15 ppm, but these were of minimal severity and partial incidence and were not seen after 13 weeks or 18 months of treatment. Thus, the minor effects at 15 ppm were adaptive responses to short-term administration that were reversible with longer-term administration.

A2.5 Temporal association

The operating principle for the key events in the MOA analysis is that the key events should occur temporally before the appearance of tumours. The identified key events all occur prior to the observation of hepatocellular tumours. Cell proliferation and enzyme induction (CYP2B10, CYP2A, CYP3A, GST, epoxide hydrolase) in the liver occurred as early as 3–7 days after dosing. Subchronic studies conducted for 28 and 90 days showed increased liver weight, histopathological changes (hepatocellular hypertrophy, vacuolation, single-cell necrosis) leading to focal hepatocytic hyperplasia and hepatocellular tumours seen in the carcinogenicity study.

A2.6 Strengths, consistency and specificity of association of tumour response with key events

A part of the Hill criteria requires that for the key events to be causally related to the formation of tumours, they must clearly be shown to be required steps that lead to the cancer, and the findings must be reproducible. The key events observed in mice fed on diets containing cyproconazole occurred in a logical temporal sequence, in a dose-dependent manner and only at the dose levels at which tumour incidences were increased. The clear establishment of CAR activation as a necessary key event for cyproconazole gives added strength to the proposed sequence of events and is supported by literature findings with other CAR agonists, such as PB. Also, concurrent experiments in CAR-null and wild-type mice with cyproconazole and PB (Milburn, 2006a,b,c) established that most of the key events are common to both compounds, including:

- CAR activation as a causal key event;
- induction of CYP isoenzymes and other metabolizing enzymes in a pattern similar to PB;
- similar progression of liver changes such as cell proliferation, hypertrophy, single-cell necrosis and fat vacuolation.

Structure–activity relationships also form part of the basis of an evaluation of the strength and consistency of a proposed MOA. In addition to cyproconazole, a wide array of other triazole fungicides have been studied for their effects on the liver in subchronic and chronic mouse and rat studies. Histopathological patterns induced by cyproconazole were similar to those induced by propiconazole, the other triazole fungicide, which also causes liver tumours in mice but not in rats (Weber, 1999). The MOA proposed for cyproconazole is consistent with the MOA for the PB-type inducer (IPCS, 1987; Trendelenburg, 2001). There is good consistency in the database for cyproconazole. The findings at early time points were reproducible across multiple studies that measured parameters such as cell proliferation, liver micropathology changes and enzyme induction (Dorobek & Müller, 1995; Warren et al., 1995b; Trendelenburg, 2001; Milburn, 2006a,b,c). Also, specificity of the MOA to mice has been demonstrated. Investigative studies with both rats and mice have shown that certain critical key events (e.g. cell proliferation, single-cell necrosis) do not occur in rats, and rats did not get tumours in the liver or any other tissue in a 2-year study with cyproconazole.

A2.7 Alternative modes of action

Based on the overall weight of evidence from the genetic toxicity battery of studies, cyproconazole is not acting via a genotoxic mode of carcinogenic action. Whereas the histology and clinical chemistry results indicate that liver toxicity occurred in both long- and short-term studies, several observations that challenge a cytotoxic MOA should be noted. For example, the increases in ALT, AST and alkaline phosphatase activities, which can be indicators of hepatocellular toxicity, were highly variable and suggest that very high doses of cyproconazole are needed to induce cytotoxicity at early time periods. In addition, although necrosis was seen in several studies with wild-type mice, it was not seen in studies with CAR-null mice. Finally, the cell proliferation data from the 28-day study are not consistent with a cytotoxic MOA, because cell proliferation decreased after 3–4 days in mice. A sustained cell proliferative response would be expected in a cytotoxic MOA. Therefore, the data do not support cytotoxicity followed by regenerative proliferation as an alternative MOA. In addition, there was no evidence of peroxisome proliferation, nor were CYP4A isoenzymes (inducible by peroxisome proliferating compounds, i.e. peroxisome proliferator-activated receptor alpha [PPARα] agonists) elevated in the mechanistic studies. However, the slight but significant increase in relative liver weight in CAR-null mice indicates that some quantitatively but not qualitatively different pathways, including PXR, might exist.

A2.8 Conclusion on the postulated mode of action in animals

The overall weight of the evidence supports a non-genotoxic mitogenic MOA for cyproconazole through activation of CAR, which is considered to be the required initiating event leading to the cascade of key events that result in the induction of liver tumours in cyproconazole-treated mice. Specifically, development of liver tumours in mice administered cyproconazole orally is considered to be initiated by activation of CAR in liver hepatocytes followed by altered gene expression, cell proliferation, suppression of apoptosis, alterations in liver function (enzyme activity) resulting in decreased plasma cholesterol (associative) and hepatocyte toxicity (hypertrophy, hyperplasia and necrosis). The data did not support peroxisome proliferation, mutagenesis or cytotoxicity followed by regenerative proliferation as alternative MOAs.

A2.9 Relevance of cyproconazole-induced tumours to humans

The human CAR is an important ligand-activated regulator of oxidative and conjugative enzymes and transport proteins. Consistent with the proposed rodent mitogenic MOA, the activation of the CAR nuclear receptor is a critical first step in the cascade of specific key events leading to tumorigenesis. Recent ligand binding studies indicate that the CAR receptor is different across human and mouse species.

There is some evidence for important species differences in signalling pathways downstream of CAR activation, affecting hepatocyte proliferation and rates of apoptosis. Studies using isolated human hepatocytes suggest that chemicals that activate CAR do not stimulate cell proliferation or inhibit apoptosis, in contrast to rodent hepatocytes (Parzefall et al., 1991; Hasmall & Roberts, 1999). More recently, Jyrkkärinne et al. (2005) published a paper on their development of well-defined homology models for human CAR. They coupled this with extensive functional analysis to suggest mechanisms that contribute to the high basal activity of human CAR and thus help to explain the wide species differences in CAR ligand specificity. The authors concluded that a single residue in helix 7 (F243) appears to explain the human/mouse species differences in response of CAR to 17α-ethynyl-3,17β-estradiol and that the differences in ligand specificity between human and mouse CAR are significant. Ross et al. (2010) reported a possibility that human CAR might support the chemically

induced hypertrophic responses but not the hyperplastic responses using double humanized PXR and CAR (huPXR/huCAR), double knockout PXR and CAR (PXRKO/CARKO) and wild-type C57BL/6J mice treated with PB or chlordane. As these papers illustrate, the understanding of the role of CAR is greatly expanding at this time, but it is not yet at the level of understanding about species specificity that has been attained for other MOA processes, such as PPARα activation (Klaunig et al., 2003).

Therefore, CAR is present in humans, and studies with other CAR agonists, such as PB, show some level of responsiveness in human liver, but other studies indicated differences between mouse and human CAR in liver effects. Although the MOA in mice suggests that CAR is involved with the liver effects of cyproconazole, the qualitative and quantitative plausibility of liver tumours to humans still remains undetermined, because there is insufficient information on the kinetic and dynamic factors of CAR in humans and different animal species.

DICAMBA

First draft prepared by
Jürg Zarn[1] and Alan Boobis[2]

[1] Nutritional and Toxicological Risks Section,
Swiss Federal Office of Public Health, Zurich, Switzerland
[2] Faculty of Medicine, Imperial College London, London, England

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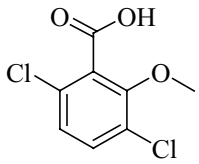
Explanation

Dicamba (Figure 1) is the International Organization for Standardization (ISO)–approved name for 3,6-dichloro-2-methoxybenzoic acid (International Union of Pure and Applied Chemistry [IUPAC]) and has the Chemical Abstracts Service (CAS) No. 1918-00-9. Dicamba is a benzoic acid auxin herbicide, mimicking the action of indolyl acetic acid in regulating plant growth.

Dicamba has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All pivotal studies contained certificates of compliance with good laboratory practice (GLP).

Figure 1. Chemical structure of dicamba



Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution, metabolism and excretion

In a published study, the distribution and excretion of [^{14}C]dicamba were investigated in male and female CD rats after administration by gavage, feeding or subcutaneous injection. In the gavage study, groups of eight females and four males received single doses of 100 mg/kg body weight (bw) or 930 mg/kg bw. Urine and faeces were collected, and, at various time points from 1 to 72 hours, individual animals were killed to collect blood and organs. In the feeding study, groups of five male and five female rats were fed diets containing 10, 100, 1000, 10 000 or 20 000 parts per million (ppm) dicamba for 4 weeks, and, at various time points from 1 to 72 hours, individual animals were killed to collect blood and organs. In the subcutaneous injection study, one male and one female rat were injected with dicamba at 0.1 mg/kg bw and killed 72 hours later.

After oral gavage or after injection, more than 90% of dicamba was excreted within 24 hours, irrespective of the dose administered. In all three dosing regimens, faecal excretion accounted for less than 5% of the dose applied. Dicamba was rapidly distributed to the organs and was excreted virtually completely within 3 days. It was estimated that about 10–20% of dicamba was glucuronidated (Tye & Engel, 1967).

The routes and rates of excretion and metabolism of ^{14}C-phenyl uniformly labelled (UL) dicamba (radiochemical purity > 99%; specific activity 0.71 GBq/mmol; lot No. not given) were

Table 1. TRR in urine and faeces in mice, rats, rabbits and dogs

Time point (h)	% of administered dose			
	Mice	Rats	Rabbits	Dogs
Urine				
0–16	67.7 ± 7.33	77.1 ± 7.7	81.5 ± 5.1	92.9 ± 5.0
0–24	72.6 ± 16.5	92.9 ± 3.7	82.6 ± 1.8	82.6 ± 20.2
24–48	11.2 ± 11.9	2.7 ± 0.5	5.5 ± 0.0	1.9 ± 0.5
48–72	3.1 ± 2.8	1.0 ± 0.4	1.0 ± 0.0	0.2 ± 0.0
72–96	—	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Total	86.9 ± 1.7	96.9 ± 4.5	89.1 ± 2.8	84.7 ± 18.3
Half-life (h)	10.2	7.0	7.4	5.4
Faeces				
0–16	1.8 ± 2.3	5.7 ± 0.4	3.6 ± 0.9	0.5 ± 0.6
0–24	3.3 ± 4.6	2.1 ± 2.0	0.0 ± 0.0	0.5 ± 0.0
24–48	5.2 ± 4.1	0.6 ± 0.1	0.8 ± 0.4	0.1 ± 0.1
48–72	1.0 ± 0.3	0.1 ± 0.0	1.0 ± 0.3	0.0 ± 0.0
72–96	—	0.0 ± 0.0	0.7 ± 0.8	0.0 ± 0.0
Total	9.4 ± 0.2	2.8 ± 1.8	2.5 ± 0.4	0.6 ± 0.1

From Atallah (1980)

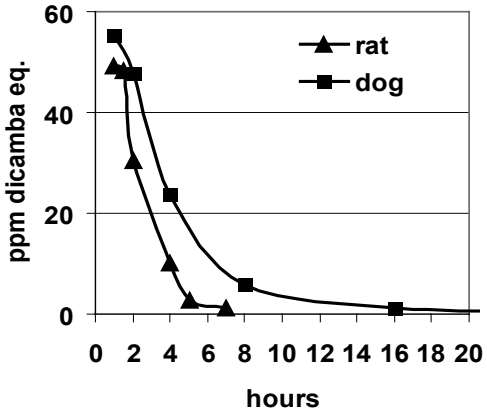
investigated in female Swiss mice, Sprague-Dawley rats, New Zealand White rabbits and Beagle dogs. By oral gavage, four mice received a single dose of 89 mg/kg bw, six rats received a single dose of 102 mg/kg bw, four rabbits received a single dose of 100 mg/kg bw and five dogs received a single dose of 88.2 mg/kg bw. The specific activities were 3.7–7.4 MBq/mmol. From each species, two animals were killed at 16 hours and 96 hours post-treatment. Urine and faeces were collected from all animals, and blood was collected from rats and dogs at several time intervals. After termination, tissues were collected for the analysis of total radioactive residues (TRR). This was a pre-GLP study of acceptable quality.

In mice, rats, rabbits and dogs, most of the radioactivity was excreted within 24 hours via urine, with elimination half-lives of 10.2, 7.0, 7.4 and 5.4 hours, respectively (Table 1). For mice, 9.4% of the administered dose was excreted in the faeces; in all other species, TRR in faeces were less than 3%. In the blood of rats and dogs, the highest concentrations of TRR were found at the first time point of measurement, at 1 hour after dose administration (Figure 2). Thereafter, radioactivity in blood declined rapidly. The decline was slightly slower in dogs than in rats. In all four species, the TRR in the whole body were 0.173–0.547% of the administered dose after 16 hours and 0.009–0.035% after 96 hours. Highest residues at 96 hours were found in kidney (0.001%) and liver (0.002–0.004%). In the urine of all four species, 97–99% of TRR was identified as dicamba, and less than 1% was the demethylated 3,6-dichloro-2-hydroxybenzoic acid (DCHBA). In faeces, 5% of TRR was not extractable in mice and rats, and up to 20% was not extractable in rabbits and dogs. Most of the TRR were identified as dicamba (70–93%). Only in dog faeces, an unknown metabolite accounted for 10%.

There were no significant species differences regarding absorption, excretion, tissue distribution or metabolism of dicamba between mice, rats, rabbits and dogs (Atallah, 1980).

In a comparative study, groups of five male CD VAF/Plus rats received by gavage a single 10 mg/kg bw dose of the free acid of dicamba (A), the dimethylamine salt (B), the isopropylamine salt (C) or the diglycolamine salt (D), respectively. Samples of approximately 1 MBq ^{14}C-phenyl UL dicamba (radiochemical purity 99.9%; specific activity 1.56 GBq/mmol; lot No. 911115) were diluted

Figure 2. Dicamba concentrations in the blood of rats and dogs



From Atallah (1980)

with non-labelled dicamba (A), dimethylamine dicamba (B), isopropylamine dicamba (C) or diglycolamine dicamba (D). Urine and faeces were collected for 24 hours after administration to analyse the absorption, metabolism and elimination of dicamba. The study complied with GLP guidelines.

The TRR in the 24-hour urine were similar in all groups, ranging from 92% to 95% of the administered dose. In the faeces, the range was 2–5%. In the blood of the four groups, the TRR at 24 hours ranged from 0.017% to 0.020% of the administered dose. In the urine of all groups, dicamba ranged from 92% to 94% of the TRR, and DCHBA accounted for 0.5–0.6%. The chemical identity of the rest of the TRR was not resolved. In the faeces of all groups, dicamba ranged from 75% to 80% of the TRR, and DCHBA accounted for 3–4%. Three to six per cent of the TRR were not extractable, and the chemical identity of the rest of the extracted TRR was not determined.

In this comparative study, no influence of the anionic moiety on the absorption, elimination or metabolism of dicamba was observed (Ekdawi, 1994b).

Urine pools from groups A, B, C and D of a previous single-dose study (Ekdawi, 1994b) were analysed for the plant metabolite 5-hydroxy-dicamba. 5-Hydroxy-dicamba was found to account for 0.0048% of the TRR in the urine pool (Ekdawi, 1994a).

The excretion kinetics of dicamba were investigated in Wistar and Sprague-Dawley rats. Groups of four male and four female rats were pretreated with unlabelled dicamba at various doses either by gavage or by feeding for 14 days, and then the animals received one dose of ^{14}C-phenyl UL dicamba (radiochemical purity > 95%; specific activity 0.59 GBq/mmol; lot No. 037H9294) (Table 2). Blood samples were taken from the animals at 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours after the labelled dose and analysed for TRR. The study complied with GLP.

All males and one female of experimental group 1 (high-dose gavage group) died within 3 hours after administration of the second dose. In experimental groups 2 and 3 (mid- and low-dose gavage groups), animals were terminated after administration of the third dose because of clear clinical signs of toxicity, such as ataxia, piloerection and respiratory sounds in group 2 and ataxia in group 3. In group 2, thickening of the wall in the forestomach and hyperaemia of the glandular stomach were observed in three animals, and erosion/ulceration was seen in one animal. In group 3, thickening of the wall in the forestomach and hyperaemia of the glandular stomach were observed in three animals. In experimental group 4 (high dose in feed group), it was shown that administration in the feed was better tolerated than administration by gavage, as no effect on body weight development and no clinical signs

Table 2. Study design

	Experiment No.										
	1	2	3	4	5	6	7	8	9	10	11
Wistar											
Group	4/sex	4/sex	4/sex	2/sex	4/sex	4/sex	4/sex	4/sex	4/sex	4/sex	4/sex
Gavage or food (14 days, non-labelled)	1000 mg/kg bw	400 mg/kg bw	150 mg/kg bw	12 000 ppm[a]	12 000 ppm	4500 ppm	1500 ppm	4500 ppm	3000 ppm	1500 ppm	900 ppm
Dose (males)	1000 mg/kg bw	400 mg/kg bw	150 mg/kg bw	710–856 mg/kg bw	988.7 mg/kg bw	366.7 mg/kg bw	122.7 mg/kg bw	369.7 mg/kg bw	248.5 mg/kg bw	120.1 mg/kg bw	73 mg/kg bw
Gavage (single dose, labelled)	1000 mg/kg bw	400 mg/kg bw	150 mg/kg bw	1000 mg/kg bw[b]	800 mg/kg bw	400 mg/kg bw	150 mg/kg bw	450 mg/kg bw	300 mg/kg bw	150 mg/kg bw	90 mg/kg bw
Analysis	TRR[c]	TRR[c]	TRR[c]	Toxicity	TRR[c]	TRR[c]	TRR[c]	TRR[c]	TRR[c]	TRR[c]	TRR[c]
	Experiment No.										
	12	13	14	15	16						
Sprague-Dawley											
Group	4/sex	4/sex	4/sex	4/sex	4/sex						
Food (14 days, non-labelled)	900 ppm	6000 ppm	3000 ppm	1500 ppm	900 ppm						
Gavage (single dose, labelled)	800 mg/kg bw	500 mg/kg bw	250 mg/kg bw	125 mg/kg bw	75 mg/kg bw						
Analysis	TRR[c]	TRR[c]	TRR[c]	TRR[c]	TRR[c]						

From Leibold (1998a)

[a] Only six doses.

[b] Not labelled.

[c] TRR in plasma.

were seen. It was therefore decided to pretreat with unlabelled dicamba in further studies by feeding instead of by gavage administration. After dosing with labelled dicamba, peak levels in plasma were reached after 0.5–1 hour, and the levels were dose proportional. This suggests that no saturation of absorption occurred. In male and female Wistar rats, the plasma area under the curve (AUC) increased linearly with dose up to 150 mg/kg bw (Table 3). At higher dose levels, the plasma AUC was disproportionately higher than the dose increase. In Sprague-Dawley rats, the plasma AUC increased linearly with dose up to 150 mg/kg bw in males and up to 250 mg/kg bw in females (Table 4). Additionally, the initial elimination half-lives increased at dose levels above 150 mg/kg bw in Wistar rats and above 125 mg/kg bw in Sprague-Dawley rats. As the absorption was not saturated, the disproportionate increase in plasma AUC, supported by increased initial elimination half-lives, indicates a reduced or delayed elimination at oral doses starting in the range of 125–300 mg/kg bw (Leibold, 1998a).

Groups of four male and four female Wistar rats were fed diets containing 600, 1200, 2400, 4800 and 9600 ppm non-labelled dicamba for 94 days. At days 29, 63 and 91, the groups received an equivalent dose (50, 100, 200, 400 or 800 mg/kg bw) by gavage of ^{14}C-phenyl UL dicamba (radiochemical purity > 99.8%; specific activity 0.63 GBq/mmol; lot No. 787-0102). At 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours post-gavage of labelled dose, blood samples were taken to analyse TRR. Additionally, a few urine and blood clinical chemistry parameters were measured in the highest and lowest dose groups. The study complied with GLP.

At all dose levels, dose-related increases in incidence and severity of convulsions were observed after the gavage dosing on days 29, 63 and 91. At the end of the study, there was no dose-related difference in blood pH, plasma creatinine or urea between the treated groups. In urine, there was a decrease (11–31% in males and 32–86% in females) in activities of alkaline phosphatase, γ-glutamyl transferase, alanine aminotransferase and *N*-acetyl-β-D-glucosaminidase. Lactate dehydrogenase activity was not changed. Initial plasma TRR level increased proportionately with dose, suggesting that oral absorption was not saturated. At all time points, there was a disproportionate increase in AUC in males at 400 mg/kg bw and above and in females at 200 mg/kg bw and above (Table 5), and increased half-lives were observed. With respect to kinetic parameters, there was no obvious effect of the pretreatment duration (Beimborn, 2003).

Two groups of four male and four female Wistar rats were administered single doses of either 0.5 mg/kg bw (groups B1) or 200 mg/kg bw (groups D1) of ^{14}C-phenyl UL dicamba (radiochemical purity > 99.2%; specific activity 0.65 GBq/mmol; lot No. ILA-72.1). Urine, faeces and expired air were collected from these animals over 7 days, and tissues were collected after termination. Two groups of 12 male and 12 female Wistar rats were administered single doses of either 0.5 mg/kg bw (groups F1 [males] and F2 [females]) or 200 mg/kg bw (groups F3 [males] and F4 [females]) of ^{14}C-phenyl UL dicamba. At 4, 8, 12 and 16 hours post-dosing, four animals per group were terminated, and tissues were collected for analysis of radioactivity. The study complied with GLP.

Based on the TRR found in urine and retained in the body, absorption is estimated to be approximately 90%. After reaching a maximum blood level at 0.5 hour in both dose groups, the TRR declined to approximately half of the initial level and thereafter increased again to reach a maximum at 2–4 hours. In high-dose males and females, the AUCs were 1.4- and 1.9-fold higher, respectively, than the low-dose AUCs. The finding of two maximum blood TRR peaks and the disproportionate AUC between high- and low-dose groups suggest an enterohepatic circulation, becoming significant in the range of 125–300 mg/kg bw (Leibold, 1998a). More than 90% of the dose was excreted via urine within 7 days, and less than 2% via faeces. Exhaled TRR were insignificant. Highest TRR in the selected organs were found 4 hours post-dosing and accounted for approximately 3% of the administered dose at both dose levels. At both dose levels, kidneys, plasma and uterus were found to

Table 3. Plasma kinetic parameters in Wistar rats

	Experiment No.													
	11		10		7		9		6		8		5	
	Gavage dose (labelled, mg/kg bw)													
	90		150		150		300		400		450		800	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
$AUC_{0-\infty}$ (µg Eq·h/g)	168	260	365	565	261	504	1005	1364	1504	1783	2167	2593	3913	4135
Plasma half-life (h)[a]	2.1	1.0	0.9	1.4	1.9	1.2	2.6	4.8	3.5	3.5	5.4	6.0	5.2	9.4

From Leibold (1998a)
Eq, equivalent; F, female; M, male
[a] Initial phase of elimination.

Table 4. Plasma kinetic parameters in Sprague-Dawley rats

	Experiment No.									
	16		15		14		13		12	
	Gavage dose (labelled, mg/kg bw)									
	75		125		250		500		800	
	M	F	M	F	M	F	M	F	M	F
$AUC_{0-\infty}$ (µg Eq·h/g)	111	115	185	197	637	390	2057	1606	3447	4763
Plasma half-life (h)[a]	1.3	1.5	2.1	0.7	2.8	1.7	7.2	4.4	12.4	1.9

From Leibold (1998a)
Eq, equivalent; F, female; M, male
[a] Initial phase of elimination.

Table 5. Kinetic parameters after administration of ^{14}C-phenyl UL dicamba in pretreated rats

	Feeding dose (ppm)									
	600		1200		2400		4800		9600	
	Gavage dose (mg/kg bw)									
	50		100		200		400		800	
	M	F	M	F	M	F	M	F	M	F
$AUC_{0-\infty}$ (µg Eq·h/g)										
Day 29	94	108	256	262	535	646	1697	2268	4774	5545
Day 63	124	105	245	248	489	637	1861	2146	4027	4766
Day 91	120	144	240	366	659	981	2382	2874	4975	6080
Plasma half-life (h)[a]										
Day 29	1.36	0.98	2.13	0.54	2.04	2.2	3.15	3.16	12.11	10.16
Day 63	0.90	1.01	16.4	2.07	1.28	1.84	3.08	2.79	3.42	5.30
Day 91	1.15	1.01	1.31	1.15	2.36	2.83	3.33	5.05	3.23	5.65

From Beimborn (2003)
Eq, equivalent; F, female; M, male
[a] Initial phase of elimination.

have the highest TRR. After 7 days, TRR in the selected organs accounted for less than 0.2% of the administered dose (Hassler, 2002).

The metabolic fate of ^{14}C-phenyl UL dicamba (radiochemical purity > 99%; specific activity 0.65 GBq/mmol; lot No. ILA-72.1) was investigated in Wistar rats. Urine, faeces, liver and kidney samples from a previous study (Hassler, 2002) were used to identify metabolites of dicamba. The study complied with GLP.

In urine and faeces at the low dose and the high dose (0.5 and 200 mg/kg bw, respectively), the majority of the TRR were accounted for by the parent dicamba (Table 6). In urine, very low amounts of glucuronidated dicamba (M1) and demethylated dicamba (3,6-dichlorosalicylic acid [DCSA], NOA 414746) were found. In high-dose pooled male and female urine, very low amounts of 5-hydroxy-dicamba (NOA 405873), accounting for 0.01% of the applied dose, were identified, and M2 (NOA 414746 glucuronidated at position 2) was found at 0.006% of the applied dose. Additionally, M3 (2-(methoxycarbonyl)phenyl derivative of M2) was identified at 0.012% of the applied dose in the urine pool. However, M3 is believed to be a by-product produced during the isolation process of M2. In faeces, the only metabolite of dicamba was NOA 414746, at low levels. In the liver and the kidneys, 84–91% of the TRR were accounted for by dicamba. In the kidney, less than 1% of the TRR were M1, and no metabolite was identified in the liver. In summary, the identified metabolites show that demethylation, hydroxylation and glucuronic acid conjugation are the metabolic pathways of dicamba (Figure 3) (Briswalter, 2003).

The dermal absorption in vivo of the dimethylamine salt of dicamba was studied in male Wistar rats using 0.01, 0.03 and 0.1 mg/cm^2 acid equivalents of ^{14}C-phenyl UL dimethylamine dicamba administered as a liquid to shaved areas of the skin. The animals were exposed for 4 or 8 hours and killed 4, 8, 24 or 72 hours after starting exposure.

The dermal absorption, expressed as a percentage of the dose, was not affected by the concentration of the sample administered, but there was a slight increase in dermal absorption with exposure time. Overall, dermal absorption over 72 hours accounted for 1% of the applied dose (Leibold, 1998b).

The dermal absorption in vivo of ^{14}C-phenyl UL dicamba in the formulation BANVEL 480 SL (48% dicamba) was studied in male Wistar rats by administering 0.012 or 4.8 mg/cm^2 as a liquid to shaved areas of the skin. The animals were exposed for 6 hours and killed 6, 24, 48 or 72 hours after starting exposure.

Irrespective of the time to termination, rats absorbed approximately 1.8% of the administered low dose and approximately 10% of the high dose (Loeffler, 2002a).

The dermal absorption in vitro of ^{14}C-phenyl UL dicamba in the formulation BANVEL 480 SL (48% dicamba) was studied by administering 0.011 or 4.35 mg/cm^2 as a liquid to rat and human skin in flow-through diffusion cells. The receptor fluid after 24 hours of exposure was collected and analysed for TRR.

At the low and the high dose levels, 21.2% and 14.8% of the applied dose penetrated through rat skin, and 5.4% and 0.01% through human skin, respectively (Loeffler, 2002b).

1.2 Effects on enzymes and other biochemical parameters

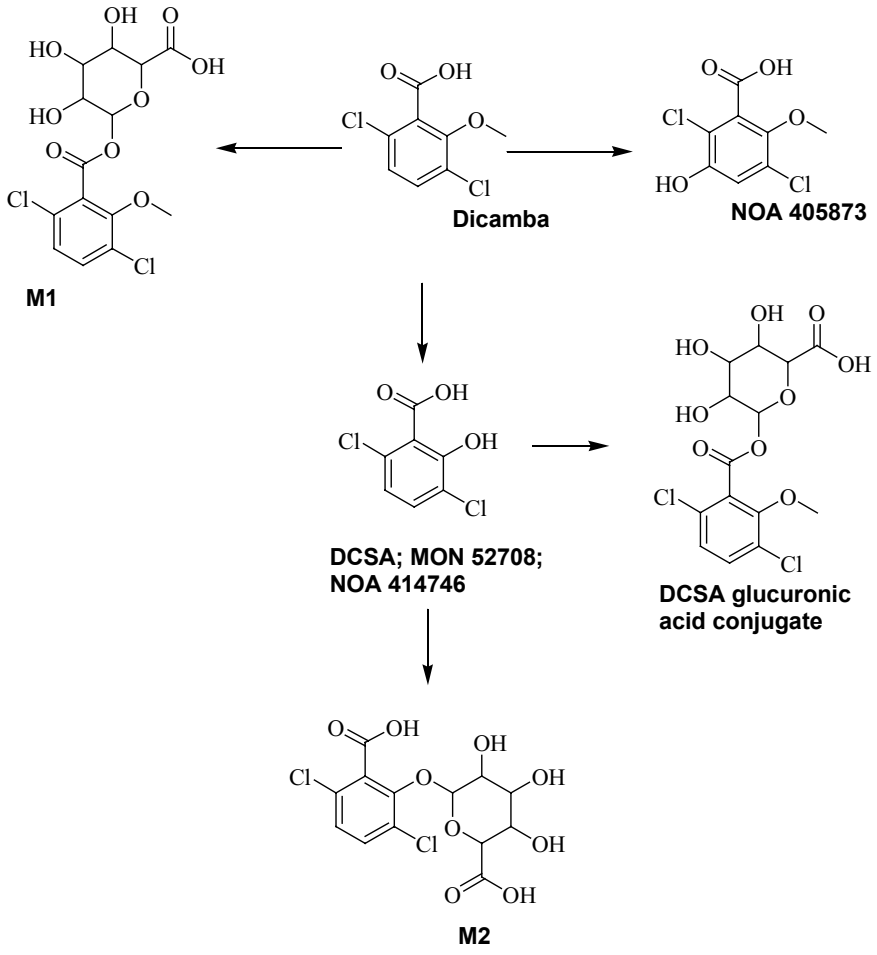
To study the peroxisome proliferator potential of dicamba, male and female Sprague-Dawley rats were fed diets for 3 weeks containing 10, 100, 1000 or 10 000 ppm dicamba. At the highest

Table 6. Metabolites identified in urine and faeces of rats

Metabolite	% of dose							
	Urine				Faeces			
	Dose (mg/kg bw)							
	0.5		200		0.5		200	
	M	F	M	F	M	F	M	F
M1	0.5	0.6	0.4	0.5	—	—	—	—
M2[a]	—	—	0.006	—	—	—	—	—
NOA 414746	0.3	0.2	0.2	0.2	0.03	0.01	0.03	0.01
NOA 405873[a]	—	—	0.01	—	—	—	—	—
Dicamba	95.6	84.2	95.7	95.7	0.45	1.32	0.18	0.37
Identified	96.4	85.0	96.3	97.4	0.48	1.34	0.20	0.39
Total	97	85.8	96.9	98.3	0.67	1.54	0.34	0.59

From Briswalter (2003)
— not identified; F, female; M, male
[a] From pooled male and female urine.

Figure 3. Proposed metabolism of dicamba in rats



From Briswalter (2003); Shah (2009a)

dose, activation of peroxisome proliferator-activated receptor alpha (PPARα), with induction of peroxisomal β-oxidation and cytochrome P450 (CYP) 4A–dependent activity, was observed. However, dicamba was not an efficient inducer of peroxisome-specific enzymes, not did it have any effect on CYP1A- or CYP2B-dependent monooxygenase activities in the rat (Espandiari et al., 1995).

2. Toxicological studies

2.1 Acute toxicity

The results of studies of acute toxicity with dicamba are summarized in Table 7.

(a) Oral administration

Groups of five male and five female Spartan rats were orally administered single doses of dicamba at 500, 794, 1250, 1984, 3150 or 5000 mg/kg bw (Banvel Tech., 85.8%). Animals were observed for 14 days, and body weight development was recorded. This was a pre-GLP study, and no quality assurance statement was provided. However, the study was of acceptable quality to derive a reliable median lethal dose (LD_{50}).

The LD_{50} in males was 1879 mg/kg bw, and in females, 1581 mg/kg bw. Surviving animals exhibited normal body weight development (Goldenthal, 1974).

(b) Dermal application

Groups of five male and five female Alpk:AP_fSD rats were dermally exposed for 24 hours to a single dose of dicamba at 2000 mg/kg bw (purity 90.4%; batch No. B2826511) and observed thereafter for 15 days. The study complied with GLP.

There were no mortalities and no signs of toxicity. Hence, the dermal LD_{50} was greater than 2000 mg/kg bw (Johnson, 2002a).

Two male and two female New Zealand White rabbits were administered single doses of dicamba dermally at 2000 mg/kg bw (Banvel Tech., 85.8%) for 24 hours. Animals were observed for 14 days. This was a pre-GLP study, and no quality assurance statement was provided.

No animals died, and therefore the dermal LD_{50} was greater than 2000 mg/kg bw (Goldenthal, 1974).

(c) Exposure by inhalation

Groups of five male and five female Spartan rats were whole-body exposed to 9.6 mg/l dicamba (Banvel Tech., 85.8%) for 4 hours. Animals were observed for 14 days. This was a pre-GLP study, and no quality assurance statement was provided

No animals died, and therefore the median lethal concentration (LC_{50}) was greater than 9.6 mg/l (Goldenthal, 1974).

Groups of five male and five female Alpk:AP_fSD rats were exposed nose-only for a single 4-hour period to dicamba (purity 91.2%; batch No. B2826511) at nominal concentrations of 1, 2.5 or 5 mg/l. Animals were observed for 14 days. The study complied with GLP.

The LC_{50} was 4.46 mg/l in males and greater than 5.19 mg/l in females (Kilgour, 2001).

(d) Dermal and ocular irritation

Three male and three female New Zealand White rabbits were dermally exposed to 500 mg dicamba (Banvel Tech., 85.8%) for 4 hours, and then the skin was washed. Examinations for skin

Table 7. Acute toxicity of dicamba

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Purity (%)	Reference
Rat	Spartan	M	Oral	1879	—	85.8	Goldenthal (1974)
		F	Oral	1581	—		
Rat	Spartan	M/F	Inhalation	—	> 9.6	85.8	Goldenthal (1974)
Rat	Alpk:AP_fSD	M/F	Inhalation	—	4.46	91.2	Kilgour (2001)
Rat	Alpk:AP_fSD	M/F	Dermal	> 2000	—	90.4	Johnson (2002a)
Rabbit	New Zealand White	M/F	Dermal	> 2000	—	85.8	Goldenthal (1974)
Hen	—	F	Oral	316	—	86.82	Roberts (1983)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male

irritation were done at 4, 24 and 72 hours. This was a pre-GLP study, and no quality assurance statement was provided.

Dicamba was only very slightly irritating to the skin (Goldenthal, 1974).

One male and two female New Zealand White rabbits were dermally exposed to 500 mg dicamba (purity 91.0%; batch No. Y01040/007) for 4 hours, and then the skin was washed. Examinations for skin irritation were done at 1, 24, 48 and 72 hours after removal of the substance. The study complied with GLP.

Dicamba was very mildly irritating to the skin (Johnson, 2002b).

Four male and four female New Zealand White rabbits were administered 47.3 mg dicamba (Banvel Tech., 85.8%) to the eyes in 0.1 ml water and were exposed for either 5 minutes or 24 hours. Animals were observed for up to 21 days. This was a pre-GLP study, and no quality assurance statement was provided.

Dicamba was severely irritating and corrosive to the eye (Goldenthal, 1974).

(e) Dermal sensitization

Twenty female guinea-pigs were used to study the sensitizing potential of dicamba (purity 86.3%; batch No. 52625110) in the Magnusson and Kligman maximization test. The study complied with GLP.

There was no evidence of sensitizing potential of a 10% dicamba challenge solution (Ullmann, 1991).

2.2 Short-term studies of toxicity

(a) Oral administration

Rats

In a 4-week range-finding study, groups of five male and five female CD rats were administered 0, 5000, 7500, 10 000, 12 500 or 15 000 ppm dicamba (purity 86.82%; lot No. 52625110) in the diet. Animals were observed for overt toxicity, and feed consumption and body weight development were recorded. This was a pre-GLP study, and no quality assurance statement was provided.

Hind leg mobility was impaired in one male at 12 500 ppm and in seven animals at 15 000 ppm. Feed consumption and body weight gain were reduced at 10 000 ppm and above, with severity increasing with dose (Goldenthal, 1979).

In a 13-week study, groups of 10 male and 10 female HanIbm: WIST rats were administered 0, 500, 3000, 6000 or 12 000 ppm dicamba (purity 89.4%; lot No. 52504710) in the diet. These feed concentrations were equal to 0, 40.1, 238.7, 479.3 and 1000 mg/kg bw per day in males and 0, 43.2, 266.4, 535.6 and 1065.3 mg/kg bw per day in females. Additional 0 and 12 000 ppm groups were kept as 4-week recovery groups fed basal diet after the treatment period. Animals were checked twice daily for signs of toxicity and for mortality. The eyes were examined prior to treatment and before scheduled termination. Body weight and feed consumption were determined weekly, and blood and urine samples for clinical chemistry and haematology were collected at study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

No mortalities occurred during the study. All effects described were confined to the 12 000 ppm groups. In both sexes, reduced activity and slowed movements were observed. Eight of 20 females showed thin retinal blood vessels, compared with 2 of 20 in the control females. Although this might be attributed to a combined effect of blood sampling and low body weight gain, a direct relationship with treatment cannot be excluded. Body weight development and feed consumption were lower in male and female animals, resulting in terminal body weights lower by 27.9% in males and 40.4% in females, respectively, compared with the controls. In the recovery groups, body weight gain in the 12 000 ppm group was higher than in the control animals. High-dose males and females had statistically significantly lower platelet counts and partial thromboplastin times, and females also had reduced red cell parameters and increased white blood cell and lymphocyte counts. All the haematological effects reverted to normal in the recovery period. Both sexes had statistically significantly increased alkaline phosphatase and alanine and aspartate aminotransferase activities. Females also had higher γ-glutamyl transpeptidase activity and higher triglyceride, cholesterol, creatinine and phosphorus levels. In males, levels of triglyceride, cholesterol and glucose were lower than in controls, and the level of urea was higher than in controls. After the recovery period, most of these clinical chemistry parameters were similar to those of control animals except for alkaline phosphatase and phosphorus levels, which were still elevated. In the urine of 15 of 20 high-dose males, triple phosphate crystals were identified, with a higher severity index, compared with 7 of 20 controls with this effect. In high-dose females, 12 of 20 urine samples had uric acid crystals, and 1 of 20 controls and 6 of 10 at 6000 ppm showed these crystals. The findings in urine returned to control levels within the recovery period. Absolute kidney weights decreased statistically significantly in both sexes of the high-dose groups, but absolute liver weights were similar to those of the control groups. Relative to body weight, only the liver weights were statistically significantly increased. Histological findings were restricted to high-dose females, in which centrilobular hepatocyte hypertrophy and hepatocellular pigmentation were observed. All liver effects were reversible.

The no-observed-adverse-effect level (NOAEL) was 6000 ppm, equal to 479.3 mg/kg bw per day in males and 535.6 mg/kg bw per day in females, based on haematological and biochemical effects at 12 000 ppm (Doubovetzky, 1997).

Dogs

Groups of four male and four female 25-week-old Beagle dogs were orally administered dicamba (purity 90.4%; lot No. B2826511) at doses of 0, 10, 50 or 300 mg/kg bw per day via gelatine capsule for 13 weeks. The capsule content was adjusted weekly based on body weight development. The control and high-dose groups contained an additional four animals of each sex, which served as 4-week recovery groups after the treatment period. Animals were checked twice daily for signs of toxicity and for mortality. A neurological and behavioural assessment of the control and the high-dose animals was performed 3 times during the study. The eyes were examined prior to treatment and before scheduled termination. Body weight was determined weekly, and blood and urine samples for clinical chemistry and haematology were collected at study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

Gait anomalies, including ataxia, stiff gait and sporadic transient collapses, were seen generally about 2 hours after dosing in all animals of the 300 mg/kg bw per day group and persisted for up to 5 hours. In a few animals, these signs persisted overnight. Occasionally, decreased activity, tremor, whimpering and salivation were observed. In the high-dose group, body weight gain was decreased by 26% in males and by 44% in females. In the 50 mg/kg bw per day group, no behavioural changes (e.g. gait anomalies) were observed. Both sexes showed lower body weight gain than in control animals during the first weeks of treatment, and body weight continued to be low (by 9% in males and by 19% in females during the whole study), whereas feed intake recovered after a short initial decrease (Figure 4). In the recovery groups, body weight development was similar to that of the controls. Red blood cell parameters were reduced during the study in males and females receiving 300 mg/kg bw per day, and the partial thromboplastin time was slightly elevated. The red blood cell parameters showed a tendency to recover after treatment cessation. One high-dose male had a marked decrease in lymphocyte, white blood cell and platelet counts. Males dosed at 50 mg/kg bw per day showed slightly lower red blood cell counts, statistically significant only at week 7. Males of the 50 mg/kg bw per day group and both sexes in the 300 mg/kg bw per day group showed lower cholesterol and phospholipid levels at 7 and 13 weeks. These lower levels showed a tendency to revert to normal in the recovery group (Table 8). Alkaline phosphatase activity in high-dose males was decreased nearly 2-fold compared with that of the controls. Absolute and relative spleen weights in high-dose males were lower than in control, and kidney weights were higher in females. These changes were reversible. There were no histological changes attributable to treatment.

The NOAEL in this study was 50 mg/kg bw per day, based on behavioural effects, changes in haematological and clinical chemistry parameters and body weight effects at 300 mg/kg bw per day (Jackson, 2003).

Groups of four male and four female approximately 39-week-old Beagle dogs were fed diets containing 0, 100, 500 or 2500 ppm dicamba (purity 86.8%; lot No. 52625110) for 52 weeks. These concentrations were equal to 0, 2, 11 and 59 mg/kg bw per day in males and 0, 2, 12 and 52 mg/kg bw per day in females, respectively. Animals were checked twice daily for signs of toxicity and for mortality. A neurological and behavioural assessment of the control and the high-dose animals was performed 3 times during the study. The eyes were examined prior to treatment and before scheduled termination. Body weight was determined weekly, and blood and urine samples for clinical chemistry and haematology were collected prior to study initiation, at 6 months and prior to study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

At 500 and 2500 ppm, some animals showed inappetance, and one high-dose male did not consume any feed for the first 3 weeks of the study. As a consequence, mean body weight development in the male high-dose group was lower in the first 5 weeks. At study termination, all groups had similar body weight gain assessed over the whole treatment period (4.3–5.8% in males and 12.3–17.4% in females). In high-dose males, erythrocyte counts, haemoglobin and haematocrit were slightly, but statistically significantly, reduced ($P < 0.05$) at 6 months but not at 12 months. There were no treatment-related organ weight changes or macroscopic or microscopic organ changes.

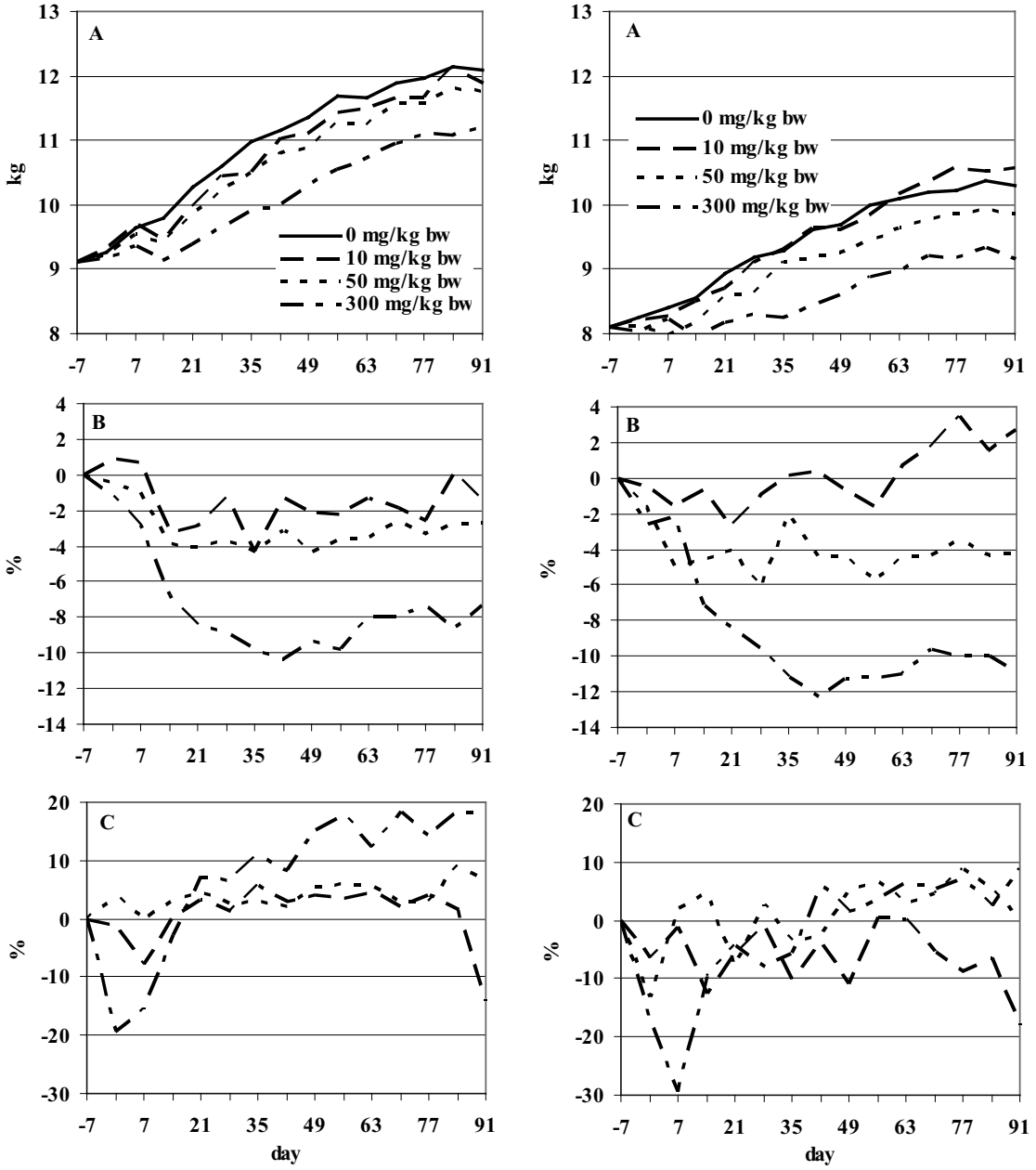
The NOAEL was 2500 ppm, equal to 52 mg/kg bw per day, the highest dose tested (Blair, 1986).

(b) Dermal application

Rats

Groups of 10 male and 10 female Alpk:AP$_f$SD rats were given dicamba at 0, 30, 300 or 1000 mg/kg bw per day (purity 91%; lot No. B2826511) by dermal application over 28 days (21 applications

Figure 4. Body weights (A), body weight changes relative to controls (B) and feed intake relative to controls (C) in male dogs (left panel) and female dogs (right panel). The body weights were normalized to the highest body weight identified in the pretreatment period.



From Jackson (2003)

of at least 6 hours). Clinical observations, body weight development and feed consumption were recorded. Urine and blood samples were analysed, and selected tissues were weighed and examined histologically at the terminal kill. The study complied with GLP.

At all dose levels, there were histological changes indicative of skin irritation at the application site. There were no treatment-related systemic effects at any dose level. Therefore, the NOAEL for systemic toxicity was 1000 mg/kg bw per day, the highest dose tested (Rattray, 2002).

Table 8. Selected haematology and clinical chemistry parameters in males of the 13-week dog study

	Red blood cells (10^{12} cells/l)				Cholesterol total (µmol/l)				Phospholipids (µmol/l)			
	Dose (mg/kg bw per day)											
	0	10	50	300	0	10	50	300	0	10	50	300
Pretest	6.11	5.82	5.76	6.14	4.84	4.61	4.25	4.47	4.89	4.73	4.45	4.65
Week 7	6.26	6.00	5.67*	5.67**	3.95	3.79	3.13*	2.98**	4.29	4.21	3.39*	3.16**
Week 13	6.60	6.39	6.04	5.43**	4.17	3.93	3.33*	2.82**	4.37	4.14	3.56*	2.96**
Recovery	6.83	nd	nd	6.10*	4.16	nd	nd	3.21	4.27	nd	nd	3.45

From Jackson (2003)
nd, no data; * $P < 0.05$; ** $P < 0.01$

Rabbits

Groups of four male and four female New Zealand White rabbits were given dicamba (purity 86.8%; lot No. 52625110) at 0, 100, 500 or 2500 mg/kg bw per day by dermal application over 3 weeks (15 applications for at least 6 hours). Clinical observations, body weight development and feed consumption were recorded, and urine and blood samples were analysed. At termination, selected organs were weighed and examined histologically. This was a pre-GLP study, but a quality assurance statement was provided.

In the control group, one animal died, and in all treatment groups, two animals each died or were killed in extremis. There was no evidence for a relationship with treatment. At all dose levels, there were dermal changes indicative of skin irritation at the application site, but no treatment-related systemic effects. Therefore, the NOAEL for systemic toxicity was 2500 mg/kg bw per day, the highest dose tested (Estes, 1979).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 52 male and 52 female Crl:CD-1 (ICR) BR mice were fed diets containing 0, 50, 150, 1000 or 3000 ppm dicamba (purity 86.8%; lot No. 52625110) for 89 weeks (males) and 104 weeks (females), respectively. These feed concentrations were equal to 0, 5.5, 17.2, 108 and 358 mg/kg bw per day in males and 0, 5.8, 18.8, 121 and 364 mg/kg bw per day in females. Groups were checked twice daily for dead or moribund animals, and feed consumption and body weight development were recorded weekly. Blood was sampled before treatment, at weeks 53 and 80 and before the terminal kill. At study termination, organs were weighed and examined histologically. The study complied with GLP.

In the 3000 ppm group, the first mortalities in males were early in the study, and the overall mortality was increased compared with the controls. Although there was no accompanying histological correlate, a relationship with treatment cannot be excluded. In high-dose females (3000 ppm), body weights were consistently lower from week 25 onwards and were 17% lower than in controls at study termination. In females at 150 ppm and above, statistically significant decreases in the neutrophil proportions and concurrent increases in lymphocyte proportions were observed (Table 9). As the total white blood cell counts were not available, the data could not be further assessed (statement by study director). The toxicological relevance of these findings is unclear. At all dose levels, the

Table 9. Differential white blood cell counts in female mice

Dietary concentration (ppm)	% white blood cells		
	Neutrophils	Lymphocytes	Eosinophils
0	46	52	2
50	45	53	1
150	33**	66**	1*
1000	31**	69**	1*
3000	34**	65**	1*

From Crome (1988)
* $P \leq 0.05$; ** $P \leq 0.01$

Table 10. Lymphoid tumour incidence in female mice found dead, killed moribund or killed at study termination

	Lymphoid tumour incidence (%)[a]				
	Dietary concentration (ppm)				
	0	50	150	1000	3000
D	9.1	12.5	29.4	17.9	19.2
T	3.3	7.4	5.6	16.7	7.7
Combined	5.8	9.8	21.2	17.3	13.5

From Crome (1988)
D, found dead or killed moribund; T, killed at study termination
[a] Historical control range: 11.1–38.7% for D and 6.9–30.4% for T.

incidence of lymphoid tumours in females was increased above that in the concurrent controls, but was within the historical control range, and no relationship with dose was evident (Table 10).

The NOAEL was 1000 ppm, equal to 108 mg/kg bw per day, based on reduced body weight gain in females at 3000 ppm (Crome, 1988).

Rats

Groups of 60 male and 60 female CD rats were fed diets containing 0, 50, 250 or 2500 ppm dicamba (purity 86.8%; lot No. 52625110) for 117 weeks, equal to 0, 2, 11 and 107 mg/kg bw per day in males and 0, 3, 13 and 127 mg/kg bw per day in females. Groups were checked twice daily for dead or moribund animals, and feed consumption and body weight development were recorded weekly for the first 13 weeks and thereafter once every 2 weeks. Blood was sampled before treatment and at 6, 12, 18 and 24 months. Ten animals of each sex per group were killed for an interim necropsy at 12 months. At interim necropsy and at study termination, organs were weighed and examined histologically. The study complied with GLP.

There were no treatment-related clinical signs or effects on clinical chemistry, haematology or urinalysis at any dose level, and survival was not affected by treatment. The body weight in all male treated groups was consistently slightly higher (approximately 10%) than that in controls. In females in the 50 and the 2500 ppm groups, body weight was approximately 10% lower than that in controls. Organ weights were not affected by treatment with dicamba. Macroscopically, increased incidences of discoloured areas and foci (red/black or white/tan/yellow) in livers of males and enlarged adrenal glands in females at all dose levels were found (Table 11). Histologically, increased incidences of liver telangiectasis and thyroid C-cell carcinoma in males were found. The incidence of mixed

Table 11. Number of rats with non-neoplastic and neoplastic changes

	Incidence of changes							
	Dietary concentration (ppm)							
	0	50	250	2500	0	50	250	2500
	Males				Females			
Animals examined	49	49	48	50	49	49	50	49
Survival at week 104 (%)	51	47	44	43	71	53	46	70
Macroscopic changes								
Discoloured areas and foci in livers	13	10	18	20	4	7	9	1
Enlarged adrenal glands	2	1	2	1	4	7	9	10
Microscopic changes								
Telangiectasis in livers	11	11	19	16	0	1	1	0
Dilatation of brain ventricles	22	16	9	11	15	18	20	30
Malignant lymphoma, mixed	0	0	0	4	0	0	0	0
Thyroid C-cell carcinoma	1	0	1	5	0	1	0	0
Thyroid C-cell adenoma	2	5	5	3	5	1	3	6

From Goldenthal (1985)

malignant lymphoma was increased in high-dose males (8% versus 0% in all other groups) and was above the historical control range of 0–1.8%. The tumours were observed in the second half of the study in animals dying before scheduled sacrifice. To overcome possible differences in diagnostic criteria of different pathologists, the company also provided aggregated historical control means and ranges for all types of malignant lymphoreticular lymphoma (3.8% and 0–8.6%, respectively, and 1.3% and 0–8.4% in another historical control group data set). The incidence of thyroid C-cell carcinoma (10%) was higher than the historical mean and range values (0.3% and 0–1.7%, respectively, and 0.2% and 0–2.0% in another historical control group data set). In the thyroids of males, no increase in the incidence of C-cell hyperplasia or adenoma was observed. In females, the incidence of dilated brain ventricles increased with dose. This effect is usually observed secondary to pituitary tumours, but these were not observed.

The NOAEL for general toxicity was 2500 ppm, equal to 107 mg/kg bw per day, the highest dose tested.

The NOAEL for the possibly increased incidences of malignant lymphoma and thyroid parafollicular cell carcinoma in males at 2500 ppm is 250 ppm, equal to 11 mg/kg bw per day (Goldenthal, 1985).

2.4 Genotoxicity

In an adequate battery of in vitro and in vivo tests, dicamba proved to be non-genotoxic (Table 12).

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of 32 male and 32 female 6-week-old Crl:CD (SD) BR VAF/Plus rats were fed diets containing 0, 500, 1500 or 5000 ppm dicamba (purity 86.9%; lot No. 52103810). The average

Table 12. Results of genotoxicity studies with dicamba

End-point	Test system	Concentration	Purity (%); lot No.	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	750–3000 µg/plate	88.5; 52504710	Negative	Ballantyne (1996)
L5178Y TK$^{+/-}$	Mouse lymphoma cells	2210 µg/ml	90.4; B2826511	Negative	Clay (2001)
Chromosomal aberration	Chinese hamster ovary cells	2330 µg/ml	86.8; 52625110	Negative	Putman (1986)
Micronucleus induction	Mouse bone marrow	1300 mg/kg bw	88.5; 52504710	Negative	Marshall (1996)

compound intake in the F_0 generation was 0, 35.1, 105 or 347 mg/kg bw per day in males and 0, 41.1, 125 or 390 mg/kg bw per day in females before mating. After 10 weeks of exposure, the F_0 generation was allowed to mate and produce the F_{1a} and F_{1b} generations. F_{1a} pups were terminated upon weaning at lactation day 21, and F_{1b} animals were used to generate the F_2 generation.

Animals were observed regularly for appearance, behaviour and clinical signs, and body weight and feed consumption were recorded during the premating phase, gestation phase and lactation phase. Pups were sexed and weighed at lactation days 4, 8, 12, 16 and 21. A complete necropsy was performed on all adult animals and on selected pups of all groups. Additionally, organs were weighed and subjected to a histological examination. The study complied with GLP.

High-dose females showed increased body tone and slow righting reflex. This had also been observed in a dose range–finding study at 6000 and 12 000 ppm. High-dose F_1 and F_2 pups had lower body weights throughout the lactation phase. At weaning, body weight was lower by more than 20% in both males and females (Table 13). Thereafter, body weight gain was not affected. At 1500 ppm, pup weights were also slightly reduced, reaching statistical significance at several time points in the lactation phase. There were no effects on mating performance or pregnancy at any dose level. In the high-dose group, there was a slight shortening of the pregnancy duration in all three matings: F_0, F_{1a} and F_{1b} (Table 14). At the high dose, the balano-preputial separation was delayed statistically significantly (45.6 days versus 43.7 days in controls). At all dose levels, the male weanlings of all three matings showed increased body weight–adjusted liver weights. Statistical significance was attained at 5000 ppm in all generations, at 500 ppm in the F_{2b} weanlings and at 1500 ppm in the F_1 and F_{2b} weanlings. In females, the effect was less pronounced. The absolute liver weights were similar in all groups.

The NOAEL for parental toxicity was 1500 ppm, equal to 105 mg/kg bw per day, based on clinical signs at 5000 ppm. The NOAEL for reproduction was 5000 ppm, equal to 347 mg/kg bw per day, the highest dose tested. The NOAEL for postnatal development was 500 ppm, equal to 35.1 mg/kg bw per day, based on reduced pup body weights (Masters, 1993).

(b) Developmental toxicity

Rats

Groups of 25 mated female CD rats were administered dicamba (purity not stated, but this batch was used in several feeding studies and was reported there to be 86.8% pure; lot No. 52625110) in corn oil by gavage at doses of 0, 64, 160 or 400 mg/kg bw per day from gestation day (GD) 6 to GD 19. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 20, animals were terminated, and the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded. The fetuses were weighed, sexed and investigated for skeletal and visceral aberrations. The study complied with GLP.

Table 13. Absolute and relative pup body weights

Dietary concentration (ppm)	Body weight (g)						Body weight (% of control)					
	Lactation day											
	0	4	8	12	16	21	0	4	8	12	16	21
F_0												
0	6.3	10.1	19	30.3	41.5	59.6	100	100	100	100	100	100
500	6.3	9.8	18.6	29.3	40	57.8	100	97	98	97	96	97
1500	6.3	10.3	19.1	29.7	40.1	57.3	100	102	101	98	97	96
5000	5.9*	9.1*	16**	24.4**	33.6**	45.4**	94	90	84	81	81	76
F_{1a}												
0	6.6	11.6	21.6	33.1	44.5	65.0	100	100	100	100	100	100
500	6.4	10.8	20.2	31.1	42.6	62.5	97	93	94	94	96	96
1500	6.4	10.4	19.4	30.2	40.7	58.4*	97	90	90	91	91	90
5000	6.1*	10.4*	18.1**	26.6**	34.1**	47.9**	92	90	84	80	77	74
F_{1b}												
0	6.6	10.7	20.1	31.5	43.3	61.8	100	100	100	100	100	100
500	6.6	10.4	19.1	30.4	41.7	59.8	100	97	95	97	96	97
1500	6.7	10.2	18.2	27.6*	37.6	52.9*	102	95	91	88	87	86
5000	6.1	9.7	16.3**	23.8**	30.5**	43.2**	92	91	81	76	70	70

From Masters (1993)
* $P < 0.05$; ** $P < 0.01$

Table 14. Duration of pregnancy in rats

Duration of pregnancy (days)	No. of animals with given duration of pregnancy			
	Dietary concentration (ppm)			
	0	500	1500	5000
F_0				
21	3	8	5	10
22	21	14	19	17
23	2	5	4	0
24	0	0	0	0
F_{1a}				
21	0	0	1	0
22	9	10	9	18
23	6	6	2	2
24	0	0	1	0
F_{1b}				
21	4	1	2	1
22	7	13	9	15
23	2	2	2	0
24	0	0	1	0

From Masters (1993)

Twenty of 25 females became pregnant at the high dose, and 23 or 24 of 25 in all other groups. Three of the pregnant high-dose females died on treatment day 2 or 3 without any obvious lesions responsible for their deaths. The mean body weight at GD 20 was lower by 8% in high-dose animals, partly due to lower gravid uterus weights in this group (62 g versus 73 g in controls). Feed consumption was reduced by approximately 20%, and animals showed behavioural changes, including ataxia, stiffening of the body, urine-soaked fur, salivation and decreased motor activity. The incidences of resorptions and the number of viable fetuses were not affected by treatment. The body weights of high-dose fetuses were reduced, but not statistically significantly, by 6%. There were no treatment-related skeletal anomalies. In the high-dose group, five fetuses had increased renal pelvic cavitation, and two fetuses of the control group had the same finding. Based on the low incidence and the fact that three of the five affected fetuses were from the same litter, this is considered not to be treatment related.

The maternal NOAEL was 160 mg/kg bw per day, based on mortality and behavioural changes at 400 mg/kg bw per day. The developmental NOAEL was 400 mg/kg bw per day, the highest dose tested (Smith, 1981).

Rabbits

Groups of 20 mated female New Zealand White rabbits were administered dicamba (purity 904%; lot No. 52625110) in capsules at 0, 30, 150 or 300 mg/kg bw per day from GD 6 to GD 18. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 29, animals were killed, and the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded. The fetuses were weighed and sexed and investigated for skeletal and visceral aberrations. The study complied with GLP.

One 150 mg/kg bw per day doe aborted on GD 22, as did four does in the high-dose group between GD 19 and GD 24. As a result of an intubation accident, one high-dose doe died on GD 12. All animals aborting showed body weight loss accompanied by reduced feed consumption and ataxia. On necropsy, no lesions were observed. Generally, the mid- and high-dose dams showed decreased motor activity and ataxia, and the high-dose animals also had rales, laboured breathing and impaired righting reflex. These clinical signs were first observed on GD 9. The body weight (Figure 5) and the feed consumption of high-dose dams were reduced throughout the study. There were no effects of treatment on the litter data. A slight increase in the incidence of irregular skull ossifications in the 30 and the 300 mg/kg bw groups but not in the 150 mg/kg bw group was considered not to be treatment related because there was no dose–response relationship and the litter incidence was not elevated. There were no other signs of possibly treatment-related fetotoxicity.

The NOAEL for dams was 30 mg/kg bw per day based on behavioural changes at 150 mg/kg bw per day. The developmental NOAEL was 300 mg/kg bw per day, the highest dose tested (Hoberman, 1992).

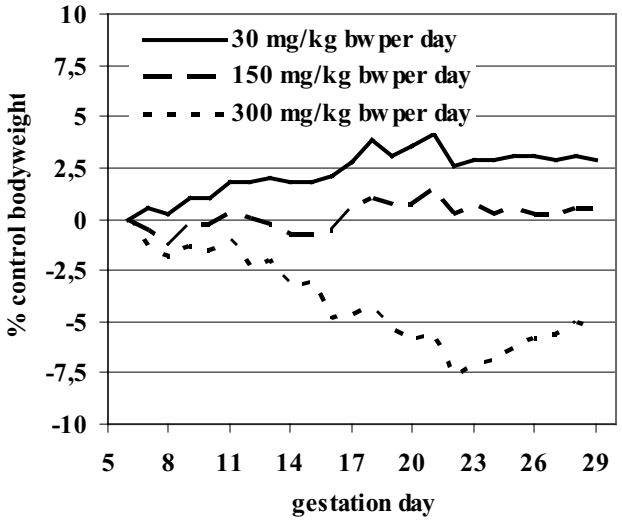
2.6 Special studies

(a) Neurotoxicity

Hen

Groups of five hens were administered single doses of dicamba (purity 86.82%; lot No. 52625110) by gavage at 0, 260, 377, 547, 793 or 1149 mg/kg bw and were observed for mortalities and signs of toxicity. Thereafter, groups of 10 hens were administered single doses of dicamba by gavage at 0, 79, 158 or 316 mg/kg bw. The highest-dose group consisted of 20 animals. As positive control, one group was treated with tri-*ortho*-cresylphosphate (TOCP) at 500 mg/kg bw. The study complied with GLP.

Figure 5. Body weight development of dams relative to control animals



From Hoberman (1992)

The LD_{50} in the first experiment was 316 mg/kg bw, and signs of toxicity included unsteadiness, inability to stand, lying on one side or lying with legs and wings outstretched. In the second experiment, signs of toxicity similar to those in the first experiment were observed at all dose levels, recumbence duration increasing with dose. In the 79 mg/kg bw group, there were no mortalities, at 158 mg/kg bw, one bird died and at 316 mg/kg bw, nine birds died. The TOCP-treated birds showed signs of ataxia. At 158 mg/kg bw and above, body weight gain was reduced, and at the highest dose level, birds even lost weight during the 21-day observation period. Sciatic nerve damage was observed in high-dose birds and may be explained by the prolonged recumbence of these animals. The TOCP-treated animals showed axonal degeneration of the spinal cord and the sciatic nerve (Roberts, 1983).

Rats

In an acute neurotoxicity study, groups of 10 male and 10 female Crl:CD BR rats were administered single doses of dicamba (purity 86.9%; lot No. 52103810) by gavage at 0, 300, 600 or 1200 mg/kg bw in corn oil. As positive control, a group was administered acrylamide intraperitoneally once daily for 7 consecutive days at 50 mg/kg bw per day. Neurobehavioural assessment using a functional observational battery (FOB) and recording of body weight and feed intake were conducted prior to treatment, on day 7 and at the end of the study on day 14. At study termination, the central and peripheral nervous tissues of at least six animals per group were examined histologically. The study complied with GLP.

One high-dose male died within 24 hours post-dosing, and the body weights and the feed consumption of high-dose males were low. Neurobehavioural effects in the FOB were apparent 1.5 ± 1 hour after dosing in all dose groups, with a dose-dependent incidence and severity. Generally, the effects were described as stimulus- or stress-induced rigidity, and males seemed to be more sensitive than females. The effect did not persist, as all animals were similar in the FOB at day 14. Histologically, the nervous tissues of treated animals were similar to the tissues in the control animals. As expected, acrylamide induced persistent neurobehavioural effects (landing on foot splay and gait), and degenerative effects in peripheral nervous tissues were found (Minnema, 1993).

In a subchronic neurotoxicity study, groups of 10 male and 10 female Crl:CD BR rats were fed diets with dicamba (purity 86.9%; lot No. 52103810) at 0, 3000, 6000 or 12 000 ppm for 13 weeks, equal to 0, 197.1, 401.5 and 767.9 mg/kg bw per day in males and 0, 253.4, 472.0 and 1028.9 mg/kg bw per day in females. Body weight and feed consumption were recorded weekly, and neurobehavioural assessments using a FOB were conducted prior to treatment and during weeks 4, 8 and 13. Prior to treatment and at the end of the study, ophthalmoscopic examinations were performed. At study termination, the central and peripheral nervous tissues of at least six animals per group from the control and the high-dose groups were histologically examined. The study complied with GLP.

The high-dose males showed lower feed intake and lower body weight gain throughout the study. In high-dose animals, with a higher incidence in females than in males, increased rigidity was observed in response to handling and touch. Rigidity was also seen upon dropping in the landing splay test and the air righting reflex test. Histologically, the nervous tissues of high-dose animals were not different from the control animal tissues (Minnema, 1994).

3. Studies on metabolites

Toxicological studies for three metabolites were submitted (Figure 6). DCSA and 3,6-dichlorogentisic acid (DCGA) have been identified as metabolites of dicamba in soya beans, sugarcane, wheat and cotton and are environmental metabolites of dicamba. DCSA has been identified in rats, cows, goats and hens, and NOA 405873 (5-hydroxy-dicamba) was found in rats.

3.1 Metabolite NOA 405873

(a) Acute toxicity

For a summary, see Table 22 below.

(b) Short-term studies of toxicity

In a poorly described subchronic study, rats and dogs were fed diets containing NOA 405873 (purity and lot No. not stated) at 100 ppm (equivalent to 10 mg/kg bw per day in rats and 2.5 mg/kg bw per day in dogs) and 250 ppm (equivalent to 25 mg/kg bw per day in rats and 6.25 mg/kg bw per day in dogs) for 90 days. Rat groups consisted of 15 male and 15 female animals and the control group of 10 animals of either sex. In the dog study, two animals of each sex per group were used.

Rats and dogs were observed daily for clinical signs, and body weight and feed consumption were measured weekly. At days 30 and 90 (dogs additionally at day 60), haematology, clinical chemistry and urinalysis were performed. At study termination, organs were weighed and examined microscopically. This was a pre-GLP study.

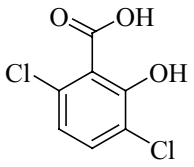
There were no treatment-related effects on any parameter investigated in the rats and the dogs, other than yellowish casts in livers of dogs of the 100 and 250 ppm groups and slightly pale livers in rats at 250 ppm. As there were no other histological or clinical chemistry correlates, these findings are considered not toxicologically relevant (Wazeter, 1966).

(c) Genotoxicity

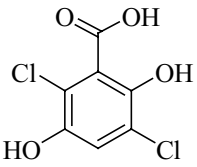
For a summary, see Table 23 below. Studies with equivocal or positive results are described below.

In a $TK^{+/-}$ mouse lymphoma mutation assay, NOA 405873 (purity 99%; lot MLA-29/21) was tested in the absence and the presence of a metabolic system (S9) up to 2370 µg/ml (10 mmol/l). The

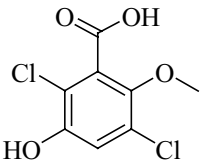
Figure 6. Structure of dicamba metabolites



DCSA; MON 52708; NOA 414746



DCGA; MON 52724



NOA 405873

positive control for assays without S9 was ethylmethanesulfonate (EMS) (500 µg/ml); for assays with S9, the positive control was benzo[*a*]pyrene (BAP) (1 µg/ml). The study complied with GLP.

Statistically significant and dose-related increases in revertants were observed in the presence and absence of metabolic activation (Table 15). Concomitantly, the cytotoxicity increased in a dose-related manner (Clay, 2002).

In a second $TK^{+/-}$ mouse lymphoma mutation assay, NOA 405873 (purity 94%; lot No. KI6212/1-18) was tested in the absence and the presence of a metabolic system (S9) up to 5000 µg/ml (10 mmol/l). The positive control for assays without S9 was EMS at about 750–1000 µg/ml; for assays with S9, the positive control was *N*-nitrosodimethylamine (NDMA) at about 2000 µg/ml. The study complied with GLP.

Statistically significant and dose-related increases in revertants were observed in the presence and absence of metabolic activation (Table 16). Concomitantly, the cytotoxicity increased in a dose-related manner (Ogorek, 2002b).

In a chromosomal aberration assay in Chinese hamster ovary (CHO) cells, NOA 405873 (purity 94%; lot No. KI6212/1-18) was tested in the absence and the presence of a metabolic system (S9) up to 5000 µg/ml. The study complied with GLP.

In the absence of metabolic activation by S9, more cells showed chromosomal aberrations (Table 17). At 5000 µg/ml, also in the presence of S9, a slight increase in cells with chromosomal aberrations was found (Ogorek, 2002a).

3.2 Metabolite 3,6-dichlorosalicylic acid (DCSA; MON 52708; NOA 414746)

(a) Absorption, distribution, metabolism and excretion

The pharmacokinetics, metabolism and elimination of DCSA were investigated in male and female Crl:CD (SD) rats following dietary exposure for 2 weeks with unlabelled DCSA (purity 97.9%; lot No. GLP-0603-16958-T) and a single oral gavage dose of ^{14}C-phenyl UL DCSA (radiochemical purity 96.9%; specific activity 1.3 GBq/mmol; lot No. 6116-01B) in corn oil. The animals were divided into five dose groups, which were further subdivided into an "A" and "B" subgroup to limit the number of blood samples taken for each animal (Table 18). For groups 4 and 5, animals were adapted to the target dose level in feed stepwise to avoid palatability problems. Animals in subgroup A of groups 1–5 were terminated at 24 hours post-dose, and animals in subgroup B of groups 1–5 were terminated at 48 hours post-dose. Blood samples were obtained from the animals in subgroup A of groups 1–5 at 1, 4, 8 and 24 hours post-dose. Similarly, blood samples were obtained from the animals in subgroup B of groups 1–5 at 0.5, 2, 6, 12 and 48 hours post-dose. The study complied with GLP.

The times to peak plasma concentrations (C_{max}) increased slightly with dose and were achieved at 0.5–2 hours post-dose in males and at 0.5–4 hours post-dose in females. Plasma half-life in

Table 15. Survival and mean mutant frequency in mouse lymphoma mutation assay

Concentration (μg/ml)	Experiment 1				Experiment 2				Historical control data (MMF)
	−S9		+S9		−S9		+S9		
	MS	MMF	MS	MMF	MS	MMF	MS	MMF	
125	100	1.3	83	1.6	103	1.7	138	1.9	—
250	101	1.1	102	1.4	95	1.5	100	1.2	—
500	90	1.3	85	1.3	95	1.4	81	2.1	—
1000	87	1.4	47	4.4**	81	1.8	57	4.4**	—
2000	55	2.7**	42	2.4*	17	12.0**	38	3.3**	—
2370	21	6.2**	43	2.0	6	[a]	40	2.6*	—
DMSO	—	1.4	—	1.5	—	1.7	—	1.6	2.1 ± 1.1
BAP	—	—	30	10.9**	—	—	51	15.4**	13.4 ± 6.4
EMS	78	9.0**	—	—	57	9.1**	—	—	14.9 ± 5.8

From Clay (2002)
DMSO, dimethyl sulfoxide; MMF, mean mutant frequency (× 10^{-4}); MS, mean survival (%); * $P < 0.05$; ** $P < 0.01$
[a] Not counted due to excessive toxicity.

Table 16. Survival and mean mutant frequency in mouse lymphoma mutation assay

Concentration (μg/ml)	Experiment 1				Experiment 2			
	−S9		+S9		−S9		+S9	
	MS	MMF	MS	MMF	MS	MMF	MS	MMF
156.25	120.09	3.1	—	—	—	—	—	—
266.7	—	—	—	—	55.18	7.5	—	—
281.25	—	—	—	—	—	—	45.71	6.8
312.5	95	4.4*	85.13	4.2	—	—	—	—
400	—	—	—	—	44.63	9.3	—	—
562.5	—	—	—	—	—	—	26.78	6.6
600	—	—	—	—	25.87	10.4*	—	—
625	26.97	8.4*	56.55	5.2	—	—	—	—
900	—	—	—	—	20.37	11.93*	—	
1125	—	—	—	—	—	—	35.18	6.4
1250	8.68	8.6*	73.07	3.2	—	—	—	—
1350	—	—	—	—	16.51	13.2*	—	—
2250	—	—	—	—	—	—	41.26	10.0*
2500	[a]	[a]	40.44	4.9	—	—	—	—
4500	—	—	—	—	—	—	21.04	10.9*
5000	—	—	0.7	12.9*	—	—	—	—
DMSO	—	4.0	—	2.4	—	7.9	—	6.9
NDMA	—	—	95.8	8.3	—	—	61.12	13.5
EMS	4.83	47.2	—	—	20.5	24.4	—	—

From Ogorek (2002b)
DMSO, dimethyl sulfoxide; MMF, mean mutant frequency (× 10^{-4}); MS, mean survival (%); * $P < 0.05$
[a] Not counted due to excessive toxicity.

Table 17. Mitotic index and chromosomal aberrations in CHO cells exposed to NOA 405873

Concentration (µg/ml)	−S9		+S9	
	Mitotic index (% of control)	Chromosomal aberrations (% of cells with specific aberration)	Mitotic index (% of control)	Chromosomal aberrations (% of cells with specific aberration)
39.06	—	—	81	—
312.5	99	4	—	—
625	98	3	—	1.5
1250	71	11.5**	82	0.5
2500	3	—	57	2.0
5000	—	—	61	6.5*
Water	100	2.0	100	2.5

From Ogorek (2002a)
—, not measured; * $P < 0.05$; ** $P < 0.01$

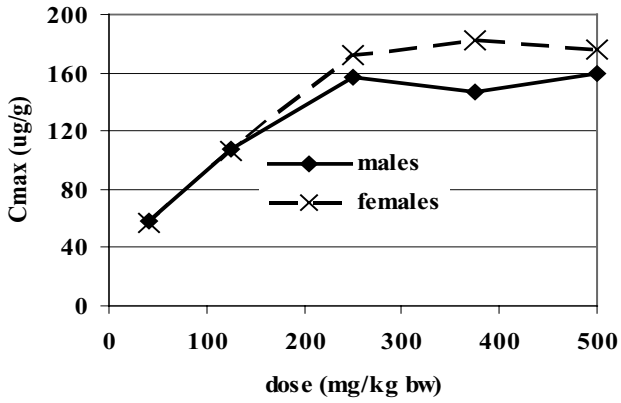
Table 18. Study design

Group	*n*	Days	Concentration (ppm)	Dose (mg/kg bw per day)[a]	Dose (mg/kg bw per day)[b]
1A	4	0–13	500	42	42
1B	4	0–13	500	42	42
2A	4	0–13	1500	125	125
2B	4	0–13	1500	125	125
3A	4	0–13	3000	250	250
3B	4	0–13	3000	250	250
4A	4	0–6	3000	250	375
		7–13	4500	375	
4B	4	0–6	3000	250	375
		7–13	4500	375	
5A	6	0–4	3000	250	500
		5–8	4500	375	
		9–13	6000	500	
5B	6	0–4	3000	250	500
		5–8	4500	375	
		9–13	6000	500	

From (Shah, 2009b)
n, number of animals per sex
[a] Non-labelled dose calculated on an assumed body weight of 250 g and feed consumption of 21 g/day.
[b] Labelled dose on day 14.

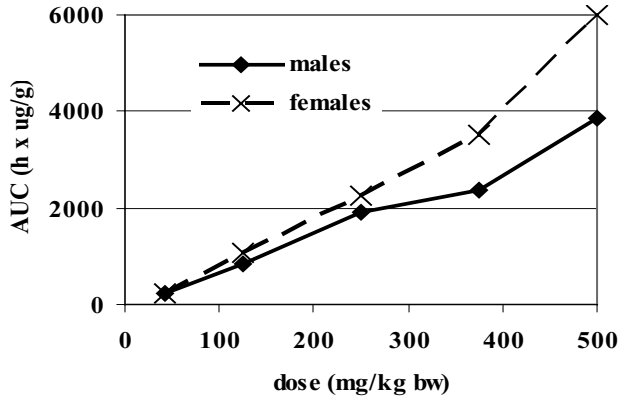
males was 5.67–7.41 hours for the dose range 42–375 mg/kg bw and then increased significantly to 12.28 hours at 500 mg/kg bw. Plasma half-life in females was 5.34–6.76 hours for the dose range 42–375 mg/kg bw and then increased significantly to 20.18 hours at 500 mg/kg bw. Absorption of [^{14}C]DCSA in males and females was saturated at doses higher than 250 mg/kg bw, as evidenced by the plateaus of the C_{max} versus dose curves (Figure 7). A similar, but less pronounced, saturation of absorption was observed at 3000 ppm and above over the 2-week dietary exposure phase with unlabelled DCSA, as shown by liquid chromatographic/tandem mass spectrometric analysis of plasma samples obtained 2 hours before dosing with [^{14}C]DCSA. The increase in AUC with dose

Figure 7. C_{max} of [^{14}C]DCSA



From Shah (2009b)

Figure 8. AUC of [^{14}C]DCSA



From Shah (2009b)

was disproportionate, particularly in females at 125 mg/kg bw and above, indicating saturation of elimination (Figure 8). Urinary excretion was the major route of elimination of DCSA. The TRR in the urine averaged 74.8% of the administered dose for group 2 males, 67.9% for group 2 females, 73.6% for group 5 males and 77.3% for group 5 females. Elimination via the faeces was 4.6% for group 2 males, 3.2% for group 2 females, 9.2% for group 5 males and 7.6% for group 5 females. On average, a slightly greater percentage of the dose was excreted in the faeces for group 5 compared with group 2. Excretion was rapid, with greater than 70% of the dose excreted by 48 hours after dosing. Excretion was somewhat faster for the lower-dose group 2 animals compared with the higher-dose group 5 animals, likely due to saturated or delayed absorption at the higher dose. For group 2, 85.3–95.5% of the excreted dose was eliminated within 24 hours, whereas for group 5, 52.7–60.0% was eliminated within 24 hours. Most of the excreted TRR was unchanged DCSA; only two metabolites were identified with levels greater than 1% of the administered dose in the excreta. The DCSA phenolic glucuronic acid conjugate was identified (metabolite M2) at 10–15% of the administered dose, and the DCSA carboxyl glucuronic acid conjugate at 1.5–16% (Shah, 2009b).

Table 19. DCSA metabolites in excreta

	% of administered dose		
	Urine + cage wash	Faeces	Total
TRR	94.5	4.2	98.7
DCSA	78.9	2.6	81.5
DCSA carboxyl glucuronide	4.4	0.2	4.6
DCSA phenolic glucuronide (M2)	10.4	—	10.4

From Shah (2009a)

In a metabolism study, single oral gavage doses of 100 mg/kg bw ^{14}C-phenyl UL DCSA (radiochemical purity 98.8%; specific activity 1.3 GBq/mmol; lot No. 6116-01A) in corn oil were given to six male Crl:CD (SD) rats. Two rats were assigned to the expired air group (group 1), and the other four animals were used for metabolism studies (group 2). For all animals, urine, cage wash and faeces were collected at 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours post-dose, and for group 1, expired air was collected at 6, 12, 24 and 48 hours. The study complied with GLP.

For group 1, the expired air collection was discontinued after 48 hours, because only 0.03% of the administered dose was identified in the expired air. Urinary excretion was the major route of elimination, with a total of 95.0% of the administered dose in the urine and cage wash and only 4.3% of the administered dose in the faeces. Excretion of radioactivity was rapid, with greater than 98% of the administered dose being excreted within 48 hours. Absorption of DCSA was extensive, as evidenced by the very small amount of the dose excreted in the faeces and the large amount of the dose excreted in the urine. At 168 hours, detectable residues were observed in liver, kidney and gastrointestinal tract, accounting for 0.01% or less of the administered dose in each tissue. TRR were highest in kidney (0.090 µg equivalent [Eq]/g), followed by liver (0.058 µg Eq/g) and gastrointestinal tract (0.027 µg Eq/g). No radioactivity was observed in spleen, fat or blood. TRR in carcass were 0.25% of the administered dose. For the period 0–48 hours, pooled urine samples accounted for 79.7%, pooled cage wash samples for 14.8% and pooled faecal samples for 4.2% of the administered dose. DCSA was the major metabolite in the urine and faeces, accounting for about 81.5%, and DCSA carboxyl glucuronide and DCSA phenolic glucuronide (M2) accounted for 4.6% and 10.4%, respectively (Table 19) (Shah, 2009a).

(b) Acute toxicity

For a summary, see Table 22 below.

(c) Repeated-dose studies

Rats

In a 13-week study, groups of 10 male and 10 female Crl:CD (SD) rats were administered 0, 500, 3000, 6000 or 12 000 ppm DCSA (purity 97.9%; lot No. GLP-0603-16958-T) in the diet. To minimize potential palatability problems, the 6000 ppm group received a dosage of 3000 ppm during study week 0 and 6000 ppm during study weeks 1 through 12, and the 12 000 ppm group received a dosage of 3000 ppm during study week 0, 6000 ppm during study week 1 and 12 000 ppm during study weeks 2 through 12. The feed concentrations were equal to 0, 32, 195, 362 and 659 mg/kg bw per day in males and 0, 37, 222, 436 and 719 mg/kg bw per day in females. Animals were checked twice daily for signs of toxicity and for mortality. The eyes were examined prior to treatment and before scheduled termination. Body weight and feed consumption were determined weekly, and blood and urine samples for clinical chemistry and haematology were collected at study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

In females at 6000 ppm, body weight gain was reduced non-significantly by 7.2%, and in males and females at 12 000 ppm, it was reduced statistically significantly by 28.1% and 29.7%, respectively. At 12 000 ppm, feed consumption was also reduced. At 12 000 ppm, both sexes had lower red blood cell parameters and lowered activated partial thromboplastin time, and males had lower platelet counts. Total protein and globulin levels in the 12 000 ppm group males and in the 6000 and 12 000 ppm group females were higher. Albumin was low in the 12 000 ppm group. Creatinine was higher in the males at 6000 and 12 000 ppm, whereas urea nitrogen was elevated in the 12 000 ppm group females. In both sexes at 12 000 ppm, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase activities were increased. In high-dose males and females and 6000 ppm females, the incidence and severity of bone marrow depletion in the sternum (or replacement of marrow with fat) were increased. No effects in an FOB or on locomotor activity were observed at any dose level.

The NOAEL was 3000 ppm, equal to 195 mg/kg bw per day, based on decreased body weight gain and effects on haematology and clinical chemistry in females at 6000 ppm (Kirkpatrick, 2009d).

In a chronic toxicity study (12 months, 20 animals of each sex per group), forming part of an as yet unfinished 24-month combined chronic toxicity/carcinogenicity study (50 animals of each sex per group), groups of 20 male and 20 female Crl:CD (SD) rats were fed diets containing 0, 10, 100, 300, 1000 or 3000 ppm DCSA (purity 97.4–97.7%; lot No. GLP-0603-16958-T) for 52 weeks. These feed concentrations were equal to 0, 0.6, 5.6, 16.9, 56.9 and 171.2 mg/kg bw per day in males and 0, 0.7, 6.9, 20.5, 68.2 and 206.2 mg/kg bw per day in females. Groups were checked twice daily for dead or moribund animals, and feed consumption and body weight development were recorded weekly for the first 13 weeks and once every 4 weeks thereafter. Ophthalmic examinations were performed during study weeks −2 and 51. Blood was sampled before treatment, at 12 and 25 weeks and at necropsy in week 52. At necropsy, organs were weighed, and organs of the control and the 3000 ppm group were examined histologically. The study complied with GLP.

There were no test substance–related clinical observations or effects on body weight, feed consumption or haematology, coagulation and urinalysis parameters. Organ weights were unaffected, and there were no treatment-related macroscopic or microscopic findings. The only effect was slightly lower serum total bilirubin in the 3000 ppm group males at study termination, in the 1000 and 3000 ppm group females at week 25 and in females at and above 300 ppm at the scheduled necropsy. As there were no corresponding changes in haematology, organ weights or histology, this effect was considered non-adverse.

The NOAEL was 3000 ppm, equal to 171.2 mg/kg bw per day, the highest dose tested (Kirkpatrick, 2009a).

In a multigeneration study, groups of 30 male and 30 female 6-week-old Crl:CD (SD) rats were fed diets containing 0, 50, 500 or 5000 ppm DCSA (purity 97.7%; lot No. GLP-0603-16958-T). Mean test substance intake for the F_0 males was 0, 4, 37 or 362 mg/kg bw per day and for the F_0 females was 0, 4, 43 or 414 mg/kg bw per day during the premating period, 0, 3, 34 and 323 mg/kg bw per day during gestation and 0, 8, 78 and 610 mg/kg bw per day during lactation. After 10 weeks of exposure, animals were allowed to mate and produce the F_1 generation. Because all surviving offspring of the F_0 animals in the 5000 ppm group were euthanized on postnatal day (PND) 21 as a result of pup mortality and a high incidence of total litter loss among the dams, no offspring of the F_0 animals in the 5000 ppm group were available for the F_1 generation. As a follow-up to a previous 13-week study in rats, three additional groups of 10 female rats per group were included for evaluation of clinical and histopathological parameters. These non-mated satellite animals were administered DCSA for at least 13 weeks at levels of 0, 50 and 500 ppm in the diet.

Animals were observed regularly for appearance, behaviour and clinical signs, and body weight and feed consumption were recorded during the premating phase, gestation phase and lactation phase. Pups were sexed and weighed at lactation days 1, 4, 7, 14 and 21. A complete necropsy was performed on all adult animals and on selected pups of all groups. Additionally, organs were weighed and subjected to a histological examination. The study complied with GLP.

No clinical effects on parental animals or any adverse effects on fertility parameters were observed at any dose level. At 5000 ppm, lower body weights (approximately 10%) and lower feed consumption in both sexes were recorded throughout the entire treatment period. In the 5000 ppm group, pup survival during PNDs 0–1, 1–4, 7–14 and 14–21 was decreased due primarily to seven females with total litter loss, and clinical signs of toxicity, including pale body, blackened ventral abdominal area, distended abdomen, uneven hair growth and desquamation, and lower body weights and weight gains during PNDs 1–21, were observed. All surviving offspring of the F_0 animals in the 5000 ppm group were terminated on PND 21. Dark green intestinal contents were noted for several of the F_1 pups that were euthanized in extremis. At necropsy on PND 21, test substance–related findings included yellow intestinal contents, desquamation, uneven hair growth and small thymus. Decreased mean absolute and relative thymus weights were noted for the 5000 ppm group F_1 males and females on PND 21. In addition, hyperkeratosis was noted upon histological evaluation of those F_1 pups in the 5000 ppm group that had gross skin lesions or clinical findings of desquamation or uneven hair loss/hair growth.

The NOAEL for parental toxicity was 500 ppm, equal to 37 mg/kg bw per day, based on lower body weight gain and lower feed consumption in both sexes at 5000 ppm. The NOAEL for offspring toxicity was 500 ppm, equal to 37 mg/kg bw per day, based on severe toxicity, including mortality, in pups during lactation. The NOAEL for reproductive performance was 5000 ppm, equal to 323 mg/kg bw per day, the highest dose tested (Coder, 2009a).

In a pilot developmental toxicity study, groups of eight mated female Crl:CD (SD) rats were administered DCSA (purity 97.9%; lot No. GLP-0603-16958-T) by gavage in corn oil at doses of 0, 50, 200, 500 or 1000 mg/kg bw per day from GD 6 to GD 19. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 20, animals were sacrificed, and the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded. The fetuses were weighed and sexed and investigated for external malformations and developmental variations. The study complied with GLP.

At 1000 mg/kg bw per day, seven animals died within 2–4 days of starting treatment, and one was euthanized in extremis. At 500 mg/kg bw per day, two animals died within 3–5 days of starting treatment. All animals dying showed weight loss and low feed consumption. Surviving animals in the 200 and 500 mg/kg bw per day groups showed salivation and red or clear material around the mouth and/or nose. At 500 mg/kg bw per day, excessive pawing and wiping the mouth on the floor were observed. In the 200 and 500 mg/kg bw per day groups, body weight losses and lower gravid uterine weights were observed, compared with controls. Absolute spleen weights were increased by 17% and absolute kidney weights by 12% in the 500 mg/kg bw per day group. Absolute liver weights were increased by 7% at 200 mg/kg bw per day and by 19% at 500 mg/kg bw per day. Surviving females in the 500 mg/kg bw per day group had 100% early resorptions of their litters. In the 200 mg/kg bw per day group, lower mean fetal weights (by 14%) were observed; however, intrauterine survival at this dose level was comparable to that in the control group. Intrauterine growth and survival at 50 mg/kg bw per day were unaffected by treatment. No malformations or developmental variations were noted in any fetuses in any group.

The maternal and developmental NOAEL was 50 mg/kg bw per day, based on severe toxicity, including mortality, in dams and lower fetal body weights in the 200 mg/kg bw per day group (Sawhney, 2007).

In the definitive developmental toxicity study, groups of 25 mated female Crl:CD (SD) rats were administered DCSA (purity 97.7%; lot No. GLP-0603-16958-T) by gavage in corn oil at doses of 0, 10, 30 or 100 mg/kg bw per day from GD 6 to GD 19. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 20, animals were terminated, and the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded. The fetuses were weighed, sexed and investigated for skeletal and visceral aberrations. The study complied with GLP.

No treatment-related effects on dams or on the development of fetuses at any dose level were observed.

In the absence of any effects, the maternal and developmental NOAEL was 100 mg/kg bw per day, the highest dose tested (Coder, 2007).

Rabbits

In a pilot developmental toxicity study, groups of six mated female New Zealand White rabbits were administered DCSA (purity 97.7%; lot No. GLP-0603-16958-T) in 0.5% carboxymethylcellulose by gavage at doses of 0, 10, 30, 100 or 300 mg/kg bw per day from GD 6 to GD 28. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 29, animals were terminated, the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded and liver, kidney, spleen and gravid uterine weights were recorded. The fetuses were weighed, sexed and investigated for external malformations and developmental variations. The study complied with GLP.

At 100 mg/kg bw per day, two females were euthanized in extremis on GD 19 and GD 20, and all females in the 300 mg/kg bw per day group were euthanized in extremis on GD 12. In these two dose groups, feed consumption was markedly reduced, and animals lost weight after the first day of treatment. In the 100 mg/kg bw per day group, the body weights were similar to control after early termination of the two dams on GD 19 and GD 20. A single female in the 300 mg/kg bw per day group had brown areas on the lungs, and another female in the 100 mg/kg bw per day group had green discoloration of the liver, distended gallbladder, dark red areas on the stomach and dark red discoloration of the stomach and caecum. A slightly lower mean kidney weight was noted in the surviving 100 mg/kg bw per day females, a difference that was not statistically significant. There were no effects on intrauterine growth or survival noted at dose levels of 10, 30 and 100 mg/kg bw per day. No external malformations or developmental variations were observed for fetuses evaluated at 10, 30 or 100 mg/kg bw per day.

The maternal NOAEL was 30 mg/kg bw per day, based on severe toxicity resulting in early termination of dams at 100 mg/kg bw per day. The developmental NOAEL was 100 mg/kg bw per day, the highest dose with dams surviving to scheduled necropsy (Coder, 2009b).

In the definitive developmental toxicity study, groups of 25 mated female New Zealand White rabbits were administered DCSA (purity 97.7%; lot No. GLP-0603-16958-T) in 0.5% carboxymethylcellulose by gavage at doses of 0, 10, 25 or 65 mg/kg bw per day from GD 6 to GD 28. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 29, animals were terminated, the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded and gravid uterine weights were recorded. The fetuses were weighed, sexed and investigated for external, visceral and skeletal malformations and developmental variations. The study complied with GLP.

One high-dose female was euthanized in extremis on GD 28 as a result of markedly reduced feed consumption and body weight loss. Generally, the body weight of the 65 mg/kg bw per day

groups was slightly and statistically not significantly lower by about 3% compared with the control group. However, body weight gain at GDs 6–9 was statistically significantly lower than in controls. Feed consumption and defecation were lower in this group. Although there were no effects on body weight or feed consumption in the 10 and 25 mg/kg bw per day groups, decreased defecation was also observed more frequently in these groups, but was not considered adverse. No effects on fetal growth, survival or the incidence of malformations or variations were observed at any dose level.

The maternal NOAEL was 25 mg/kg bw per day, based on generally very mild effects on body weight at 65 mg/kg bw per day. The developmental NOAEL was 65 mg/kg bw per day, the highest dose tested (Coder, 2009c).

Dogs

Groups of five male and five female 18-week-old Beagle dogs were orally administered DCSA (purity 97.7%; lot No. GLP-0603-16958-T) via gelatine capsule at doses of 0, 15, 50 or 150 mg/kg bw per day for 13 weeks. The capsule content was adjusted weekly based on body weight development. Animals were checked twice daily for signs of toxicity and for mortality. The eyes were examined prior to treatment and before scheduled termination. Body weight was determined weekly, and blood (collected prior to study initiation, at week 6 and at study termination) and urine samples for clinical chemistry and haematology were collected at study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

One high-dose female was killed in extremis in poor condition, probably due to repeated emesis and dehydration. Treatment-related clinical observations included abnormal excreta (diarrhoea, soft faeces and/or mucoid faeces) and/or emesis, primarily in the 50 and 150 mg/kg bw per day test substance–treated groups, and were most commonly observed prior to dose administration for both sexes. High-dose animals of both sexes had 11% lower body weights at study termination. Slightly increased activated partial thromboplastin times were observed in 150 mg/kg bw per day group males at study week 13 and in 150 mg/kg bw per day group females at study week 6. In all dosed males, mean corpuscular volume and mean corpuscular haemoglobin were slightly but dose-relatedly and statistically significantly increased. High-dose animals of both sexes had slightly lower liver enzyme activity. Relative liver weights in high-dose males and females were increased and showed a high incidence of periportal hepatocellular hypertrophy.

The NOAEL was 50 mg/kg bw per day, based on lower body weight gain and increased liver weights accompanied by hepatocellular hypertrophy in both sexes at 150 mg/kg bw per day (Kirkpatrick, 2009c).

(d) Genotoxicity

For a summary, see Table 23 below. Studies with equivocal or positive results are described below.

DCSA (purity 97.9%; lot No. GLP-0603-16958-T) was tested in cultured peripheral human lymphocytes for its potential to induce chromosomal aberrations. The study complied with GLP.

DCSA increased the number of cells with chromosomal aberrations in the presence and absence of metabolic activation by S9 after 3 hours or 22 hours of exposure (Tables 20 and 21). DCSA is damaging to deoxyribonucleic acid (DNA) at cytotoxic levels (Murli, 2007).

3.3 Metabolite 3,6-dichlorogentisic acid (DCGA; MON 52724)

(a) Acute toxicity

For a summary, see Table 22.

Table 20. Chromosomal aberrations in peripheral human lymphocytes: 3 hours of treatment, harvest at 22 hours

Concentration	−S9			+S9		
	Mitotic index (% of cells in mitosis)	Aberrations (% of cells)		Mitotic index (% of cells in mitosis)	Aberrations (% of cells)	
		−gaps	+gaps		−gaps	+gaps
DMSO (10 µl/ml)	10.3	1.0	1.5	10.8	0	1.0
Historical control data (DMSO)	10.2 (3.2–17.7)	0.6 (0–2.5)	1.8 (0–5.5)	10.2 (4.3–16.5)	0.4 (0.0–3.0)	1.4 (0.0–3.0)
294 µg/ml	—	—	—	10.8	0.5	1.0
420 µg/ml	10.4	0.0	1.5	9.2	0.5	1.5
600 µg/ml	9.0	0.5	1.0	7.2	2.5	3.5
858 µg/ml	6.4	1.0	1.0	4.7	9.5	10.0
1230 µg/ml	3.8	3.0	3.5	0.1	—	—

From Murli (2007)
— not measured

Table 21. Chromosomal aberrations in peripheral human lymphocytes: 22 hours of treatment, harvest at 22 hours

Concentration	−S9			+S9		
	Mitotic index (% of cells in mitosis)	Aberrations (% of cells)		Mitotic index (% of cells in mitosis)	Aberrations (% of cells)	
		−gaps	+gaps		−gaps	+gaps
DMSO (10 µl/ml)	10.4	0.0	1.0	13.6	0.0	0.0
Historical control data (DMSO)	9.6 (3.0–15.2)	0.5 (0–2.5)	1.6 (0.0–5.0)	—	—	—
250 µg/ml	8.9	1.0	2.5	—	—	—
350 µg/ml	6.6	2.5	4.5	—	—	—
400 µg/ml	7.2	4.0	8.5	—	—	—
450 µg/ml	4.5	4.5	9.0	—	—	—
750 µg/ml	—	—	—	14.4	2.5	2.5
800 µg/ml	—	—	—	14.9	4.0	7.0
850 µg/ml	—	—	—	11.1	2.0	2.0
925 µg/ml	—	—	—	8.4	2.0	3.5
1000 µg/ml	—	—	—	6.1	8.0	8.0

From Murli (2007)
— not measured

Table 22. Summary of acute toxicity of metabolites of dicamba

Compound	Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	Purity (%); lot No.	Reference
NOA 405873	Rat	?	M/F	Oral	> 2000	94; KI 6212/1-18	Sommer (2001)
DCSA	Rat	SD	F	Oral	2641	97.9; GLP-0603-16958-T	Smedley (2007)
DCGA	Rat	SD	F	Oral	1460	96.3; GLP-0903-19699-T	Oley (2009)

F, female; M, male

(b) Short-term studies of toxicity

In a 4-week study, groups of 10 male and 10 female Crl:CD (SD) rats were administered 0, 500, 3000, 6000 or 12 000 ppm DCGA (purity 98.1%; lot No. GLP-0904-19809-T) in the diet. These feed concentrations were equal to 0, 40, 240, 474 and 956 mg/kg bw per day in males and 0, 45, 265, 519 and 1063 mg/kg bw per day in females. Animals were checked twice daily for signs of toxicity and for mortality. An evaluation of potential neurotoxicity by FOB and motor activity assessment was done. Body weight and feed consumption were determined weekly and twice weekly, respectively, and blood and urine samples for clinical chemistry and haematology were collected at study termination. At study termination, organs were weighed and histology was performed. The study complied with GLP.

All animals survived to the scheduled necropsy, and no test substance–related clinical observations, effects on organ weights or histological or macroscopic findings were seen. The 12 000 ppm group males and females had lower mean body weights (9.2% and 6.0% lower in males and females, respectively, compared with controls) and feed consumption throughout the study. High-dose males had statistically significantly lower lymphocyte counts by 32% compared with controls. Lymphocyte counts in high-dose females were also low, but not statistically significantly.

The NOAEL in this study was 6000 ppm, equal to 474 mg/kg bw per day, based on reduced body weight gain and lower lymphocyte counts in high-dose animals at 12 000 ppm (Kirkpatrick, 2009b).

(c) Developmental toxicity

In a pilot developmental toxicity study, groups of eight mated female Crl:CD (SD) rats were administered DCGA (purity 96.3%; lot No. GLP-0903-19699-T) by gavage in corn oil at doses of 0, 50, 200, 500 or 1000 mg/kg bw per day from GD 6 to GD 19. During gestation, animals were observed for mortality, morbidity and overt signs of toxicity, and body weight development and feed consumption were recorded. On GD 20, animals were terminated, and the number and distribution of implantation sites, early and late resorptions, live and dead fetuses and corpora lutea were recorded. The fetuses were weighed, sexed and investigated for external developmental variations and malformations. The study complied with GLP.

Four high-dose females had significantly lower body weight gain and lower feed consumption and died between GD 12 and GD 19, and one was killed in extremis. No animals aborted. Clinical signs in high-dose animals were rales and hypoactivity. On necropsy, the dead animals had red vaginal contents and clear fluid in the thoracic cavity. Dose-related increases in rale incidences were also observed in animals at 200 mg/kg bw per day and above. The 500 mg/kg bw per day group also had lower body weights from GD 9 onwards. Intrauterine growth, survival and development of the fetuses were unaffected by test substance administration at all dose levels.

The maternal NOAEL in this pilot study was 50 mg/kg bw per day, based on increased incidence of rales at 200 mg/kg bw per day and above. The developmental NOAEL was 1000 mg/kg bw per day, the highest dose tested (Coder, 2009d).

(d) Genotoxicity

For a summary, see Table 23.

4. Observations in humans

No reports were submitted.

Table 23. Summary of results of genotoxicity tests with metabolites of dicamba

End-point	Test system	Concentration	Purity (%); lot No.	Result	Reference
NOA 405873					
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537 and *Escherichia coli* WP2 *uvrA*	312.5–5000 µg/plate	94; KI 6212/1-18	Negative	Deparade (2001)
L5178Y TK$^{+/-}$	Mouse lymphoma cells	156–5000 µg/ml	94; KI 6212/1-18	Positive	Ogorek (2002b)
L5178Y TK$^{+/-}$	Mouse lymphoma cells	125–2370 µg/ml	99; MLA-29/21	Positive	Clay (2002)
Chromosomal aberration	CHO cells	312.5–5000 µg/ml	94; KI 6212/1-18	Positive	Ogorek (2002a)
Micronucleus induction	Mouse bone marrow	2000 mg/kg bw	99; MLA-29/21	Negative	Fox (2003)
Unscheduled DNA synthesis in vivo	Rat liver	2000 mg/kg bw	99; MLA-29/21	Negative	Clay (2004)
DCSA					
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvrA*	6.67–5000 µg/plate	97.9; GLP-0603-16958-T	Negative	Mecchi (2006)
HGPRT	CHO cells	200–1600 µg/ml	97.9; GLP-0603-16958-T	Negative	Cifone (2006)
Chromosomal aberration	Human peripheral lymphocytes	17–2500 µg/ml	97.9; GLP-0603-16958-T	Positive	Murli (2007)
Chromosomal aberration	Rat bone marrow in vivo	400–1600 mg/kg bw	97.9; GLP-0603-16958-T	Negative	Xu (2008)
Micronucleus induction	Mouse bone marrow	250–1000 mg/kg bw	97.9; GLP-0603-16958-T	Negative	Xu (2007)
DCGA					
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvrA*	1.6–5000 µg/plate	96.3; GLP-0903-19699-T	Negative	Stankowski (2009)
Chromosomal aberration	Male rat bone marrow in vivo	375–1500 mg/kg bw	96.3; GLP-0903-19699-T	Negative	Murli (2009)

HGPRT, hypoxanthine–guanine phosphoribosyl transferase

Comments

Biochemical aspects

The absorption, distribution, metabolism and excretion of dicamba after oral dosing were investigated in several studies in rats and one study using mice, rabbits and dogs. Generally, there were no differences in the toxicokinetics of dicamba between species and sexes. Furthermore, it was shown that the anionic counter-ion of dicamba salts did not influence the absorption, metabolism or elimination of dicamba. Dicamba was rapidly absorbed, with peak levels occurring within the first hour after administration. The absorption was not saturated at doses up to 1000 mg/kg bw. Between 2 and 4 hours after the first absorption peak, a second peak was observed, indicative of enterohepatic recirculation of dicamba. At doses greater than 125 mg/kg bw, the elimination half-life of dicamba equivalents increased, and the AUC increased disproportionately with dose, indicating saturation of

elimination at higher doses. Only 3% of a low or high dose (0.5 or 200 mg/kg bw) was found in the tissues 4 hours post-dosing, with highest residues in the kidneys, plasma and uterus. After 7 days, only 0.2% of the administered dose was found in the tissues. More than 95% of the administered dose is excreted in the urine, with less than 5% in the faeces. Excretion by expired air is negligible. In urine and faeces, more than 90% of the radioactivity found was accounted for by unchanged dicamba. In urine, very low amounts of glucuronidated dicamba (M1), DCSA (NOA 414746), 5-hydroxy-dicamba (NOA 405873) and M2 (NOA 414746) were found. In the liver and kidneys, 84–91% of TRR were identified as dicamba. In summary, dicamba is poorly metabolized, and the pathways involved include demethylation, hydroxylation and glucuronic acid conjugation.

Toxicological data

Dicamba is of low acute toxicity. The lowest oral LD_{50} was approximately 1600 mg/kg bw in female rats. By dermal application, the LD_{50} was greater than 2000 mg/kg bw, and the LC_{50} in an inhalation study was 4.46 mg/l. Dicamba was only slightly irritating to the skin but severely irritating to the eye. Dicamba did not show skin sensitizing potential.

In repeated-dose studies in rats and dogs, the mostly mild effects observed included lower body weight gains, haematological and clinical chemistry effects and clinical signs of toxicity.

In a 13-week rat feeding study with dietary concentrations up to 12 000 ppm, reduced activity, lower body weight development and reduced feed consumption were observed. Animals had significantly lower platelet counts and partial thromboplastin times; females also had reduced red cell parameters and increased white blood cell and lymphocyte counts, and clinical chemistry parameters were changed. After a recovery period, most of the haematological and clinical chemistry parameters were similar to those in control animals. Relative liver weights were statistically significantly increased. Histological findings were restricted to high-dose females, which showed reversible centrilobular hepatocyte hypertrophy and hepatocellular pigmentation. The NOAEL was 6000 ppm (equal to 479.3 mg/kg bw per day), based on haematological and biochemical effects at 12 000 ppm. In a 13-week dog study with administration in capsules of doses up to 300 mg/kg bw per day, including a high-dose recovery group, behavioural changes (i.e. ataxia, stiff gait and sporadic transient collapses approximately 2 hours after dosing) at 300 mg/kg bw per day were observed, and body weight gain was decreased by 26% in males and by 44% in females. In the high-dose recovery group, body weight development was similar to that in the controls. Red blood cell parameters were reduced and the partial thromboplastin time was slightly elevated in males and females receiving 300 mg/kg bw per day. These effects were partially reversible within 4 weeks. The NOAEL in this study was 50 mg/kg bw per day, based on behavioural effects at 300 mg/kg bw per day. In a 52-week feeding study in dogs with dietary concentrations up to 2500 ppm, animals in the 500 ppm and 2500 ppm groups showed initially lower feed consumption and lower body weight gain. This effect was transient and is not considered to be adverse. The NOAEL was 2500 ppm (equal to 52 mg/kg bw per day), the highest dose tested.

In a 24-month feeding study (89 weeks for males) in mice with dietary concentrations up to 3000 ppm, the onset of mortality was early in the study, and the overall mortality was increased in males. At 3000 ppm, body weights in females were lower from week 25 onwards. The NOAEL was 1000 ppm (equal to 108 mg/kg bw per day), based on reduced body weight gain in females at the highest dose.

Dicamba was not carcinogenic in mice.

A carcinogenicity study in rats with dietary concentrations up to 2500 ppm was considered adequate to assess carcinogenicity at 104 weeks, although survival was low at study termination (week 117). The incidences of mixed malignant lymphoma (8% versus 0% in all other groups) and thyroid C-cell carcinoma were increased in the high dose group males, although not statistically significantly. Although the incidence of malignant lymphoma was higher than the historical control

range of 0–1.8%, it was at the upper bound of the historical control ranges aggregated for all types of malignant lymphoreticular lymphoma (0–8.6% and 0–8.4% in another historical control group data set). There was no increase in the incidence of C-cell hyperplasia or adenoma, which are part of the progression to carcinoma. The NOAEL for general toxicity was 2500 ppm (equal to 107 mg/kg bw per day), the highest dose tested.

Dicamba was an equivocal carcinogen in rats.

The potential genotoxicity of dicamba was tested in an adequate battery of in vitro and in vivo studies, providing no evidence of genotoxic potential.

The Meeting concluded that dicamba was unlikely to be genotoxic.

On the basis of the absence of genotoxicity and the absence of carcinogenicity in mice and the fact that an equivocal increase in the incidences of lymphoid tumours and of thyroid C-cell carcinoma in male rats occurred only at the highest dose, the Meeting concluded that dicamba is unlikely to be carcinogenic at human dietary exposure levels.

In a two-generation study of reproductive toxicity in rats at dietary concentrations up to 5000 ppm, high-dose females showed increased body tone and slow righting reflex, and high-dose F_1 and F_2 pups had lower body weights throughout the lactation phase. At weaning, their body weights were lower by more than 20%. Thereafter, body weight gain was not affected. At 1500 ppm, pup weights were also slightly reduced, attaining statistical significance at several time points in the lactation phase. There were no effects on mating performance or pregnancy at any dose level. At the high dose, balano-preputial separation was delayed statistically significantly (45.6 days versus 43.7 days in controls). The NOAEL for parental toxicity was 1500 ppm (equal to 105 mg/kg bw per day), based on behavioural effects at 5000 ppm. The NOAEL for reproductive toxicity was 5000 ppm (equal to 347 mg/kg bw per day), the highest dose tested. The NOAEL for effects on postnatal development was 500 ppm (equal to 35.1 mg/kg bw per day), based on reduced pup body weights.

In a study on developmental toxicity in rats at dose levels up to 400 mg/kg bw per day, 3 of 25 females died on treatment day 2 or 3. The mean body weight on GD 20 was lower (by 8%) in high-dose animals. Feed consumption was reduced by approximately 20%, and animals showed behavioural changes, such as ataxia and stiffening of the body. The body weights of high-dose fetuses were (not statistically significantly) reduced by 6%. There were no treatment-related skeletal anomalies. The maternal NOAEL was 160 mg/kg bw per day, based on mortality and behavioural changes at 400 mg/kg bw per day, and the developmental NOAEL was 400 mg/kg bw per day, the highest dose tested.

In a study on developmental toxicity in rabbits at dose levels up to 300 mg/kg bw per day, one 150 mg/kg bw per day doe aborted on GD 22, and four does in the high-dose group aborted between GD 19 and GD 24. All animals aborting showed body weight loss accompanied by reduced feed consumption and ataxia. On necropsy, no lesions were observed. Generally, the mid- and high-dose dams showed decreased motor activity and ataxia, and the high-dose animals also had rales, laboured breathing and impaired righting reflex. These clinical signs were first observed on GD 9. The body weights and the feed consumption of high-dose dams were reduced. There were no effects of treatment on the litter data. The NOAEL for dams was 30 mg/kg bw per day, based on behavioural changes at 150 mg/kg bw per day. The developmental NOAEL was 300 mg/kg bw per day, the highest dose tested.

The Meeting concluded that dicamba was not teratogenic.

In an acute neurotoxicity study in rats at doses ranging from 300 to 1200 mg/kg bw, nonspecific and transient neurobehavioural effects were apparent within 1.5 hours after dosing in all dose groups, with a dose-dependent incidence and severity of rigidity.

In a 13-week rat feeding study of neurotoxicity with dietary concentrations up to 12 000 ppm (equal to 767.9 mg/kg bw per day), no behavioural or histological evidence for neurotoxicity was observed.

Toxicological studies for three metabolites were submitted. DCSA and DCGA have been identified as metabolites of dicamba in soya beans, sugarcane, wheat and cotton and are also environmental metabolites of dicamba. DCSA has been identified in rats, cows, goats and hens, and 5-hydroxy-dicamba was found in rats.

In a poorly described 13-week feeding study in rats and dogs, 5-hydroxy-dicamba showed no toxicity up to 250 ppm (equivalent to 25 mg/kg bw per day in rats and 6.25 mg/kg bw per day in dogs), the highest dietary concentration tested. 5-Hydroxy-dicamba gave positive results in mouse lymphoma assays and in a CHO chromosomal aberration test at cytotoxic levels, in the absence and presence of metabolic activation (S9). 5-Hydroxy-dicamba was negative in a mouse micronucleus test.

DCSA showed pharmacokinetic behaviour very similar to that of the parent dicamba and was excreted mainly unchanged and to a minor extent as DCSA carboxyl glucuronide and DCSA phenolic glucuronide (M2, also identified as a rat metabolite of dicamba). In a 13-week feeding study in rats at dietary DCSA concentrations up to 12 000 ppm, reduced body weight gain and haematological and clinical chemistry effects were observed at 6000 and 12 000 ppm. The NOAEL was 3000 ppm (equal to 195 mg/kg bw per day), based on reduced body weight gain at 6000 ppm. In a 13-week study in dogs administered up to 150 mg/kg bw per day by capsule, the NOAEL was 50 mg/kg bw per day, based on reduced (by 11%) body weight gain and liver effects at 150 mg/kg bw per day. In a 52-week feeding study in rats, the NOAEL was 3000 ppm (equal to 171.2 mg/kg bw per day), the highest dose tested. In a two-generation study in rats at dietary concentrations of DCSA up to 5000 ppm, the NOAEL for parental toxicity was 500 ppm (equal to 37 mg/kg bw per day), based on lower body weight gain and reduced feed consumption in both sexes at 5000 ppm. The NOAEL for offspring toxicity was 500 ppm (equal to 37 mg/kg bw per day), based on severe toxicity, including mortality, in pups during lactation. The NOAEL for reproductive performance was 5000 ppm (equal to 323 mg/kg bw per day), the highest dose tested. In a pilot and a definitive rat developmental study on DCSA, the overall NOAEL for maternal and developmental toxicity was 100 mg/kg bw per day, based on severe dam toxicity, including mortality and lower fetal body weight, at 200 mg/kg bw per day. In a pilot and a definitive rabbit developmental study on DCSA, the overall NOAEL for maternal toxicity was 30 mg/kg bw per day, based on reduced feed consumption and lower body weight gain at 65 mg/kg bw per day. The NOAEL for developmental toxicity was 65 mg/kg bw per day, the highest dose tested. In genotoxicity studies, including a mouse micronucleus test, DCSA was negative. However, in human peripheral lymphocytes, DCSA increased the number of cells with chromosomal aberrations in the presence and absence of metabolic activation by S9 after 3 or 22 hours of exposure at cytotoxic levels.

The metabolite DCGA was evaluated in a 4-week feeding study in rats at dietary concentrations up to 12 000 ppm. The NOAEL was 6000 ppm (equal to 474 mg/kg bw per day), based on reduced body weight gain and lower lymphocyte counts in animals at 12 000 ppm. In a pilot rat developmental study with doses up to 1000 mg/kg bw per day, the maternal NOAEL was 50 mg/kg bw per day, based on increased incidences of rales at 200 mg/kg bw per day, and the developmental NOAEL was 1000 mg/kg bw per day, the highest dose tested. DCGA was negative in an Ames and a rat chromosomal aberration test.

It was concluded that DCSA and DCGA have toxicities similar to or lower than that of dicamba. Based on available data, 5-hydroxy-dicamba appears to be of lower toxicity than the parent.

The Meeting concluded that the existing database on dicamba was adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.3 mg/kg bw on the basis of a NOAEL of 30 mg/kg bw per day in a rabbit developmental toxicity study, based on maternal toxicity (behavioural changes) at 150 mg/kg bw per day. A safety factor of 100 was applied. The ADI is

supported by a postnatal developmental NOAEL of 35.1 mg/kg bw per day in the rat multigeneration study, on the basis of reduced pup body weights at 105 mg/kg bw per day. This ADI would also be protective against the equivocal increase in the incidences of malignant lymphoma and thyroid parafollicular cell carcinoma in male rats at 107 mg/kg bw per day.

The Meeting established an acute reference dose (ARfD) of 0.5 mg/kg bw based on a NOAEL of 50 mg/kg bw per day in the 13-week dog study, based on behavioural effects observed shortly after dosing at 300 mg/kg bw per day. A safety factor of 100 was applied.

The behavioural effects seen in a study on developmental toxicity in rabbits at dose levels of 150 mg/kg bw per day and above are not considered to be an adequate basis for an ARfD because the clinical signs were observed first after four applied doses.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	1000 ppm, equal to 108 mg/kg bw per day	3000 ppm, equal to 358 mg/kg bw per day
		Carcinogenicity	3000 ppm, equal to 358 mg/kg bw per day[b]	—
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	2500 ppm, equal to 107 mg/kg bw per day[b]	—
		Carcinogenicity	250 ppm, equal to 11 mg/kg bw per day	2500 ppm, equal to 107 mg/kg bw per day[c]
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	5000 ppm, equal to 347 mg/kg bw per day[b]	—
		Parental toxicity	1500 ppm, equal to 105 mg/kg bw per day	5000 ppm, equal to 347 mg/kg bw per day
		Offspring toxicity	500 ppm, equal to 35.1 mg/kg bw per day	1500 ppm, equal to 105 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	160 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	400 mg/kg bw per day[b]	—
Rabbit	Developmental toxicity study[d]	Maternal toxicity	30 mg/kg bw per day	150 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day[b]	—
Dog	Thirteen-week study of toxicity[e]	Toxicity	50 mg/kg bw per day	300 mg/kg bw per day
	One-year study of toxicity[a]	Toxicity	2500 ppm, equal to 52 mg/kg bw per day[b]	—

[a] Dietary administration.
[b] Highest dose tested.
[c] Equivocal increase in the incidences of malignant lymphoma and thyroid parafollicular cell carcinoma in male rats.
[d] Gavage administration.
[e] Administration by gelatine capsule.

Estimate of acceptable daily intake for humans

0–0.3 mg/kg bw

Estimate of acute reference dose

0.5 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to dicamba

Absorption, distribution, excretion and metabolism in mammals

Rate and extent of oral absorption	Rapid, > 90% within 24 h
Distribution	Extensive, highest levels in kidneys, plasma and uterus
Potential for accumulation	None
Rate and extent of excretion	Rapid, close to 100% within 48 h, mainly via urine
Metabolism in animals	Poorly metabolized, primarily via demethylation, hydroxylation and glucuronidation
Toxicologically significant compounds in animals, plants and the environment	Dicamba, DCSA, DCGA

Acute toxicity

Rat, LD_{50}, oral	1600 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	4.46 mg/l
Rabbit, dermal irritation	Slightly irritating
Rabbit, eye irritation	Severely irritating
Guinea-pig, dermal sensitization	Not a sensitizer (Magnusson and Kligman test)

Short-term studies of toxicity

Target/critical effect	Body weight reduction, haematology and clinical chemistry (dogs)
Lowest relevant oral NOAEL	50 mg/kg bw per day (dogs)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, the highest dose tested (rats)
Lowest relevant inhalation NOAEL	No data

Long-term studies of toxicity and carcinogenicity

Target/critical effect	Reduced body weight gain
Lowest relevant NOAEL	1000 ppm, equal to 108 mg/kg bw per day (mice)
Carcinogenicity	Equivocal increase in malignant lymphoma and thyroid C-cell carcinoma (rats) at 2500 ppm (equal to 107 mg/kg bw per day); unlikely to be carcinogenic at human dietary exposure levels

Genotoxicity

	Not genotoxic

Reproductive toxicity

Reproduction target/critical effect	No reproductive effects, offspring toxicity (rats)

Lowest relevant reproductive NOAEL	347 mg/kg bw per day, the highest dose tested (rats); 30 mg/kg bw per day (maternal toxicity in rabbits)
Lowest relevant offspring NOAEL	35.1 mg/kg bw per day (rats)
Developmental target/critical effect	No developmental effects (rats, rabbits)
Lowest relevant developmental NOAEL	300 mg/kg bw per day (rabbits), the highest dose tested

Neurotoxicity/delayed neurotoxicity

Not neurotoxic

Other toxicological studies

Metabolism, pharmacokinetic, toxicity and genotoxicity studies with metabolites

Medical data

No data

Summary

	Value	Study	Safety factor
ADI	0–0.3 mg/kg bw	Developmental toxicity study in rabbit	100
ARfD	0.5 mg/kg bw	Thirteen-week study of toxicity in dog	100

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DITHIANON

First draft prepared by
D. Kanungo[1] and Les Davies[2]

[1] Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India, New Delhi, India
[2] Chemical Review – Australian Pesticides & Veterinary Medicines Authority, Kingston, ACT, Australia

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Explanation

Dithianon ($C_{14}H_4N_2O_2S_2$) is the International Organization for Standardization (ISO)–approved name for 5,10-dihydro-5,10-dioxonaphtho[2,3-b]-1,4-dithiine-2,3-dicarbonitrile (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) No. 3347-22-6. Dithianon is used on a range of fruits and vegetables as a multisite contact fungicide that inhibits spore germination.

Dithianon was evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1992, when an acceptable daily intake (ADI) of 0–0.01 mg/kg body weight (bw) was established. It is being reviewed at the present Meeting as part of the periodic re-evaluation programme of the Codex Committee on Pesticide Residues (CCPR). All the pivotal studies met the basic requirement of the relevant guideline and contained certificates of compliance with good laboratory practice (GLP) or quality assurance (QA).

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral administration

To obtain information on the absorption, distribution, rate and route of excretion and biotransformation of dithianon, studies were performed in adult male and female Sprague-Dawley rats (body weight 200 ± 50 g; 7–9 weeks old) using [^{14}C]dithianon and [^{13}C/^{14}C]dithianon (Figure 1).

In a preliminary excretion study, two rats (one male and one female) were given a single oral dose of [^{14}C]dithianon at a dose of 200 mg/kg bw. As the animals suffered from severe diarrhoea, the study was terminated. Subsequently, another two rats were given a single oral dose of 50 mg/kg bw. Urine was collected at 8, 24, 48, 72, 96 and 120 hours. Faeces were collected at 24-hour intervals up to 120 hours. Expired air was monitored at 24-hour intervals up to 48 hours.

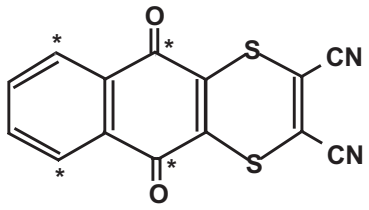
In the main study, oral doses of 10 mg/kg bw (low dose) and 50 mg/kg bw (high dose) were used; the 50 mg/kg bw dose was an effect level, the 10 mg/kg bw dose a no-effect level. Each animal in the test groups received approximately 370 kBq of [^{14}C]dithianon. The test substance was delivered in 1.0% (weight per volume [w/v]) sodium carboxymethylcellulose in water.

The test groups included in the blood kinetics, absorption, distribution, metabolism and excretion studies are shown in Table 1.

Total radioactive residues (TRR) in plasma, urine, cage washings and tissues were determined by liquid scintillation counting or combustion of aliquots to yield $^{14}CO_2$. Liquid scintillation counting was used to analyse trapped $^{14}CO_2$. The TRR in the carcasses were determined by digestion and liquid scintillation counting. The TRR in the faeces were determined by extraction and liquid scintillation counting of the faecal extracts. Thin-layer chromatography was used to characterize and identify extractable residue components.

In the preliminary study, it was found that faecal excretion was the predominant route (62.8% and 70.96% of the dose for males and females, respectively). No detectable radioactivity was found in the expired air.

Figure 1. Chemical structure of dithianon and position of radiolabel



* Indicates the position of ^{14}C and ^{13}C labels

Table 1. Test groups, number of animals, target dose rate and euthanasia time

Test groups	Radiolabel	Number of rats		Target dose (mg/kg bw)	Euthanasia at time post-dosing
		Male	Female		
Pilot excretion study (SOHD)	5, 6, 9, 10-[^{14}C]	1	1	50	At least 120 h
SOLD	5, 6, 9, 10-[^{14}C]	5	5	10	120 h
SOHD	5, 6, 9, 10-[^{14}C]	5	5	50	120 h
MOLD	5, 6, 9, 10-[^{14}C]	5	5	10	120 h after last dose
Pharmacokinetics (SOLD)	5, 6, 9, 10-[^{14}C]	5	5	10	240 h
Pharmacokinetics (SOHD)	5, 6, 9, 10-[^{14}C]	5	5	50	240 h
Tissue and organ distribution (SOLD)	5, 6, 9, 10-[^{14}C]	5	5	10	One male and one female at 6, 24, 48, 96 and 168 h
Biliary excretion (SOLD)	5, 6, 9, 10-[^{14}C]	3	3	10	48 h
Biliary excretion (SOHD)	5, 6, 9, 10-[^{14}C]	3	3	50	48 h
Metabolite identification (SOHD)	5, 6, 9, 10-[^{13}C/^{14}C]	5	5	50	120 h

From Holmsen (2009)
MOLD, multiple oral low dose; SOHD, single oral high dose; SOLD, single oral low dose

In the main study, the actual doses administered were 9.34–11.82 mg/kg bw for the 10 mg/kg bw groups and 46.44–54.45 mg/kg bw for the 50 mg/kg bw groups.

Following administration of a single oral dose of [^{14}C]dithianon, ^{14}C residues were observed in blood within 15 minutes, and mean peak plasma levels of 0.813–0.992 parts per million (ppm) for the low-dose groups and 3.81–3.89 ppm for the high-dose groups occurred at 6 hours. The differences between males and females were insignificant. The radioactive residues in the blood declined quickly, with an elimination half-life of approximately 46–57 hours for the low- and high-dose groups. The pharmacokinetic parameters following administration of a single oral dose of [^{14}C]dithianon are summarized in Table 2.

The extent of absorption of [^{14}C]dithianon by the rat following administration of single oral doses (low and high) was determined in a biliary excretion study. The percentage absorbed at 48 hours after dosing is shown in Table 3. The amount of absorption (sum of the amounts in urine, bile, gastrointestinal tract tissues, carcass and liver) was 52% and 48% of the administered dose for male and female rats in

Table 2. Pharmacokinetic parameters following a single oral dose of [^{14}C]dithianon

Pharmacokinetic parameter	Mean value (n = 5)			
	SOLD (10 mg/kg bw)		SOHD (50 mg/kg bw)	
	Male	Female	Male	Female
T_{max} (h)	6.0	6.0	6.0	6.0
C_{max} (g/g)	0.992	0.813	3.89	3.81
$T_{½\ elim}$ (h)	55.8	56.8	46.4	56.7
AUC (g·h/ml)	25.4	31.6	155.7	209.8
MRT (h)	NR	NR	NR	NR

From Holmsen (2009)
AUC, area under the blood concentration versus time curve calculated for the time interval between 0 and 240 h; C_{max}, maximum blood concentration; MRT, mean residence time; NR, not reported; SOHD, single oral high dose; SOLD, single oral low dose; $T_{½\ elim}$, elimination half-life; T_{max}, time of maximum blood concentration

Table 3. Extent of absorption following a single oral dose of [^{14}C]dithianon

Matrix	% of administered radioactive dose			
	SOLD (10 mg/kg bw)		SOHD (50 mg/kg bw)	
	Male	Female	Male	Female
Urine	31.38	30.10	33.02	23.54
Bile	11.59	9.52	7.21	7.49
Gastrointestinal tract	6.17	5.79	2.22	8.85
Carcass	2.36	2.64	1.11	6.53
Liver	0.05	0.03	0.02	0.03
Total absorption	51.55	48.08	43.58	46.44

From Holmsen (2009)
SOHD, single oral high dose; SOLD, single oral low dose

the low-dose bile group, respectively. For the high-dose bile group, the absorption amounted to 44% of the dose for male rats and 46% of the dose for female rats. The results indicate that a small portion (7–12%) of the dose eliminated in the faeces of intact animals was excreted via the bile. The remaining faecal excretion (50–60% of the dose) would appear to represent unabsorbed dithianon.

The distribution of the TRR in various tissues is presented in Tables 4 and 5. Results are reported for male and female rats at different time intervals following administration of 10 mg/kg bw. TRR distribution in tissues was examined at approximately 6, 24, 48, 96 and 168 hours post-dosing.

The TRR data from the low-dose groups showed that the highest ^{14}C residues were initially present in the gastrointestinal tract, kidneys and plasma. The highest TRR levels were observed at 6 hours post-dosing in all tissues. At 168 hours, the TRR were highest in the kidneys (0.127–0.149 ppm), gastrointestinal tract (0.028–0.059 ppm) and blood (0.026–0.041 ppm). The TRR in the remaining tissues were less than or equal to 0.026 ppm or below the limits of detection at 168 hours. In summary, the tissue retention of orally administered dithianon was low. Higher levels of radioactivity were found in the kidney and gastrointestinal tract. The accumulation of radioactivity was observed mainly in the gastrointestinal tract, kidneys and liver.

The details of the excretion balance are shown in Table 6. Within 48 hours after dosing, more than 90% of the applied dose was excreted in urine and faeces, the majority of radioactivity being eliminated via faeces (64% and 68% of dose for females and males, respectively). In urine, 26–27% of

Table 4. Total radioactive residues in tissues of male rats given 10 mg/kg bw

Matrix	TRR (ppm)				
	6 h	24 h	48 h	96 h	168 h
Adrenal glands	0.290	< 0.398	< 0.178	< 0.105	< 0.027
Bone marrow	0.337	< 0.151	< 0.231	< 0.173	< 0.176
Brain	< 0.016	< 0.011	< 0.012	< 0.010	< 0.010
Eyes	0.087	0.045	< 0.023	0.020	< 0.017
Fat	0.067	< 0.040	< 0.028	< 0.026	< 0.031
Gastrointestinal tract	97.0	3.00	0.307	0.085	0.028
Heart	0.327	0.0.052	0.018	0.018	0.022
Kidneys	2.73	0.813	0.359	0.268	0.127
Liver	0.558	0.101	0.037	0.023	0.010
Lungs	0.241	0.097	0.038	0.030	0.023
Muscle	0.060	0.022	< 0.012	< 0.009	< 0.008
Pancreas	0.156	0.050	0.031	0.025	0.026
Plasma	0.757	0.175	0.056	0.033	0.010
Spleen	0.085	0.034	0.018	< 0.015	0.015
Testes	0.102	0.031	0.015	0.013	< 0.008
Thyroid gland	< LOD	< LOD	< LOD	< LOD	< LOD
Whole blood	0.549	0.154	0.067	0.048	0.026

From Holmsen (2009)
LOD, limit of detection

the dose was recovered after 48 hours. Total recovery of radioactivity (including cage wash) reached 96.1% and 98.8% of the administered dose in the females and males, respectively. No sex-specific differences in the route or rate of excretion were observed.

A summary of the distribution of the radioactivity after administration of a single oral dose of 10 or 50 mg/kg bw to rats is presented in Table 7.

The excretion of radioactivity during a 5-day period following administration of a 10 or 50 mg/kg bw single dose of [^{14}C]dithianon and a 10 mg/kg bw repeated dose of dithianon is shown in Table 8. From this, it can be seen that the majority of the administered dose was excreted through the faeces (64.0–72.2%) and urine (26.7–31.4%).

After 14 daily oral doses of non-radioactive dithianon (10 mg/kg bw per day) followed by a single oral dose of [^{14}C]dithianon (10 mg/kg bw), the pattern of excretion of radioactivity in both males and females 120 hours post-dosing was very similar to that obtained from animals receiving single oral doses of [^{14}C]dithianon at both dose levels.

In conclusion, the biokinetics of dithianon were studied in rats following oral administration of ^{14}C-labelled dithianon at nominal doses of 10 and 50 mg/kg bw. The material balance from the preliminary study showed that dithianon was not metabolized to volatile compounds, including carbon dioxide, as negligible amounts of radioactivity (< 0.05% of dose) were detected in the expired air from treated animals during the 5-day period following treatment at 50 mg/kg bw. Therefore, it was not necessary to collect carbon dioxide or volatile organics in the excretion–retention study.

Orally administered dithianon was about 40–50% absorbed in rats. There were no substantial dose- or sex-related differences in the absorption, elimination or distribution of radioactivity following oral administration of [^{14}C]dithianon.

Table 5. Total radioactive residues in tissues of female rats given 10 mg/kg bw

Matrix	TRR (ppm)				
	6 h	24 h	48 h	96 h	168 h
Adrenal glands	< 0.257	0.183	< 0.009	< 0.155	< 0.073
Bone marrow	< 0.523	0.255	< 0.275	< 0.357	< 0.229
Brain	0.018	0.016	< 0.010	< 0.010	< 0.008
Eyes	0.057	0.066	< 0.020	0.016	< 0.021
Fat	< 0.034	< 0.032	< 0.025	< 0.023	< 0.019
Gastrointestinal tract	110	11.8	1.03	0.271	0.059
Heart	0.164	0.074	0.031	0.020	< 0.011
Kidneys	2.01	0.757	0.438	0.219	0.149
Liver	0.585	0.133	0.048	0.025	0.012
Lungs	0.325	0.116	0.051	0.030	0.017
Muscle	0.054	0.030	0.012	< 0.009	< 0.010
Ovaries	0.549	0.269	0.117	< 0.085	< 0.070
Pancreas	0.135	0.098	0.025	0.024	< 0.012
Plasma	0.754	0.239	0.096	0.035	< 0.011
Spleen	0.108	0.058	0.029	0.027	0.019
Thyroid gland	0.805	< LOD	< LOD	< LOD	< LOD
Uterus	0.256	0.116	0.036	0.019	< 0.015
Whole blood	0.519	0.225	0.114	0.066	0.041

From Holmsen (2009)
LOD, limit of detection

Table 6. Excretion of radioactivity in urine and faeces after oral administration in rats (50 mg/kg bw)

Matrix	Time interval (h)	Excretion of radioactivity (% of dose)	
		Male	Female
Urine	0–24	18.62	21.09
	24–48	7.36	6.29
	48–72	0.61	0.55
	72–96	0.33	0.16
	Total	26.92	28.09
Faeces	0–24	31.80	30.36
	24–48	36.14	34.02
	48–72	2.43	2.79
	72–96	0.27	0.34
	Total	70.64	67.51
Cage wash	—	1.22	0.46
Total		98.78	96.06

From Bross & Seiferlein (2009)

Table 7. Tissue distribution at maximum plasma level (T_{max} = 6 hours)

Matrix	% of dose			
	10 mg/kg bw		50 mg/kg bw	
	Male	Female	Male	Female
Liver	0.304	0.267	0.111	0.137
Kidney	0.425	0.353	0.120	0.105
Bone marrow	0.000 11	0.000 13	0.000 05	0.000 06
Plasma	0.132	0.061	0.027	0.042
Total	0.861	0.681	0.258	0.284

From Bross & Seiferlein (2009)

Table 8. Mean excretion and retention of radioactivity by male and female rats after a single oral dose of [^{14}C]dithianon at a dose level of 10 or 50 mg/kg bw and after 14 daily doses of dithianon followed by a single oral dose of [^{14}C]dithianon at a dose level of 10 mg/kg bw per day

Dose regime	Mean % excretion and retention of radioactivity					
	Single (10 mg/kg bw)		Single (50 mg/kg bw)		Repeated (10 mg/kg bw)	
	Male	Female	Male	Female	Male	Female
Tissues	0.08	0.20	0.11	0.18	0.17	0.15
Urine (0–120 h)	31.04	31.43	29. 93	31.28	30.80	26.66
Cage washing	0.11	0.16	0.60	0.68	0.27	0.37
Faeces (0–120 h)	65.98	64.03	66.67	65.44	67.04	72.15
Total recovery	97.21	95.82	97.30	97.57	98.28	99.33

From Hawkins et al. (1988)

The majority of the administered dose was recovered in faeces (64.0–72.2%) and urine (26.7–31.4%).

In the low-dose bile group, approximately 10% of the radioactivity was eliminated in the bile and 30% was eliminated in the urine, indicating that at least 40% of the applied dose was absorbed. In the high-dose bile group, a comparable percentage of the dose was absorbed. Of the absorbed dose, urinary excretion predominated over biliary excretion. There was no accumulation of radioactivity in tissues and, except for the gastrointestinal tract and kidneys, very little residue remained in the tissues at 168 hours post-dosing. The sex difference was not significant overall. Following oral administration, dithianon was absorbed quickly into the blood and reached a maximum concentration (C_{max}) at 6 hours in both the low-dose and high-dose groups. C_{max} was 0.813–0.992 g/g in the low-dose group and 3.81–3.89 g/g in the high-dose group. The elimination half-life ($T_{½ elim}$) was between 55.8 and 56.8 hours (low dose) and 46.4 and 56.7 hours (high dose). The area under the curve (AUC) values were 25.4–31.6 g·h/ml for the low dose and 155.7–209.8 g·h/ml for the high dose. The label was mostly excreted as polar metabolites and glucuronic acid conjugates in bile and urine, indicating an extensive metabolism of the parent compound. Residues in faeces consisted of polar metabolites, but there were negligible amounts of parent compound.

The studies described above (Hawkins et al., 1988; Hawkins & Elsom, 1993; Bross & Seiferlein, 2009; Holmsen, 2009) were conducted in compliance with GLP of the United States Environmental Protection Agency (USEPA), Japan, the Organisation for Economic Co-operation and Development (OECD), the European Commission (EC) and/or the United Kingdom. A QA statement was attached.

Table 9. Percentage of dithianon absorbed, remaining in the treated skin and removed from the site of application following a single topical application of [^{14}C]dithianon aqueous paste

Application rate (mg/cm^2)	Absorbed[a]		Treated skin		Skin swabs/gauze wash		Total
	%	mg equivalent[b]	%	mg equivalent[b]	%	mg equivalent[b]	%
0.16	0.32	0.006	3.14	0.06	95.6	1.83	99.1
1.66	0.27	0.054	1.91	0.38	101.0	20.10	103.2

From Bounds (1998)

[a] Urine, faeces, cage wash, carcass, blood and untreated skin.

[b] Calculated from the group mean achieved dose.

(b) Dermal application

The extent of absorption and excretion of radiolabelled [^{14}C]dithianon (AC 37114) up to 168 hours following a single topical application as an aqueous paste at two concentrations to male rats was determined. The low dose was approximately 0.16 mg/cm^2 [^{14}C]dithianon (equal to 8.0 mg/kg bw), and the high dose was approximately 1.7 mg/cm^2 [^{14}C]dithianon (equal to 79 mg/kg bw). The preparation was applied for 8 hours under semiocclusion with a stainless steel gauze held in place over the silicone saddle by surgical tape and vet-wrap bandage, and then the application site was washed with 1% Tween 80 in distilled water to remove any unabsorbed material.

The majority of the applied radioactivity (group mean > 95%) was recovered in the skin swabs and gauze washes for both the low- and high-dose preparations. Very little of the dose was absorbed (approximately 0.3%), and only a small proportion of the dose remained in the treated skin at the end of the experiment (3% and 2% for the low-dose and high-dose groups, respectively) for both dose preparations. Table 9 summarizes the findings.

In conclusion, [^{14}C]dithianon applied as an aqueous paste at two dose levels had a very low potential for dermal absorption in the rat. Only approximately 0.3% of the applied dose was absorbed.

The study was conducted in compliance with GLP, and a QA statement was attached (Bounds, 1998).

1.2 Biotransformation

The objective of this study was to investigate the metabolites of dithianon in excreta, selected tissues and plasma from male and female Sprague-Dawley (Crl:CD(SD)) rats after a single oral dose of 10 mg/kg bw (one low-dose group) or 50 mg/kg bw (two high-dose groups). In order to achieve the required specific activity, ^{14}C-labelled dithianon (96.1% radiochemical purity labelled in the 5 and 10 positions) was mixed with non-radiolabelled compound (^{12}C and ^{13}C) at a ratio of 1:1. Animals were dosed by gavage using a 0.5% aqueous solution of carboxymethylcellulose containing 1% Cremophor. The purity and the isotope ratio were checked by high-performance liquid chromatography (HPLC) and HPLC with tandem mass spectrometry (MS/MS) prior to dosing. The study was conducted between 12 January 2007 and 28 April 2009.

As dithianon is subject to extensive metabolic degradation, identification of its metabolites is challenging and has not been successfully achieved in previous investigations. Urine and faecal samples of one high-dose group were used for isolation and identification of metabolites. In one low-dose group and the other high-dose group, liver and kidney tissues as well as plasma of male and female rats were analysed for metabolites at maximum plasma levels of radioactivity.

More than 14 metabolites were detected in urine of rats given [^{14}C]dithianon as a single oral low dose, a single oral high dose and multiple oral low doses. However, identification of dithianon

Table 10. Summary of identified metabolites in urine, faeces, liver, kidney and plasma of rats

Metabolite designation	Urine	Faeces	Liver	Kidney	Plasma
Dithianon (M216F000)					
M216F001		x	x		
M216F002	x			x	x
M216F003	x				
M216F004		x	x		
M216F005		x			
M216F006	x				
M216F007		x			
M216F008	x	x	x	x	x
M216F009	x	x			
M216F011	x	x			
M216F012	x	x	x	x	x
M216F013	x				
M216F014	x				
M216F015	x	x	x	x	x
M216F016	x	x			
M216F017		x	x		
M216F018	x				
M216F019	x			x	
M216F020	x			x	x
M216F022	x			x	x
M216F023	x				
M216F024	x				
M216F025	x				
M216F026	x			x	
M216F027		x	x		

From Bross & Seiferlein (2009)

metabolites was not possible due to their detection in minor quantities. The sum of the detected metabolites approached about 25–30% of the dose. The patterns of metabolites detected in rat urine samples were very similar with respect to dose level or duration of administration.

The structures and codes of all identified metabolites in urine, faeces, liver, kidney and plasma are summarized in Table 10.

The HPLC chromatograms of urine of the first 48 hours were dominated by one major peak, representing 0.9–10% of the administered dose. This metabolite was identified as M216F020 (glucuronic acid conjugate of 1,4-dihydroxynaphthalene) and is the only one present in amounts above 2% of the dose. Besides this metabolite, the patterns indicated the presence of many other metabolites (up to 16 further peaks), which were all insignificant in quantitative terms, as none of them amounted to more than 2% of the dose, with most of them accounting for less than 1%. Among these, the following metabolites were identified: M216F006 (accounting for 0.30–1.69% of the dose), M216F022 (0.71–1.29%), M216F012/M216F019 (0.28–1.11%), M216F003 (0.21–0.49%) and M216F025 (0.36–0.50%).

Table 11. Composition of radioactivity in rat urine following a 50 mg/kg bw oral dose

Metabolite identity / designation	Time interval (h)	% of dose in urine	
		Male	Female
Identified			
M216F025	0–24	0.50	0.49
	24–48	0.38	0.36
M216F006	0–24	1.28	1.69
	24–48	0.42	0.30
M216F020	0–24	10.06	9.00
	24–48	2.86	0.92
M216F022	0–24	1.09	1.29
	24–48	0.71	0.76
M216F012/M216F019	0–24	0.52	1.11
	24–48	0.28	0.41
M216F026	0–24	0.96	n.d.
	24–48	0.20	n.d.
M216F008	0–24	n.d.	1.08
	24–48	n.d.	0.39
M216F003	0–24	n.d.	0.49
	24–48	0.21	0.38
M216F015	0–24	0.20	n.d.
	24–48	n.d.	n.d.
M216F002	0–24	n.d.	0.35
	24–48	n.d.	0.12
Total identified	0–48	19.67	19.14
Characterized by HPLC[a]			
Polar substances (0–20 min)	0–24	2.92	n.d.
	24–48	0.86	0.12
Semipolar substances (20–40 min)	0–24	1.09	5.32
	24–48	1.44	2.53
Non-polar substances (> 40 min)	0–24	n.d.	0.27
	24–48	n.d.	n.d.
Total characterized	0–48	6.31	8.24
Total identified and characterized	0–48	25.98	27.38

From Bross & Seiferlein (2009)

n.d., not detected

[a] Characterized by HPLC, meaning sum of all peaks in the respective retention time area from the chromatograms; each of them comprised not more than 1.3% of the dose, with most of them being below 1% of the dose.

The composition of radioactivity in urine samples in the 50 mg/kg bw group is given in Table 11. Only the time intervals 0–24 hours and 24–48 hours were considered, as the total excreted dose in all later sampling intervals was below 1%.

The acetonitrile extracts of faeces of the first 48 hours show a multitude of metabolites with up to 30 peaks. The patterns were dominated by five peaks, four of which could be identified. These were M216F012, accounting for 0.37–0.55% of the dose (sum of both tautomeric forms), M216F015,

accounting for 0.28–0.78% of the dose, and M216F027, which occurred in two isomeric forms that together represent between 0.99% and 1.95% of the dose. The unidentified peak, which was non-polar (retention time [t_R] = 60 minutes), accounted for 0.20–0.47% of the dose. Besides these five more dominant metabolites, many further peaks were present in the chromatograms; however, all of them were less significant in quantitative terms. Among these, the following metabolites were identified: M216F007 (accounting for 0.04–0.14% of the dose), M216F016 (0.06–0.13%), M216F004 (0.13–0.35%), M216F008 (0.09–0.18%), M216F001 (0.07–0.14%) and M216F017 (0.18–0.30%). The unchanged parent compound was not detected in the acetonitrile extracts.

The investigation of water, ammonia and microwave extracts as well as of the protease supernatants with two different HPLC systems characterized the released radioactivity as being composed of a multitude of metabolites with a broad spectrum of polarity, all present only in insignificant amounts. None of the patterns showed any sex-related differences. The composition of radioactivity in faecal samples is summarized in Table 12. Only the time intervals 0–24 hours and 24–48 hours were considered, as the total excreted dose in all later sampling intervals was below 3.2%. Total identified metabolites in faeces accounted for 5.8% of the dose in females and 7.3% in males. The total degree of identification and characterization amounted to 56% and 54% of the dose in males and females, respectively.

A summary of the composition of radioactivity in liver, kidney and plasma is presented in Table 13 for the 10 mg/kg bw dose group and in Table 14 for the 50 mg/kg bw dose group.

In conclusion, dithianon was rapidly and extensively metabolized to a very large number of mainly polar metabolites in rats. There were no fundamental sex differences in metabolism. As a result of its different functional groups (CO, CN, S), the molecule is exposed to chemical and enzymatic attacks at various sites of the molecule, leading to many reactive primary products that undergo further transformation by reacting with endogenous compounds (e.g. glucosidation, sulfonylation, acetylation, coupling to amino acids). A key step in its metabolic degradation is the oxidation of the sulfur atoms, leading to a cleavage of the dithiine ring and, after further reactions, resulting in a 1,4-naphthoquinone moiety that bears various substituents, such as carboxy groups, hydroxyl groups (M216F024, in this case already conjugated with glucuronic acid), amino acids (M216F016, bearing a glycine moiety) or amino groups (M216F012) and sulfonate groups (M216F005) or both (M216F007) at the quinone part of the ring system. The amino groups can be further acetylated (M216F003, M216F011).

Many identified metabolites show a reduction of the 1,4-naphthoquinone moiety to a 1,4-dihydroxynaphthalene ring that is still bearing the above-mentioned substituents (M216F002, M216F006, M216F013, M216F014, M216F018). One hydroxyl group of this dihydroxynaphthalene ring is further conjugated with glucuronic acid, leading, for example, to the metabolites M216F020, M216F022, M216F023 or M216F025. M216F020 represents the glucuronide of the 1,4-dihydroxynaphthalene ring without any further substituents and was found to be the only metabolite of quantitative significance (occurring in amounts > 5% of the dose in urine).

Furthermore, opening of the dithiine ring and transformation into a thiazine ring (M216F004, M216F008 with an additional carboxy group) were observed, as well as further conjugation with glycine (M216F019). Another metabolite after cleavage of the dithiine ring is M216F027, a copper(II) complex in which two dimercapto-naphthoquinone units bind to one copper ion, which was found to be a main metabolite of faeces. However, it cannot be excluded that this metabolite represents an artefact generated during sample workup. Whereas the vast majority of the identified metabolites show the cleavage of the dithiine ring, demonstrating the importance of this degradation route for dithianon, some metabolites still maintaining the intact three-ring system (i.e. without cleavage of the dithiine ring) were identified. Metabolite M216F026 represents a glucuronide of the intact parent compound having a glucuronic acid unit at the 1,4-dihydroxynaphthalene moiety, which was yielded by prior reduction of the naphthoquinone system. The substitution of the carbonitrile groups in the

Table 12. Composition of radioactivity in faeces of the 50 mg/kg bw dose group

Metabolite identity / designation	Time interval (h)	% of dose in faeces	
		Male	Female
Identified (in acetonitrile extracts)			
M216F007	0–24	0.14	0.07
	24–48	0.04	0.04
M216F016	0–24	0.13	n.d.
	24–48	n.d.	0.06
M216F012 (sum of both tautomers)	0–24	0.47	0.37
	24–48	0.55	0.45
M216F004	0–24	0.33	0.13
	24–48	0.32	0.35
M216F008	0–24	0.18	0.16
	24–48	0.09	0.13
M216F001	0–24	0.14	0.07
	24–48	0.08	n.d.
M216F017	0–24	0.28	0.18
	24–48	0.18	0.30
M216F015	0–24	0.69	0.28
	24–48	0.78	0.38
M216F027 (sum of both isomers)	0–24	0.99	1.12
	24–48	1.95	1.73
Total identified	0–48	7.34	5.82
Characterized by HPLC (in acetonitrile extracts)[a]			
Polar substances (0–20 min)	0–24	0.09	n.d.
	24–48	n.d.	n.d.
Semipolar substances (20–40 min)	0–24	0.44	0.36
	24–48	0.37	0.15
Non-polar substances (> 40 min)	0–24	1.46	2.14
	24–48	1.27	1.70
Characterized by extraction			
Water extracts	0–24	9.89	9.57
	24–48	10.61	8.89
Ammonia extracts	0–24	6.94	7.30
	24–48	9.06	9.54
Protease supernatants	0–24	1.51	1.29
	24–48	1.71	1.61
Microwave extracts	0–24	2.59	2.58
	24–48	2.80	3.43
Total characterized	0–48	48.74	48.56
Total identified and characterized	0–48	56.08	54.38
Final residue (RRR)	0–48	7.52	8.49

From Bross & Seiferlein (2009)

n.d., not detected; RRR, residual radioactive residue

[a] Characterized by HPLC means sum of all peaks (all of them being below 0.5% dose) in the respective retention time area from the patterns of acetonitrile extracts.

Table 13. Composition of radioactivity in tissues and plasma of the 10 mg/kg bw dose group

Metabolite identity / designation	% of dose					
	Liver		Kidney		Plasma	
	Male	Female	Male	Female	Male	Female
Identified (in acetonitrile extracts)						
M216F012/M216F019	0.006	0.004	0.006	n.d.	0.005	n.d.
M216F004	0.011	0.007	n.d.	n.d.	n.d.	n.d.
M216F008	n.d.	0.005	0.004	0.008	0.010	0.001
M216F001	0.009	0.006	n.d.	n.d.	n.d.	n.d.
M216F017	n.d.	0.003	n.d.	n.d.	n.d.	n.d.
M216F015	0.013	0.011	0.004	0.006	n.d.	0.004
M216F027	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M216F020	n.d.	n.d.	0.059	0.020	0.034	n.d.
M216F022	n.d.	n.d.	0.009	n.d.	0.013	n.d.
M216F026	n.d.	n.d.	0.010	0.003	n.d.	n.d.
M216F002	n.d.	n.d.	0.004	0.003	n.d.	n.d.
Total identified	0.039	0.036	0.096	0.040	0.062	0.005
Characterized by HPLC[a]						
Acetonitrile extracts (sum of further peaks)	0.080	0.073	0.026	0.050	0.030	0.028
Characterized by extraction						
Water extracts	0.106	0.049	0.171	0.115	0.029	0.020
Total characterized	0.186	0.122	0.197	0.165	0.059	0.048
Total identified and characterized	0.225	0.158	0.293	0.205	0.121	0.053
Final residue (RRR)	0.003	0.002	0.006	0.004	0.000	0.000

From Bross & Seiferlein (2009)
n.d., not detected; RRR, residual radioactive residue
[a] Characterized by HPLC means sum of all further peaks that were not identified in the patterns of acetonitrile extract.

intact parent molecule by carboxy and amino moieties (M216F015, M216F017) was observed as a further metabolic conversion of dithianon.

The main transformation steps observed are oxidation of the sulfur atom, cleavage of the dithiine ring, resulting in a naphthoquinone moiety (bearing various substituents at the quinone part of the ring system), and reduction of the naphthoquinone ring to a 1,4-dihydroxynaphthalene moiety, with further glucuronidation and substitution of the carbonitrile groups by amino and carboxy units in the intact parent molecule. The metabolic pathway is shown in Figure 2.

The studies described above in this section (Schlueter & Memmesheimer, 1994a,b; Bross & Schreiner, 2007; Bross & Seiferlein, 2009) were conducted in compliance with GLP according to guidelines of the USEPA, Japan, the OECD, the EC and/or the United Kingdom. A QA statement was attached.

2. Toxicological studies

2.1 Acute toxicity

The results of the studies on the acute toxicity of dithianon are presented in Table 15. All the studies were conducted according to OECD, EC and USEPA guidelines and complied with GLP.

Table 14. Composition of radioactivity in tissues and plasma of the 50 mg/kg bw dose group

Metabolite identity / designation	% of dose					
	Liver		Kidney		Plasma	
	Male	Female	Male	Female	Male	Female
Identified (in acetonitrile extracts)						
M216F012/M216F019	0.002	n.d.	n.d	n.d.	n.d.	0.001
M216F004	0.003	0.003	n.d	n.d.	n.d.	n.d.
M216F008	0.004	0.002	n.d	0.002	0.001	0.001
M216F001	n.d.	n.d.	n.d	n.d.	n.d.	n.d.
M216F017	n.d.	0.002	n.d	n.d.	n.d.	n.d.
M216F015	0.005	0.004	n.d.	0.001	0.002	0.002
M216F027 (sum of both isomers)	n.d.	0.003	n.d.	n.d.	n.d.	n.d.
M216F020	n.d.	n.d.	0.021	0.014	n.d.	n.d.
M216F022	n.d.	n.d.	0.004	0.004	n.d.	n.d.
M216F026	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M216F002	n.d.	n.d.	n.d.	0.003	0.001	n.d.
Total identified	0.014	0.014	0.025	0.024	0.004	0.004
Characterized by HPLC[a]						
Acetonitrile extracts (sum of further peaks)	0.027	0.035	0.005	0.005	0.011	0.019
Characterized by extraction						
Water extracts	0.039	0.048	0.045	0.040	0.006	0.013
Total characterized	0.066	0.083	0.050	0.045	0.017	0.032
Total identified and characterized	0.080	0.097	0.075	0.069	0.021	0.036
Final residue (RRR)	0.001	0.001	0.001	0.002	0.000	0.000

From Bross & Seiferlein (2009)
n.d., not detected; RRR, residual radioactive residue
[a] Characterized by HPLC means sum of all further peaks that were not identified in the patterns of acetonitrile extract.

2.2 Short-term studies of toxicity

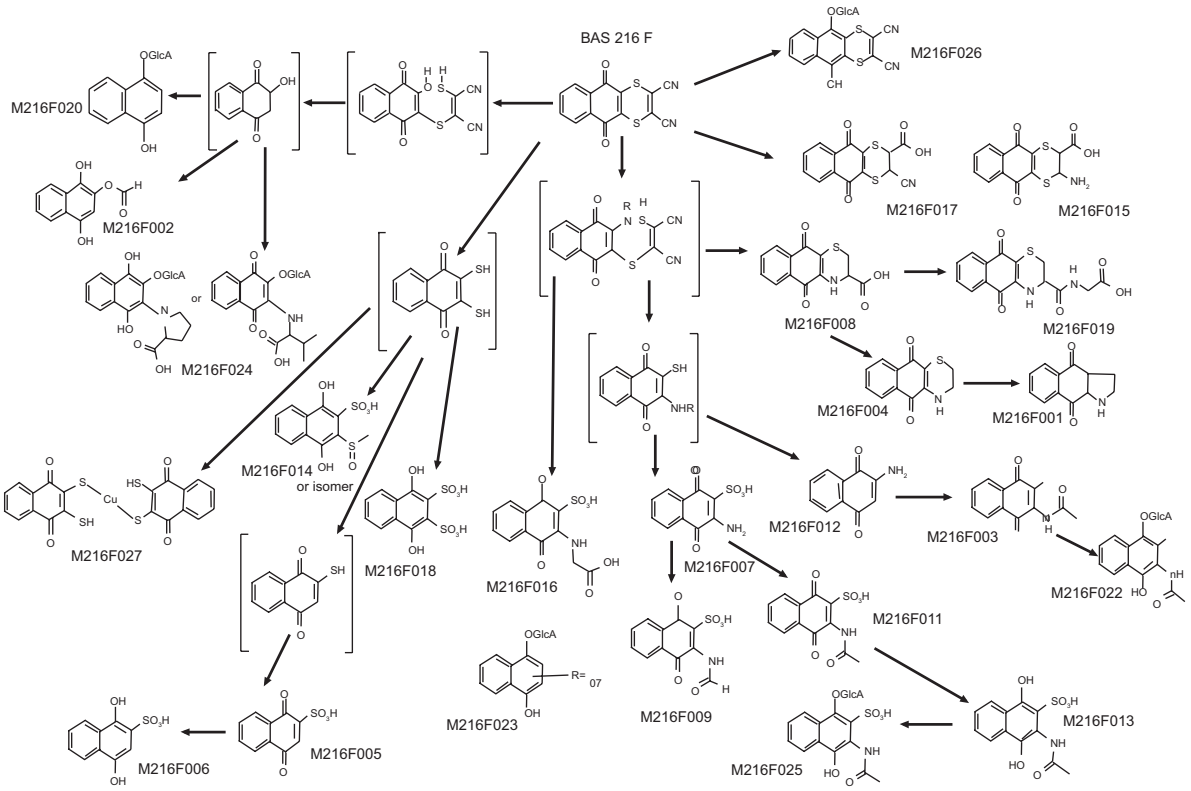
(a) Oral administration

Mice

In a 4-week range-finding study performed to determine the dose levels for a subsequent 80-week study, dithianon technical (92% active ingredient) was fed to four groups of Crl:CD-1(ICR) BR strain mice (six of each sex per group) at a dietary concentration of 0, 100, 500 or 1000 ppm (equivalent to 0, 15, 75 and 150 mg/kg bw per day for both sexes) for 28 consecutive days. The mice were 41 days old, and body weights were between 23 and 30 g (males) and 20 and 24.1 g (females). Feed consumption, body weight and substance intake were determined. The animals were examined regularly for signs of toxicity or mortality. Haematology and thyroid hormone levels were determined after 4 weeks on test. Surviving animals were subjected to gross pathological assessment, and liver and kidneys were weighed (females), followed by histopathological examination of the liver and kidney (females).

No mortalities and no treatment-related clinical signs were observed during the study. Body weights in the 1000 ppm group (males and females) were consistently lower than in the controls throughout the study period, with overall decreased body weight gains of approximately 35% for

Figure 2. Metabolic pathway for dithianon in rats



[BAS 216 F is dithianon]

From Bross & Seiferlein (2009)

Table 15. Acute toxicity of dithianon

Species	Strain	Sex	Route	Batch No., purity (%)	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Results	Reference
Rat	Wistar	F	Oral	DT2003-2035, 96.9	~300	—	[a]	Gamer & Leibold (2005b)
Rat	Wistar	M & F	Oral	Charge 15C/86, 92 ± 0.6	720 (M) 678 (F) 702 (M & F)	—	[b]	Ullmann (1987a)
Rat	Wistar	M & F	Dermal	DT2003-2035, 96.9	> 2000	—	[c]	Gamer & Leibold (2005c)
Rat	Wistar	M & F	Dermal	15C/86, 92 ± 0.6	> 2000	—	[c]	Ullmann (1986a, 1993)
Rat	Wistar	M & F	Inhalation, 4 h (dust exposure, nose only)	DT 2003-2035, 96.9	—	M, 0.31 F, 0.58 Combined, 0.33 (MMAD ranged from 2.0 to 3.2 µm)	—	Gamer & Leibold (2005a)

Table 15 (continued)

Species	Strain	Sex	Route	Batch No., purity (%)	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Results	Reference
Rat	Wistar	M & F	Inhalation, 4 h (aerosol exposure, nose only)	30/84, 94.7	—	M, 1.8 F, 2.4 Combined, 2.1 (MMAD ranged from 3 to 7 µm)	—	Ullmann (1984a)
Rabbit	New Zealand White	M & F	Acute dermal irritation / corrosivity	DT2003-2035, 96.9	—	—	Non-irritant	Remmele & Leibold (2005a)
Rabbit	New Zealand White	M & F	Primary skin irritation (4 h occlusive application)	15C/86, 92 ± 0.6	—	—	Non-irritant	Ullmann (1986b)
Rabbit	New Zealand White	M & F	Acute eye irritation	DT2003-2035, 96.9	—	—	Severe eye irritation potential	Remmele & Leibold (2005b)
Rabbit	New Zealand White	M & F	Primary eye irritation	15C/86, 92 ± 0.6	—	—	Irreversibly irritating to the rabbit eye (not rinsed)	Ullmann (1987b)
Guinea-pig	HsdPoc: DH	F	Skin sensitization effects (guinea-pig maximization)	DT2003-2035, 96.9	—	—	Has a sensitizing effect on the skin of the guinea-pig	Gamer & Leibold (2005d)
Guinea-pig	Dunkin-Hartley	M & F	Delayed hypersensitivity in the albino guinea-pig (guinea-pig maximization)	30/84, 94.7	—	—	Not considered to be a skin sensitizer after challenge or rechallenge	Ullmann (1984b)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male; MMAD, mass median aerodynamic diameter

[a] Three dose levels were administered (50, 300 and 500 mg/kg bw). Clinical observations in the 500 and 300 mg/kg bw groups revealed impaired and poor general state, dyspnoea, staggering, piloerection, smeared fur and diarrhoea; findings were observed from hour 1 until the end of study day 6 after administration. Clinical observations in the 50 mg/kg bw groups revealed impaired general state, dyspnoea and diarrhoea; findings were observed from hour 1 after administration until the end of study day 1. The mean body weights of the surviving animals of the administration groups increased throughout the study period. During necropsy, findings in the animals that died comprised few or several black erosions/ulcers in the glandular stomach and slight red discoloration of contents of the small intestine (500 mg/kg bw, two females; 300 mg/kg bw, two females). No macroscopic pathological abnormalities were noted in one animal of the 500 mg/kg bw group that died and in the surviving animals examined at the end of the observation period (300 mg/kg bw, four females; 50 mg/kg bw, six females).

[b] Signs of toxicity, which were first observed at 1 or 2 h after dosing in all groups, included sedation and ruffled fur. Other clinical observations, which were seen at 400 mg/kg bw and above, included curved body position, dyspnoea and diarrhoea.

[c] No systemic clinical observations were noted in the animals. At the application site, very slight or well-defined erythema and a distinct brown discoloured application area were observed on days 1 and 2.

both males and females, compared with controls, at study termination. Feed consumption was decreased in the 1000 ppm group (males and females) only during the first week of treatment, compared with controls. There were no effects on feed consumption or body weights in the other treatment groups.

In the 1000 ppm group (males and females) and in the 500 ppm group (females only), haemoglobin concentrations were lower than in the controls, indicating a slight anaemia for these animals. There were no other treatment-related effects on haematology. However, females of the two higher dose groups showed a dose-related decrease in plasma triiodothyronine (T_3) and thyroxine (T_4) levels, whereas treated males from the same groups showed a dose-related decrease in plasma T_4 levels only. No other effects on thyroid hormone parameters were observed. A protocol amendment was added to measure liver and kidney weights in females, but not in males. The reason for the amendment is not specified in the study report. However, it may be related to the reductions in haemoglobin that occurred at two dose levels in the females and were more pronounced than those that occurred at only one dose level in males. There were no treatment-related effects on organ weights in females. Microscopic examination of the liver and kidney of females revealed an increased incidence of iron deposition in Kupffer cells of the liver (haemosiderin) in six of six females in the 1000 ppm group and in four of six females in the 500 ppm group. Haemosiderin deposition was slightly increased in severity at 1000 ppm compared with 500 ppm. It was concluded that 500 ppm (75 mg/kg bw) should be selected as the high dose for an 18-month study, because it was associated with slight toxicity that would probably not alter the normal lifespan of animals. The no-observed-adverse-effect level (NOAEL) was 100 ppm (equivalent to 15 mg/kg bw per day), based on slight anaemia and histopathological effects in the livers of females at 500 ppm.

The study was conducted prior to the implementation of GLP, but a QA statement was attached (Brown, 1987).

Rats

In a preliminary range-finding study, dithianon technical (batch/purity not reported) was fed to four groups of Sprague-Dawley rats (four of each sex per group) at a dietary concentration of 0, 315, 1250 or 5000 ppm (equivalent to 0, 30, 119 and 476 mg/kg bw per day for both sexes) for 28 consecutive days. Feed consumption and body weight were determined. The animals were examined regularly for mortality or signs of toxicity. After 4 weeks on test, surviving animals were subjected to gross pathological assessment, followed by histopathological examinations of the gastrointestinal tract. According to protocol, haematology and clinical chemistry parameters and organ weights were not measured. Animals in the 5000 ppm group exhibited a loss of body weight and markedly reduced feed consumption. Consequently, at 5000 ppm, seven of eight animals died during weeks 3 and 4. There was a decrease in overall body weight gain of 31% for males and 46% for females in the 1250 ppm group compared with the controls, with a resultant decrease in mean absolute body weight at study termination. There was a reduction in feed consumption in the 1250 ppm group (males and females) during the first half of the study, compared with controls, but feed consumption in this group recovered to near control levels during the second half of the study. There were no effects on body weights or feed consumption in the 315 ppm group. Macroscopic examination showed empty gastrointestinal tracts and lung congestion for animals in the 5000 ppm group that died during the study. At terminal necropsy, animals in the 1250 ppm group showed intestines containing liquid orange-coloured contents. There were no other abnormalities in any of the groups. Microscopic examination of the selected tissues revealed no treatment-related histopathological effects in any of the groups. The NOAEL for dithianon was 315 ppm (equivalent to 30 mg/kg bw per day), based on decreased overall body weight gains and decreased feed consumption for males and females at 1250 ppm. Based on these results, the highest dietary concentration of 1000 ppm was selected for use in a 2-year dietary toxicity study in order to avoid undue anorexia.

The study was pre-GLP, and no QA statement was attached (Wheldon & Frohberg, 1966).

Dithianon technical (purity 92%; batch No. 15C/86) was fed to four groups of Sprague-Dawley rats (10 of each sex per group) for 90 days at a dietary concentration of 0, 30, 180 or 1080 ppm (equal

to 0, 2.5, 14.6 and 86.7 mg/kg bw per day for males and 0, 3.0, 16.3 and 99.5 mg/kg bw per day for females). Another group of 10 animals of each sex was assigned to a 4-week recovery period. This dose schedule was selected on the basis of a 14-day pilot study performed with dithianon dietary concentrations of 180, 1080, 1440 and 2160 ppm. At 1440 ppm, the body weights were decreased by 5% in the males and 18.6% in the females, and at 2160 ppm, by 31.8% in the males and 25.8% in the females, after 2 test weeks. In view of this, the highest dose selected was 1080 ppm in the 90-day study. Test substance/diet mixtures were freshly prepared each day. The age of male and female rats at the start of the study was 32–33 days and 34–35 days, respectively. Body weights at study initiation were 91–124 g (males) and 89–124 g (females). Feed consumption, body weight and substance intake were determined weekly. Ophthalmoscopy was performed prior to treatment and at 13 weeks. The animals were examined for mortality or signs of toxicity at least once a day. During week 13 on test, the animals were subjected to a comprehensive clinical examination that included urinalysis, clinicochemical and haematological examinations. All animals were subjected to gross pathological assessment, followed by histopathological examinations.

No treatment-related mortalities occurred during the treatment period. No overt signs of toxicity were observed that could be attributed to administration of the test material. Feed consumption values for males and females in all treated groups were comparable to those of controls during the treatment period. There were no treatment-related effects on body weight in the 30 or 180 ppm dose groups. Mean body weights at study termination were statistically significantly lower in males and females receiving 1080 ppm, and overall body weight gains were decreased by 14% in males and 11% in females, compared with controls.

An evaluation of haematological parameters showed that in animals of both sexes receiving 1080 ppm, erythrocyte count, packed cell volume and haemoglobin concentration were slightly but statistically significantly reduced, and the reticulocyte count was slightly but statistically significantly increased, compared with controls. Other haematological parameters were within normal limits. An evaluation of clinical chemistry parameters showed slight increases in blood urea nitrogen in males and females (statistically significant in males only) at 1080 ppm. Increases in total protein and albumin levels in males and decreases in T_4 levels in males and in chloride levels in females were also noted at 1080 ppm. T_3 levels were statistically significantly decreased in the males at all tested doses. The decrease in T_4 levels was not considered to be toxicologically significant because of the absence of any corresponding pathological findings or thyroid gland weight changes. All animals showed normal urinary status following 90 days of treatment. For the 1080 ppm group, absolute and relative (to body weight) kidney, liver and adrenal gland weights were elevated in males and females compared with controls, whereas absolute heart weight was statistically significantly decreased in females in comparison with controls. Gross necropsy, ophthalmoscopy and microscopic evaluation performed by the original study pathologist revealed no treatment-related findings after the 90-day exposure. However, an independent microscopic re-evaluation of the kidney tissue sections from females in the 1080 ppm group (Grasso, 1991a) revealed tubular epithelial cell damage (hydropic degeneration) in 4 of 10 animals and tubular epithelial cell hyperplasia (tubular hyperplasia) in 2 of 10 animals, compared with 0 of 10 control animals for both findings.

In conclusion, the NOAEL in rats fed dithianon for 90 days was 180 ppm (equivalent to 14.6 mg/kg bw per day), based on decreased overall body weight gains, increased absolute and relative kidney, liver and adrenal gland weights, and changes in haematological and clinical chemistry parameters in males and females, as well as histopathological findings of renal tubular epithelial cell degeneration and regenerative hyperplasia (females only), at 1080 ppm.

This GLP study was conducted according to OECD and USEPA test guidelines (Leuschner, 1987, 1990a,b).

Dogs

Dithianon technical (purity 92 ± 0.6%; batch No. 15 c/86) was fed to four groups of Beagle dogs (four of each sex per group) for 90 days at a dietary concentration of 0, 40, 200 or 1000 ppm (equal to 0, 0.63, 2.95 and 12.6 mg/kg bw per day, respectively, for males and 0, 0.67, 3.0 and 12.6 mg/kg bw per day, respectively, for females). The test substance/diet mixtures were prepared daily. At the beginning of the experiment, the dogs were 8 months (male) and 12 months (females) of age. Initial body weights were 9.2–11.6 kg (males) and 7.8–10.4 kg (females). Feed consumption and general health status of the animals were determined daily, and body weights were measured once a week. Clinical chemistry and haematological examinations were carried out once before treatment began and during weeks 6 and 13. Urinalyses were carried out before treatment and during weeks 5 and 12. Ophthalmological examinations were carried out before dosing commenced and during week 13 of treatment. All animals were subjected to complete gross and histopathological examinations, and selected organs were weighed.

All animals survived up to study termination, and no overt signs of toxicity were observed that could be attributed to administration of the test material. For animals in the 1000 ppm group, feed consumption and body weight were slightly, but consistently, reduced throughout the study, compared with controls. Overall body weight gains were decreased during the treatment period for males and females at 1000 ppm. By study termination, the mean body weight gain in male and female controls was 1.1 kg (11% increase), whereas the mean body weight gain in 1000 ppm males was 0.1 kg (1% increase). The 1000 ppm females lost approximately 0.35 kg (4%) by study termination. There were no treatment-related effects on feed consumption, body weight or body weight gain in the other dose groups. There were no treatment-related effects on haematological or urinalysis parameters. Analysis of clinical chemistry parameters showed that alkaline phosphatase activity was statistically significantly elevated in the 1000 ppm group in both males and females, compared with controls. In the 1000 ppm group, absolute and relative kidney weights were slightly and generally statistically significantly increased in both males and females. However, as there were no macroscopic or microscopic changes in this organ that were attributable to treatment and because there were no corresponding blood or urinary clinical chemistry changes, slight increases in kidney weight were difficult to interpret as to their clinical significance. Three addenda were submitted with this study (Leuschner, 1989, 1990c, 1991). None of the addenda changed the interpretation of the study, except one addendum noted that there was a statistically significant increase in thromboplastin time for females at 1000 ppm.

It was concluded that the subchronic (90-day) NOAEL for dithianon technical in dogs was 200 ppm (equal to 2.95 mg/kg bw per day), based on a decrease in body weight (females only), decreased body weight gain (males) and decreased feed consumption, increases in alkaline phosphatase activity and kidney weights for males and females and an increase in thromboplastin time for females at 1000 ppm. The study was conducted in compliance with GLP regulations of the United States Food and Drug Administration, the OECD and Japan. A QA statement was attached (Neuwann, 1987).

Four groups of Beagle dogs (four of each sex per group) were fed dithianon technical (purity 92%; batch No. 15C/86) at a dietary concentration of 0, 40, 200 or 1000 ppm (equal to 0, 1.6, 7.3 and 37.1 mg/kg bw per day, respectively, for males and 0, 1.6, 7.9 and 37.5 mg/kg bw per day, respectively, for females) for 52 consecutive weeks. The age of the dogs at study commencement was 5–7 months, including an acclimatization period of 5 weeks. At the start of treatment, the males weighed 5.6–8.8 kg and females 5.45–8.6 kg. Diets were prepared once per week. Feed consumption for all groups, including the controls, was approximately 3-fold greater than in the 90-day study in dogs. Thus, dogs in the 52-week study received higher doses compared with dogs in the 90-day study. The differences in feed consumption between the 52-week and the 90-day studies may be related to the use of different diets. Feed consumption and the state of health of the animals were determined daily,

and body weights were measured once a week. Haematological examinations as well as urinalyses were carried out once before treatment began and during study weeks 13, 26 and 52. Clinical chemistry examinations were carried out once before treatment began and during study weeks 26 and 52. Ophthalmological examinations were carried out before the beginning of treatment and during study weeks 13 and 52. All animals were subjected to complete gross and histopathological examinations, and selected organs were weighed.

All animals survived to study termination, and there were no clinical observations that were considered to be related to treatment. Body weight gains for males and females at 1000 ppm were reduced by 20% and 15%, respectively, compared with controls, at week 13 of the study. Overall body weight gains for treatment weeks 1–52 were decreased by 20% in the males at 1000 ppm, compared with the controls. Females in the 1000 ppm group gained more weight than the controls during weeks 26–52 of treatment (Tables 16 and 17), resulting in no effect on overall body weight gain at study termination. In the 1000 ppm group, there was a statistically significant decrease in feed consumption in males (13%) between weeks 1 and 13 of treatment. There were slight decreases in feed consumption of approximately 7% during the remainder of the treatment period in males and females at 1000 ppm. There were no treatment-related effects on body weight gain or feed consumption in the other treatment groups. No treatment-related ocular lesions were observed.

Haematological evaluations (Table 18) revealed a mild anaemia at the 1000 ppm concentration in both sexes. This was characterized by slight reductions in haemoglobin concentration, red blood cell count and packed cell volume, which were apparent by week 13 of treatment and reached statistical significance by week 26, compared with the controls. The reductions in red blood cell parameters were still present at week 52. At week 52, the 1000 ppm treatment level showed immature red blood cells in three of four males and in three of four females, and the mean platelet counts and white blood cell counts were slightly elevated (males only). In contrast, anaemia was not observed at the 1000 ppm level in the 90-day feeding study in dogs. The anaemia observed in males and females at 1000 ppm at week 13 in the 52-week study may be due to the approximately 3-fold greater feed consumption in the 52-week study, which resulted in a higher dose, compared with the 90-day study.

Clinical chemistry findings (Table 19) consisted of statistically significant increases in alkaline phosphatase activity at 1000 ppm in both sexes at weeks 26 and 52. There were no treatment-related effects noted in the urinalyses.

Organ weight data (Table 20) showed increases in absolute and relative liver, kidney and thyroid gland weights for males and females in the 1000 ppm group, compared with controls. For the liver and kidneys, these increases were statistically significant, except for the absolute organ weights in males. For the thyroid glands, only the increase in relative organ weight in females was statistically significant. Increases in thyroid gland weights, noted in Table 20 for 1000 ppm males and females, may be related to hepatic enzyme induction.

There were no treatment-related macroscopic findings at necropsy. Microscopically, hepatocellular hypertrophy was observed in the 200 ppm (females only) and 1000 ppm groups (males and females) (Table 21). In the absence of any degenerative changes in hepatocytes, this was considered to reflect possible enzyme induction. For males and females at 1000 ppm, there were occasional foci of pigmented histiocytes (Kupffer cells) in the sinusoids, which stained positively for iron. In addition, a slight increase in brown pigment in the proximal tubular epithelial cells in the kidney was observed in the 200 ppm (females only) (although there is no statistically significant variation in weight of kidney) and 1000 ppm groups (males and females).

In view of the above, the NOAEL for dithianon fed to dogs in their diet for 52 weeks was 200 ppm, equal to 7.3 mg/kg bw per day, based on increases in kidney and liver weights in both sexes at 1000 ppm.

The study was conducted in accordance with GLP standards as per the USEPA. A QA statement was attached (Clay, 1991).

Table 16. Mean body weights of dogs after 13, 26 and 52 weeks of dithianon administration in the diet

Dietary concentration (ppm)	Mean body weight (kg)					
	Males			Females		
	Week 13	Week 26	Week 52	Week 13	Week 26	Week 52
0	10.01	10.41	11.05	8.73	9.36	9.33
40	9.56	9.94	10.30	8.16	8.95	9.38
200	10.40	11.19	11.30	8.28	8.55	8.73
1000	9.26	9.64	10.16	8.34	8.79	9.59

From Clay (1991)

Table 17. Body weight gain in dogs over selected intervals of dithianon administration

Dietary concentration (ppm)	Mean body weight gain (kg)			
	Weeks 1–13	Weeks 13–26	Weeks 26–52	Weeks 1–52
Males				
0	2.14	0.40	0.64	3.18
40	2.18	0.38	0.36	2.91
200	2.79	0.79	0.11	3.69
1000	1.71	0.38	0.53	2.61
Females				
0	1.84	0.64	−0.04	2.44
40	1.76	0.79	0.43	2.98
200	1.60	0.28	0.18	2.05
1000	1.58	0.45	0.80	2.83

From Clay (1991)

(b) Dermal application

Rats

In a 21-day dermal toxicity study in rats, dithianon technical (purity 92%; batch No. 15C/86) was suspended in polyethylene glycol 200 and applied dermally to Sprague-Dawley rats (five of each sex per group) at a dose of 40, 200 or 1000 mg/kg bw per day for 6 hours per day for 22 consecutive days. Control rats (10 of each sex per dose) received daily dermal doses of the vehicle alone. A dose volume of 4 ml/kg bw was applied to shaven skin, covered by a gauze patch held in place by an occlusive wrap of tin foil and an elastic bandage. At the end of each treatment period, test sites were wiped with moist gauze and dried. Animals were about 8–10 weeks old at the time of initiation of treatment; males weighed 286.9–359 g, and females, 186–223.9 g. Skin reactions were recorded daily. Feed consumption and body weight were determined weekly. The state of health was checked at least daily. Clinicochemical and haematological examinations of samples taken on day 23 were carried out. At necropsy, all animals were assessed by gross pathological examination, and selected organs were weighed. Histopathological examinations were also performed on all animals.

There were no mortalities during the study, and daily dermal exposure to dithianon at doses up to 1000 mg/kg bw per day did not cause any behavioural changes or clinical signs of toxicity. Animals receiving dithianon showed signs of skin irritation at the application site from about day 6. These signs

Table 18. Changes in haematological parameters after dietary administration of dithianon to dogs for 52 weeks

Dietary concentration (ppm)	Mean haematology values											
	Males						Females					
	Hb (g/dl)	RBC (10^6/mm^3)	PCV (%)	MCHC (g/dl)	L (%)	N (%)	Hb (g/dl)	RBC (10^6/mm^3)	PCV (%)	MCHC (g/dl)	L (%)	N (%)
Week 26												
0	15.8	6.68	45.8	34.5	3.93	6.23	16.6	6.82	47.7	34.9	3.60	6.68
40	16.2	6.66	46.7	34.6	3.88	6.28	15.8	6.40	45.3	34.7	5.18*	7.73
200	15.9	6.60	46.4	34.4	4.35	7.43	15.8	6.42	45.5	34.7	3.95	7.40
1000	13.9*	5.86*	41.0*	33.9*	4.20	7.83	14.1**	5.88**	41.8**	33.7*	5.18*	10.08**
Week 52												
0	16.5	7.41	48.9	33.8	3.10	5.58	17.9	7.64	52.2	34.4	3.63	7.03
40	17.04	7.38	50.2	33.9	3.45	7.38*	16.5	7.03	48.8	33.8	3.83	7.38
200	17.4	7.39	51.0	34.0	3.60	6.98	16.8	7.17*	49.2	34.2	3.30	7.00
1000	15.0	6.64*	45.4	33.1	4.25DR*	8.83*	14.3	6.23	43.5*	32.9*	4.20	8.10

From Clay (1991)
Hb, haemoglobin; L, lymphocytes; MCHC, mean corpuscular haemoglobin concentration; N, neutrophils; PCV, packed cell volume; RBC, red blood cells; * $P < 0.05$; ** $P < 0.01$; DR, significant using the dose–response test

Table 19. Changes in clinical chemistry parameters after dietary administration of dithianon to dogs for 52 weeks

	Dietary concentration (ppm)							
	0		40		200		1000	
	M	F	M	F	M	F	M	F
Week 26								
AP (IU/l)	121	158	102	115	166	154	200DR*	338*
BUN (mg/dl)	16	18	13	17	14	15	12*	11***
Creatinine (mg/dl)	1.0	1.0	0.9	0.9	0.9	0.9	0.8**	0.8***
Total protein (g/dl)	5.3	5.5	5.4	5.6	5.8*	5.7	5.7	5.7
Albumin (g/dl)	3.4	3.5	3.4	3.5	3.6	3.6	3.6	3.6
Week 52								
AP (IU/l)	94	147	89	77	98	113	186	265*
BUN (mg/dl)	15	16	11	15	15	14	11	11**
Creatinine (mg/dl)	1.0	1.0	0.9**	1.0	0.9	0.9*	0.8***	0.8***
Total protein (g/dl)	5.9	5.8	5.9	6.1	6.3	5.8	6.3	6.1
Albumin (g/dl)	3.0	3.1	2.9	3.1	3.3	3.0	3.2DR*	3.1

From Clay (1991)
AP, alkaline phosphatase; BUN, blood urea nitrogen; F, female; IU, international units; M, male; * $P < 0.05$;** $P < 0.01$; *** $P < 0.001$; DR, significant using the dose–response test

Table 20. Mean organ weight after dietary administration of dithianon to dogs for 52 weeks

	Dietary concentration (ppm)							
	0		40		200		1000	
	M	F	M	F	M	F	M	F
Body weight (g)	10 738	9200	10 075	9088	11 125	8638	10 025	9413
Adrenal (left)								
Absolute weight (g)	0.549	0.668	0.615	0.612	0.530	0.585	0.588	0.608
Relative (to body) weight (%)	0.0051	0.0073	0.0061	0.0067	0.0048	0.0069	0.0059	0.0068
Adrenal (right)								
Absolute weight (g)	0.620	0.689	0.616	0.649	0.503	0.600	0.588	0.601
Relative (to body) weight (%)	0.0058	0.0075	0.0061	0.0071	0.0045	0.0071	0.0059	0.0066
Kidney (left)								
Absolute weight (g)	24.934	20.844	27.418	20.865	25.750	22.017	30.752	28.301*
Relative (to body) weight (%)	0.2319	0.2268	0.2720	0.2295	0.2330	0.2539	0.3079***	0.3067**
Kidney (right)								
Absolute weight (g)	24.259	20.486	26.314	21.533	25.552	21.449	30.586	25.936DR**
Relative (to body) weight (%)	0.2254	0.2234	0.2615	0.2372	0.2309	0.2480	0.3061***	0.2805**
Liver								
Absolute weight (g)	315.1	268.7	309.3	311.3	326.2	322.6	443.3	452.0DR*
Relative (to body) weight (%)	2.93	2.93	3.08	3.42	2.95	3.73	4.38*	4.81*
Testes (left)								
Absolute weight (g)	10.529	—	10.202	—	9.851	—	10.351	—
Relative (to body) weight (%)	0.983	—	0.1015	—	0.0885	—	0.1029	—
Testes (right)								
Absolute weight (g)	10.773	—	9.946	—	9.543	—	10.39	—
Relative (to body) weight (%)	0.1007	—	0.989	—	0.0857	—	0.1006	—
Ovary (left)								
Absolute weight (g)	—	0.569	—	0.805	—	0.438	—	0.916
Relative (to body) weight (%)	—	0.0060	—	0.0088	—	0.0050	—	0.0110
Ovary (right)								
Absolute weight (g)	—	0.456	—	0.631	—	0.438	—	0.601
Relative (to body) weight (%)	—	0.0049	—	0.0070	—	0.0040	—	0.0073
Thyroid (left)								
Absolute weight (g)	0.407	0.350	0.420	0.417	0.385	0.346	0.491	0.511
Relative (to body) weight (%)	0.0038	0.0039	0.0041	0.0045	0.0035	0.0040	0.0049	0.0054DR*
Thyroid (right)								
Absolute weight (g)	0.424	0.351	0.378	0.347	0.388	0.363	0.463	0.481
Relative (to body) weight (%)	0.0040	0.0038	0.0037	0.0038	0.0035	0.0043	0.0047	0.0051DR*

From Clay (1991)
F, female; M, male; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; DR, significant using the dose–response test

include erythema, oedema, atonia, fissuring and desquamation. At terminal sacrifice (day 23), body weight gain was reduced by 47% for males (only) receiving 1000 mg/kg bw per day, compared with controls. For females (only) at 1000 mg/kg bw per day, haematological parameters revealed a slight

Table 21. Histopathological data in dogs after administration of dithianon for 52 weeks

	Incidence of finding							
	Dietary concentration (ppm)							
	0		40		200		1000	
	M	F	M	F	M	F	M	F
Number of animals examined	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
Liver								
Hepatocyte pigment	4	4	4	4	4	4	4	4
Histiocyte pigment	0	0	0	0	0	0	3	3
Hepatocellular hypertrophy	0	0	0	0	0	4	4	4
Intracellular inclusions	0	0	0	0	0	0	0	1
Kidney								
Mineralization	4	3	4	4	4	4	4	4
Pigment	4	1	3	1	4	3	4	4
Basophilic tubules	0	0	0	0	0	0	1	0
Focal nephropathy	0	0	1	0	0	0	0	1
Hyaline droplets	0	0	0	0	1	0	0	0
Testis								
Atrophy	1	—	0	—	0	—	2	—
Orchitis	0	—	0	—	0	—	1	—
Thyroid								
Leukocyte foci	2	0	1	0	0	0	0	0
Cyst	2	0	0	1	1	0	2	0
Lymphocytic thyroiditis	0	0	0	1	0	0	1	2
Parathyroid								
Cyst	1	0	2	2	1	2	0	2

From Clay (1991)
F, female; M, male

anaemia, as indicated by slight decreases in red blood cell count, haemoglobin concentration and packed cell volume, compared with controls. For males and females, no treatment-related effects were noted for feed consumption, clinical chemistry and urinalysis parameters, absolute and relative (to body weight) organ weights or gross necropsy findings. For females (only) at 1000 mg/kg bw per day, histopathology revealed slight nephrotoxicity, as indicated by increased incidences of regenerative basophilic tubules of moderate severity with occasional mitotic figures and luminal eosinophilic material.

In conclusion, the NOAEL in this 21-day dermal toxicity study was 200 mg/kg bw per day, based on decreased body weight change for males and on slight anaemia and histopathological effects in the kidneys of females at 1000 mg/kg bw per day (the highest dose tested).

The study was conducted prior to implementation of GLP. A QA statement was attached (Brown, 1989).

(c) Exposure by inhalation

Rats

In a 14-day inhalation study, three groups of Wistar rats (five of each sex per group) were exposed via nose-only inhalation to dithianon technical (purity not reported) (administered as an

aerosol in water) for 6 hours per day, 5 days per week, for 2 weeks (total of 10 exposures) at a concentration of 110, 310 or 1070 mg/m^3 (0.110, 0.310 or 1.070 mg/l) (gravimetric). Additionally, one control group of five rats of each sex was exposed to filtered air, and another control group of five rats of each sex was exposed to water (vehicle). An intermediate-dose recovery group of five rats of each sex was exposed to dithianon technical at a concentration of 310 mg/m^3 (0.310 mg/l) for 6 hours per day, 5 days per week, for 2 weeks and then allowed a 2-week recovery period. Rats were about 7 weeks old at the start of the experiment.

Feed consumption was measured daily, whereas body weights were measured prior to exposure, every fourth day and on the day of sacrifice. The animals were examined individually before and during the exposures and also following exposure each day. Animals in the recovery group were further examined daily during the observation period of 14 days to determine their general health, behaviour and condition. After 2 weeks on test, the animals were subjected to a comprehensive clinical examination, which included urinalysis, clinicochemical and haematological examinations. All animals were subjected to gross pathological assessment, including organ weights of selected tissues, followed by histopathological examinations. The mass median aerodynamic diameters (MMADs) for all concentrations of dithianon technical tested were obtained during the second and fourth hours of each exposure. The MMADs for the 110, 310 and 1070 mg/m^3 groups were 3.1, 3.5 and 4.1 µm, respectively. These particle size data indicate that respirable concentrations of the test material were administered to the test animals, thereby validating the delivery method used in this study.

Results from this study showed neither mortality nor treatment-related clinical signs of toxicity and no treatment-related effects on feed consumption, body weight or organ weights. Additionally, no treatment-related effects on haematological, clinical chemistry or urinalysis parameters and no treatment-related macroscopic or microscopic findings were noted in this study.

Based on these results, the no-observed-adverse-effect concentration (NOAEC) from this study is 1070 mg/m^3 (1.07 mg/l) (gravimetric), the highest concentration tested. In addition, based on route-to-route extrapolation, the NOAEC of 1070 mg/m^3 (1.07 mg/l) from the 14-day inhalation toxicity study in rats can be converted to a systemic NOAEL using the following equation:

> NOAEC (mg/l) × (% inhalation absorption) × breathing rate (l/h) × exposure time (h/day) / average body weight of the rat during the treatment period (kg) = NOAEL (mg/kg bw per day)

Assuming a 100% inhalation absorption value, a breathing rate for the Wistar rat of 8.46 litres per hour, an exposure time of 6 hours per day and an average body weight for the Wistar rat from the 14-day inhalation toxicity study of 0.0865 kg, a systemic NOAEL of 628 mg/kg bw per day is obtained:

> (1.07 mg/l) × (1) × (8.46 l/h) × (6 h/day) / 0.0865 kg = 627.9 mg/kg bw per day

The study was conducted prior to implementation of GLP. A QA statement was not attached (Bhide, 1986).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

A chronic dietary study was conducted to evaluate the effect of dithianon on the incidence and morphology of tumours following administration to mice for 80 weeks. Groups of Crl:CD-1(ICR)BR strain mice (51 of each sex per group) received dithianon technical (purity 92%; batch No. 15C/86) in the diet at a concentration of 0, 20, 100 or 500 ppm (equivalent to 0, 3, 15 and 75 mg/kg bw per day) for 80 consecutive weeks. Diets were prepared weekly. At the start of the study, the animals were 46 days old and weighed 20.8–34 g (males) and 19.5–26.8 g (females). Feed consumption and body weight were determined once a week for the first 16 weeks and at 4-week intervals thereafter. Water

consumption was determined daily during weeks 23–26. The animals were examined for mortality twice a day and for signs of toxicity once a day; moreover, comprehensive clinical examinations of the animals were performed once a week. Blood smears were prepared from all mice killed during the study and from all surviving mice at weeks 52 and 78 and at terminal sacrifice (week 80). At the end of the treatment period, all surviving animals were sacrificed and subjected to gross pathological assessment, and selected organs were weighed. Histopathological examinations were performed on all control and high-dose animals and on animals that died during the study. In addition, lungs, liver and kidneys and any macroscopically abnormal tissues were examined from surviving low- and intermediate-dose animals.

There was a slight increase in mortality during the last 25 weeks of the study in the males receiving 500 ppm, compared with the controls. There was no effect on mortality in females. Survival rates at study termination in the animals receiving 0, 20, 100 and 500 ppm were 73%, 75%, 71% and 63%, respectively, for males and 80%, 86%, 71% and 82%, respectively, for females.

Females in the 500 ppm group had an increased incidence of fur staining, compared with the controls. There were no treatment-related effects on body weight gain, feed consumption or water consumption in males or females. Absolute and relative kidney weights were increased in a concentration-related manner in the 100 and 500 ppm groups in males and females, and these increases were generally statistically significant.

An evaluation of haematological parameters showed no treatment-related effects on differential white blood cell count. According to protocol, erythrocyte parameters were not measured. Microscopic examination revealed an exacerbation of spontaneous chronic nephropathy in males at 500 ppm and in females at 100 and 500 ppm. For males at 500 ppm, there was an association between the observed kidney damage (chronic nephropathy) and an increased incidence of early mortality, indicating that the maximum tolerated dose (MTD) was exceeded at 500 ppm. There were no unusual tumours noted in any of the treatment groups. The incidence and spectrum of the spontaneous background tumours observed were comparable among all groups.

In conclusion, the NOAEL for chronic toxicity was 20 ppm (approximately 3 mg/kg bw per day), based on increased absolute and relative kidney weights for males and females and an exacerbation of spontaneous chronic nephropathy for females at 100 ppm. Dithianon was not carcinogenic to mice at a dietary concentration of 500 ppm, which exceeded the MTD. Thus, the NOAEL for carcinogenicity in this study was 500 ppm (equivalent to 75 mg/kg bw per day).

This study was conducted in compliance with GLP, and a QA statement was attached (Brown, 1990).

Rats

In a 2-year carcinogenicity study, Sprague-Dawley (Crl:CD(SD)BR) rats (70 of each sex per group) received diets containing dithianon technical (purity 92%; batch No. 15C/86) at a concentration of 0, 20, 120 or 600 ppm (equivalent to 0, 1, 6 and 30 mg/kg bw per day) for a period of 104 weeks. Satellite groups of animals (20 of each sex per dose) were used in the chronic toxicity evaluations. At the initiation of the study, the animals were 46–47 days old and weighed 162.7–254.8 g (males) and 135.9–199.2 g (females). Feed consumption and body weight were determined once a week for the first 16 weeks and at 4-week intervals thereafter. Water consumption was determined daily for the satellite animals during weeks 44–47. The animals were examined for mortality and overt toxicity once a day, and comprehensive clinical examinations were performed once a week. Ophthalmological examinations were carried out prior to the start of the study and at week 102. Urinalyses, clinicochemical and haematological examinations were carried out during weeks 13, 26, 52, 78 and 103 using 20 animals of each sex per group when possible. All animals were subjected to gross pathological assessment, and selected organs were weighed. Histopathological examinations were performed on all control and high-dose animals; animals that died during the study; lungs, liver,

kidneys and thyroid glands and any macroscopically abnormal tissues from low- and intermediate-dose animals; and tissues from low- and intermediate-dose animals for any treatment-related changes that were noted in the high-dose group.

Survival rates at study termination for the animals receiving 0, 20, 120 and 600 ppm were 37%, 43%, 29% and 29%, respectively, for males and 34%, 54%, 51% and 62%, respectively, for females. Clinical observations showed a progressive increase in the incidence of fur staining and rough haircoat during the course of the study in 600 ppm females. Body weight gain and feed consumption were slightly decreased in 600 ppm males during the initial months of the study. The overall body weight gain depression was approximately 12% for males receiving 600 ppm. In females receiving 600 ppm, body weight gain and feed consumption were decreased throughout the duration of the study; overall body weight gain was depressed 25–32%, compared with controls, beginning at week 13. This deviation indicated that the high dose level of 600 ppm exceeds the MTD for female rats (Tables 22 and 23).

Haematological evaluations (Table 24) consistently showed slight decreases in haemoglobin, red blood cell counts and packed cell volume in animals receiving 600 ppm. Clinicochemical determinations (Table 25) revealed consistent increases in γ-glutamyl transpeptidase in 600 ppm males towards the end of the study. Blood urea nitrogen values were increased consistently in 600 ppm males and females beginning at week 52 as well as at week 13 for 600 ppm males. In addition, inorganic phosphorus concentration was increased in 600 ppm males at termination, compared with controls. There was a consistently decreased cholesterol concentration in animals receiving 600 ppm. At termination of the study, an assay of thyroid hormones (thyroid stimulating hormone, T_4 and T_3) showed a decreased T_3 level, but not a decreased T_4 level, in the 600 ppm male group. Urinalysis revealed an increase in epithelial cells on examination of urinary sediments in 600 ppm females at week 26 and an increased protein content in 600 ppm males and females during the second half of the study. Organ weight analysis (Table 26) showed that absolute and relative kidney weights and relative liver weights of 600 ppm animals were increased.

At terminal necropsy, a higher incidence of gross kidney lesions (pallor, cysts, masses and an irregular surface) was noted in the 600 ppm animals. Histopathological examination confirmed that the kidney was the target organ for the toxicity of dithianon. The treatment-related renal effects consisted of an exacerbation of a chronic and progressive glomerulonephropathy and tubular nephrosis, which occur spontaneously in rats. At the 600 ppm level, predominantly in males (16/69), the effects had progressed to end-stage kidneys with extensive cortical destruction. Although no statistically significant treatment-related effects on survival were evident in male rats, the incidence of morbidity and mortality associated with renal and urogenital tract lesions (especially in males with end-stage kidneys) was increased at 600 ppm. In fact, half of the cases of end-stage kidneys in the 600 ppm males were considered to have been the primary cause of death. In addition, increased incidences of parathyroid hyperplasia, urogenital tract infections, aortic mineralization and arteritis in the testes and pancreas were observed at 600 ppm in males, which were probably secondary to the chronic renal effects (Table 27).

Data on the incidence of neoplastic changes in rats after chronic administration of dithianon are provided in Table 28. The incidence of renal adenoma and carcinoma was increased in high-dose females when compared with the respective control group.

For female rats, an independent review (Grasso, 1991b) of all female kidney sections from the rat carcinogenicity/chronic toxicity study was conducted. This evaluation showed that the high concentration level (600 ppm) in the rat carcinogenicity study induced tissue necrosis (increased incidences of severe glomerulonephropathy [9/70 versus 0/70 in controls], glomerular sclerosis [21/70 versus 4/70 in controls] and fibrosis [23/70 versus 3/70 in controls]) (Table 29). This re-evaluation also revealed an increase in the incidence of chronic progressive nephropathy in 120 ppm and 600 ppm females.

Table 22. Mean body weight in rats after 72, 84 and 104 weeks of dithianon administration

Dietary concentration (ppm)	Mean body weight (g)					
	Males			Females		
	Week 72	Week 84	Week 104	Week 72	Week 84	Week 104
0	681.4	695.4	647.1	422.7	457.6	463.9
20	688.2	705.3	676.2	428.1	454.8	458.8
120	689.5	705.1	692.0	434.9	459.1	475.6
600	655.5	648.7	589.5	338.6	364.0	371.9

From Brown (1991)

Table 23. Statistically significant differences in body weight gain of rats after dithianon administration

Dietary concentration (ppm)	Sex	Mean body weight (g)				
		Weeks 0–12	Weeks 12–24	Weeks 24–36	Weeks 36–48	Weeks 48–60
120	Males	** +	—	—	—	—
600		* +	—	—	—	—
120	Females	*	**	—	—	—
600		***	***	***	***	**

From Brown (1991)
+ Increasing with dose; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 24. Changes in haematological parameters after chronic administration of dithianon to rats

Parameter	Dietary concentration (ppm)							
	0		20		120		600	
	M	F	M	F	M	F	M	F
Week 52								
MCV (fl)	55.5	58.3	54.7	58.7	54.0*	58.6	51.9***	57.7
MCH (pg)	18.2	19.4	18.1	19.4	17.9	19.6	17.6	19.3
MCHC (g/dl)	32.8	33.3	33.1	33.1	33.1*	33.4	34.0***	33.4
RBC (10^6/cm^3)	8.24	7.28	8.11	7.29	8.17	7.10	8.23	6.96**
Hb (g/dl)	15.0	14.1	14.7	14.1	14.6	13.9	14.5	13.4***
PCV (%)	45.7	42.4	44.3	42.8	44.1*	41.6	42.6***	40.1***
Week 103								
MCV (fl)	58.6	63.1	57.3	60.8**	58.1	61.2*	57.3	60.5**
MCH (pg)	19.6	21.4	19.2	20.7	19.5	20.9	19.2	20.6
MCHC (g/dl)	33.4	33.8	33.5	34.0	33.6	34.2	33.5	34.1
RBC (10^6/cm^3)	7.63	7.10	7.58	7.13	7.68	7.02	7.00	7.01
Hb (g/dl)	14.9	15.1	14.5	14.7	15.0	14.7	13.3**	14.5
PCV (%)	44.7	44.6	43.3	44.3	44.5	42.9	39.7**	42.3

From Brown (1991)
F, female; Hb, haemoglobin; M, male; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cells; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 25. Changes in clinical chemistry parameters after chronic administration of dithianon to rats

Parameter	Dietary concentration (ppm)							
	0		20		120		600	
	M	F	M	F	M	F	M	F
Week 52								
GGT (IU/l)	3	3	3	2	6	4	7	3
BUN (mg/dl)	13	16	13	17	12	16	14**	20**
Cholesterol (mg/dl)	128	143	135	134	135	136	97**	90***
AP (IU/l)	110	56	118	52	147*	51	109	52
P (mg/dl)	4.7	3.5	4.9	3.9	5.0	3.7	5.0	4.1
Glucose (mg/dl)	76	109	95	118	101**	125	117***	110
CPK (IU/l)	652	279	542	360	360	222	270*	270
Week 103								
GGT (IU/l)	6	5	7	5	7	4	14**	8
BUN (mg/dl)	16	14	15	14	16	15	19**	19*
Cholesterol (mg/dl)	90	90	109	105	106*	91	82	75*
AP (IU/l)	89	48	105	41	131	51	101	67
P (mg/dl)	4.5	4.6	4.6	4.0**	4.7*	4.7	6.0***	4.9
Glucose (mg/dl)	72	74	91*	88*	89*	88*	105***	88*
CPK (IU/l)	520	569	563	491	240**	562	178**	327
Week 105								
T_3 (ng/dl)	55.2	58.6	54.8	62.3	46.4	63.6	36.6**	49.1
T_4 (g/dl)	2.2	1.6	2.0	1.6	2.5	1.8	2.1	1.4
TSH (ng/ml)	3.1	2.2	3.1	1.9	2.1	2.2	4.1	2.3

From Brown (1991)
AP, alkaline phosphatase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; GGT, γ-glutamyl transferase; IU, international units; P, phosphorus; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid stimulating hormone; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The severity of the chronic progressive nephropathy was also increased in a concentration-related manner at 120 and 600 ppm. Furthermore, there was evidence of a secondary, proliferative syndrome, which was limited to the females receiving 600 ppm. The proliferative findings consisted of a late manifestation of chronic tubular hyperplasia (atypical hyperplasia), preneoplastic lesions (proliferative tubules), benign tumours (tubular adenomas) and malignant tumours (adenocarcinoma) (Table 30).

The NOAEL for the late proliferative process in which the atypical hyperplasia progresses to proliferative tubules and in turn to tubular adenomas and adenocarcinomas in female rats was 120 ppm, compared with 600 ppm (highest dose tested) in male rats. Thus, for female rats, there was a steep dose–response relationship for the induction of neoplastic lesions. There were no other treatment-related tendencies observed in the type, distribution or incidence of other tumours.

The NOAEL for chronic toxicity was 20 ppm (equivalent to 1 mg/kg bw per day), based on histopathological kidney lesions (females) at 120 ppm. The NOAEL for carcinogenicity was 120 ppm (equivalent to 6 mg/kg bw per day), based on increased incidences of preneoplastic lesions (proliferative tubules), benign tumours (tubular adenomas) and malignant tumours (adenocarcinomas) in females at 600 ppm (equivalent to 30 mg/kg bw per day).

Importantly, the proliferative process in female rats demonstrates a progression of hyperplastic and preneoplastic lesions to neoplasia following long-term exposure to the compound at an

Table 26. Variations in organ weights after chronic administration of dithianon to rats

	Dietary concentration (ppm)							
	0		20		120		600	
	M	F	M	F	M	F	M	F
Body weight (g)	621.9	453.0	605.0	455.0	671.4	415.3	545.2**	321.4***
Liver								
Absolute weight (g)	14.139	10.539	13.726	10.177	14.698	9.502	15.130	10.316
Relative (to body) weight (%)	2.2740	2.3459	2.2800	2.2458	2.2260	2.2927	2.8233**	3.2884**
Kidney (left)								
Absolute weight (g)	1.724	1.199	1.703	1.182	1.849	1.169	1.994	1.413
Relative (to body) weight (%)	0.2769	0.2679	0.2828	0.2672	0.2851	0.2880	0.3741***	0.4544***
Kidney (right)								
Absolute weight (g)	1.870	1.225	1.697	1.211	1.848	1.219	2.030	1.603
Relative (to body) weight (%)	0.3009	0.2737	0.2815	0.2737	0.2843	0.2981	0.3791***	0.5052***
Adrenal (left)								
Absolute weight (g)	0.037	0.049	0.039	0.039	0.082	0.035	0.041	0.037
Relative (to body) weight (%)	0.0060	0.0112	0.0064	0.0086	0.0109	0.0085	0.0079	0.0119
Adrenal (right)								
Absolute weight (g)	0.032	0.065	0.047	0.038	0.035	0.030	0.037	0.043
Relative (to body) weight (%)	0.0051	0.0147	0.0077	0.0084	0.0053	0.0072	0.0070	0.133
Brain								
Absolute weight (g)	2.252	2.016	2.213	2.009	2.231	1.999	2.247	1.984
Relative (to body) weight (%)	0.3625	0.4453	0.3670	0.4561	0.3441	0.4934	0.4234*	0.6301***
Testis (right)								
Absolute weight (g)	2.014	—	1.531	—	1.989	—	1.607	—
Relative (to body) weight (%)	0.325	—	0.2512	—	0.3135	—	0.2869	—
Testis (left)								
Absolute weight (g)	2.005	—	1.629	—	1.784	—	2.232	—
Relative (to body) weight (%)	0.3215	—	0.2673	—	0.2833	—	0.3933	—
Ovary (right)								
Absolute weight (g)	—	0.037	—	0.095	—	0.043	—	0.034
Relative (to body) weight (%)	—	0.082	—	0.0208	—	0.0103	—	0.0102
Ovary (left)								
Absolute weight (g)	—	0.070	—	0.066	—	0.037	—	0.106
Relative (to body) weight (%)	—	0.154	—	0.138	—	0.089	—	0.0335
Thyroid (left)								
Absolute weight (g)	0.022	0.016	0.021	0.016	0.017	0.015	0.025	0.014
Relative (to body) weight (%)	0.0035	0.0039	0.0030	0.0039	0.0028	0.0033	0.0041	0.0042
Thyroid (right)								
Absolute weight (g)	0.038	0.014	0.021	0.015	0.019	0.016	0.028	0.014
Relative (to body) weight (%)	0.0054	0.0032	0.0030	0.0034	0.0031	0.0034	0.0046	0.0045

From Brown (1991)
F, female; M, male; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 27. Non-neoplastic findings in rats after chronic administration of dithianon

	Dietary concentration (ppm)							
	0		20		120		600	
	M	F	M	F	M	F	M	F
Kidney and urinary tract								
Renal lesion as cause of demise	0/44	0/46	0/40	0/33	0/50	0/34	11/50	0/28
Urogenital tract lesion as cause of demise	2/44	1/46	0/40	0/33	0/50	1/34	6/50	0/28
Aorta								
Mineralization	1/70	0/69	0/39	0/33	0/50	0/34	6/70	0/70
Seminal vesicle								
Vesiculitis	3/68	—	2/43	—	2/48	—	11/69	—
Parathyroid								
Hyperplasia	0/66	0/69	0/65	0/66	0/64	0/64	8/65	0/64
Prostate								
Prostatitis	6/70	—	5/40	—	6/49	—	14/69	—
Testis								
Atrophy	16/70	—	13/47	—	6/54	—	23/70	—
Arteritis	2/70	—	1/47	—	3/54	—	17/70	—
Leydig cell hyperplasia	2/70	—	4/47	—	2/54	—	6/70	—
Pancreas								
Arteritis	2/69	0/67	0/39	0/33	0/50	1/32	8/69	1/69
Fatty atrophy	1/69	2/67	0/39	2/33	1/50	0/32	5/69	1/69
Kidney								
Glomerulonephropathy	19/70	18/70	25/68	25/68	23/70	33/70	25/69	40/69
Tubular nephrosis	0/70	0/70	0/68	0/68	0/70	3/70	31/69	29/69
Proliferative tubules	0/70	0/70	0/68	0/68	0/70	3/70	0/69	9/69
End-stage kidney	0/70	0/70	0/68	0/68	0/70	0/70	16/69	2/69
Cyst formation	2/70	3/70	1/68	0/68	1/70	0/70	11/69	3/69

From Brown (1991)
F, female; M, male

excessively toxic dose (600 ppm), which exceeded the MTD. Moreover, the histological morphology depicting the cell of origin (proximal tubular epithelial cell) is common to all of the proliferative lesions (i.e. atypical hyperplasia, proliferative tubules, tubular adenoma and adenocarcinoma). Moreover, it appears likely that a secondary mechanism involving repeated episodes of nephrotoxicity in female Sprague-Dawley rats eventually leads to the induction of kidney tumours following long-term (2-year) dietary exposure to dithianon at a concentration exceeding the MTD. The results from the two short-term mechanistic studies cited in section 2.6 and the 90-day subchronic and 2-year carcinogenicity rat studies support the weight of evidence that the increased incidence of kidney tumours in female rats occurs at an excessively toxic dose and is a secondary proliferative response to this toxicity. It was considered that this mechanism is not relevant to humans at potential levels of exposure to dithianon.

The study complied with GLP requirements, and a QA statement was attached (Brown, 1991; Grasso, 1991b).

Table 28. Neoplasms (benign and malignant) that occurred in rats after chronic administration of dithianon

	Incidence of neoplasms							
	Dietary concentration (ppm)							
	0		20		120		600	
	M	F	M	F	M	F	M	F
Kidney								
B-adenoma	0/70	0/70	1/68	0/68	0/70	0/70	0/69	10/69
M-carcinoma	0/70	0/70	1/68	0/68	0/70	0/70	0/69	2/69
M-sarcoma	0/70	1/70	0/68	0/68	0/70	0/70	0/69	0/69
B-lipomatous tumour	0/70	0/70	2/68	0/68	0/70	0/70	1/69	0/69
N-metastatic tumour	0/70	2/70	1/68	1/68	1/70	0/70	2/69	0/69
Skin								
Squamous cyst	6/70	1/70	7/58	1/56	4/62	0/51	11/70	1/70
B-papilloma	5/0	1/70	10/58	2/56	3/62	0/51	13/70	0/70
Seminal vesicle								
N-metastatic tumour	0/68	—	0/43	—	0/48	—	1/69	—
Testis								
B-Leydig cell tumour	7/70	—	5/47	—	3/54	—	7/70	—
Prostate								
N-metastatic tumour	0/70	—	0/40	—	0/49	—	1/69	—
Parathyroid								
B-adenoma	1/66	1/69	0/65	0/66	1/64	0/64	1/65	0/64
Pancreas								
B-islet cell adenoma	6/69	1/67	2/39	1/33	3/50	2/32	1/69	2/69

From Brown (1991)
B, benign; F, female; M, male; malignant; N, lymph node

Another independent review of all kidney slides from the 2-year carcinogenicity study in rats was performed by Dr Gordon Hard, a pathology expert with special expertise in nephrotoxicity, to get a more detailed picture of the type of histopathological changes induced in the rat kidney after 2 years of dietary treatment. The aim of the review was to provide a more detailed histological description based on in-depth microscopic examination and to attempt to define a mode of action underlying the observed increase in tumour incidence occurring only in the high-dose female rats. Kidneys from all animals in the control, low-dose, mid-dose and high-dose female groups and control and high-dose male groups were examined. Kidneys from a major portion of the mid-dose males were also evaluated, as well as those from the two renal tumour–bearing low-dose males.

In the review of the histopathological kidney slides, findings in females were in accordance with the findings in the original study with regard to tumour incidence. A single adenoma was observed in 10 of 69 high-dose females. A renal tubule carcinoma was diagnosed in two females of the high-dose group, both of which also had an adenoma. In one animal, a small proliferative lesion was borderline between atypical tubule hyperplasia and incipient adenoma, and a single focus of atypical tubule hyperplasia was observed in a further two animals. The renal tubule tumours appeared to show a similar morphological pattern of attempted tubular differentiation within lobular clusters, suggesting a common mode of initiation and subsequent pathogenesis. Furthermore, foci of atypical tubule

Table 29. Incidence of kidney lesions in female rats after chronic administration of dithianon

	Incidence (number of lesions observed)			
	Dietary concentration (ppm)			
	0	20	120	600
No. of tissues examined	*70*	*70*	*70*	*70*
Atypical hyperplasia	0	0	4	10
Proliferative tubules	0	0	0	6
Adenoma	0	0	0	7
Adenocarcinoma	0	0	0	2
Transitional cell carcinoma	0	0	0	1
Eosinophilic inclusions	0	2	3	9
Glomerular sclerosis	4	6	5	21
Glomerular fibrosis	3	5	3	23
Glomerulonephropathy				
- Minimal	42	42	41	24
- Mild	16	10	15	20
- Moderate	7	9	7	10
- Severe	0	1	0	9

From Grasso (1991b)

Table 30. Incidence of proliferative lesions in the kidneys of female rats (re-evaluation of female kidney sections)

	Incidence (number of lesions observed)			
	Dietary concentration (ppm)			
	0	20	120	600
No. of tissues examined	*70*	*70*	*70*	*70*
Microscopic observations				
Basophilic tubules[a]	24	24	34	39
Atypical hyperplasia	0	0	4	10
Proliferative tubules	0	0	0	6
Adenoma	0	0	0	7
Adenocarcinoma	0	0	0	2

From Grasso (1991b)

[a] Associated with chronic progressive nephropathy.

hyperplasia and adenomas of small size were located within the outer stripe of outer medulla, which conformed with the site of compound-related tubule change. Dithianon exposure in the high dose group females was associated with nephrosis localized to the proximal part of the straight segment or P3 of the proximal tubule, mainly in the outer part of the outer stripe of outer medulla and in the medullary rays. Changes consisted of scattered cells with enlarged nuclei (usually with hypertrophied nucleoli), most being 2–3 times larger than normal, but with a few that were very large and represented karyomegaly (4 times larger or more). Although the nuclear change was relatively frequent, it was not scattered diffusely through all tubules of the outer stripe of outer medulla; instead, several

enlarged nuclei were present together in specific tubules, but not in neighbouring P3 tubules. In addition, examination of the P3 tubules revealed the presence of occasional nuclear fragments within condensed hyaline cytoplasm in the P3 lumen, representing degenerate cells, and an occasional P3 cell undergoing mitosis. In comparison with the control females, chronic progressive nephropathy was exacerbated in the high-dose females, with a mode of severity at grade 3, but (in contrast to the high-dose males) with only one animal having an end-stage kidney. In the mid-dose females, similar renal damage was not observed. In accordance with the original study report, no renal tubule tumours were present in this group. Contrary to the re-evaluation of Grasso (1991b), no foci of atypical tubule hyperplasia were observed in the mid-dose females. In low-dose females, kidney slides were not different from those of the control animals.

In male rats, the major finding in the high-dose animals was exacerbated chronic progressive nephropathy. The direct treatment-related effect was limited to the outer stripe of outer medulla, as in the females. The majority of high-dose males had either patent (open) P3 tubules as a variation from normal or obviously dilated P3 tubules. In a few cases, there were narrow tracts of strongly dilated tubules (representing P3 tubules) with more flattened epithelial lining and sometimes modest thickening of the basement membrane running through the outer stripe of outer medulla. In addition, there was a scatter of cells with minimal to mild nuclear enlargement in the P3 tubules of most high-dose males. The extent of nuclear enlargement in male kidney was limited and not as prominent as in the high-dose females. Nuclei of the dimensions constituting karyomegaly were not seen. In rats with end-stage kidney, some of the tubules of the outer stripe of outer medulla were widely dilated or cystic. In mid-dose males, no treatment-related findings were noted. In low-dose males, two animals had a renal tubule tumour, one with an adenoma and one with a carcinoma. Both of these tumours had a distinctive appearance of amphophilic/vacuolar and lobular morphology (unlike the high-dose female tumours). This distinctive phenotype recently has been identified as a spontaneous neoplasm unrelated to chemical treatment. Therefore, these tumours had no relationship to those occurring in the high-dose female group.

Details of the distribution provided by the reviewer with regard to chronic progressive nephropathy are presented in Table 31. Chronic progressive nephropathy was much more prominent in males than in females and was considered not to be treatment related.

The mode of action involving key histopathology events leading to the development of renal tubule tumours exclusively in the high-dose female rats receiving 600 ppm of dithianon in the diet for 2 years appeared to involve nuclear enlargement with chronic cell loss and sustained cell proliferation localized to the P3 segment of proximal tubule in the outer stripe of outer medulla. Both the observed compound-related nephrosis and the increased incidence of renal tumours were restricted to the high-dose females, and there was concordance between the site of chronic injury and the site of tumour development. This mode of action is compatible with a non-genotoxic pathway of renal tumour development, implying a threshold. The histological re-evaluation indicated that 120 ppm was a no-observed-effect level (NOEL) for lesions associated with tumour development, but 20 ppm was a NOAEL for exacerbation of chronic progressive nephropathy, a rat-specific disease process not considered by the reviewer to be an expression of nephrotoxicity **(**Hard, 2009).

2.4 Genotoxicity

Dithianon was evaluated for its potential genotoxicity in a battery of 11 genotoxicity studies, 5 of which were in vitro studies and 6 were in vivo studies (Table 32). Most of the studies complied with GLP, and QA statements were attached.

On the basis of these studies, dithianon is concluded not to have mutagenic or genotoxicity properties either in vitro or in vivo.

Table 31. Group distribution of chronic progressive nephropathy severity

Dietary concentration (ppm)	Total number of rats in group	Total number of rats graded[a]	Number of rats with chronic progressive nephropathy grade of severity						
			0	1	2	3	4	5	6
Females									
0	70	65	6	36	14	9	0	0	0
20	70	68	11	28	14	9	6	0	0
120	70	68	4	21	21	17	5	0	0
600	70	68	0	15	19	27	5	1	1
Males									
0	70	66	4	20	29	10	2	1	0
20	70	na	na	na	na	na	na	na	na
120	70	45	3	16	14	10	1	1	0
600	70	66	0	6	14	18	10	2	16

From Hard (2009)

na, not assessed for chronic progressive nephropathy

[a] Excludes rats that could not be graded for chronic progressive nephropathy because of other intercurrent disease process or postmortem autolysis.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a two-generation reproduction study, dithianon technical (purity 91.6%; batch No. 15C/86) was administered to Crl:CD(SD)BR strain Sprague-Dawley rats (28 of each sex per group and 25 of each sex per group in the P and F_1 generations, respectively) at a dietary concentration of 0, 35, 200 or 600 ppm during the premating (100 days), mating, gestation and lactation periods. At the start of the treatment, animals were 6 weeks old and weighed 163–207 g (males) and 121–166 g (females). The dietary concentrations were selected based on the results of a 90-day feeding study in rats, which showed decreased overall body weight gains, increased kidney, liver and adrenal gland weights and changes in haematological and clinical chemistry parameters for males and females and histopathological findings in the kidney in females at 1080 ppm. The dams (P generation) were allowed to rear their offspring (F_{1a} generation) until weaning. The F_1 parental animals were selected from the F_{1a} offspring and treated for a 100-day premating period and then mated. The F_1 parental animals were also allowed to rear their offspring (F_{2a} generation) until weaning. The litters for the F_{1a} and F_{2a} generations were culled on postnatal day 4 to a maximum of eight pups per litter. Feed consumption of the P and F_1 parents was determined weekly up to the mating period. After mating, female feed consumption was determined on days 0, 7, 14 and 20 of gestation and on days 1, 4, 7 and 14 postpartum.

For P and F_1 generation males treated with 35, 200 or 600 ppm dithianon in their diets, mean compound intake at the time of mating (week 14) was estimated to be equal to 1.7, 9.0 and 27.6 mg/kg bw per day, respectively. For females of both generations, mean compound intake at the time of mating was comparable to that during gestation and was estimated to be equal to 2.1, 11.4 and 34.9 mg/kg bw per day, respectively. During lactation, compound consumption increased for females of both generations. On average, dithianon consumption during lactation for P and F_1 females treated with 35, 200 and 600 ppm was 4.5, 22.3 and 72.6 mg/kg bw per day, respectively (Table 33).

In general, body weights of P and F_1 parents were determined once weekly during the premating and mating periods. P/F_1 females were weighed on days 0, 7, 14 and 20 of gestation and on days

Table 32. Results of genotoxicity studies on dithianon

Test system	Assay/strain/species	Batch No., purity (%)	Result	Reference
Gene mutation in bacterial cells[a]	Ames mutagenicity test, *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	15C/86, 92	Negative	Mueller (1986, 1993)
Gene mutation in mammalian cells	HGPRT mammalian cell mutation, V79 Chinese hamster cells	30/84, 94.7	Negative	Miltenburger (1984a,b, 1993)
Gene mutation in mammalian cells	HGPRT mammalian cell mutation, V79 Chinese hamster cells	15C/86, 90	Negative without S9; equivocal with S9 (positive responses only at cytotoxic doses that exceeded target cytotoxicity)	van de Waart (1993a)
Cytogenetic tests	Chromosomal aberration assay, V79 Chinese hamster cells	15C/86, 92	Positive at cytotoxic concentration	Heidemann (1988)
DNA damage and repair	Unscheduled DNA synthesis, Wistar CF HB rat hepatocytes	15C/86, 92	Negative	Timm (1986)
Cytogenetic tests	Micronucleus, EMD: NMRI(SPF) mice	162/83, purity not stated	Negative	Schulze Schencking & Unkelbach (1984)
Chromosomal damage (clastogenicity)	Micronucleus, Wistar rat	DT2008-3473, 95.5	Negative	Schulz,& Landsiedel (2009a)
Chromosomal aberration	Bone marrow cells of Wistar rat	15C/86, 91.6	Negative	Voelkner (1990, 1993)
DNA damage and repair	Comet assay, Wistar rat[b]	DT2008-3473, 95.5	Equivocal	Schulz & Landsiedel (2009b); Schulz & Mellert (2009a)
Potential for DNA binding	Covalent binding index, Sprague-Dawley rat[c]	903-1101, > 95	Negative	Sagelsdorff (2009)
Potential to induce DNA repair synthesis	Unscheduled DNA synthesis, Wistar rat	DT2008-3473, 95.5	Negative	Schulz & Mellert (2009b)

DNA, deoxyribonucleic acid; S9, 9000 × *g* rat liver supernatant

[a] Because of cytotoxicity observed with several of the bacterial strains (some cultures with no viability at the highest doses tested), the experiment was repeated with lower dose levels.

[b] In the present comet assay in kidney cells of male and female Wistar rats in vivo, single oral administration of dithianon did not lead to a clear and reproducible increase in the mean relative tail intensity at any dose level or exposure time. Increased levels of DNA damage observed only in single female rats are considered to be due to secondary effects induced by organ toxicity that is known to be expressed at more pronounced levels in the female than in the male rat with special patterns of cellular degeneration in the female rat kidney. Thus, under the experimental conditions of this study, dithianon is considered to be equivocal with respect to DNA damaging potential in the comet assay using kidney cells of male and female Wistar rats in vivo.

[c] No DNA adduct formation could be detected in the liver or in the kidney of female rats after treatment with [ring-^{14}C]- or [cyano-^{14}C]-dithianon at a limit of detection of about 0.1 covalent binding index. This maximum possible DNA binding ability of dithianon is more than 5 orders of magnitude lower than the corresponding value for the strong genotoxic compound aflatoxin B_1 and more than 4000 times lower than the covalent binding index for the genotoxic compound 2-acetylaminofluorene. [^{3}H]Acetylaminofluorene showed levels of DNA binding that were well within the expected range, thus confirming the sensitivity of the test system. Under the experimental conditions of this assay, dithianon was not considered to have DNA binding–mediated genotoxic potential.

1, 4, 7, 14 and 21 after birth. The F_1 and F_2 pups were weighed on the day of or the day after birth and on days 4, 7, 14 and 21 postpartum.

The state of health of parents and pups was checked at least once each day, and parental animals were examined for their mating and reproductive performances. Pups were sexed and evaluated as to health, and pup viability was recorded. All pups were examined macroscopically at necropsy. All P and F_1 parental animals were assessed by gross pathology (including testes and epididymides weight

Table 33. Mean dithianon intake for P and F_1 generation animals

Dietary concentration (ppm)	Mean dose (mg/kg bw per day)							
	Premating period (males)		Premating period (females)		Gestation period (females)		Lactation period (females)	
	Week 1	Week 14[a]	Week 1	Week 14[a]	Week 1	Week 3	Week 1	Week 3
P generation								
35	3.2	1.6	3.2	2.1	2.1	2.1	3.4	5.8
200	18.1	8.8	18.5	11.8	11.7	11.1	17.7	29.9
600	52.2	26.9	53.4	34.8	35.0	33.8	55.3	91.9
F_1 generation								
35	3.5	1.8	3.6	1.9	2.1	2.0	3.1	5.6
200	20.4	9.1	21.0	11.2	11.7	11.1	15.6	26.0
600	61.1	28.3	59.6	34.6	37.4	33.5	55.6	87.8

From Osterburg (1991)

[a] Week 14 of the premating period was the last week prior to mating.

determination) and subjected to a histopathological examination of the organs of the reproductive system.

There were no treatment-related mortalities observed for either generation of parental animals. No treatment-related clinical signs were observed at any stage of the study. During the premating period, there were decreases in overall body weight gain for parental animals of the P generation (8.3% for males, 4.3% for females) and F_1 generation (4.3% for males, 3.9% for females) at 600 ppm, compared with the controls. The decreases in overall body weight gain were statistically significant for the P generation males. Feed consumption was slightly decreased during the premating period for males and females in the P and F_1 parental animals at 600 ppm. In the 600 ppm group, feed consumption for the P females was also reduced during the gestation period, compared with the controls (Tables 34–36).

There were no effects of treatment on mating performance or fertility indices for the P and F_1 parental animals. There were no treatment-related effects on gestational length, pup sex ratio, pup viability at birth and at weaning, and pup body weights for either generation. There were also no effects on physical or functional development of the offspring. At necropsy, no treatment-related macroscopic effects were observed in the parental animals or in the pups. There were no effects on the weight of the testes or the epididymides in the P or F_1 parental males; these were the only organs weighed. No treatment-related histopathological findings were observed in the reproductive organs of P and F_1 parental animals. Furthermore, no treatment-related malformations were observed in the F_{1a} or F_{2a} pups.

Based on these results, the NOAEL for parental toxicity was 200 ppm (approximately 9 mg/kg bw per day), based on reductions in body weight gain and feed consumption for P and F_1 parental animals at 600 ppm. The NOAEL for fertility and reproductive function was 600 ppm (equal to 27.6 mg/kg bw per day), the highest concentration tested.

The study complied with GLP requirements, and a QA statement was attached (Osterburg, 1991).

(b) Developmental toxicity

Rats

In a preliminary oral (gavage) embryotoxicity study, dithianon technical (purity 91.6%; batch No. 15C/86) was administered by intragastric intubation to groups of eight sexually mature and

Table 34. Mean body weight and feed consumption data for P males during the premating and mating periods[a]

	Dietary concentration (ppm)			
	0	35	200	600
Number of rats dosed	28	28	28	28
Mean body weight (g)				
- Day 1	182.8	183.7	181.9	182.6
- Day 43	410.6	407.9	408.0	389.2*
- Day 99	518.6	521.9	518.3	490.3*
- Day 120	537.3	540.0	539.5	508.5*
- Days 1–99	335.8	338.2	336.4	307.4*
Mean feed consumption (g)				
- Days 1–3	18.0	18.0	17.7	18.1
- Days 8–10	24.6	24.2	24.1	22.8**
- Days 43–45	28.6	28.1	28.0	26.0**
- Days 99–101	26.8	26.1	26.0	24.8**

From Osterburg (1991)
Significantly different from the vehicle control values: * $P < 0.05$; ** $P < 0.01$
[a] Premating period: days 1–99 from the beginning of the study; mating period: days 100–120 from the beginning of the study.

Table 35. Mean body weight and feed consumption data for P females during the premating, gestation and lactation periods

	Dietary concentration (ppm)			
	0	35	200	600
Premating period (days 1–99 from the beginning of the study)				
Number of rats dosed	28	28	28	28
Mean body weight (g)				
- Day 1	139.3	140.6	140.2	138.3
- Day 43	223.8	229.2	239.0**	219.2
- Day 99	259.7	266.8	281.0**	252.5
- Days 1–99	119.5	126.2	140.8**	114.3
Mean feed consumption (g)				
- Days 1–3	14.6	14.7	14.4	13.9
- Days 8–10	16.9	16.9	17.7	16.2
- Days 43–45	20.9	18.6	20.0	17.3
- Days 99–101	18.8	18.2	17.4	16.0**
Gestation period (gestation days 0–20)				
Number of rats dosed	27	27	26	26
Mean body weight change (g)	105.91	105.36	103.32	106.42
Mean feed consumption (g)	21.57	21.21	20.97	19.52**
Lactation period (lactation days 1–21)				
Number of rats dosed	27	27	26	26
Mean body weight change (g)	39.73	40.90	33.91	33.56
Mean feed consumption (g)	38.30	45.96*	41.43	38.45

From Osterburg (1991)
Significantly different from the vehicle control values: * $P < 0.05$; ** $P < 0.01$

Table 36. Mean body weight and feed consumption data for F_1 parental animals

	Dietary concentration (ppm)							
	0	35	200	600	0	35	200	600
	Males				Females			
Premating period (days 1–99 post-weaning)								
Number of rats dosed	25	25	25	25	24	25	25	25
Mean body weight (g)								
- Day 1	113.9	117.9	114.1	111.0	101.4	107.1	100.5	95.1
- Day 99	504.5	526.9	518.1	484.6	266.4	271.5	274.0	253.7
- Days 1–99	390.6	409.0	404.0	373.6	165.1	164.5	173.4	158.6
Mean feed consumption (g)								
- Days 1–3	17.4	18.3	17.2	16.9	16.3	15.4	15.4	13.4**
- Days 43–45	28.0	28.1	27.0	26.1	18.8	18.2	19.1	17.3
- Days 99–101	26.3	27.9	26.6	26.4	17.4	17.7	16.8	15.4**
Gestation period (gestation days 0–20)								
Number of rats dosed	—	—	—	—	22	22	23	25
Mean body weight change (g)	—	—	—	—	99.99	100.25	99.26	103.78
Mean feed consumption (g)	—	—	—	—	20.44	20.29	20.48	19.86
Lactation period (lactation days 1–21)								
Number of rats dosed	—	—	—	—	22	22	23	25
Mean body weight change (g)	—	—	—	—	35.57	39.89	27.75	40.75
Mean feed consumption (g)	—	—	—	—	Spilled			

From Osterburg (1991)
** Significantly different from the vehicle control values ($P < 0.01$)

mated female Sprague-Dawley Crl:CD(SD)BR strain rats at a dose of 20, 40, 70 or 100 mg/kg bw per day for 10 consecutive days from days 6 to 15 of gestation. A control group of eight rats received vehicle (1% carboxymethylcellulose in water). The dose volume was 10 ml/kg bw, adjusted daily on the basis of the individual body weights. The male rats were 14 weeks old when mating started, and the weight range was approximately 400–600 g. Female rats were approximately 8–12 weeks old and weighed between 170 and 254 g. All animals were examined at least once daily for signs of ill-health and behavioural changes; mortality checks were performed twice daily. Body weights were recorded on days 0, 6, 9, 12, 16 and 20 of gestation. On day 20 of pregnancy, the animals were sacrificed and examined macroscopically for pathological changes. The ovaries and uteri were removed and examined, and the following data were recorded: number of corpora lutea, number and position of implantations (live fetuses, early resorptions, late resorptions and dead fetuses), individual fetal weights and sex of the fetuses. All fetuses were examined for external malformations.

The results from this pilot range-finding study indicated maternal toxicity (markedly decreased [by approximately 85% of control] body weight gain during days 6–15) and developmental toxicity (slightly decreased [by approximately 14% of control] mean fetal weight) in the 100 mg/kg bw per day group (the highest dose tested). Maternal toxicity (moderately decreased [by approximately 40% of control] body weight gain during days 6–15) in the absence of developmental toxicity was observed at the next lower dose of 70 mg/kg bw per day.

Based on these results, the dose levels selected for the definitive developmental toxicity study in rats were 0, 20, 50, 70 and 100 mg/kg bw per day. The study was conducted in compliance with GLP, and a QA statement was attached (Mueller, 1989a, 1993b).

In a teratogenicity study, pregnant Sprague-Dawley Crl:CD(SD)BR strain rats (25–32 animals per group) received daily gavage doses of dithianon technical (purity 91.6%; batch No. 15C/86) at 0, 20, 50, 70 or 100 mg/kg bw per day during days 6–15 of gestation. The 0 and 70 mg/kg bw per day groups contained 32 rats, at the request of the study sponsor, in order to ensure that 20 pregnant animals were obtained in these groups. The 20 and 100 mg/kg bw per day groups contained 25 rats. The 50 mg/kg bw per day group (25 animals) was added after the initiation of the study, at the request of the sponsor, to provide an additional intermediate-dose group, and a second control group consisting of an additional 25 animals was added at the same time. The test material was administered as a suspension in 1% carboxymethylcellulose, at a dose volume of 10 ml/kg bw. The male rats were 14 weeks old when mating started, and the weight range was approximately 400–600 g. Female rats were approximately 8–12 weeks old and weighed between 170 and 254 g. Doses were selected for the definitive study based on results from the preliminary study described above (Mueller, 1989a). For the definitive study, all surviving animals were necropsied on day 20 of gestation, and the fetuses were removed and examined. All animals were examined at least once daily for signs of ill-health, toxicity and behavioural changes; mortality checks were performed twice per day. Body weights were recorded on days 0, 6, 9, 12, 16 and 20 of gestation. On day 20 of pregnancy, the animals were sacrificed and examined macroscopically for pathological changes. The ovaries and uteri were removed and examined, and the following data were recorded: number of corpora lutea, number and position of implantations (live fetuses, early resorptions, late resorptions and dead fetuses), individual fetal weights and sex of the fetuses. All fetuses were examined for external malformations and for skeletal or visceral abnormalities.

Treatment-related mortalities occurred during the gestation period in 1 of 32 and 5 of 25 animals at 70 and 100 mg/kg bw per day, respectively. Prior to their deaths, these animals appeared thin and pale and exhibited diarrhoea, lethargy and vaginal discharge. There were no abortions in any of the groups. Decreases in body weight gain (44%, 43% and 94%) and feed consumption (31%, 27% and 48%) were observed in the 50, 70 and 100 mg/kg bw per day groups, respectively, compared with the controls, during the dosing period. There were actual mean body weight losses observed at 70 mg/kg bw per day from days 6 to 9 of gestation (3 g) and at 100 mg/kg bw per day from days 6 to 12 of gestation (33 g). Necropsy of the maternal animals revealed a discoloration of the gastric wall in several animals given 70 and 100 mg/kg bw per day (Table 37).

Treatment with dithianon did not affect pregnancy status, as the pregnancy rates for initial control, 20, 70 and 100 mg/kg bw per day groups were 72%, 100%, 81% and 79%, respectively. Similarly, the pregnancy rates for the second control and 50 mg/kg bw per day groups were both 80%. The mean numbers of corpora lutea and total implantation sites for the treated groups were similar to those of their respective control groups, and there was no treatment-related increase in preimplantation loss. An increased number of resorptions (early plus late resorptions) was observed in the 70 and 100 mg/kg bw per day groups compared with the control group. A total of 12 resorptions (from 11 of 23 litters) was observed in the initial control group. The total number of resorptions for the 70 and 100 mg/kg bw per day groups was 67 (from 17 of 25 litters) and 97 (from 11 of 15 litters), respectively. The total number of resorptions was also increased, relative to the controls, at 50 mg/kg bw per day. A total of 72 resorptions (from 13 of 20 litters) was observed at 50 mg/kg bw per day, compared with a total of 19 resorptions (from 10 of 20 litters) in the second control group. For the 50, 70 and 100 mg/kg bw per day groups, the increases in total resorptions reflect an increase in the number of females with 100% resorptions. No litter in either control group had a 100% resorption rate. However, for the 50, 70 and 100 mg/kg bw per day groups, the incidence of litters with 100% resorptions was 6 of 20 litters (30%), 5 of 25 litters (20%) and 8 of 15 litters (53%), respectively, and the number of resorptions attributed to these litters was 60, 53 and 78 early resorptions, respectively. Because of the increase in total resorptions, the mean number of resorptions (defined as the total number of resorptions divided by the total number of litters per dose group) was increased in the 50, 70 and 100 mg/kg

Table 37. Summary toxicity data of dams treated with dithianon

Maternal findings	Dose (mg/kg bw per day)					
	0	20	70	100	0	50
No. of rats in the study	32	25	32	25	25	25
No. of non-pregnant rats	9	0	6	5	5	5
No. of rats died or killed moribund	0	0	1*	5[a]	0	0
No. of dams with total litter loss	0	0	5	8	0	6
No. of rats with live fetuses at necropsy (day 20)	23	25	20	7	20	14
No. of thin animals	0	0	3	13	0	2
Mean feed consumption (g)						
- Days 6–16	23.29	22.56	16.93**	12.10**	24.09	16.60**
- Days 0–20	23.05	22.72	20.16**	16.35**	23.73	19.97**
Mean body weight gain (g)						
- Days 0–6	24.58	25.58	25.83	31.61	29.54	30.01
- Days 12–16	26.85	24.91	23.74	28.23	25.96	21.90
- Days 16–20	49.87	47.03	50.09	58.09	44.92	48.14
- Days 6–16	51.09	50.01	29.26**	3.14**	49.25	27.53**
- Days 0–20	125.54	122.62	105.18**	93.50*	123.71	105.68*
Necropsy data						
Stomach – lumen, material	0	0	0	3	0	0
Gastric wall – greyish layer	0	0	4	5	0	0
100% intrauterine deaths	0	0	5	8	0	6

From Mueller (1991)

* $P < 0.05$; ** $P < 0.01$

[a] Pregnancy status not determined for the rat of the 70 mg/kg bw per day group and for one of the dead rats of the 100 mg/kg bw per day group.

bw per day groups compared with controls. The mean numbers of resorptions for the initial control, 70 and 100 mg/kg bw per day groups were 0.52 (12 resorptions divided by 23 litters), 2.68 (67 resorptions divided by 25 litters) and 6.47 (97 resorptions divided by 15 litters), respectively. Similarly, the mean numbers of resorptions for the second control and 50 mg/kg bw per day groups were 0.95 (19 resorptions divided by 20 litters) and 3.60 (72 resorptions divided by 20 litters), respectively. Therefore, developmental toxicity, with a subsequent reduction in the mean number of live fetuses, was indicated at dose levels of 50 mg/kg bw per day and above. The mean number of live fetuses at 70 and 100 mg/kg bw per day was 9.4 and 5.6, respectively, versus 12.1 in the initial control group. Similarly, the mean number of live fetuses at 50 mg/kg bw per day was 7.5 versus 10.7 in the second control group. The total number of resorptions (10 resorptions from 6 of 25 litters), the mean number of resorptions (0.40, or 10 resorptions divided by 25 litters) and the mean number of live fetuses (11.3) for the 20 mg/kg bw per day group were comparable to controls, and no developmental toxicity was indicated at the lowest dose tested. Mean fetal weight was statistically significantly lower at 100 mg/kg bw per day (2.9 g), compared with the initial controls (3.3 g). Mean fetal weights for the 20 and 70 mg/kg bw per day groups were 3.3 g and 3.2 g, respectively, and were not different from those of the initial controls. Similarly, mean fetal weights for the 50 mg/kg bw per day group and the second control group were both 3.5 g. There were no differences in the type or incidences of fetal external, visceral or skeletal malformations or variations in any of the treatment groups (Table 38).

In summary, the NOAEL for both maternal and developmental toxicity for dithianon (technical) in rats was 20 mg/kg bw per day, based on decreases in body weight gain and feed consumption

Table 38. Summary of caesarean section and litter data

	Dose (mg/kg bw per day)					
	0	20	70	100	0	50
No. of surviving pregnant rats intubated	23	25	25	15	20	20
No. of dams with total litter loss	0	0	5	8	0	6
No. of dams with live fetuses at necropsy (day 20)	23	25	20	7	20	14
Mean no. of corpora lutea per litter	15.4	14.7	14.4	17.1	13.9	14.4
Mean no. of implantations per litter	12.6	11.7	12.4	14.7	11.7	11.5
% preimplantation loss per litter	18.7	19.0	14.1	13.0	15.8	19.9
No. of early resorptions per litter – fetal incidence						
- Animals with live fetuses at necropsy	0.5	0.4	0.7	2.7	1.0	0.9
- All animals	0.5	0.4	2.6	6.5	1.0	3.6
No. of late resorptions per litter – fetal incidence						
- Animals with live fetuses at necropsy	0	0	0.1	0	0.1	0
- All animals	0	0	0	0	0.1	0
No. of dead fetuses	0	0	0	0	0	0
No. of live fetuses per litter (litter size)						
- Animals with live fetuses at necropsy	12.1	11.3	11.7	12.0	10.7	10.6
- All animals	12.1	11.3	9.4	5.6	10.7	7.5
% postimplantation loss per litter						
- Animals with live fetuses at necropsy	3.7	2.9	5.8	16.5	8.2	6.6
- All animals	3.7	2.9	24.6*	61.0*	8.2	34.6
Mean proportion of live male fetuses	50.3	47.1	49.6	47.0	51.4	52.9
Live fetal body weight (g)	3.3	3.4	3.2	2.9*	3.5	3.5

From Mueller (1991)
* $P < 0.05$

in the dams and increased number of resorptions and a subsequent reduction in the mean number of fetuses per dam at 50 mg/kg bw per day and above. Because developmental toxicity was observed only at dose levels that were maternally toxic, dithianon is not selectively toxic to the fetus. Dithianon is not teratogenic in Sprague-Dawley rats.

The study was conducted in compliance with GLP, and a QA statement was attached (Mueller, 1991).

Rabbits

In a preliminary oral (gavage) embryotoxicity study, dithianon technical (purity 91.6%; batch No. 15C/86) was administered to groups of eight sexually mature and mated female New Zealand White rabbits at a dose level of 10, 20 or 40 mg/kg bw per day for 13 consecutive days from day 6 to day 18 of gestation. A control group of eight rabbits received vehicle (1% carboxymethylcellulose in water). The dose volume was 10 ml/kg bw, adjusted daily on the basis of the individual body weights. The male rabbits were at least 30 weeks old when mating started, and the weight range was approximately 5–5.5 kg. The sexually mature female rabbits were approximately 14–17 weeks old and weighed between 3.1 and 4 kg. All animals were examined at least once daily for signs of ill-health and behavioural changes; daily mortality checks were performed twice per day. Body weights were recorded on days 0, 6, 9, 12, 15, 19, 24 and 28 of gestation. On day 28 of pregnancy, the animals were sacrificed and examined macroscopically for pathological changes. The ovaries and uteri were

removed and examined, and the following data were recorded: number of corpora lutea, number and position of implantations (live fetuses, early resorptions, late resorptions and dead fetuses), individual fetal weights and sex of the fetuses. All fetuses were examined for external malformations.

The results of this range-finding study indicated moderate maternal toxicity (i.e. decreased body weight gain with a body weight loss between days 6 and 9 and abortions in two of nine rabbits) and developmental toxicity (i.e. increased post-implantation loss resulting from an increased number of early resorptions) at a dose of 40 mg/kg bw per day. The range-finding study also showed that an intermediate dose of 20 mg/kg bw per day did not produce any adverse maternal or developmental effects.

Based on the results indicated above, the doses selected for the definitive developmental toxicity study in the rabbit were 0, 10, 25 and 40 mg/kg bw per day. The study was conducted in compliance with GLP, and a QA statement was attached (Mueller, 1989b, 1993a).

On the basis of the doses selected from the range-finding study**,** four groups of pregnant New Zealand White rabbits (20 per group) were used in a definitive developmental toxicity study. Dithianon technical (purity 91.6%; batch No. 15C/86) was administered by oral gavage at doses of 0, 10, 25 or 40 mg/kg bw per day during days 6–18 of gestation. The test material was administered once per day as a suspension in aqueous carboxymethylcellulose (1%) at a dose volume of 10 ml/kg bw. The male rabbits were healthy and sexually mature. The sexually mature female rabbits were approximately 14–17 weeks old and weighed between 2.8 and 4.4 kg. All animals were examined at least once daily for signs of ill-health and behavioural changes; daily mortality checks were performed twice per day. Body weights were recorded on days 0, 6, 9, 12, 15, 19, 24 and 28 of gestation. On day 28 of pregnancy, the animals were sacrificed and examined macroscopically for pathological changes. The ovaries and uteri were removed and examined, and the following data were recorded: number of corpora lutea, number and position of implantations (live fetuses, early resorptions, late resorptions and dead fetuses), individual fetal weights and sex of the fetuses. All fetuses were examined for external malformations and for skeletal or visceral abnormalities.

No treatment-related mortalities were reported. Three abortions were noted at 40 mg/kg bw per day, compared with no abortions at 25 mg/kg bw per day, one abortion in the 10 mg/kg bw per day group and one abortion in the control group. The abortions in the 40 mg/kg bw per day group were considered to be treatment related, whereas the abortion in the 10 mg/kg bw per day group was considered incidental because the incidences were comparable in the 10 mg/kg bw per day and control groups.

Maternal toxicity, expressed as dose-dependent decreases in body weight gain and feed consumption compared with controls, was observed for the 25 and 40 mg/kg bw per day groups. In the 40 mg/kg bw per day group, between gestation days 6 and 19, body weight gain was decreased by 58% and feed consumption was decreased by 38% compared with controls, with an actual 108 g loss in body weight from days 6 to 19. In the 25 mg/kg bw per day group, a 48% decrease in body weight gain and a 29% decrease in feed consumption were noted, compared with controls, during gestation days 6–19. In the 10 mg/kg bw per day group, there was a slight decrease in feed consumption (4% compared with controls) during the treatment period, but this was not considered adverse because it did not result in a decrease in body weight gain in this group (Table 39). No treatment-related macroscopic effects were observed at necropsy in the maternal animals.

Developmental toxicity was observed in the 40 mg/kg bw per day group and consisted of a statistically significant increase in the incidence of postimplantation loss (41.5% versus 7.7% in the controls) due to an increase in the number of early resorptions (31 from 10 litters versus 8 from 4 litters in the controls). This resulted in a statistically significant reduction in the mean number of fetuses per doe in the 40 mg/kg bw per day group (3.7 versus 6.9 in the controls). There were no treatment-related effects on preimplantation or postimplantation losses in the other groups. There

Table 39. Maternal parameters measured in a rabbit developmental study with dithianon

Maternal findings	Dose (mg/kg bw per day)			
	0	10	25	40
No. of females in the study	20	20	20	20
No. of non-pregnant females	4	1[a]	0	1
No. of pregnant females that died	0	4	3	4
No. of females that aborted and were therefore killed	1	1	0	3
No. of females with 100% intrauterine deaths	1	0	0	2
No. of females with live fetuses at necropsy	14	15	17	10
Mean feed consumption (g/day per animal)				
- Days 6–19	211.45	190.58	150.79	131.88
- Days 0–28	206.56	186.21	163.50	167.51
Mean body weight gain (g)				
- Days 0–6	303.39	265.34	234.14	282.46
- Days 6–19	366.85	350.92	191.31	153.87
- Days 19–24	142.32	64.77	154.11	135.98
- Days 24–28	138.59	53.30	102.56	95.88
- Days 0–28	968.14	787.40	675.65	675.71

From Mueller (1990)

[a] This animal was also found dead during the study.

were no effects of treatment on fetal weight or on the incidence of malformations or variations at any of the doses tested.

In summary, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on the reductions in body weight gain and feed consumption at 25 mg/kg bw per day. The NOAEL for developmental effects was 25 mg/kg bw per day, based on an increased incidence of postimplantation loss resulting from an increase in abortions and resorptions and a subsequent reduction in the mean number of fetuses per doe at 40 mg/kg bw per day. There were no developmental effects in the absence of maternal toxicity. Thus, dithianon (technical) is not teratogenic in New Zealand White rabbits.

The study was conducted in compliance with GLP, and a QA statement was attached (Mueller, 1990).

2.6 Special studies

(a) Subacute neurotoxicity

Rats

Dithianon technical (purity 95.5%; batch No. AC12395-26) was administered to groups of 10 male and 10 female Wistar CrlGlxBrlHan:WI strain rats by gavage for 4 weeks at a dose of 0 (Group 0, vehicle control), 15 (Group 1), 30 (Group 2) or 60 (Group 3) mg/kg bw per day. Feed and water consumption were determined once a week. Body weight was determined once a week and on the days when functional observational batteries (FOBs) were performed. The animals were examined for clinical signs of toxicity or mortality at least once a day. The ages of the animals were 32–36 days (males) and 31–35 days (females). Only animals free from clinical signs of disease were used for the study. Detailed clinical examinations in an open field were conducted prior to the start of

the administration period and weekly thereafter, except for the weeks when FOBs were carried out. FOBs (including auditory startle response test and grip strength tests of forelimbs and hindlimbs) and motor activity measurements were performed on days −1 and 27. At the end of the study, the first five animals of each sex per dose were sacrificed by perfusion fixation and subsequently examined neuropathologically (brain, spinal cord, optic nerve, peripheral nervous system). The remaining animals were sacrificed without further examination.

During week 1, feed consumption was statistically significantly reduced only for animals at 60 mg/kg bw per day (highest dose tested), specifically by 16.9% for males and by 13.9% for females, compared with controls. This treatment-related effect resulted in a statistically significantly decreased body weight change at 60 mg/kg bw per day for the 4-week treatment period, specifically by 25.3% for males and by 17.5% for females. No treatment-related effect on water consumption was observed. No clinical signs of neurotoxicity were observed in treated animals, up to 60 mg/kg bw per day (the highest dose tested), for the following sets of detailed measurements: daily observations, weekly clinical examinations, FOB (including home cage observations, open-field observations and sensorimotor tests/reflexes) and motor activity assessment. Other non-neurotoxic, treatment-related clinical observations (including open-field observations) were noted, indicative of general systemic toxicity, for males and females at both 30 and 60 mg/kg bw per day. Specifically, slight to severe urine-smeared anogenital region was seen in over half of the males and females at 60 mg/kg bw per day during weeks 3 and 4, whereas this observation was noted in only a few rats at 30 mg/kg bw per day, and only with slight severity. In addition, piloerection was observed sporadically in half of the males and females at 60 mg/kg bw per day. Also, several male animals at 60 mg/kg bw per day showed slight to moderate salivation predominately before treatment for several days of the study. Because this finding was primarily observed before administration of the test compound, it was assessed as a conditioning reflex rather than an adverse or toxic effect. In addition, dark discoloured urine was observed in over half of the males and females at 60 mg/kg bw per day, whereas it was observed in only a few male and female rats at 30 mg/kg bw per day. Importantly, this observation is probably related to the physical property (solid brown colour) of the test article. Therefore, this finding was considered to be substance related but not adverse.

For the FOB parameters, only rearing (an open-field observation) was statistically significantly reduced in males at 60 mg/kg bw per day on day 27 (−79.6%), which was considered to be substance related. Importantly, this reduction in rearing was considered to be related to general systemic toxicity rather than neurotoxicity. Other open-field observations (including abnormal movements and impairment of gait), home cage observations (including abnormal movements and impairment of gait) and sensorimotor tests/reflexes (including coordination of movements such as righting response) in the FOB demonstrated no test substance–related findings up to 60 mg/kg bw per day (the highest dose tested). There were no neuropathological changes and no specific clinical signs of neurotoxicity observed up to 60 mg/kg bw per day (the highest dose tested). For motor activity measurements, a statistically significantly reduced value was obtained only in male animals at 60 mg/kg bw per day on day 27, including the earliest 5–6 intervals considered to assess exploration behaviour. Importantly, this reduction in motor activity was considered to be related to general systemic toxicity rather than neurotoxicity. As mentioned above, no treatment-related effects were found in the neuropathological examinations, including organ weights, gross lesions and detailed neuropathology of perfusion-fixed tissues (including brain, spinal cord, optic nerve and peripheral nervous system).

Based on these results (including clinical observations of smeared anogenital region with urine and dark discoloured urine at 30 mg/kg bw per day), the NOAEL for general systemic toxicity was 15 mg/kg bw per day. For neurotoxicity, the NOAEL was 60 mg/kg bw per day (the highest dose tested) for both sexes.

The study was conducted in compliance with GLP, and a QA statement was attached **(**Kaspers, Kaufmann & Ravenzwaay, 2003).

(b) Short-term study (7 days) on nephrotoxicity

Rats

In a short-term study, groups of 15 male (approximate weight 75 g) and 15 female (approximate weight 125 g) Crl:CD(SD)BR strain Sprague-Dawley rats received dithianon technical (purity 90%; batch No. 15C/86) in the diet at a concentration of 0, 120, 600 or 1080 ppm (equivalent to 0, 12, 60 and 108 mg/kg bw per day, respectively, in both sexes). After exposure for 2, 4 or 7 days, five animals of each sex per group were killed. The objectives of the study were to 1) define the nephrotoxic potential of dithianon in Sprague-Dawley rats; 2) establish a no-effect level for renal damage; and 3) investigate further the sex difference in the renal toxicity of dithianon.

The kidneys were examined macroscopically, microscopically and at the ultrastructural level. Exposure to dithianon at levels up to 1080 ppm did not result in any adverse clinical signs. Body weight gain was decreased for males and females at 1080 ppm at each time interval during the study. The decreases in body weight gain for males and females, respectively, were 61% and 31% at 2 days, 43% and 43% at 4 days and 25% and 45% at 7 days, compared with controls. A transient, slight reduction in feed consumption was noted in animals receiving 1080 ppm. There was also an increased incidence of pale kidneys at necropsy in the 600 and 1080 ppm treatment groups.

Histopathological evaluation of the kidneys revealed hydropic degeneration of the proximal tubular epithelial cells in both males and females receiving 600 or 1080 ppm at 4 days and 7 days. The incidence and severity of the lesions were increased in females compared with the corresponding males. At day 7, there was evidence of active, tubular epithelial cell degeneration. In females, the newly regenerated tubular epithelial cells showed signs of hydropic degeneration, demonstrating the continued susceptibility of these cells to damage. In contrast, males showed no evidence of further degeneration in any regenerative tubular epithelial cells, suggesting that adaptation to the toxic effects had occurred at day 7. Electron microscopy of sections from animals at 600 and 1080 ppm demonstrated that the mitochondria in the proximal tubular cells were the target for the toxic effects of dithianon. In females fed 1080 ppm, further change consisting of dilatation of the endoplasmic reticulum was observed. The ultrastructural changes were consistent with the findings observed histopathologically as hydropic degeneration. Although slight focal changes were seen ultrastructurally at 120 ppm in female rats, these changes were described as having only minimal severity, both quantitatively and qualitatively, and occurred only at 2 days. Therefore, these minimal focal changes at 120 ppm were considered to be unrelated to treatment (Table 40).

In conclusion, the NOAEL for light and electron microscopic changes in the kidney was 120 ppm (equivalent to 12 mg/kg bw per day) in females for short-term exposure of dithianon technical for up to 7 days.

The study was conducted in compliance with GLP, and a QA statement was attached **(**Price, 1991).

(c) Short-term study (28 days) on renal cell turnover

Rats

Dithianon technical (purity 90%; batch No. 15C/86) was administered to female Crl:CD(SD)BR strain Sprague-Dawley rats (approximate weight 125 g) (five per group per time point) at a dose of 20, 120 or 600 ppm (equivalent to 2, 12 and 60 mg/kg bw per day) for 7, 14 and 28 days in order to measure effects on renal cell turnover and to correlate any findings with light microscopic observations. Continuous labelling of deoxyribonucleic acid (DNA) was achieved with Alzet osmotic pumps containing 5-bromo-2′-deoxyuridine implanted subcutaneously in anaesthetized rats 7 days prior to necropsy.

At each of the time points (7, 14 and 28 days), all designated rats were sacrificed. Animals were examined for signs of macroscopic external and internal abnormalities; body and individual

Table 40. Incidence of hydropic degeneration and multiple foci of basophilic tubules in male and female rats fed dithianon for 2, 4 and 7 days

	Exposure time (days)											
	2				4				7			
	Dietary concentration (ppm)											
	0	120	600	1080	0	120	600	1080	0	120	600	1080
Males												
No. of animals examined	5	5	5	5	5	5	5	5	5	5	5	5
No abnormalities	4	2	4	4	5	5	3	0	4	4	1	2
Tubular dilatation	0	2	1	1	0	0	0	0	0	0	0	0
Hydropic degeneration	0	0	0	0	0	0	1	3	0	0	2	1
Interstitial cell infiltration	1	1	0	0	0	0	0	0	0	0	0	0
Basophilic tubules												
- Few	0	2	0	0	0	0	1	2	1	1	1	1
- Several	0	0	0	0	0	0	0	0	0	0	1	2
Females												
No. of animals examined	5	5	5	5	5	5	5	5	5	5	5	5
No abnormalities	3	3	3	3	3	4	2	1	4	5	1	0
Tubular dilatation	1	1	0	0	0	0	0	0	0	0	0	0
Hydropic degeneration	0	0	0	0	0	0	3	4	0	0	4	4
Interstitial cell infiltration	0	0	2	1	0	1	0	0	0	0	0	0
Basophilic tubules												
- Few	2	0	1	2	2	1	1	0	1	0	0	0
- Several	0	0	0	0	0	0	0	0	0	0	1	4

From Price (1991)

kidney weights were recorded. Sections of both kidneys were fixed in 10% neutral buffered formalin for histological examination after staining with haematoxylin and eosin. Sections of the cortex of the right kidney were fixed in 4% glutaraldehyde buffered with sodium cacodylate at 0.1 mol/l, pH 7.4, for ultrastructural examination. Sections of kidney (4 μm) were cut, dewaxed and incubated with antibodies and stained with diaminobenzidine. The number of dark-staining nuclei was counted and compared with control values.

Hydropic degeneration was observed in proximal tubular epithelial cells after dietary exposure to 600 ppm dithianon for 7, 14 and 28 days. This lesion was accompanied by islands of basophilic tubules, indicative of active regeneration. Continuous labelling techniques using 5-bromo-2′-deoxyuridine showed an increase in tubular cell turnover rate at 600 ppm, consistent with the histopathological results. No increase in tubular cell turnover rate was observed at 20 or 120 ppm.

It was concluded that persistent cellular damage to proximal tubular epithelial cells triggered a regenerative response of basophilic tubules, which is the basis of the indirect mechanism in the development of proliferative lesions (including tumours) following longer-term (2-year) dietary exposure of female rats to 600 ppm. In this study, the NOAEL for the repeated cellular degenerative/regenerative responses was 120 ppm (approximately 12 mg/kg bw per day), according to histopathological and DNA labelling criteria. Neither the degenerative/regenerative (cell turnover) responses nor renal tumours (in the carcinogenicity study) were observed at concentrations of 20 and 120 ppm.

Although atypical hyperplasia was observed at 120 ppm in 4 of 70 female rats following 104 weeks of treatment, progression to neoplasia did not occur at this dose.

The study was conducted in compliance with GLP, and a QA statement was attached (Price, 1993).

(d) S-phase response study (7 days and 28 days)

Rats

The aim of these studies was to determine whether dithianon induced a cell proliferation response in the kidneys of rats after oral administration over periods of 7 and 28 days. Dithianon (purity 95.5%; batch No. DT2008-3473) was administered to groups of eight male and eight female CrI:CD (SD) strain Sprague-Dawley rats (61–63 days old) at a dietary concentration of 0, 120 or 600 ppm (equal to 0, 6.5 and 32.6 mg/kg bw per day for males and 0, 8 and 40.6 mg/kg bw per day for females) over a period of 7 days. In another study, similar dietary concentrations (equal to 0, 6.8 and 33.5 mg/kg bw per day for males and 0, 8.2 and 43.5 mg/kg bw per day for females) were administered over a period of 28 days. Feed consumption and body weights were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. One week prior to necropsy, osmotic minipumps containing 5-bromo-2′-deoxyuridine were implanted subcutaneously. Cell proliferation (S-phase response) and apoptosis (TUNEL)[1] were evaluated in the kidneys.

Irrespective of the duration of administration of dithianon (7 days or 28 days), dietary concentrations of 120 and 600 ppm in male Sprague-Dawley rats caused a marginal exacerbation of basophilic tubules, cell proliferation and secondary apoptosis without overt cytotoxicity of the kidney parenchyma, representing a first-grade chronic progressive nephropathy. Chronic progressive nephropathy is a rat-specific, spontaneously occurring kidney alteration.

In female Sprague-Dawley rats given similar dietary concentrations, the 7-day study showed specific degenerative lesions in the kidney parenchyma (outer stripe of outer medulla and cortex) as well as a significant increase of cell proliferation in high-dose animals. Administration of the high dose to female rats for 28 days affected body weight. Moreover, specific degenerative lesions in the kidney parenchyma (outer stripe of outer medulla and cortex) as well as a significant increase of cell proliferation were observed. Additionally, the high dose significantly increased apoptosis in the kidney cortex.

The studies were conducted in compliance with GLP, and QA statements were attached (Kaspers et al., 2009a,b,c,d).

2.7 Studies on metabolites

No known toxic metabolites were found in either animal or plant studies.

3. Observations in humans

Personnel handling dithianon in manufacturing, research and product formulation underwent regular medical examinations. This surveillance programme was not aimed to specifically identify

[1] TUNEL, or terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick end labelling, is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. The assay relies on the presence of nicks in the DNA that can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyse the addition of dUTPs that are secondarily labelled with a marker. It may also label cells that have suffered severe DNA damage (Gavrieli, Sherman & Ben Sasson, 1992).

dithianon-related symptoms or diseases. The only effects reported have been skin-related effects, such as erythema, swelling and itching. The information available does not allow a firm conclusion as to whether all these skin reactions should be interpreted as primary irritation or whether some may be true allergic reactions.

Skin and eye irritation have been repeatedly observed in dithianon-exposed workers. In operators spraying dithianon-containing products, erythema, swelling, itching, blistering and peeling of the skin have been reported. Epicutaneous patch testing has demonstrated that sensitization to products containing dithianon may occur. However, it is unclear whether pure dithianon is a potential sensitizer or whether the allergic reactions are due to excipients, impurities or decomposition products.

No observations regarding health effects after exposure of the general public are known (Holmsen, 2009).

4. Literature review

A literature review was performed to find an explanation for the observed sex- and species-specific induction of kidney tumours (seen only in female rats after long-term treatment at high doses, not in male rats and not in male or female mice). No straightforward explanation for the sex- and organ-specific occurrence of dithianon-induced tumours in the female rat kidney was found. Moreover, no other chemical was found that exclusively induces kidney tumours in female rats. An expert reviewer has discussed possible reasons for the sex-specific occurrence of dithianon-induced tumours in the female rat kidney. The most promising hypothesis explaining the sex-specific induction of kidney tumours was associated with the involvement of cyclooxygenase-2 (COX-2). Prostaglandin H-synthase can oxidize lipophilic xenobiotics if they have a sufficiently low redox potential, which is the case for many phenolic compounds and aromatic amines. This is assumed to be possible for dithianon or dithianon metabolites, which undergo oxidative metabolism. COX-2 is present in the proximal straight tubules of female but not male F344/NSlc rats (immunohistochemically and morphometrically determined, but no activities were measured; the rats were 3 months old) (Ichii et al., 2008). As the proximal straight tubules appear to be a site of origin of the sex-specific dithianon-induced kidney tumours of the female rats, this difference in the occurrence of COX-2 may be significant. The metabolic formation of the many terminal sulfonic acid derivatives observed in the metabolism studies (M216F005, M216F006, M216F007, M216F009, M216F011, M216F013, M216F014, M216F016, M216F018, M216F025) may occur via reactive intermediates (sulfenic and sulfinic acid derivatives). If COX-2 is a significant contributor to this metabolic route of dithianon, the occurrence of COX-2 in the proximal straight tubules exclusively in female compared with male rat kidney may be related to the sex-specific occurrence of kidney tumours in the female rat (Oesch, 2009). However, this hypothesis for the involvement of COX-2 in kidney tumour formation in female rats cannot be correct, as the kidney tumour has been reported in Sprague-Dawley rats and not in F344/NSlc rats. Further evidence from experimental results for the involvement of COX-2 in kidney tumour formation is not available at the present time.

Comments

Biochemical aspects

At tested doses of 10 and 50 mg/kg bw, orally administered dithianon was about 40–50% absorbed in rats, with a time to maximum concentration in blood of approximately 6 hours. There were no substantial dose- or sex-related differences in the absorption, elimination or distribution of radioactivity following oral administration of [^{14}C]dithianon. The majority of the administered dose was recovered in faeces (64.0–72.2%) and urine (26.7–31.4%).

The material balance from a preliminary study showed that dithianon was not metabolized to volatile compounds, including carbon dioxide. The elimination half-life was between 46 and 57 hours. There was no bioaccumulation of dithianon in tissues. The parent compound was extensively metabolized by the following key transformation steps: oxidation of the sulfur atoms, cleavage of the dithiine ring, reduction of the 1,4-naphthoquinone moiety and further glucuronidation, as well as substitution of the carbonitrile moieties by amino and carboxy groups. The only metabolite in rat urine at a level greater than 2% was M216F020 (glucuronic acid conjugate of 1,4-dihydroxynaphthalene). All other identified metabolites were present in insignificant amounts. The metabolic pathways were similar in male and female rats.

Toxicological data

Dithianon technical has moderate acute toxicity in rats (LD_{50} approximately 300 mg/kg bw). The dermal LD_{50} in rats was greater than 2000 mg/kg bw. Dithianon is slightly to moderately toxic by acute inhalation in rats, with an LC_{50} between 0.31 and 2.1 mg/l, depending on particle size. Dithianon is non-irritating to rabbit skin but is a severe eye irritant. It was found to be a skin sensitizer (guinea-pig maximization test).

Short-term oral toxicity studies were conducted in mice, rats and dogs. These studies indicate that the kidney is the main target organ. A 4-week (range-finding) study in mice with dithianon administered in the diet resulted in slight anaemia and haemosiderin deposition in the liver of females at 500 ppm, with a NOAEL of 100 ppm (equivalent to 15 mg/kg bw per day). A 90-day rat oral toxicity study revealed slight anaemia (both sexes) as well as histopathological findings of renal tubular epithelial cell degeneration and regenerative hyperplasia (females only) at 1080 ppm, with a NOAEL of 180 ppm (equal to 14.6 mg/kg bw per day).

Studies in dogs included a 90-day dietary study and a 1-year dietary study. In the 90-day study, the doses used were 0, 40, 200 and 1000 ppm (equal to 0, 0.63, 2.95 and 12.6 mg/kg bw per day in males and 0, 0.67, 3.0 and 12.6 mg/kg bw per day in females). In the 1-year study, the same dietary doses were used, but the compound intakes were 0, 1.6, 7.3 and 37.1 mg/kg bw per day in males and 0, 1.6, 7.9 and 37.5 mg/kg bw per day in females, respectively. In the 90-day study, the NOAEL was 2.95 mg/kg bw per day, based on decreases in body weight (females), decreased body weight gain (males), decreased feed consumption, increases in alkaline phosphatase activity and increased kidney weights (males and females) and increased thromboplastin time (females) at 12.6 mg/kg bw per day. Oral administration of dithianon for 1 year resulted in slight anaemia, liver impairment and effects on kidney and thyroid. The NOAEL for dithianon fed to dogs in their diets for 1 year was 200 ppm (equal to 7.3 mg/kg bw per day), based on increases in kidney and liver weights in both sexes at 1000 ppm (equal to 37.1 mg/kg bw per day).

Long-term toxicity and carcinogenicity studies were undertaken in mice (80 weeks) and rats (104 weeks). In male mice at 500 ppm in the diet, there was an association between the observed kidney damage (chronic nephropathy) and an increased incidence of early mortality, indicating that the MTD was exceeded at 500 ppm. No increases in tumour incidence were noted in any of the treatment groups. The NOAEL for chronic toxicity in mice was 20 ppm (equivalent to 3 mg/kg bw per day), based on increased absolute and relative kidney weights for males and females and an exacerbation of spontaneous chronic nephropathy in females at 100 ppm. Dithianon was not carcinogenic in mice at a dietary concentration of 500 ppm, the highest dose tested. In the rat study, the NOAEL for chronic toxicity was 20 ppm (equivalent to 1 mg/kg bw per day), based on histopathological kidney lesions (females) at 120 ppm. Increased incidences of renal tubule adenomas and carcinomas in kidney were observed in female rats at 600 ppm (equivalent to 30 mg/kg bw per day). The highest dietary concentration (600 ppm) also resulted in a consistently significant lower body weight (19.8–20.5%) in female rats compared with controls at weeks 72, 84 and 104. Moreover, only the highest dose (i.e.

600 ppm) in female rats demonstrated severe glomerulonephropathy with sclerosis. The tumours in rat kidneys were associated with severe nephrotoxicity in proximal tubular cells. This dose exceeded the MTD in females.

In a 7-day dietary study in rats, hydropic degeneration of the proximal tubular epithelial cells was seen in both males and females receiving 600 ppm (equivalent to 60 mg/kg bw per day) or 1080 ppm (equivalent to 108 mg/kg bw per day) at 4 and 7 days, with significantly greater incidence and severity in females. In females at day 7, the newly regenerated tubular epithelial cells with signs of hydropic degeneration demonstrated the susceptibility of renal tubules to damage. In contrast, males showed no evidence of further degeneration, suggesting adaptation to the toxic effects by day 7. Electron microscopy suggested that the mitochondria in the proximal tubular cells were the target. The NOAEL for this study was 120 ppm (equivalent to 12 mg/kg bw per day) in males and females.

These findings were further substantiated by a 28-day dietary renal turnover study in female rats. Continuous labelling techniques using 5-bromo-2′-deoxyuridine showed an increase in tubular cell turnover at 600 ppm (equivalent to 60 mg/kg bw per day), consistent with the histopathological results. The NOAEL for the repeated cellular degenerative/regenerative responses was 120 ppm (equivalent to 12 mg/kg bw per day). It appears that persistent cellular damage to proximal tubular epithelial cells triggers a regenerative response in basophilic tubules, which is the basis for the development of proliferative lesions following long-term (2-year) dietary exposure of female rats to 600 ppm. Neither the degenerative/regenerative (cell turnover) responses nor renal tumours (in the carcinogenicity study) were observed at a concentration of 20 or 120 ppm. The high susceptibility of female rats to renal effects (proximal tubular degeneration, regeneration and tumours) might be related to the involvement of COX-2, but experimental evidence for this as an explanation for the sex difference is not available.

Dithianon was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. The majority of results were negative, including in vivo studies. There were some positive results in vitro that occurred only at cytotoxic doses.

The Meeting concluded that dithianon is unlikely to be genotoxic in vivo.

In view of the lack of in vivo genotoxicity, the lack of any tumorigenic response in mice and male rats and the fact that the kidney tumours in female rats occurred only at doses that were cytotoxic, the Meeting concluded that dithianon is unlikely to pose a carcinogenic risk at human dietary exposure levels.

In a multigeneration study in rats, the NOAEL for fertility and reproductive functions was 600 ppm (equal to 27.6 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 200 ppm (equal to 9.0 mg/kg bw per day), based on reductions in feed consumption and body weight gain in parental animals of both generations at 600 ppm.

Developmental toxicity studies have been carried out in rats and rabbits. In a rat developmental toxicity study, the NOAEL for maternal toxicity and embryo and fetal toxicity was 20 mg/kg bw per day, based on decreases in body weight gain and feed consumption in the dams and increased number of resorptions and a subsequent reduction in the mean number of fetuses per dam at 50 mg/kg bw per day and above. In a rabbit developmental toxicity study, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reductions in body weight gain and feed consumption at 25 mg/kg bw per day. The NOAEL for developmental effects was 25 mg/kg bw per day, based on an increased incidence of postimplantation loss resulting from an increase in abortions and resorptions and a subsequent reduction in the mean number of fetuses per doe at 40 mg/kg bw per day. There were no developmental effects in the absence of maternal toxicity.

The Meeting concluded that dithianon did not cause developmental toxicity at doses that were not toxic to the dams and that it was not teratogenic.

In a 4-week neurotoxicity study in rats, a NOAEL of 15 mg/kg bw per day was identified based on clinical observations of smeared anogenital region with urine and dark discoloured urine at 30 mg/kg bw per day. There were no signs of neurotoxicity in any other study.

Skin and eye irritation have been repeatedly observed in dithianon-exposed workers. In operators spraying dithianon-containing products, erythema, swelling, itching, blistering and peeling of the skin have been reported.

The Meeting concluded that the existing database on dithianon was adequate to characterize the potential for hazard to fetuses, infants and children.

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.01 mg/kg bw for dithianon based on a NOAEL of 1 mg/kg bw per day for histopathological kidney lesions in females at 6 mg/kg bw per day in a 2-year toxicity study of rats and using a 100-fold safety factor.

The Meeting established an acute reference dose (ARfD) of 0.1 mg/kg bw for dithianon, taking into account a NOAEL of 12 mg/kg bw and using a safety factor of 100. The NOAEL was based on a mechanistic study in which nephrotoxicity was assessed in rats following 4 and 7 days of dosing. At these time points, a dietary intake of 60 mg/kg bw per day of dithianon induced repeated cellular degenerative/regenerative responses in kidney tubular cells of female rats.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighty-week study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equivalent to 3 mg/kg bw per day	100 ppm, equivalent to 15 mg/kg bw per day
		Carcinogenicity	500 ppm, equivalent to 75 mg/kg bw per day[b]	—
Rat	Seven-day study of toxicity[a]	Nephrotoxicity	120 ppm, equivalent to 12 mg/kg bw per day	600 ppm, equivalent to 60 mg/kg bw per day
	Ninety-day study of toxicity[a]	Toxicity	180 ppm, equal to 14.6 mg/kg bw per day	1080 ppm, equal to 86.7 mg/kg bw per day
	Twenty-four-month study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equivalent to 1 mg/kg bw per day	120 ppm, equivalent to 6 mg/kg bw per day
		Carcinogenicity	120 ppm, equivalent to 6 mg/kg bw per day	600 ppm, equivalent to 30 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Offspring toxicity	600 ppm, equal to 27.6 mg/kg bw per day[b]	—
		Parental toxicity	200 ppm, equal to 9.0 mg/kg bw per day	600 ppm, equal to 27.6 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	10 mg/kg bw per day	25 mg/kg bw per day
		Embryo and fetal toxicity	25 mg/kg bw per day	40 mg/kg bw per day
Dog	Twelve-month study of toxicity[a]	Toxicity	200 ppm, equal to 7.3 mg/kg bw per day	1000 ppm, equal to 37.1 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.

Estimate of acceptable daily intake for humans

0–0.01 mg/kg bw

Estimate of acute reference dose

0.1 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to dithianon

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	T_{max} approximately 6 h, 42–52% absorbed
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Excreted in faeces (64.0–72.2%) and urine (26.7–31.4%)
	Half-life 46–57 h
Metabolism in animals	Extensive
Toxicologically significant compounds in animals, plants and the environment	Dithianon

Acute toxicity	
Rat, LD_{50}, oral	300 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	0.31 mg/l
Rabbit, dermal irritation	Non-irritant
Rabbit, eye irritation	Severe irritant
Guinea-pig, dermal sensitization	Sensitizer (Magnusson & Kligman test)

Short-term studies of toxicity	
Target/critical effect	Kidney, tubular damage
Lowest relevant oral NOAEL	12 mg/kg bw per day (7-day study in rats)
	7.3 mg/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	Not relevant
Lowest relevant inhalation NOAEC	Not relevant

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Kidney, tubular damage
Lowest relevant NOAEL	1 mg/kg bw per day (24-month study in rats)
Carcinogenicity	Only in kidneys of female rats at cytotoxic doses

Genotoxicity	
	Not genotoxic in vivo

Reproductive toxicity	
Reproduction target/critical effect	None
Lowest relevant reproductive NOAEL	27.6 mg/kg bw per day, the highest dose tested, in rats
Developmental target/critical effect	Increase in postimplantation loss in the presence of maternal toxicity; not teratogenic

Lowest relevant developmental NOAEL	25 mg/kg bw per day in rabbits
Neurotoxicity/delayed neurotoxicity	
	Not neurotoxic
Other toxicological studies	
	Mechanistic studies on kidney toxicity
Medical data	
	Local skin and eye irritation effects in exposed plant workers and operators

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw	Two-year dietary toxicity study in rats	100
ARfD	0.1 mg/kg bw	Four/seven-day nephrotoxicity study in rats	100

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ETOXAZOLE

First draft prepared by
C. Adcock[1] and V. Dellarco[2]

[1] Health Evaluation Directorate, Pest Management Regulatory Agency, Health Canada, Ottawa, Canada
[2] Office of Pesticides, Environmental Protection Agency, Washington, DC, United States of America (USA)

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Explanation

Etoxazole is the International Organization for Standardization (ISO)–approved name for (*RS*)-5-*tert*-butyl-2-[2-(2,6-difluorophenyl)-4,5-dihydro-1,3-oxazol-4-yl]phenetole (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) No. 153233-91-1. Etoxazole is a new acaricide that belongs to the diphenyloxazole class of miticides/ovicides, possibly acting by inhibiting chitin biosynthesis and causing adults to lay sterile eggs. Etoxazole has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All the pivotal studies contained certificates of compliance with good laboratory practice (GLP).

Evaluation for acceptable daily intake

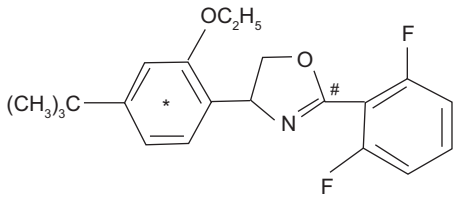
1. Biochemical aspects

The absorption, distribution, metabolism and excretion, as well as the toxicokinetics, of etoxazole have been investigated in Sprague-Dawley rats. Summaries of the relevant data are presented below.

1.1 Absorption, distribution and excretion

In a rat metabolism study, [^{14}C-*t*-butylphenyl]etoxazole or [^{14}C-oxazole]etoxazole (≥ 97.5% radiochemical purity) (Figure 1) was administered to Sprague-Dawley (Hsd/Ola:CD) rats (3–12 of each sex per dose) as a single oral gavage dose at 5 or 500 mg/kg body weight (bw) or as a repeated oral gavage dose of equimolar [^{14}C-*t*-butylphenyl/^{14}C-oxazole]etoxazole at 5 mg/kg bw per day for 14 days. In addition, groups of bile duct–cannulated rats (three of each sex per dose) received a single dose via stomach cannula of [^{14}C-*t*-butylphenyl]etoxazole or [^{14}C-oxazole]etoxazole at 5 or 500 mg/kg bw.

Figure 1. Radiolabelled forms of etoxazole used in absorption, distribution, metabolism and excretion studies: Structure and position of label



* [^{14}C-*t*-butylphenyl]etoxazole
\# [^{14}C-oxazole]etoxazole

Within 168 hours of administering a single oral dose of [^{14}C-*t*-butylphenyl]etoxazole or [^{14}C-oxazole]etoxazole at 5 or 500 mg/kg bw, 92.0–97.6% of the administered dose was recovered from male and female rats. There were no major sex- or ^{14}C label–related differences in the pattern of excretion. For both low- and high-dose rats, faecal excretion was the primary route of elimination, and excretion of the ^{14}C dose was essentially complete within 120 hours of dosing. In the low-dose

Table 1. Average recovery of radioactivity in bile duct–cannulated rats 48 hours after a single oral dose of ^{14}C-labelled etoxazole (5 mg/kg bw)

Sample	Recovery of radioactivity (% of administered dose)			
	[^{14}C-*t*-butylphenyl]etoxazole		[^{14}C-oxazole]etoxazole	
	Male	Female	Male	Female
Carcass	1.37	1.59	1.85	1.26
Bile	40.25	53.95	29.80	36.80
Urine	12.13	13.53	18.37	24.11
Faeces	46.55	33.99	50.46	39.14
Cage washings	0.14	0.28	0.22	0.71
Total	100.44	103.33	100.70	102.0

From Elsom (1996)

Table 2. Average recovery of radioactivity in bile duct–cannulated rats 48 hours after a single oral dose of ^{14}C-labelled etoxazole (500 mg/kg bw)

Sample	Recovery of radioactivity (% of administered dose)			
	[^{14}C-*t*-butylphenyl]etoxazole		[^{14}C-oxazole]etoxazole	
	Male	Female	Male	Female
Carcass	1.03	8.05	0.92	5.94
Bile	12.54	11.87	9.77	10.91
Urine	4.29	6.03	5.38	8.17
Faeces	80.32	70.97	79.35	74.32
Cage washings	0.07	0.59	0.23	0.14
Total	98.24	97.51	95.66	99.48

From Elsom (1996)

groups (both ^{14}C labels), 77.1–88.3% of the dose was recovered in the faeces and 7.6–16.6% of the dose was recovered in the urine. In the high-dose groups (both ^{14}C labels), 90.9–93.8% of the dose was recovered in the faeces and 1.6–3.2% in the urine. For all dose groups, radioactivity recovered in exhaled air accounted for less than or equal to 0.05% of the dose, and radioactivity remaining in tissues and the residual carcass by 168 hours post-dosing accounted for 0.06–0.76% of the dose.

^{14}C-labelled etoxazole was rapidly absorbed from the gastrointestinal tract of rats following oral dosing. Maximum concentrations of radioactivity in plasma were observed within 2–4 hours of dosing for the low-dose groups and within 4–6 hours of dosing for the high-dose groups (Tables 1 and 2). Based on the recovery of radioactivity in the urine and bile of the bile duct–cannulated rats, a substantial portion (48.2–67.5%) of the dose was absorbed by rats in the low-dose groups. However, absorption (15.2–19.1% of the dose) was limited in the high-dose groups. The position of the ^{14}C label within the molecule had no effect on the level of absorption, but there was a minor sex-related effect. Absorption, particularly at the low dose level, was slightly higher (1.3 times) in females than in males. In addition, biliary excretion was slightly higher (1.2–1.3 times) in females than in males and slightly higher (1.1–1.5 times) in the [^{14}C-*t*-butylphenyl]etoxazole groups than in the [^{14}C-oxazole]etoxazole groups. Urinary excretion was higher (1.5–1.8 times) in the [^{14}C-oxazole]-etoxazole groups.

Concentrations of radioactivity in the plasma over time were not affected by position of the ^{14}C label, but were affected by sex and dose level. In each dose group, concentrations of plasma radio-

activity were approximately 2 times higher in males than in females. For the single low-dose groups, maximum concentrations in the plasma (C_{max}) were 0.961–1.51 µg equivalent (Eq)/g for males and 0.631–0.646 µg Eq/g for females. For the single high-dose groups, C_{max} was 15.8–16.4 µg Eq/g for males and 5.3–5.6 µg Eq/g for females. Maximum plasma concentrations were 7–18 times higher in the high-dose rats than in the low-dose rats, but the increase was not proportional to the 100-fold increase in the dose level. With the exception of the [^{14}C-oxazole]etoxazole high-dose rats, terminal half-lives were similar between the sexes within each group (28.2–88.8 hours). For the [^{14}C-oxazole]-etoxazole high-dose rats, the terminal half-life was 101.9 hours for males and 7.1 hours for females. The area under the curve (AUC) values were approximately 2–3 times higher in males than in females for each group, but were similar between ^{14}C labels. AUC values were 23.9–33.0 µg Eq·h/g for low-dose males, 15.6–16.4 µg Eq·h/g for low-dose females, 425–464 µg Eq·h/g for high-dose males and 121–143 µg Eq·h/g for high-dose females. Although the dose level was increased by 100 times, AUC values were only 7–19 times higher in the high-dose rats, further indicating that absorption was limited at the higher dose level (Tables 3 and 4).

Maximum concentrations of radioactivity in most tissues were observed at the 3- and 6-hour sampling interval for the low- and high-dose groups, respectively; concentrations in tissues declined steadily at longer post-treatment intervals. The relative distribution of radioactivity between tissues was similar regardless of sex, ^{14}C label and dose level. Excluding the gastrointestinal tract, concentrations of radioactivity over time were generally highest in liver, lymph nodes, thyroid and fat and were lowest in brain for both sexes in each dose group. For each tissue, the actual concentrations of radioactivity were similar between the two ^{14}C labels, but concentrations were generally 1.5–2 times higher in tissues of males than in tissues of females from the same group. Increasing the dose level by 100 times increased the concentration of radioactivity in tissues. However, with the exception of the gastrointestinal tract, the approximately 20 times increase in tissue concentrations in the high-dose groups did not reflect the 100 times increase in the dose level. By 168 hours post-dosing, concentrations of radioactivity remaining in liver, thyroid and fat of repeated-dose rats were 3.9–7.8 times higher than in the same tissues from the [^{14}C-*t*-butylphenyl]etoxazole single-dose group.

1.2 Biotransformation

Analyses of urine and faeces identified or characterized 69.6–76.3% of the dose in excreta from the single low-dose groups and 89.1–91.1% of the dose in excreta from the single high-dose groups. With the exception of minor urinary metabolites, the metabolite profile in excreta was qualitatively similar between the sexes, dose levels and ^{14}C labels. Etoxazole was eliminated primarily in the faeces as parent compound. Unchanged parent accounted for 17.8–29.1% of the dose in faeces from the low-dose groups and 74.7–80.2% of the dose in faeces from the high-dose groups. Two other minor metabolites were also identified in faeces: 4-(4-*tert*-butyl-2-ethoxyphenyl)-2-(2,6-difluorophenyl)-oxazole (R-13; 0.3–1.8% of the dose) and 2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethyl-2′,6′-difluorobenzoate hydrochloride (R-7; 1.2–6.1% of the dose). Urinalyses identified two metabolites in the urine of [^{14}C-*t*-butylphenyl]etoxazole-dosed rats, 2-amino-2-(2-ethoxy-4-(1′-hydroxymethyl-1′-methylethyl)phenyl)ethanol (R-24) and 2-amino-2-(2-ethoxy-4-(1′-hydroxycarbonyl-1′-methylethyl)-phenyl)ethanol (Met 1), and one metabolite in the urine of [^{14}C-oxazole]etoxazole-dosed rats, 2,6-difluorobenzoic acid (R-11). At the low dose, levels of Met 1 were higher in the urine of males (5.4% of the dose) than in the urine of females (2.0% of the dose), whereas levels of R-24 were higher in the urine of females (4.1% of the dose) than in the urine of males (0.5% of the dose). For the high-dose rats, Met 1 accounted for 0.2–0.5% of the dose, and R-24 accounted for 0.4–0.9% of the dose. In the urine of the [^{14}C-oxazole]etoxazole-dosed rats, metabolite R-11 accounted for 12.7–14.6% of the dose in low-dose rats and 1.7–2.4% of the dose in high-dose rats.

Table 3. Radioactive inventory (n = 5) and selected pharmacokinetic parameters in rats following oral administration of [^{14}C-t-butylphenyl]etoxazole (n = 3)

Matrix	Radioactive inventory (% of administered dose at day 7)			
	Single dose 5 mg/kg bw Male	Single dose 500 mg/kg bw Male	Single dose 5 mg/kg bw Female	Single dose 500 mg/kg bw Female
Expired air	< 0.01	< 0.03	< 0.01	< 0.03
Urine	8.49	1.55	7.55	1.65
Faeces	88.3	91.6	86.8	93.8
Bile	ND	ND	ND	ND
Carcass/organs[a]	0.76	0.05	0.24	0.12
Cage wash	0.07	0.01	0.05	0.02
Total	97.6	93.2	94.7	95.6
C_{max} (µg Eq/g)	1.51	16.4	0.631	5.3
T_{max} (h)	3.0	6.0	4.0	6.0
AUC (µg Eq·h/g)	33.0	424.5	15.6	142.7

From Elsom (1996)
ND, not determined; T_{max}, time to C_{max}
[a] Carcass/gastrointestinal tract, kidneys and liver.

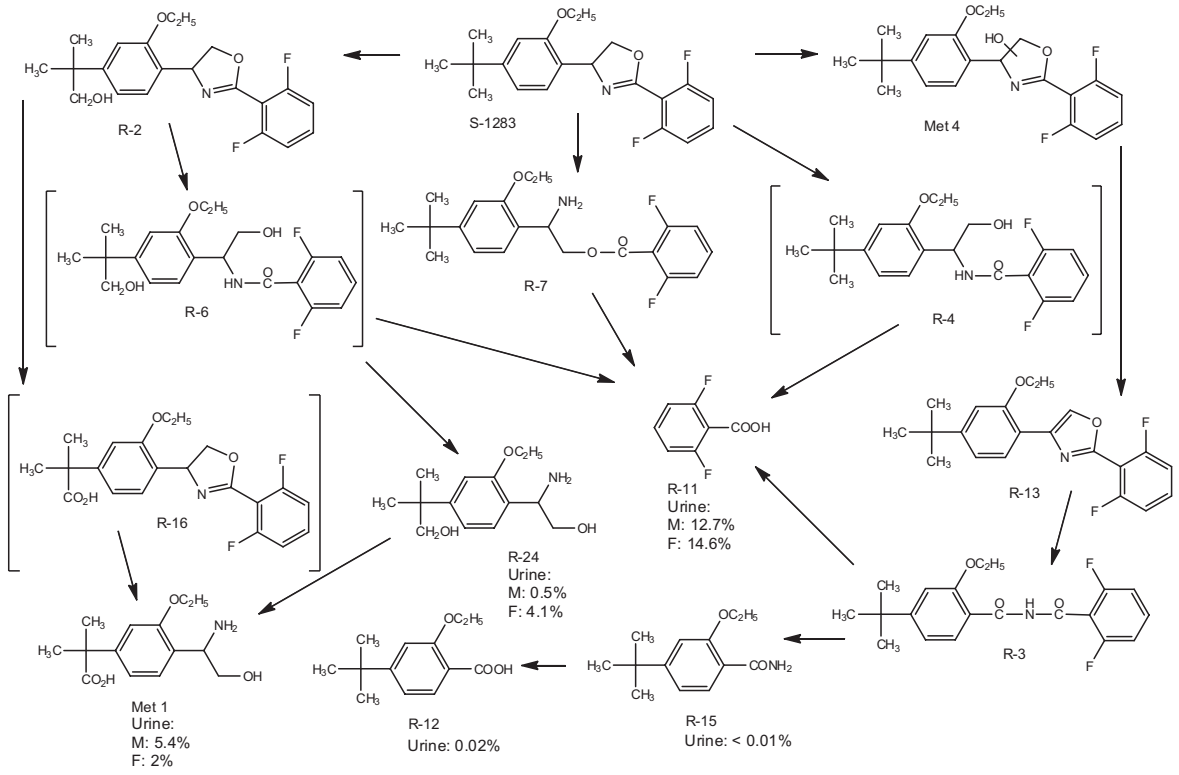
Table 4. Radioactive inventory (n = 5) and selected pharmacokinetic parameters in rats following oral administration of [^{14}C-oxazole]etoxazole (n = 3)

Matrix	Radioactive inventory (% of administered dose at day 7)			
	Single dose 5 mg/kg bw Male	Single dose 500 mg/kg bw Male	Single dose 5 mg/kg bw Female	Single dose 500 mg/kg bw Female
Expired air	0.04	< 0.01	0.05	< 0.01
Urine	14.2	3.23	16.6	1.94
Faeces	77.1	91.0	77.6	90.9
Bile	ND	ND	ND	ND
Carcass/organs[a]	0.65	0.06	0.25	0.07
Cage wash	0.04	0.01	0.08	0.01
Total	92.0	94.3	94.6	92.9
C_{max} (µg Eq/g)	0.961	15.8	0.646	5.6
T_{max} (h)	2.0	6.0	3.0	4.0
AUC (µg Eq·h/g)	23.9	464.0	16.4	121.1

From Elsom (1996)
ND, not determined; T_{max}, time to C_{max}
[a] Carcass/gastrointestinal tract, kidneys and liver.

Analyses of bile from each group detected two additional metabolites: 2-(2,6-difluorophenyl)-4-[2-ethoxy-4-(1-hydroxymethyl-1-methylethyl)phenyl]-4,5-dihydrooxazole (R-2; ≤ 1.6% of the dose) and 4-(4-*tert*-butyl-2-ethoxyphenol)-2-(2,6-difluorophenyl)-oxazole (Met 4; 1.4–16.3% of the dose). Met 4 accounted for 5.9–6.9% of the dose in bile of low-dose males, 10.3–16.3% of the dose in bile of low-dose females and 1.4–4.6% of the dose in bile of high-dose rats.

Figure 2. Postulated major metabolic pathways for etoxazole in rats



From Elsom (1996)

Metabolite R-2 was also detected in plasma sampled at T_{max} (the time to C_{max}), along with etoxazole. The levels of R-2 and etoxazole in plasma were similar between the dose levels and ^{14}C labels, but different between the sexes. In males, R-2 was the major radioactive component in plasma (45.8–50.0%), and etoxazole accounted for 2.8–3.6%. However, in females, metabolite R-2 accounted for 1.6–6.9% and etoxazole accounted for 2.8–11.6%. Unknown components having retention values around 0.27–0.32 comprised a much greater proportion of the radioactivity in plasma of females (36.3–55.0%) than in plasma of males (14.7–24.4%).

Analyses of liver extracts detected etoxazole, along with five metabolites: R-2; *N*-(2,6-difluorobenzoyl)-2-amino-2-[2-ethoxy-4-(1′-hydroxymethyl-1′-methylethyl)phenyl]ethanol (R-6); 2-(2,6-difluorophenyl)-4-[2-ethoxy-4-(1-hydroxycarbonyl-1-methylethyl)phenyl]-4,5-dihydrooxazole (R-16); R-24; and Met 1. The metabolite profile in liver was qualitatively similar between the sexes, dose levels and ^{14}C labels, with the exceptions of metabolites Met 1 and R-24. Etoxazole (5.1–23.3% of liver radioactivity) was detected in liver in each group, along with the metabolites R-2 (7.3–18.9%), R-6 (6.2–16.3%) and R-16 (1.2–10.2%). However, metabolites Met 1 (4.3–6.9%) and R-24 (10.5–18.8%) were detected only in liver of [^{14}C-*t*-butylphenyl]etoxazole-dosed rats.

Based on the analyses of excreta, bile and liver, the biotransformation of etoxazole in rats primarily involves the hydroxylation of the 4,5-dihydrooxazole ring, followed by cleavage of the metabolite and hydroxylation of the *tertiary*-butyl side-chain (Figure 2) (Elsom, 1996).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with etoxazole administered orally, dermally or by inhalation are summarized in Table 5. All of the studies were certified to comply with GLP.

Table 5. Acute toxicity of etoxazole

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Sprague-Dawley	Male and female	Oral	95.57	LD_{50} > 5000 mg/kg bw	Allan (1992a)
Mouse	ICR (Hsd/Ola:ICR)	Male and female	Oral	95.57	LD_{50} > 5000 mg/kg bw	Allan (1992b)
Rat	Sprague-Dawley	Male and female	Dermal	95.57	LD_{50} > 2000 mg/kg bw	Allan (1992c)
Rat	Fischer	Male and female	Inhalation	95.39	LC_{50} > 1.09 mg/l (maximal attainable concentration)	Ebino (1994)

LC_{50}, median lethal concentration; LD_{50}, median lethal dose

Table 6. Irritation and skin sensitization potential of etoxazole

Species	Strain	Sex	End-point (method)	Purity (%)	Result	Reference
Rabbit	New Zealand White	Male and female	Skin irritation	95.57	Not irritating	Liggett (1992a)
Rabbit	New Zealand White	Male and female	Eye irritation	95.57	Not irritating	Liggett (1992b)
Guinea-pig	Dunkin-Hartley	Female	Skin sensitization (Magnusson & Kligman)	95.96	Not sensitizing	Allan (1995)

The results of studies of the irritation and skin sensitization potential of etoxazole are summarized in Table 6.

(a) Oral administration

In an acute oral toxicity study, fasted young adult Sprague-Dawley (Hsd/Ola:Sprague-Dawley (CD)) rats (five of each sex) were orally gavaged with a single dose of etoxazole (purity 95.57%) in 1% weight per volume (w/v) aqueous methylcellulose. Animals were then observed for 14 days. Technical etoxazole is of low acute toxicity by the oral route, based on the lack of mortality at 5000 mg/kg bw. All rats showed piloerection within 5 minutes of dosing; all subsequently showed hunched posture and abnormal gait (waddling). Lethargy was observed in 9 of 10 rats. All females (but no males) showed a decreased respiratory rate 4–5 hours after dosing. The rats had all recovered by day 2 (the day following dosing). One male and one female had slightly reduced body weight gains at termination (day 15). No macroscopic abnormalities were observed following sacrifice on day 15 (Allan, 1992a).

In an acute oral toxicity study, fasted young adult ICR (Hsd/Ola:ICR) mice (five of each sex) were orally gavaged with a single dose of etoxazole (purity 95.57%) in 1% w/v aqueous methylcellulose. Animals were then observed for 14 days. Technical etoxazole is of low acute toxicity by the oral route, based on the lack of mortality at 5000 mg/kg bw. All mice showed piloerection within 5 minutes of dosing; all subsequently showed hunched posture and abnormal gait (waddling). The mice had all recovered by day 2 (the day following dosing). All mice showed satisfactory weight gains during the 14-day observation period. No macroscopic abnormalities were observed following sacrifice on day 15 (Allan, 1992b).

(b) Dermal application

In an acute dermal toxicity study, etoxazole (purity 95.57%) was applied to the skin of male and female Sprague-Dawley rats as a 64% w/v dilution in distilled water at the limit dose of 2000 mg/kg bw and at an application volume of 3.13 ml/kg bw. No mortality occurred, and no clinical signs or dermal responses were observed during the study. Slightly lower (compared with other rats in the same group) body weight gains were recorded for one male and three females on day 8 and for one male on day 15, although these animals achieved anticipated body weight gains on days 15 and 8, respectively. All other rats achieved anticipated body weight gains throughout the study. No macroscopic abnormalities were observed for animals killed on day 15. The acute dermal median lethal dose (LD_{50}) for etoxazole in male and female rats was greater than 2000 mg/kg bw (Allan, 1992c).

(c) Exposure by inhalation

In an acute inhalation toxicity study, male and female Fischer rats were exposed whole body to an aerosol generated with undiluted etoxazole (purity 95.39%) at a concentration of 1.09 mg/l (mean atmospheric concentration, maximum attainable concentration) at an air flow rate of 100 l/min. The mean values of mass median aerodynamic diameter and geometric standard deviation were 3.6 μm and 1.5 μm, respectively. Thus, more than 99% of the test substance consisted of particles with an aerodynamic diameter of 10 μm or less. No mortality occurred. No clinical signs of toxicity were observed in any animal during exposure; slight reddish adhesive materials in the naso-rostral region were observed in four of five males immediately after the termination of exposure and in all females 1 day after exposure; this sign disappeared 1 day after exposure in the males and by 4 days after exposure in the females. All animals showed an increase in body weight 7 and 14 days after exposure. No macroscopic abnormalities were observed for animals killed on day 15. The acute inhalation median lethal concentration (LC_{50}) for etoxazole in male and female rats was greater than 1.09 mg/l, the maximum attainable concentration (Ebino, 1994).

(d) Ocular irritation

Rabbits

In a primary eye irritation study, approximately 53 mg (the weight of test substance occupying a volume of 0.1 ml) of etoxazole (purity 95.57%) was applied into the lower reverted lid of one eye of one male and five female New Zealand White rabbits. Mild conjunctival irritation was observed in all rabbits 1 hour after instillation (redness degree 1 and chemosis degree 1 in all animals; discharge degree 1 in five rabbits, degree 2 in the remaining one). All reactions had resolved 1 day after instillation. There were no signs of toxicity or ill-health in any rabbits during the 7-day observation period. Etoxazole was judged to be not irritating to the rabbit eye (Liggett, 1992b).

(e) Dermal irritation

Rabbits

In a primary skin irritation study, 0.5 g of etoxazole (purity 95.57%) moistened with 0.5 ml distilled water was applied to the shorn skin of one female and five male New Zealand White rabbits. The treated skin area was 25 mm × 25 mm, and exposure lasted 4 hours. No dermal responses were observed in any rabbits during the observation period, and there were no signs of toxicity or ill-health. Etoxazole was not irritating to rabbit skin (Liggett, 1992a).

(f) Dermal sensitization

The skin sensitization potential of etoxazole (purity 95.96%) was investigated using the Magnusson & Kligman maximization method. Groups of 20 female albino Dunkin-Hartley guinea-pigs

were tested according to the following dosing regimen: 1) induction by intradermal injection of 0.25% w/v in 5% acetone in Alembicol D associated with Freund's complete adjuvant; 2) induction by topical application of 80% w/v in acetone; and then 3) topical challenge with 40% w/v and 80% w/v in acetone. Groups of 10 animals were similarly treated in a positive control test with formalin. There were no dermal reactions seen in any of the test or control animals challenged with etoxazole at 40% w/v and 80% w/v in acetone. Dermal reactions were seen in all 10 animals in the positive control group compared with none in the controls. Etoxazole was not sensitizing to skin in the Magnusson & Kligman test in guinea-pigs (Allan, 1995).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 4-week range-finding study, groups of six ICR (Crj:CD-1) mice of each sex per dose received etoxazole (purity 96.26%) in the diet at a concentration of 0, 80, 400, 2000 or 10 000 parts per million (ppm) (equal to 0, 12.1, 58.2, 289.4 and 1465 mg/kg bw per day for males and 0, 11.8, 59.7, 294.1 and 1476 mg/kg bw per day for females). There was no mortality, and there were no clinical signs. Body weights of male mice treated at 10 000 ppm were slightly, but consistently, lower than those of controls during the study. Feed efficiency was also slightly decreased. Haematology and urinalysis did not reveal any effect of treatment. Clinical chemistry showed a significant increase in alkaline phosphatase activity in males and increasing trends in activities of glutamic oxaloacetic transaminase (aspartate aminotransferase [AST]) and glutamic pyruvic transaminase (alanine aminotransferase [ALT]) in mice of both sexes treated at 10 000 ppm. In addition, females showed a significant increase in blood urea nitrogen and an increasing trend in triglyceride levels. At necropsy, the livers of the six males and five of the six females treated at 10 000 ppm were noted to be dark-coloured. In mice of both sexes treated at 10 000 ppm, liver weights were statistically significantly increased (only relative liver weights in males), and males also showed increased absolute and relative kidney weights. At 2000 ppm, relative liver weights were increased in mice of both sexes, whereas at 400 ppm, only males exhibited an increasing trend in relative liver weight. The no-observed-adverse-effect level (NOAEL) was established at 2000 ppm (equal to 289.4 mg/kg bw per day in males and 294.1 mg/kg bw per day in females), based on decreased body weight (males only), changes in clinical chemistry parameters and increased liver weight at 10 000 ppm (Enomoto, 1992a).

In an oral subchronic toxicity study, etoxazole (purity 97.99%) was administered in the diet for 13 weeks to 12 ICR (Crj:CD-1) mice of each sex per dose at a concentration of 0, 100, 400, 1600 or 6400 ppm (equal to 0, 13.42, 55.13, 213.6 or 878.4 mg/kg bw per day in males and 0, 15.15, 62.00, 250.5 or 994.5 mg/kg bw per day in females). Mortality, body weight, body weight gain, feed consumption, feed efficiency, and haematological and urinalysis parameters were unaffected by the test substance. No treatment-related effects were observed in males and females at doses of up to 400 ppm.

Most treatment-related effects indicated hepatotoxicity. Increased absolute and relative liver weights were observed in males at 1600 ppm and in both sexes at 6400 ppm (Table 7). Liver enlargement was observed in females at 1600 and 6400 ppm. Increased incidences of microscopic hepatic lesions were also observed: centrilobular hepatocellular swelling in males at 1600 ppm (slight) and in both sexes at 6400 ppm (slight to moderate) and slight periportal hepatocellular necrosis at 6400 ppm in both sexes (Table 8). Increased triglyceride levels were also observed in females at 1600 and 6400 ppm, whereas males and females from the 6400 ppm group also exhibited increased alkaline phosphatase levels. In females at 6400 ppm, slight increases in ALT and AST activities were also

Table 7. Mean absolute and relative liver weights in mice fed etoxazole for 13 weeks[a]

Organ	Mean (± SD) liver weight (g)				
	Dietary concentration (ppm)				
	0	100	400	1600	6400
Males					
Terminal body weight	44.1 ± 3.3	44.7 ± 5.1	44.1 ± 3.9	45.4 ± 3.4	44.0 ± 2.3
Liver					
- Absolute	2.27 ± 0.19	2.38 ± 0.36	2.37 ± 0.23	2.64 ± 0.32** (↑16)	2.60 ± 0.12** (↑15)
- Relative	5.16 ± 0.32	5.32 ± 0.40	5.37 ± 0.23	5.82 ± 0.47** (↑13)	5.92 ± 0.33** (↑15)
Females					
Terminal body weight	34.1 ± 2.8	35.0 ± 3.9	34.2 ± 3.0	35.4 ± 4.0	33.5 ± 2.2
Liver					
- Absolute	1.80 ± 0.23	1.70 ± 0.23	1.80 ± 0.17	1.98 ± 0.28	2.19 ± 0.20** (↑22)
- Relative	5.26 ± 0.38	4.88 ± 0.49	5.27 ± 0.40	5.59 ± 0.38	6.53 ± 0.41** (↑24)

From Inui (1994)

SD, standard deviation; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 21-1 through 22-2 on pages 71–74; per cent change from controls is presented parenthetically.

Table 8. Incidence of microscopic liver lesions in mice fed etoxazole for 13 weeks[a]

Observation	Incidence of microscopic liver lesions (no. affected/12)									
	Dietary concentration (ppm)									
	0	100	400	1600	6400	0	100	400	1600	6400
	Males					Females				
Centrilobular hepatocellular swelling	0	0	0	5*	10**	0	0	0	0	12**
Slight periportal hepatocellular necrosis	0	0	0	0	4*	0	0	0	0	6**

From Inui (1994)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 23-1 through 24-2 on pages 75–78.

observed. The treatment-related effects noted at 1600 ppm are considered to be non-adverse, adaptive responses to exposure to etoxazole. Also noted at 6400 ppm was a decrease in absolute and relative kidney weights in males.

The NOAEL was established at 1600 ppm (equal to 213.6 mg/kg bw per day in males and 250.5 mg/kg bw per day in females), based on periportal hepatocellular necrosis, increased alkaline phosphatase levels, increased ALT and AST levels (females only), increased liver weight, liver enlargement (females only) and centrilobular hepatocellular swelling at 6400 ppm (Inui, 1994).

Rats

In a 4-week range-finding study, groups of six Fischer rats of each sex per dose received etoxazole (purity 96.26%) in the diet at a concentration of 0, 80, 400, 2000 or 10 000 ppm (equal to 0, 6.1, 30.1, 150.5 and 761 mg/kg bw per day for males and 0, 6.4, 31.9, 160.5 and 732 mg/kg bw per day for females). No unscheduled deaths occurred during the study. Clinical signs, body weight, feed consumption, feed efficiency, haematological parameters and urinalysis parameters were unaffected

by the test substance. No treatment-related effects were observed in the 80 ppm groups. The only finding at 400 ppm that was possibly related to treatment was increased relative liver weight in the females. At 2000 and 10 000 ppm, hepatotoxicity was indicated. Increased γ-glutamyl transpeptidase activity was observed in females at 2000 and 10 000 ppm, and total cholesterol level was increased in females at 2000 and 10 000 ppm. An increased incidence of liver enlargement was observed in males at 2000 and 10 000 ppm and in females at 2000 and 10 000 ppm. An increased incidence of centrilobular hepatocellular swelling was observed in both sexes (2/2 treated versus 0/2 controls in each case). Increased absolute and relative liver weights were observed in males and females at 2000 and 10 000 ppm. Absolute and relative adrenal weights were increased in males at 2000 ppm and above and in females at 10 000 ppm. At 2000 ppm and above, triglygeride levels were increased in females, and blood urea nitrogen level was increased in males. At 10 000 ppm, platelet count and total protein and globulin levels were increased in males, and globulin level was increased in females.

The NOAEL for this study is 400 ppm (equal to 30.1 mg/kg bw per day in males and 31.9 mg/kg bw per day in females), based on liver enlargement, centrilobular hepatocellular swelling and increased liver weights in both sexes and increased γ-glutamyl transpeptidase activity in females at 2000 ppm (Nakashima, 1992).

Sprague-Dawley rats (12 of each sex per dose) were given diets containing etoxazole (purity 96.26% and 95.57%) at a concentration of 0, 100, 300, 1000 or 3000 ppm (equal to 0, 6.12, 18.28, 61.8 and 183.7 mg/kg bw per day in males and 0, 6.74, 20.50, 69.0 and 204.8 mg/kg bw per day in females) for 13 weeks. Mortality, clinical signs, body weight, body weight gain, feed consumption, feed efficiency, ophthalmoscopy and urinalyses were unaffected by the test substance. Most adverse effects indicated hepatotoxicity. Increased total cholesterol level was observed in 3000 ppm males (Table 9). Increased absolute and relative (to body) liver weights were observed in males and females at 1000 and 3000 ppm (Table 10). An increased incidence of liver enlargement was observed in 3000 ppm males and in 1000 and 3000 ppm females. Slight centrilobular hepatocellular swelling was observed in males at 1000 and 3000 ppm and in females at 3000 ppm (Table 11). Absolute liver weight was increased in females at 100 and 300 ppm. In 300 ppm males, increased absolute liver weight and relative (to body) liver weight were observed. However, there was no corroborating evidence for toxicity at 100 and 300 ppm in either sex. Thus, these findings are considered non-adverse. Treatment-related haematological effects were noted in males. Haematocrit and haemoglobin were slightly decreased at 1000 and 3000 ppm. Although treatment related, these changes were not considered adverse because of the small magnitude of change.

In males, the NOAEL for this study was determined to be 1000 ppm (equal to 61.8 mg/kg bw per day), based on the increases in cholesterol level, liver weight, liver size and centrilobular hepatocellular swelling. The lowest-observed-adverse-effect level (LOAEL) for males was 3000 ppm (equal to 183.7 mg/kg bw per day).

In females, the NOAEL was determined to be 3000 ppm (equal to 204.8 mg/kg bw per day). The LOAEL for females was not established, as the effects noted in females (increases in centrilobular hepatocellular swelling and in liver weight and size) at 3000 ppm are considered non-adverse (Nakashima, 1994).

In an additional study, testes samples (from paraffin blocks) obtained from eight male rats of the control and high dose level (3000 ppm) groups in the previous study were immunohistochemically stained for proliferating cell nuclear antigen (PCNA) by the avidin biotin peroxidase complex method using an anti-PCNA antibody. The stained sections were examined for proliferative activity of testicular interstitial cells. Sections of paraffin-embedded duodenum from the same animals were used as a positive control for the PCNA staining (i.e. to verify the integrity of the PCNA staining).

Table 9. Selected clinical chemistry findings in male rats fed etoxazole for 13 weeks[a]

Parameter	Dietary concentration (ppm)				
	0	100	300	1000	3000
AST (U/l)	101 ± 17	103 ± 22	95 ± 15	92 ± 16	142 ± 23** (↑41)
Total cholesterol (mg/dl)	55 ± 8	59 ± 10	63 ± 12	66 ± 16	76 ± 15** (↑38)
γ-Glutamyl transpeptidase (U/l)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	2 ± 0** (↑100)

From Nakashima (1994)

** $P \leq 0.01$

[a] Data were extracted from Table 19-1 in the study report, page 72; $n = 12$; per cent difference from control is presented parenthetically.

Table 10. Absolute and relative liver weights in rats fed etoxazole for 13 weeks[a]

	Mean (± SD) weight (g)				
	Dietary concentration (ppm)				
	0	100	300	1000	3000
Males					
Terminal body weight	487 ± 32	503 ± 43	528 ± 41	504 ± 51	485 ± 43
Liver					
- Absolute	12.59 ± 1.46	13.42 ± 1.71	14.89 ± 1.62* (↑18)	15.00 ± 2.52* (↑19)	15.44 ± 2.00** (↑23)
- Relative	2.58 ± 0.20	2.66 ± 0.20	2.82 ± 0.16* (↑9)	2.97 ± 0.34** (↑15)	3.17 ± 0.17** (↑23)
Females					
Terminal body weight	278 ± 18	292 ± 21	285 ± 21	280 ± 27	274 ± 25
Liver					
- Absolute	6.68 ± 0.55	7.36 ± 0.57* (↑10)	7.38 ± 0.70* (↑10)	7.54 ± 1.24	8.06 ± 0.87** (↑21)
- Relative	2.41 ± 0.14	2.53 ± 0.18	2.59 ± 0.20	2.69 ± 0.21** (↑12)	2.95 ± 0.24** (↑22)

From Nakashima (1994)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from Tables 23-1, 23-2, 24-1 and 24-2 in the study report, pages 78–81; $n = 12$; per cent difference from control is presented parenthetically.

Table 11. Incidence of centrilobular hepatocellular swelling in rats fed etoxazole for 13 weeks[a]

	Incidence (no. of animals/12)									
	Dietary concentration (ppm)									
	0	100	300	1000	3000	0	100	300	1000	3000
	Males					Females				
Centrilobular hepatocellular swelling	0	0	0	5*	11**	0	0	0	0	9**

From Nakashima (1994)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from Tables 25 and 26 in the study report, pages 82–83.

Table 12. Selected clinical chemistry findings in rats fed etoxazole for 13 weeks[a]

Parameter	Dietary concentration (ppm)		
	0	5000	10 000
Males			
Creatine phosphokinase (U/l)	122 ± 21	130 ± 35	164** ± 38 (↑34)
Total protein (g/dl)	6.32 ± 0.13	6.52* ± 0.15 (↑3)	6.69** ± 0.23 (↑6)
Globulin (g/dl)	3.43 ± 0.10	3.61* ± 0.15 (↑5)	3.71** ± 0.20 (↑8)
Total cholesterol (mg/dl)	63 ± 13	71 ± 13	92** ± 16 (↑46)
Females			
Serum glutamic oxaloacetic transaminase (AST) (U/l)	75 ± 9	73 ± 10	66* ± 6 (↓12)
Globulin (g/dl)	3.23 ± 0.13	3.38* ± 0.14 (↑5)	3.40* ± 0.14 (↑5)
Total bilirubin (g/dl)	0.16 ± 0.03	0.14* ± 0.02 (↓13)	0.13** ± 0.01 (↓19)

From Nakashima (1998)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 19-1, 19-2, 20-1 and 20-2, pages 77–80; $n = 12$; per cent difference from control is listed parenthetically.

The mean PCNA labelling index in the testicular interstitial cells was 0.16 for the controls and 0.19 for the 3000 ppm dose group. Therefore, it was concluded that etoxazole administered in the diet for 13 weeks had no proliferative effects on the testicular interstitial cells (Nakashima, 1996a).

In another oral subchronic toxicity study, etoxazole (purity 95.2%) was administered in the diet to 12 Sprague-Dawley rats of each sex per group at a concentration of 0, 5000 or 10 000 ppm (equal to 0, 300.4 or 610 mg/kg bw per day for males and 0, 336.6 or 692 mg/kg bw per day for females) for 13 weeks. The objective of this study was to evaluate the subchronic toxicity following oral administration of doses of etoxazole higher than those administered in the previous 90-day oral subchronic toxicity study in rats and thus to find dose levels for an oral chronic toxicity and oncogenicity study in rats.

There were no mortalities during the study. Body weights, body weight gains, feed consumption, feed efficiency, ophthalmoscopy and urinalysis were unaffected by the test substance. At 5000 and 10 000 ppm, changes in clinical chemistry, organ weights and histopathology indicated possible hepatotoxicity. In clinical chemistry, total protein level was increased in the 5000 and 10 000 ppm males, and globulin level was increased in the 5000 and 10 000 ppm animals. Although not statistically significant, a dose-related increase in cholesterol level at 5000 and 10 000 ppm was noted in both males and females (Table 12).

Absolute and relative (to body) liver weights were increased in the 5000 and 10 000 ppm animals. Microscopically, centrilobular hepatocellular hypertrophy was observed: hypertrophy was moderate in the 10 000 ppm animals, slight to moderate in the 5000 ppm males and slight in the 5000 ppm females. Aside from the direct evidence of possible hepatotoxicity, some dose-related haematological effects were also noted. Haematocrit was decreased in males and females at 5000 and 10 000 ppm. Decreased haemoglobin was noted in males and females at 10 000 ppm. Platelet counts were increased in the 5000 ppm females and 10 000 ppm animals. Mean corpuscular volume and prothrombin time were decreased in the 5000 and 10 000 ppm females. Treatment-related increases were noted in thyroid and kidney weights in this study. Increases were observed in absolute and relative thyroid weights in the males at the high dose level. Absolute and relative kidney weights were also increased in the females at the high dose level. These findings were not considered adverse, however,

as no corroborating evidence of toxicity in these organs was observed. Several other parameters differed from controls. At 10 000 ppm, creatine phosphokinase activity was increased in the males. Elongation of the incisors was observed in gross pathology and during clinical observations towards the end of the study, yet no histologically discernible alterations were noted.

The LOAEL for this study was determined to be 5000 ppm (equal to 300.4/336.6 mg/kg bw per day for males/females), based on clinical chemistry, increased liver weights and histopathology. A NOAEL was not observed for this study (Nakashima, 1998), as adverse effects were observed at the lowest dose tested.

In a further study, groups of six Sprague-Dawley rats of each sex per dose received etoxazole (purity 95.39%) in the diet at a concentration of 0, 1000 or 2000 ppm (equal to 0, 74.75 and 152.35 mg/kg bw per day at week 4 and 0, 59.6 and 119.5 mg/kg bw per day at week 13 for males and 0, 76.2 and 155.1 mg/kg bw per day at week 4 and 0, 66.7 and 133.5 mg/kg bw per day at week 13 for females) for either 4 or 13 weeks. At each sacrifice, the liver was analysed to determine cytochrome P450 content and enzyme activities (ethoxycoumarin *O*-dealkylase and pentoxyresorufin *O*-dealkylase). Histopathological examination of the liver was also performed. No mortality occurred, and no clinical signs related to treatment were noted. Body weight and feed consumption were not affected by treatment. No macroscopic change attributable to treatment was noted at necropsy. Male rats treated at 2000 ppm showed statistically significant increases in liver weights after 4 or 13 weeks of treatment. Female rats of this same group showed increases in absolute and relative liver weights, compared with controls, after 4 weeks, but both absolute and relative liver weights were comparable to those of the control group after 13 weeks of treatment. In animals treated at 1000 ppm, only female rats showed increases in absolute and relative liver weights, compared with controls, after 4 weeks, but again both absolute and relative liver weights were comparable to those of the control group after 13 weeks of treatment. Histopathology showed centrilobular hepatocellular swelling in the liver of all male rats sacrificed after 4 weeks of treatment at 2000 ppm, whereas no treatment-related findings were ever detected in female rats or in male rats after 13 weeks of treatment at either dose level. Results of liver biochemistry did not show any significant change in any of the treated groups compared with the controls, with the exception of a statistically significant increase in microsomal protein content in male rats treated for 13 weeks at 2000 ppm. In conclusion, hepatomegaly could not be associated with an effect on the cytochrome P450 system (Inui, 1995).

Dogs

In a 4-week range-finding study, groups of one male and one female Beagle dog received etoxazole (purity 96.26%) in pulverized basal diet (offered moistened with water to avoid spillage) at a concentration of 0, 1000, 3000, 10 000 or 30 000 ppm (mean substance intakes: 0, 30.5, 99.7, 321 and 968 mg/kg bw per day for males and 0, 35.7, 102, 340 and 594 mg/kg bw per day for females) for 28 days. The animals were subjected to ophthalmology, urinalysis before beginning treatment and at week 4, and haematology and blood chemistry examinations before beginning treatment and at weeks 2 and 4. All animals were necropsied at the end of the study and examined for gross pathology. Absolute and relative organ weights were calculated; liver sections from all animals were examined microscopically.

No mortality occurred. Clinical signs were limited to vomit of feed or foamy fluid and/or watery stool or loose stool on occasion in animals from all groups, including the control, but more frequently reported for the female dog treated at 30 000 ppm. Body weight loss was observed in the female treated at 30 000 ppm, whereas reduced body weight gains were recorded in the male dog treated at 30 000 ppm and in dogs of both sexes receiving 10 000 or 3000 ppm, compared with the body weight gains of control dogs. Average feed consumption was markedly reduced only in the female dog treated at 30 000 ppm and was comparable to that of control animals for all the

other treated dogs. Ophthalmology and haematology did not reveal any change in treated dogs. At urinalysis, absence of protein was noted in the female dog treated at 30 000 ppm and in dogs of both sexes treated at 10 000 ppm. Clinical chemistry showed increases in alkaline phosphatase in both dogs treated at 30 000 ppm and in glutamic oxalic transaminase (AST) and glutamic pyruvic transaminase (ALT) in the female at weeks 2 and 4. Decreases in total protein and albumin levels were also observed at week 4 of treatment in dogs of both sexes, accompanied by a reduced albumin to globulin ratio in the male. Total cholesterol level was reduced in the male at both weeks 2 and 4 and in the female at week 4. In addition, the female showed an increase in creatine phosphokinase activity at week 4 and decreases in glucose levels at weeks 2 and 4 of treatment. Similarly, in dogs of both sexes treated at 10 000 ppm, alkaline phosphatase activity was increased at weeks 2 and 4, total protein and albumin levels were reduced at week 4 (with a low albumin to globulin ratio in the male) and total cholesterol level was reduced in the male at week 4 and at both time points in the female. In addition, the female dogs showed a decrease in glucose level and an increase in triglyceride level at both weeks 2 and 4 of treatment. In dogs treated at 3000 ppm, increases in alkaline phosphatase activity were noted at weeks 2 and 4 of treatment. In dogs treated at 1000 ppm, alkaline phosphatase activity was also increased, but at week 4 only and with the increase in the female still within normal range. The only necropsy observation was emaciation noted in the female dog treated at 30 000 ppm. Organ weight analysis showed increased absolute and relative liver weights and reduced absolute and relative spleen weights in the male treated at 30 000 ppm, decreased absolute pancreas weight and increased relative weights of the brain, pituitary and liver of the female treated at 30 000 ppm, increased absolute and relative liver weights in dogs of both sexes and decreased absolute and relative thyroid weights of the male treated at 10 000 ppm, increased absolute and relative liver weights in dogs treated at 3000 ppm and increased absolute and relative liver weights in the male treated at 1000 ppm. In the female treated at 1000 ppm, only a slight increase in relative liver weight was recorded. Histopathology of the livers revealed centrilobular hepatocellular swelling in dogs treated at 30 000 ppm, accompanied by bile duct proliferation in the male and focal haemorrhage and inflammatory cell infiltration in the female; centrilobular hepatocellular swelling in dogs treated at 10 000 ppm accompanied by focal necrosis in the male; and centrilobular hepatocellular swelling only in dogs treated at 3000 and 1000 ppm.

No NOAEL was established in this study, which was designed only to explore appropriate high doses for further study (Enomoto, 1992b).

In an oral subchronic toxicity study, etoxazole (purity 95.39%) was administered in the diet to four Beagle dogs of each sex per group at a concentration of 0, 200, 2000 or 10 000 ppm (equal to 0, 5.33, 53.7 and 268 mg/kg bw per day in males and 0, 5.42, 55.9 and 277 mg/kg bw per day in females) for 13 weeks. Males from the 2000 and 10 000 ppm dose groups exhibited mucous stool, and one male from the 10 000 ppm dose group exhibited bloody mucous stool. In both males and females, absolute and relative liver weights were increased at 2000 ppm and above. Slight to moderate centrilobular hepatocellular swelling was observed in both sexes at 2000 ppm and above, whereas slight centrilobular inflammatory cell infiltration was observed in females from the 10 000 ppm dose group only. Males from the 2000 ppm dose group exhibited elevated triglyceride levels; this parameter was elevated in both sexes from the 10 000 ppm dose group. Alkaline phosphatase levels were increased in both sexes at 2000 ppm and above (Table 13). Additionally at 10 000 ppm, the activated partial thromboplastin time was reduced, albumin levels were decreased, globulin levels were increased and the albumin to globulin ratio was decreased in both males and females. Slight reductions in red cell indices (red blood cell count, haemoglobin concentration, haematocrit) were observed in males as well, and glucose levels were reduced in females. Prostate weights (absolute and relative) were decreased in males from the 2000 ppm dose groups and above (Table 14). Histologically, acinar cell atrophy was observed in the prostate of one and three males from the 2000 and

Table 13. Selected clinical chemistry findings in male dogs fed etoxazole for 13 weeks[a]

Clinical chemistry parameter	Study week	Dietary concentration (ppm)			
		0	200	2000	10 000
Males					
Triglycerides (mg/dl)	Pretest	45 ± 4	47 ± 6	42 ± 9	37 ± 13
	7	47 ± 7	45 ± 4	49 ± 14	54 ± 9
	13	39 ± 10	40 ± 5	58* ± 11 (↑49)	54 ± 10 (↑38)
Alkaline phosphatase (U/l)	Pretest	128 ± 34	116 ± 10	132 ± 19	132 ± 13
	7	109 ± 24	98 ± 6	168 ± 41 (↑54)	365* ± 246 (↑235)
	13	95 ± 18	81 ± 5	163 ± 57 (↑72)	281* ± 73 (↑196)
Albumin (g/dl)	Pretest	3.08 ± 0.14	3.12 ± 0.14	3.19 ± 0.23	3.21 ± 0.13
	7	3.35 ± 0.11	3.37 ± 0.19	3.24 ± 0.23	2.89* ± 0.28 (↓14)
	13	3.27 ± 0.13	3.46 ± 0.23	3.24 ± 0.21	2.64* ± 0.46 (↓19)
Females					
Triglycerides (mg/dl)	Pretest	43 ± 12	47 ± 6	41 ± 6	40 ± 8
	7	52 ± 20	48 ± 9	49 ± 12	92* ± 24 (↑77)
	13	39 ± 11	44 ± 5	44 ± 6	88** ± 22 (↑126)
Alkaline phosphatase (U/l)	Pretest	110 ± 9	141 ± 29	121 ± 21	99 ± 23
	7	96 ± 15	122 ± 45	158 ± 53 (↑64)	587** ± 243 (↑511)
	13	73 ± 15	106 ± 44	151 ± 64 (↑106)	697** ± 332 (↑855)
Albumin (g/dl)	Pretest	3.42 ± 0.05	3.35 ± 0.19	3.24 ± 0.14	3.27 ± 0.24
	7	3.55 ± 0.13	3.48 ± 0.04	3.40 ± 0.16	3.16** ± 0.14 (↓11)
	13	3.56 ± 0.06	3.49 ± 0.03	3.40 ± 0.20	3.04** ± 0.22 (↓15)

From Kitazawa (1995)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 17-1 through 17-6, pages 87–92; $n = 4$; per cent difference from controls is provided parenthetically.

Table 14. Selected organ weights of dogs fed etoxazole for 13 weeks[a]

Organ weight	Organ weight (g)			
	Dietary concentration (ppm)			
	0	200	2000	10 000
Males				
Liver				
- Absolute	264 ± 30	263 ± 15	324** ± 13 (↑23)	351** ± 21 (↑33)
- Relative	2.72 ± 0.20	2.60 ± 0.19	3.23** ± 0.25 (↑19)	3.54** ± 0.12 (↑30)
Prostate				
- Absolute	5.1 ± 0.6	5.1 ± 0.6	3.8 ± 1.8 (↓25)	1.9 ± 0.2 (↓63)
- Relative	0.052 ± 0.006	0.051 ± 0.015	0.038 ± 0.020 (↓25)	0.019* ± 0.002 (↓63)
Females				
Liver				
- Absolute	247 ± 21	253 ± 15	300** ± 11 (↑21)	373** ± 29 (↑51)
- Relative	2.53 ± 0.17	2.55 ± 0.27	3.19* ± 0.36 (↑26)	3.86** ± 0.37 (↑53)

From Kitazawa (1995)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 21-1, 21-2, 22-1 and 22-2, pages 101–104; $n = 4$; per cent difference from control is provided parenthetically.

Table 15. Selected histopathological findings in dogs fed etoxazole for 13 weeks[a]

Microscopic finding	Incidence (no./4)			
	Dietary concentration (ppm)			
	0	200	2000	10 000
Males				
Liver: centrilobular hepatocellular swelling	0	0	4*	4*
Prostate: acinar cell atrophy	0	0	1	3
Colon/rectum: enteritis	0	0	0	1
Lymph nodes: (omental) – lymphadenitis	0	0	0	1
Lymph nodes: (iliac) – lymphoid cell hyperplasia	0	0	0	1
Females				
Liver: centrilobular hepatocellular swelling	0	0	4*	4*
Liver: centrilobular inflammatory cell infiltration	0	0	0	2

From Kitazawa (1995)

* $P \leq 0.05$

[a] Data were obtained from the study report, Tables 23 and 24, pages 105–106.

10 000 ppm dose groups, respectively. Thyroid gland weights (absolute and relative) were elevated in males and females from the 10 000 ppm dose group; however, no corresponding histological findings were observed. One male from the 10 000 ppm dose group exhibited elevated white blood cell and segmented neutrophil counts and was observed to have severe lymphadenitis of the omental lymph node, moderate lymphoid cell hyperplasia of the iliac lymph node and moderate enteritis of the colon and rectum (Table 15).

The NOAEL in this study is 200 ppm (equal to 5.33/5.42 mg/kg bw per day in males/females), based on clinical signs of toxicity (bloody/mucous stool), hepatotoxicity (increased alkaline phosphatase and triglyceride levels, increased liver weight, centrilobular hepatocellular swelling) and effects on the prostate (decreased weight, acinar cell atrophy) at the LOAEL of 2000 ppm (equal to 53.7/55.9 mg/kg bw per day in males/females) (Kitazawa, 1995).

In a 1-year oral toxicity study, etoxazole (purity 95.96%) was administered in the diet to four Beagle dogs of each sex per group at a concentration of 0, 200, 1000 or 5000 ppm (equal to 0, 4.62, 23.5 and 116 mg/kg bw per day in males and 0, 4.79, 23.8 and 117 mg/kg bw per day in females) for 52 weeks. Findings noted at 200 ppm were limited to slight increases in absolute and relative liver weights in males and females. At 1000 ppm, levels of alkaline phosphatase were increased in males and females throughout treatment. Absolute and relative liver weights were also increased in males and females. Liver enlargement was observed in females, and slight centrilobular hepatocellular swelling was observed in all animals at this dose level. At 5000 ppm, the highest dose tested, alkaline phosphatase and triglyceride levels were increased in males and females throughout treatment (Table 16). Absolute and relative liver weights were also increased in males and females (Table 17). Liver enlargement and severe centrilobular hepatocellular swelling were observed in all animals at this dose level. Additionally in 5000 ppm males, mucous stool and slight decreases in activated partial thromboplastin time, red blood cell count, haemoglobin concentration and haematocrit were observed. Albumin levels were decreased in males, globulin levels were increased in females and the albumin to globulin ratio was decreased in both sexes at this dose (Table 18). One male exhibited slight atrophy of the glandular epithelium of the prostate gland.

Table 16. Selected clinical chemistry findings in dogs fed etoxazole for 52 weeks[a]

Clinical chemistry parameter	Study week	Dietary concentration (ppm)			
		0	200	1000	5000
Males					
Alkaline phosphatase (U/l)	Pretest	100 ± 12	129 ± 40	113 ± 40	102 ± 22
	13	73 ± 12	109 ± 28	186 ± 102 (↑155)	344** ± 47 (↑371)
	26	63 ± 19	79 ± 20	191 ± 99 (↑203)	367** ± 59 (↑483)
	52	58 ± 20	78 ± 17	210 ± 122 (↑262)	398** ± 70 (↑586)
Triglycerides (mg/dl)	Pretest	34 ± 4	46 ± 15	34 ± 7	41 ± 6
	13	36 ± 10	49 ± 7	50 ± 18	115** ± 18 (↑219)
	26	42 ± 12	46 ± 7	52 ± 12	108** ± 19 (↑157)
	52	43 ± 14	47 ± 12	52 ± 13	106** ± 14 (↑147)
Females					
Alkaline phosphatase (U/l)	Pretest	107 ± 8	91 ± 25	92 ± 13	96 ± 23
	13	87 ± 7	79 ± 19	180 ± 44 (↑107)	608* ± 290 (↑599)
	26	63 ± 5	66 ± 14	215 ± 62 (↑241)	507* ± 232 (↑705)
	52	65 ± 7	50 ± 6	197 ± 61 (↑203)	686* ± 330 (↑955)
Triglycerides (mg/dl)	Pretest	41 ± 9	35 ± 7	33 ± 5	39 ± 10
	13	45 ± 7	45 ± 9	47 ± 7	110* ± 32 (↑144)
	26	50 ± 7	64 ± 20	41 ± 6	107** ± 20 (↑114)
	52	49 ± 17	46 ± 5	47 ± 14	107** ± 19 (↑118)
Creatinine (mg/dl)	Pretest	0.97 ± 0.03	0.91 ± 0.03	0.94 ± 0.16	0.96 ± 0.08
	13	1.13 ± 0.05	1.02 ± 0.04	1.00 ± 0.12	1.07 ± 0.06
	26	1.14 ± 0.05	0.96** ± 0.03 (↓16)	1.01 ± 0.10	1.02 ± 0.09
	52	1.20 ± 0.06	1.01** ± 0.06 (↓16)	1.04* ± 0.07 (↓13)	1.05* ± 0.07 (↓13)

From Kitazawa (1996a)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 17-1, 17-5, 18-1, 18-2 and 18-5, pages 98, 102, 105, 106 and 109, respectively; $n = 4$; per cent difference from controls is provided parenthetically.

Table 17. Liver weights of dogs fed etoxazole for 52 weeks[a]

	Liver weight (g)			
	Dietary concentration (ppm)			
	0	200	1000	5000
Males				
Absolute	248 ± 36	288* ± 18 (↑16)	319** ± 11 (↑29)	435** ± 6 (↑75)
Relative	2.15 ± 0.13	2.42 ± 0.10	2.71** ± 0.27 (↑21)	3.60** ± 0.17 (↑67)
Females				
Absolute	244 ± 19	268 ± 24	326* ± 53 (↑34)	429** ± 59 (↑76)
Relative	2.13 ± 0.14	2.44 ± 0.21	2.74* ± 0.34 (↑29)	3.65** ± 0.25 (↑71)

From Kitazawa (1996a)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] Data were obtained from the study report, Tables 21-1, 21-2, 22-1 and 22-2, pages 114–117; $n = 4$; per cent difference from control is provided parenthetically.

Table 18. Selected clinical chemistry findings in dogs fed etoxazole for 52 weeks[a]

Clinical chemistry parameter	Study week	Dietary concentration (ppm)			
		0	200	1000	5000
Males					
Albumin (g/dl)	Pretest	3.07 ± 0.22	3.2 ± 0.14	3.04 ± 0.23	3.1 ± 0.14
	13	3.09 ± 0.18	3.28 ± 0.16	2.85 ± 0.41	2.7 ± 0.25 (↓13)
	26	3.1 ± 0.22	3.13 ± 0.26	3.02 ± 0.08	2.76 ± 0.24 (↓11)
	52	3.22 ± 0.35	3.36 ± 0.22	3.08 ± 0.04	2.91 ± 0.24 (↓10)
Albumin/globulin ratio	Pretest	1.39 ± 0.19	1.4 ± 0.18	1.39 ± 0.09	1.41 ± 0.1
	13	1.2 ± 0.25	1.32 ± 0.19	1.04 ± 0.08	0.94 ± 0.19 (↓22)
	26	0.96 ± 0.21	1.06 ± 0.21	0.93 ± 0.08	0.84 ± 0.16 (↓12.5)
	52	0.91 ± 0.15	1.01 ± 0.19	0.86 ± 0.09	0.84 ± 0.22 (↓8)
Females					
Albumin (g/dl)	Pretest	3.14 ± 0.28	3.13 ± 0.15	3.01 ± 0.27	3.13 ± 0.22
	13	3.21 ± 0.24	3.12 ± 0.18	2.97 ± 0.13	2.90 ± 0.07 (↓10)
	26	3.29 ± 0.21	3.06 ± 0.23	3.04 ± 0.15	3.06 ± 0.2 (↓7)
	52	3.22 ± 0.12	3.08 ± 0.34	3.14 ± 0.06	3.11 ± 0.23 (↓3.5)
Globulin (g/dl)	Pretest	2.18 ± 0.28	2.18 ± 0.3	2.1 ± 0.35	2.05 ± 0.21
	13	2.74 ± 0.29	2.57 ± 0.41	2.61 ± 0.36	2.95 ± 0.3 (↑7)
	26	2.91 ± 0.22	2.87 ± 0.58	3.0 ± 0.49	3.1 ± 0.17 (↑6.5)
	52	3.38 ± 0.53	2.92 ± 0.56	3.22 ± 0.31	3.79 ± 0.43 (↑12)
Albumin/globulin ratio	Pretest	1.46 ± 0.2	1.46 ± 0.21	1.46 ± 0.24	1.53 ± 0.07
	13	1.18 ± 0.15	1.24 ± 0.23	1.15 ± 0.16	1.0 ± 0.12 (↓15)
	26	1.14 ± 0.13	1.09 ± 0.18	1.04 ± 0.18	0.99 ± 0.12 (↓13)
	52	0.97 ± 0.16	1.07 ± 0.11	0.99 ± 0.12	0.83 ± 0.14 (↓14.5)

From Kitazawa (1996a)

[a] Data were obtained from the study report, Tables 17-3, 17-4, 18-3 and 18-4, pages 100, 101, 107 and 108, respectively; $n = 4$; per cent difference from control is provided parenthetically.

The NOAEL is 200 ppm (equal to 4.62/4.79 mg/kg bw per day in males/females), based on elevated alkaline phosphatase levels, increased liver weights, liver enlargement (females) and an increased incidence of slight hepatocellular swelling in the liver at the LOAEL of 1000 ppm (equal to 23.5/23.8 mg/kg bw per day in males/females) (Kitazawa, 1996a).

(b) Dermal application

Rats

In a 28-day dermal toxicity study, groups of 10 Sprague-Dawley (Crj:CD) rats of each sex per dose were treated dermally with etoxazole (purity 95.2%) as a suspension in 0.5% methylcellulose at a dose level of 0, 30, 100 or 1000 mg/kg bw per day for 4 weeks. The volume of application was 2 ml/kg bw, and the duration of treatment was 6 hours daily. All animals were subjected to functional observations and motor activity assessment during week 4. No mortality occurred, and no clinical signs or skin reactions were found during the study. Body weights and feed consumption were not affected by treatment. Functional observational batteries, motor activity evaluation, ophthalmology, haematology, clinical chemistry and necropsy did not reveal any treatment-related effect. The NOAEL in rats was 1000 mg/kg bw per day, the highest dose texted (Ichiki, 1999).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a mouse oncogenicity study, etoxazole (purity 96.01%) was administered in the diet for 78 weeks to 52 ICR (Crj:CD-1) mice of each sex per dose at nominal doses of 0, 15, 60 or 240 mg/kg bw per day (actual doses of 0, 15.1, 60.1 and 241 mg/kg bw per day in males and 0, 15.1, 60.5 and 243 mg/kg bw per day in females). Average concentrations in the diet were 0, 143, 564 and 2285 ppm. A satellite group of 12 mice of each sex per dose were treated similarly, of which 10 mice of each sex per dose were sacrificed at 52 weeks. There were no treatment-related effects on mortality, clinical signs, body weight, feed consumption, feed efficiency, haematology, clinical chemistry, urinalysis, gross pathology or neoplastic microscopic pathology. Relative (to body weight) liver weight was increased in males and females from the 240 mg/kg bw per day dose group at week 52, whereas an increased incidence of centrilobular fatty change was observed at week 52 in males from the 240 mg/kg bw per day dose group. Although there appeared to be some dose-related findings indicative of hepatotoxicity in animals sacrificed at week 52 in this study, the relationship to treatment is uncertain, as these findings were not observed at week 78 in this study or at a similar dose level in a second mouse oncogenicity study at week 52 and were observed only at the next higher dose of 480 mg/kg bw per day at week 78 in the second study. These findings likely represent an adaptive response of the liver.

Therefore, the NOAEL in this study is considered to be 241 mg/kg bw per day (dietary concentration of 2285 ppm), the highest dose tested (Kitazawa, 1996b). The LOAEL was not established. Under the conditions of this study, there was no evidence of carcinogenic potential. However, it is evident that higher doses should have been tested to elicit toxicologically significant effects.

In a carcinogenicity study, etoxazole (purity 95.2%) was administered in the diet to 50 ICR (Crj:CD-1) mice of each sex per group for up to 78 weeks at a concentration of 0, 2250 or 4500 ppm (equal to 0, 242 and 484 mg/kg bw per day in males and 0, 243 and 482 mg/kg bw per day in females, respectively). An additional 12 mice of each sex per group were treated for up to 52 weeks. There were no treatment-related effects on mortality, clinical signs, body weight, body weight gain, feed consumption, feed efficiency, haematology or gross or neoplastic lesions. In the main study (animals sacrificed at 78 weeks), an increased incidence of fatty change in the centrilobular hepatocytes of males from the 4500 ppm dose group was observed when all animals were considered (15/50 treated versus 5/50 in controls) and at the terminal sacrifice (12/33 treated versus 4/34 in controls). Absolute and relative liver weights were also increased in males at 52 and 78 weeks of treatment. Absolute and relative liver weights of females were also increased at weeks 52 and 78. Spleen weights appeared to be decreased at week 78 in both male and female mice. The NOAEL in males is 2250 ppm (equal to 242 mg/kg bw per day), based on an increased incidence of centrilobular hepatocellular fatty change and increased absolute liver weight at the LOAEL of 4500 ppm (equal to 484 mg/kg bw per day). In females, the NOAEL is 4500 ppm (equal to 482 mg/kg bw per day), and the LOAEL was not established because of the absence of adverse effects up to the highest dose tested (Nakashima, 2001a).

There were no treatment-related increases in tumour incidence in either sex when compared with controls. Dosing was considered adequate in males based on the finding of hepatocellular fatty change. No adverse toxicity was observed in females; however, a study using higher doses is not required based on a consideration of the weight of the evidence. There was no evidence of carcinogenicity in this study.

Rats

In a combined chronic toxicity/carcinogenicity study, etoxazole (purity 95.39%) was administered in the diet to 50 Crj:CD(SD) rats of each sex per dose for up to 104 weeks at nominal doses of 0, 4, 16 or 64 mg/kg bw per day (average concentrations in the diet were 0, 112, 449 and 1786 ppm).

Table 19. Incidence and first detection time of testicular interstitial cell tumour

Time point	Incidence			
	Dose (mg/kg bw per day)			
	0	4	16	64
Interim sacrifice week 26	0/10	0/10	0/10	0/10
Interim sacrifice week 52	0/10	1/10	0/10	0/10
Interim sacrifice week 78	0/10	1/10	2/10	2/10
Terminal sacrifice week 104	1/31	5/25	5/23*	8/28**
Rats killed in extremis or found dead	0/19	3/25	3/27	1/20
Overall incidence	1/80	10/80**	10/80**	11/78**

From Nakashima (1996b)
* $P \leq 0.05$; ** $P \leq 0.01$

Table 20. Incidence of testicular tubular atrophy

Time point	Incidence			
	Dose (mg/kg bw per day)			
	0	4	16	64
Interim sacrifice week 26	0/10	1/10	0/10	1/10
Interim sacrifice week 52	0/10	0/10	0/10	0/10
Interim sacrifice week 78	1/10	1/10	0/10	0/10
Terminal sacrifice week 104	2/31	6/25	7/23*	10/28**
Rats killed in extremis or found dead	3/19	3/25	3/27	6/20
Overall incidence	6/80	11/80	10/80	17/78

From Nakashima (1996b)
* $P \leq 0.05$; ** $P \leq 0.01$

The actual test material intake was 0, 4.01, 16.1 and 64.4 mg/kg bw per day for the males and 0, 4.03, 16.1 and 64.5 mg/kg bw per day for the females. Additionally, 10 rats of each sex per dose per interim sacrifice time were treated in a similar manner and sacrificed at weeks 26, 52 and 78. Treatment-related findings were limited to reduced overall body weight gain (6–8%) in both sexes from the 64 mg/kg bw per day dose group and signs of hepatotoxicity in males at week 26 only (liver enlargement at 16 and 64 mg/kg bw per day and centrilobular hepatocellular swelling at 64 mg/kg bw per day). Transient changes in clinical chemistry parameters were also observed. Increased albumin levels were observed in females from the 16 and 64 mg/kg bw per day dose groups at week 78. Levels of the enzymes creatine phosphokinase and lactate dehydrogenase were elevated in females from the 64 mg/kg bw per day dose group at week 26. Also at week 26, males from the 16 and 64 mg/kg bw per day dose groups exhibited increased cholesterol levels, whereas males from the 64 mg/kg bw per day dose group showed only increased bilirubin levels. Overall, these findings are not considered to be adverse.

An increase in benign interstitial cell tumours was observed in the testis at all doses tested compared with the controls (1/80, 10/80, 10/80 and 11/78) (Table 19). In male rats treated at 16 and 64 mg/kg bw per day, the incidence of testicular tubular atrophy was significantly increased at terminal sacrifice. At 64 mg/kg bw per day, the overall incidence of this lesion was also significantly increased (Table 20). Tubular atrophy was not observed when an interstitial cell tumour was simultaneously observed in the same side of the testis. A comparison with historical control data from either the performing laboratory or the animal supplier (Charles River) is given in Table 21.

Table 21. Comparison of testicular interstitial cell tumour incidences

Dose (mg/kg bw per day)	Incidence of interstitial cell tumour			
	Carcinogenicity phase only		All animals (including interim sacrifices)	
	Number	%	Number	%
0	1/50	2.0	1/80	1.3
4	8/50	16.0**	10/80	12.5
16	8/50	16.0**	10/80	12.5
64	9/48	18.8**	11/78	14.1
Historical controls				
- In house (controls used in three toxicity studies)	10/149	6.7 (2.0–10.2)	—	—
- In house (controls used in two toxicity studies)	—	—	9/159	5.7 (5.0–6.3)
- Charles River (laboratory animal supplier)	59/1260	4.7 (1.4–10.0)	—	—

From Nakashima (1996b)
** $P < 0.01$

Table 22. Incidence of pancreatic islet cell adenomas and carcinomas (main and interim sacrifice groups; all animals examined)

Site and lesion	Incidence							
	Dose (mg/kg bw per day)							
	Male				Female			
	0	4	16	64	0	4	16	64
Number of animals examined	*80*	*80*	*80*	*79*	*80*	*80*	80	80
Islet cell adenoma	5	2	1	6	1	1	0	5
Mixed acinar–islet cell adenoma	0	1	0	0	0	0	0	0
Islet cell carcinoma	0	0	0	0	2	1	1	0

From Nakashima (1996b)

Benign islet cell adenoma in the pancreas was observed in the 64 mg/kg bw per day females (1/80, 1/80, 0/80 and 5/80) (Table 22). No increase in either of these tumours was observed in the second carcinogenicity study (see below; Nakashima, 2001b) at higher doses. The tumours are not considered to be treatment related.

The NOAEL in this study is 64 mg/kg bw per day, the highest dose tested. A LOAEL was not observed in this study, as no adverse findings were noted (Nakashima, 1996b).

In a combined chronic toxicity/carcinogenicity study, etoxazole (purity 95.2%) was administered to groups of 50 Crj:CD(SD) rats of each sex per dose at a dietary concentration of 0, 50, 5000 or 10 000 ppm (equal to 0, 1.83, 187 and 386 mg/kg bw per day in males and 0, 2.07, 216 and 445 mg/kg bw per day in females) for 104 weeks. Additional groups of 15 rats of each sex per dose (satellite study) were similarly dosed and sacrificed at 52 weeks. A functional observational battery was conducted at 52 weeks on 10 rats of each sex per dose. Mean absolute body weights were decreased by as much as 9% in the 10 000 ppm males up to week 100 and by 15% at study termination. Overall weight gain in the 10 000 ppm group males was reduced by 20% because of a marked weight loss after week 76 compared with that of controls. Female rats fed the 10 000 ppm diet weighed up to

Table 23. Statistically significant changes in incidences of clinical signs (main group)

Clinical sign	Incidence							
	Dietary concentration (ppm)							
	0	50	5000	10 000	0	50	5000	10 000
	Males				Females			
Incisor: whitening of upper incisor	0/50	0/50	49/50**	50/50**	0/49	0/50	50/50**	50/50**
Incisor: whitening of lower incisor	0/50	0/50	49/50**	50/50**	0/49	0/50	50/50**	50/50**
Incisor: elongation of upper incisor	14/50	12/50	37/50**	50/50**	9/49	13/50	47/50**	50/50**
Incisor: elongation of lower incisor	—	—	—	—	6/49	8/50	10/50	17/50**
Incisor: abrasion of upper incisor	0/50	1/50	1/50	5/50*	4/49	2/50	6/50	23/50**
Incisor: abrasion of lower incisor	2/50	3/50	6/50	49/50**	5/49	3/50	27/50**	49/50**
Appearance: emaciation	—	—	—	—	2/49	7/50	8/50*	14/50**

From Nakashima (2001b)
* $P \leq 0.05$; ** $P \leq 0.01$

15% less than controls from week 1 to week 84 and up to 17% less after week 84 and gained 15% less weight than controls during the first year and 22% less overall. Feed consumption by the 10 000 ppm females was 15% less than that of controls during the first 12 weeks of the study and 7% less than controls over the entire study. Feed efficiency was also 7% less than that of controls during the first 13 weeks of the study when feed efficiency was measured. At 5000 ppm, body weights at week 104 and overall body weight gains were also reduced by 4% and 5%, respectively, in males and by 7% and 10%, respectively, in females.

Clinical signs observed in almost all male and female rats in the satellite and main studies at 5000 and 10 000 ppm included whitening of the upper and lower incisors, elongation of the upper incisor and abrasion (secondary to elongation) of the upper (52 weeks only) and/or lower incisors. Similar observations were made during gross examination of the satellite and main study rats of both sexes. An increased incidence of abnormal amelogenesis (formation of tooth enamel) of the upper incisor was observed microscopically in the 10 000 ppm group males and females at 52 weeks and in both sexes of the 5000 and 10 000 ppm groups in the main study. Female rats fed the 5000 and 10 000 ppm diets also had a significantly increased incidence of emaciation that was confirmed during gross examination of the 10 000 ppm dose group (Table 23).

Evaluation of clinical chemistry parameters revealed that platelet counts were elevated in females from the 5000 and 10 000 ppm dose groups, whereas the activated partial thromboplastin time was elongated in males from the 5000 and 10 000 ppm dose groups at week 104 only. Levels of cholesterol, serum protein, albumin (week 13 only), globulin and γ-glutamyl transferase were consistently elevated in males and females from the 10 000 ppm dose group, whereas urinary protein level was elevated in 10 000 ppm females only. At 5000 ppm, albumin and protein levels were elevated in males and females at week 13 only. Slight decreases in haematocrit and haemoglobin levels were observed in the 5000 ppm females only at week 26, whereas at 10 000 ppm, these changes, along with reduced mean cell volume, were observed in both sexes throughout the study. Mean cell volume was also reduced in the 5000 ppm group males. Postmortem examination showed significantly increased absolute and relative liver weights in both sexes from the 5000 and 10 000 ppm dose groups at 52 weeks and in males from the 10 000 ppm dose group at 104 weeks, as well as an increased incidence of liver spots in males from the 10 000 ppm dose group. Thyroid weights (absolute and relative) were elevated in males from the 5000 and 10 000 ppm dose groups as well, with no corroborating evidence of thyroid toxicity. In addition to abnormalities in the incisors, gross examination revealed that male and female rats had thickening of the bone in the parietal region at 10 000 ppm.

Table 24. Statistically significant treatment-related non-neoplastic findings (overall incidence)

Site/lesion	Incidence							
	Dietary concentration (ppm)							
	0	50	5000	10 000	0	50	5000	10 000
	Males				Females			
Liver: centrilobular hepatocellular hypertrophy	0/50	0/50	0/50	8/50**	0/49	0/50	0/50	28/50**
Upper incisor: abnormal amelogenesis	1/50	0/50	10/50**	32/50**	3/49	4/50	16/50**	40/50**
Bone (parietal region): hyperplasia of bone tissue	—[a]/0[b]	—/0	1/1	15/15	—/0	—/0	1/1	13/13

From Nakashima (2001b)
** $P < 0.01$
[a] Number of animals bearing the histopathological lesion in bone in parietal region.
[b] Number of animals bearing the gross pathological lesion in bone in parietal region.

Table 25. Testicular effects, non-neoplastic and neoplastic

Effects	Incidence			
	Dietary concentration (ppm)			
	0	50	5000	10 000
Seminiferous tubule atrophy total	23/50	12/50	20/50	24/50
Seminiferous tubule atrophy in animals sacrificed at week 104	4/16	4/17	7/20	14/23*
Interstitial cell tumour	5/50	2/50	4/50	1/50

From Nakashima (2001b)
* $P < 0.05$

Treatment-related microscopic findings observed at 10 000 ppm, other than abnormal amelogenesis, included hyperplasia of the bone in the parietal region in both sexes, bile duct hyperplasia in females and centrilobular hepatocellular hypertrophy in both sexes (Table 24). There was a statistically significant increase in the incidence of seminiferous tubular atrophy at terminal sacrifice only at 10 000 ppm. This effect was not observed when all animals were included (Table 25).

The NOAEL in this study is 50 ppm (equal to 1.83/2.07 mg/kg bw per day in males/females), based on effects on the incisors, including abnormal amelogenesis, observed at the LOAEL of 5000 ppm (equal to 187/216 mg/kg bw per day in males/females).

At the doses tested, there was no significant increase in the incidence of neoplastic lesions in male or female rats receiving any dose of the test material. The animals were adequately dosed based on decreased weight gain and bone effects in both sexes at 10 000 ppm and effects on the incisors at 5000 and 10 000 ppm (Nakashima, 2001b).

2.4 Genotoxicity

A battery of GLP-compliant studies of mutagenicity with etoxazole was conducted to assess its potential for inducing gene mutation, chromosomal aberration and unscheduled deoxyribonucleic acid (DNA) synthesis. The study results (summarized in Table 26) were negative, with the exception of the mammalian cell gene mutation assay and a published study with human lymphocytes, which

Table 26. Genotoxicity of etaxazole

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 313, 625, 1250, 2500 or 5000 µg/plate	96.26	Negative	Watanabe (1992)
Reverse mutation	*S. typhimurium* TA102	0, 313, 625, 1250, 2500 or 5000 µg/plate	95.2	Negative	Ota (1999)
Mammalian cell gene mutation	Mouse lymphoma L5178Y cells	0, 0.5, 1, 2.5, 3, 4, 5 or 10 µg/ml (+S9)	95.4	Positive (+S9)	Adams (1996)
		0, 10, 35, 40, 50, 55 or 60 µg/ml (−S9)		Inconclusive (−S9)	
Chromosomal aberration	Chinese hamster lung cells	0, 22.5, 45, 90 or 180 µg/ml (+S9, 6–18 h, 6–42 h)	95.39	Negative	Matsumoto (1994)
		0, 15.6, 31.3, 62.5 or 125 µg/ml (−S9, 24 h)			
		0, 12.5, 25, 50 or 100 µg/ml (−S9, 48 h)			
Chromosomal aberration, sister chromatid exchange, micronucleus	Human lymphocytes	0, 5, 10 or 20 µg/ml	Not provided	Positive	Rencüzoğullari et al. (2004)
In vivo					
Mouse micronucleus	CD-1 mice, male and female	0, 1250, 2500 or 5000 mg/kg bw (sampling times: 24, 48, 72 h)	95.4	Negative	Odawara (1996)
Unscheduled DNA synthesis	CD rats, male	0, 2500 or 5000 mg/kg bw (sampling times: 12–14 h, 2–4 h)	95.3	Negative	Clare (1997)

demonstrated mutagenicity at cytotoxic doses. Overall, etoxazole did not demonstrate any genotoxic potential.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a non-guideline reproduction range-finding study, etoxazole (purity 97.99%) was administered continuously in the diet to eight Crj:CD(SD) rats of each sex per group at a dietary concentration of 0, 100, 300, 1000 or 3000 ppm (actual intakes shown in Table 27). Parental animals were given test article diet formulations for 3 weeks prior to mating. On postnatal day (PND) 21, all pups and their respective dams were sacrificed.

When compared with concurrent controls, no treatment-related changes were observed in the following parameters: mortality, clinical signs, body weights, feed consumption, and reproductive

Table 27. Etoxazole intakes during the different phases of a range-finding one-generation study in rats

Phase of the study	Dose (mg/kg bw per day)			
	Dietary concentration (ppm)			
	100	300	1000	3000
Males				
Premating	6.35–7.47	19.4–23.4	64.7–77.0	185–229
Breeding	4.71–5.72	14.7–17.9	48.8–61.6	145–182
Females				
Premating	7.30–7.51	22.4–23.2	78.1–80.3	221–223
Gestation	6.50–7.82	18.5–22.6	66.0–78.0	191–225
Lactation	11.89–22.92	35.1–65.2	120–227.2	369–658

From Hatakenaka (1994a)

function and performance in the parental animals; and mean number of pups delivered, survival indices, sex ratios, clinical signs and gross pathology in the offspring. At 300, 1000 and 3000 ppm, relative (to body weight) liver weights were increased in the males and females. Additionally at 3000 ppm, enlarged liver was noted in four of eight males and two of eight females (versus 0/16 controls). No treatment-related findings were noted at 100 or 300 ppm. Although relative liver weights were increased at 300 and 1000 ppm, they were not considered to be biologically significant in the absence of other liver findings. In the offspring at 3000 ppm, decreased body weights were noted in both sexes on lactation days 14 and 21.

The NOAEL for parental toxicity is 1000 ppm, based on enlarged livers at 3000 ppm. The NOAEL for reproductive effects is 3000 ppm, the highest dose tested, and the NOAEL for offspring toxicity is 1000 ppm, based on decreased body weight at 3000 ppm (Hatakenaka, 1994a).

In a two-generation reproductive toxicity study, etoxazole (purity 96.01%) was administered continuously in the diet to Crj:CD(SD) rats (24 of each sex per dose) at a dietary concentration of 0, 80, 400 or 2000 ppm (equivalent to premating doses of 0/0, 5.6/6.7, 28.2/33.4 and 139/159 mg/kg bw per day in males/females of the F_0 parental generation and 0/0, 6.3/6.8, 31.7/35.6 and 157/172 mg/kg bw per day in males/females of the F_1 parental generation) (Tables 28 and 29). In the parental animals, there were no treatment-related effects on mortality, clinical signs, body weight, body weight gain, feed consumption, reproductive function or performance, or gross pathology in either the F_0 or the F_1 generation. At 2000 ppm, non-adverse changes in organ weight were observed in the F_0 generation (increased absolute and relative liver weights in males, increased absolute [not statistically significant] and relative adrenal gland weights in females), with no corresponding pathology noted in these tissues upon necropsy. Liver weights (absolute and relative) were also increased in F_1 males at 2000 ppm. Two males of the F_1 generation from the 2000 ppm dose group exhibited slight centrilobular hepatocellular fatty change; no other animals from any dose group exhibited this lesion.

Therefore, the NOAEL for parental toxicity is 400 ppm in males (31.7 mg/kg bw per day in the F_1 generation), based on liver toxicity (increased weight, fatty change) noted at the LOAEL of 2000 ppm (157 mg/kg bw per day in the F_1 generation). For females, the parental NOAEL is 2000 ppm (159 mg/kg bw per day in F_0 females), the highest dose tested, as no adverse effects were noted in females.

In the offspring, no treatment-related effects on sex ratio, body weight or gross pathology were noted in either generation. As this study was conducted prior to the most current test guidelines,

Table 28. Etoxazole intakes by male rats during the different phases of a two-generation study

Dietary concentration (ppm)	Generation	Dose (mg/kg bw per day)	
		Premating period	Breeding period
80	F_0	4.20–8.36	3.52–4.05
	F_1	4.29–9.57	3.45–3.94
400	F_0	21.5–41.4	17.2–20.5
	F_1	21.8–48.6	17.0–19.0
2000	F_0	105.0–209.1	89.1–101.9
	F_1	110.1–233.3	86.4–99.0

From Hatakenaka (1996)

Table 29. Etoxazole intakes by female rats during the different phases of a two-generation study

Dietary concentration (ppm)	Generation	Dose (mg/kg bw per day)		
		Premating period	Gestation period	Lactation period
80	F_0	5.27–8.92	4.77–5.41	8.26–16.38
	F_1	5.12–9.73	4.82–5.56	7.89–16.21
400	F_0	26.5–45.1	23.3–26.9	41.8–82.3
	F_1	26.4–50.5	23.6–25.6	37.9–82.5
2000	F_0	127–223	116–131	217–427
	F_1	128–240	113–124	201–410

From Hatakenaka (1996)

developmental landmarks, organ weights and histopathology were not assessed in the offspring, as the older test guidelines in force at the time at which this study was conducted did not require these assessments.

At 2000 ppm, there was an increase in the number of pup deaths and number of litters with pup deaths between lactation days 0 and 4 in both generations, resulting in decreased viability indices on lactation day 4 (Tables 30 and 31). Therefore, the NOAEL and LOAEL for offspring toxicity are 400 ppm (equivalent to 28.2/33.4 mg/kg bw per day in F_0 generation males/females) and 2000 ppm (equivalent to 139/159 mg/kg bw per day in F_0 generation males/females), respectively. A LOAEL for reproductive performance was not observed. The NOAEL for reproductive performance is 2000 ppm (Hatakenaka, 1996).

(b) Developmental toxicity

Rats

In a preliminary study of prenatal developmental toxicity, groups of seven mated Sprague-Dawley female rats were given etoxazole (purity 97.99%) suspended in 1% aqueous solution of sodium carboxymethylcellulose by oral gavage at a dose of 0, 10, 100, 300 or 1000 mg/kg bw per day from gestation day (GD) 6 to GD 15. All dams were sacrificed on GD 20, and the uterine contents were examined. There was no evidence of maternal toxicity, and caesarean sectioning at day 20 also revealed no treatment-related changes in ovaries and uteri. The per cent incidence of resorptions and fetal deaths was statistically significantly increased at 1000 mg/kg bw per day (14.5% versus 1.5% in controls).

Table 30. F_1 generation mean litter size and viability[a]

Observation	Dietary concentration (ppm)			
	0	80	400	2000
Mean litter size[b]				
- Day 0	14.4	13.8	12.7	13.7
- Day 4[c]	14.3	13.0	12.4	12.2
- Day 4[d]	8	7.7	7.9	7.5
- Day 4[e]	8	8	7.9	7.9
- Day 21	8	7.7	7.9	7.5
- Day 21[e]	8	8	7.9	7.9
Number of live pups[f]				
- Day 0	331	332	266	301
- Day 4[c]	328	313	261	269
- Day 4[d]	184	184	166	166
- Day 21	184	184	166	166
Number of deaths				
- Days 0–4[c]	3	19	5	32
- Days 4[d]–21	0	0	0	0
Dams with total litter loss days 0–4	0	1	0	1
Viability index (%)				
- Day 0	96.2	97.8	95.8	93.5
- Day 4	99.1	94.3	98.3	89.5*
- Day 21	100	100	100	100
Lactation index (%)	NR	NR	NR	NR
Sex ratio	0.499	0.485	0.459	0.579

From Hatakenaka (1996)

NR, not reported; * $P \leq 0.05$

[a] Data extracted from the study report, Table 12, page 70.

[b] Calculated from the number of live pups presented in this table.

[c] Pre-cull.

[d] Post-cull, including dams with total litter loss between day 0 and day 4.

[e] Post-cull, excluding dams with total litter loss between day 0 and day 4.

[f] Tabulated from individual data presented in the study report, Appendix 65, pages 156–163.

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested, and the NOAEL for developmental toxicity was 300 mg/kg bw per day, based on an increase in resorptions and fetal death at 1000 mg/kg bw per day (Hatakenaka, 1993).

In a developmental toxicity study, etoxazole (purity 95.39%) was administered to pregnant Crj:CD (SD) rats (24 per dose) in 1% carboxymethylcellulose via gavage at a dose of 0, 40, 200 or 1000 mg/kg bw per day from GD 6 through GD 15. All dams were sacrificed on GD 20. There were no unscheduled deaths during the study. When compared with concurrent controls, no treatment-related changes in clinical signs, body weights or gross pathology were noted at any dose level tested. Slight, non-adverse decreases in feed consumption and body weight gain were noted in the dams during the dosing period at 1000 mg/kg bw per day. No treatment-related effects on reproductive parameters were observed, and no treatment-related external, visceral or skeletal variations or malformations were noted in the fetuses.

Table 31. F_2 generation mean litter size and viability[a]

Observation	Dietary concentration (ppm)			
	0	80	400	2000
Mean litter size[b]				
- Day 0	13.2	13.2	12.7	14.0
- Day 4[c]	11.9	12.9	11.5	11.8
- Day 4[d]	7.8	7.8	7.2	7.2
- Day 4[e]	7.8	7.8	8.0	7.9
- Day 21	7.8	7.8	7.2	7.2
- Day 21[e]	7.8	7.8	7.9	7.9
Number of live pups[f]				
- Day 0	290	304	241	322
- Day 4[c]	261	296	219	272
- Day 4[d]	163	180	136	166
- Day 21	163	180	134	166
Number of deaths				
- Days 0–4[c]	29	8	22	50
- Days 4[d]–21	0	0	2	0
Dams with total litter loss days 0–4	1	0	2	2
Viability index (%)				
- Day 0	92.0	96.1	97.7	94.9
- Day 4	90.0	97.7	87.1	85.5
- Day 21	100	100	98.5	100
Lactation index (%)	NR	NR	NR	NR
Sex ratio	0.511	0.497	0.506	0.510

From Hatakenaka (1996)

NR, not reported

[a] Data extracted from the study report, Table 12, page 70.

[b] Calculated from the number of live pups presented in this table.

[c] Pre-cull.

[d] Post-cull, including dams with total litter loss between day 0 and day 4.

[e] Post-cull, excluding dams with total litter loss between day 0 and day 4.

[f] Tabulated from individual data presented in the study report, Appendix 65, pages 156–163.

The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on reduced feed consumption and body weight gain observed during the treatment period at the LOAEL of 1000 mg/kg bw per day. The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested; a LOAEL for developmental toxicity was not established, as no adverse effects on prenatal development were noted (Hatakenaka, 1994b).

Rabbits

In a preliminary study of prenatal developmental toxicity, groups of five inseminated female Japanese White rabbits (Kbl:JW) were given etoxazole (purity 95.39%) suspended in a 1% aqueous solution of sodium carboxymethylcellulose by oral gavage at a dose of 0, 10, 100, 300 or 1000 mg/kg bw per day from GD 6 to GD 18. All does were sacrificed on GD 27, and the uterine contents were examined. There was no evidence of maternal toxicity, but at necropsy of does, enlarged liver was observed in three of the five does treated at 1000 mg/kg bw per day, and this finding was considered

Table 32. Summary of survival and pregnancy status in does

Dose (mg/kg bw per day)	No. of mated females	No. of pregnant females	No. of females that died or were killed in extremis[a]	No. of females that aborted	No. of females that survived[a]	No. of females with resorptions only	No. of females with live fetuses
0	18	18	0	0	18	4	14
40	18	18	0	1	17	2	15
200	18	18	0	1	17	0	17
1000	17	17	1	0	16	1	15

From Hojo (1994)

[a] All females were pregnant.

Table 33. Incidence of 27 presacral vertebrae with 13th ribs in fetuses

	Dose (mg/kg bw per day)				Historical control
	0	40	200	1000	
Number of fetuses examined (number of litters examined)	117 (14)	121 (15)	149 (17)	129 (15)	—
Fetal (litter) incidence of 27 presacral vertebrae	0 (0)	2 (2)	2 (2)	4 (3)	Not provided
Fetal (litter)[a] incidence of 27 presacral vertebrae with 13th ribs	5 (2)	10 (6)	5 (3)	14* (9)	2–15 (2–7)

From Hojo (1994)

* $P \leq 0.05$

[a] Statistical analyses were not performed for litter incidences.

likely to be related to treatment. Caesarean sectioning at day 27 did not reveal any treatment-related changes in ovaries, uteri or fetuses.

The NOAEL for both maternal and developmental toxicity was 1000 mg/kg bw per day, the highest dose tested (Hojo, 1993).

In a developmental toxicity study, etoxazole (purity 95.39%) was administered in 1% aqueous sodium carboxymethylcellulose via gavage to pregnant Kbl:Japanese White rabbits (18 per dose) at a dose level of 0, 40, 200 or 1000 mg/kg bw per day on GD 6 through GD 18. All does were sacrificed on GD 27 (Table 32). At 1000 mg/kg bw per day, body weight gains were decreased during the treatment and post-treatment intervals, resulting in a decreased overall body weight gain in these animals. The does in this dose group also lost considerable weight during the post-treatment period. Feed consumption was similarly decreased throughout the treatment and post-treatment intervals. Liver enlargement was observed in 2 of 17 does from the 1000 mg/kg bw per day dose group, compared with 0 in controls. At 1000 mg/kg bw per day, incidences of skeletal variations (27 presacral vertebrae and 27 presacral vertebrae with 13th rib) were increased on a fetal and litter basis when compared with controls (Table 33).

The maternal NOAEL is 200 mg/kg bw per day, based on liver enlargement and decreased body weight gain and feed consumption at the LOAEL of 1000 mg/kg bw per day. The developmental NOAEL is 200 mg/kg bw per day, based on an increased incidence of skeletal variations at the LOAEL of 1000 mg/kg bw per day (Hojo, 1994).

2.6 Special studies

(a) Liver effects

Groups of six Sprague-Dawley rats of each sex per dose received etoxazole (purity 95.39%) in the diet at a concentration of 0, 1000 or 2000 ppm (equal to 0, 74.75 and 152.35 mg/kg bw per day at week 4 and 0, 59.6 and 119.5 mg/kg bw per day at week 13 for males and 0, 76.2 and 155.1 mg/kg bw per day at week 4 and 0, 66.7 and 133.5 mg/kg bw per day at week 13 for females) for either 4 or 13 weeks. At each sacrifice, the liver was analysed to determine cytochrome P450 content and enzyme activities (ethoxycoumarin *O*-dealkylase and pentoxyresorufin *O*-dealkylase). Histopathological examination of the liver was also performed. No mortality occurred, and no clinical signs related to treatment were noted. Body weight and feed consumption were not affected by treatment. No macroscopic change attributable to treatment was noted at necropsy. Male rats treated at 2000 ppm showed statistically significant increases in liver weights after 4 or 13 weeks of treatment. Female rats of this same group showed increases in absolute and relative liver weights, compared with controls, after 4 weeks, but both absolute and relative liver weights were comparable to those of the control group after 13 weeks of treatment. In animals treated at 1000 ppm, only female rats showed increases in absolute and relative liver weights, compared with controls, after 4 weeks, but again both absolute and relative liver weights were comparable to those of the control group after 13 weeks of treatment. Histopathology showed centrilobular hepatocellular swelling in the liver of all male rats sacrificed after 4 weeks of treatment at 2000 ppm, whereas no treatment-related findings were ever detected in female rats or in male rats after 13 weeks of treatment at either dose level. Results of liver biochemistry did not show any significant change in any of the treated groups compared with the controls, with the exception of a statistically significant increase in microsomal protein content in male rats treated for 13 weeks at 2000 ppm. In conclusion, hepatomegaly could not be associated with an effect on the cytochrome P450 system (Inui, 1995).

(b) Testicular effects

A supplementary 4-week study was performed to assess the effects of etoxazole on proliferative activity of interstitial testicular cells, spermatogenesis and related serum hormone levels in male rats in order to provide supplementary data to a combined chronic toxicity/carcinogenicity study (Nakashima, 1996b). Groups of 14 male Sprague-Dawley rats, 14 weeks old, received etoxazole (purity 95.39%) in the diet at concentrations adjusted weekly to achieve intakes of 4, 16 or 64 mg/kg bw per day (the same dose levels used in the combined study). Actual intakes were 3.9, 16.0 and 64.1 mg/kg bw per day. After 4 weeks of treatment, serum levels of estradiol, luteinizing hormone, prolactin and testosterone were analysed in 10 animals in each group. For these same animals, testis weight was recorded, light microscopy of the testis and epididymis was performed and the cell index of germ cells (number of germ cells/number of Sertoli cells in seminiferous tubules) was determined. The remaining four animals per group were used to examine the labelling index with 5-bromo-2′-deoxyuridine (BrdU) in testicular interstitial cells.

There were no statistically significant differences in any of the examined parameters (body weight, feed consumption, serum hormone levels, absolute and relative testis weights, histopathology, cell index of germ cells and BrdU labelling index in interstitial cells) between the treated and the control groups. Dietary administration of etoxazole in the diet to male Sprague-Dawley rats for 4 weeks at doses up to and including 64 mg/kg bw per day revealed no effects on serum hormone levels reflecting testicular function, proliferative activity of testicular interstitial cells or spermatogenesis (Yoshida, 1996).

(c) Neurotoxicity

The clinical observations of the repeated-dose studies did not reveal any evidence of neurotoxicity. In addition, a functional observational battery, which included an assessment of motor activity,

grip strength and sensorimotor reaction to stimuli, conducted at 1 year in the 2-year study in rats, yielded negative results for neurotoxicity.

(d) Studies on impurity 2,5-YI

The acute oral toxicity and the genotoxic potential of the manufacturing impurity 2,5-YI were investigated. The studies were reported to comply with GLP and performed according to internationally accepted guidelines.

A group of 10 fasted Sprague-Dawley rats (five males and five females) was given a single dose by oral gavage of 2,5-YI (purity 98.52%), formulated in 1% w/v aqueous methylcellulose, at the limit dose of 5000 mg/kg bw. There were no mortalities during the study. Clinical signs of reaction to treatment included piloerection, soft to liquid faeces and gasping/noisy respiration, seen in all or the majority of rats. Less commonly observed were hunched posture, waddling gait, lethargy, decreased respiratory rate, pallor of the extremities, increased urine production, increased salivation, walking on toes, unsteadiness, hair loss and red/brown staining around nose and mouth. Recovery was complete in all instances by day 12. Slightly lower (compared with other rats in the same group) body weight gains were recorded for one male and one female on day 8, but these rats achieved satisfactory body weight gains as measured on day 15. All other rats achieved satisfactory body weight gains throughout the study.

The acute oral LD_{50} for 2,5-YI was greater than 5000 mg/kg bw (McRae, 1996).

In two independent microbial mutagenicity tests, *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 *uvrA* were exposed to 2,5-YI (purity 98.52%) in dimethyl sulfoxide at a concentration of 312.5, 625, 1250, 2500 or 5000 µg/plate in the presence or absence of exogenous metabolic activation (±S9). 2,5-YI was tested up to the limit of solubility (625 µg/plate) and the limit dose (±S9). No cytotoxicity was observed at any dose level in either test. No increases in the number of revertants per plate were observed in any bacterial strain at any dose level of 2,5-YI in the presence or absence of S9 activation when compared with vehicle controls. The efficacy of the S9 mix and the sensitivity of the test system to detect mutagenic agents were adequately demonstrated by the responses obtained with the non-activated and S9-activated positive controls. Under the conditions of this study, 2,5-YI is considered to be non-mutagenic in *S. typhimurium* or *E. coli* when tested up to 5000 µg/plate, with or without exogenous metabolic activation (Jones, 1996).

3. Observations in humans

Medical data on etoxazole are limited, but no reports of adverse effects were identified during routine monitoring of production plant workers or among personnel involved in the experimental biological testing or field trials. There is no evidence available to support any findings in relation to poisoning with etoxazole.

Comments

Biochemical aspects

The absorption, distribution, metabolism and excretion of etoxazole were investigated in rats. ^{14}C-labelled etoxazole was rapidly but only moderately absorbed from the gastrointestinal tract of rats following oral dosing. Maximum concentrations of radioactivity in plasma were observed within 2–4 hours of dosing for the low-dose group (5 mg/kg bw) and within 4–6 hours for the high-dose group (500 mg/kg bw). At the low dose, the degree of absorption in males (50–54%) was less than

that in females (63–70%), but there were no major sex-related differences in the pattern of excretion. Saturation of absorption occurred at a high dose (500 mg/kg bw per day), with less than 30% absorbed. Faecal excretion was the primary route of elimination, and excretion was essentially complete within 120 hours after dosing. Very little etoxazole was retained in the tissues. By 168 hours post-dosing, concentrations of radioactivity remaining in liver, thyroid and fat of rats were 3.9–7.8 times higher in the repeated-dose experiment than in the same tissues from the single-dose group.

The parent compound was the major component in the faeces, at 17.8–29.1% in the low-dose groups and 74.7–80.2% in the high-dose groups. Based on the analyses of excreta, bile and liver, the biotransformation of etoxazole in rats primarily involves the hydroxylation of the 4,5-dihydrooxazole ring, followed by cleavage of the metabolite and hydroxylation of the *tert*-butyl side-chain.

Toxicological data

Etoxazole had low acute toxicity in rats, causing no mortality at the limit dose after oral (LD_{50} > 5000 mg/kg bw), dermal (LD_{50} > 2000 mg/kg bw) or inhalation (LC_{50} > 1.09 mg/l air, highest attainable concentration) exposure. Etoxazole was not irritating to the skin or eyes of rabbits and not sensitizing under the conditions of the Magnusson & Kligman maximization test in guinea-pigs.

Following repeated dietary dosing, the liver was the main target organ in mice, rats and dogs. Hepatotoxicity was manifest as increased liver weight, liver enlargement and centrilobular hepatocellular hypertrophy, as well as alterations in clinical chemistry (elevated serum levels of liver enzymes, cholesterol, triglycerides and protein). In several studies, effects on the liver were mild and considered to be non-adverse, reflecting an adaptive response of the liver rather than overt hepatotoxicity. The spectrum of liver effects and the doses eliciting hepatotoxicity did not change significantly with duration of dosing, although the severity of the histopathological lesions observed in the liver did increase slightly with longer-term dosing. For example, fatty change of the liver was observed in mice only after exposure to doses of 2285 ppm (equal to 241 mg/kg bw per day) and higher for 18 months and in rats in the second generation of a multigeneration reproduction study at 2000 ppm (equal to 157 mg/kg bw per day), and the degree of centrilobular hepatocellular hypertrophy was graded as severe only in high-dose dogs after 12 months of dosing at 5000 ppm (equal to 116 mg/kg bw per day). Generally, the macroscopic observation of liver enlargement was more evident in females than in males, whereas the microscopic observation of hepatocellular hypertrophy was more prominent in males. Periportal necrosis of the liver was observed only in mice at 6400 ppm (equal to 878 mg/kg bw per day), the highest dose tested. An increased incidence of hyperplasia of the bile duct was observed at high doses in female rats only after dosing for 2 years. A special study revealed that drug metabolizing enzymes were not induced following exposure of rats to etoxazole for 4 or 13 weeks.

Dental and bone abnormalities were observed in rats after repeated dosing. The dental abnormalities included elongation of the upper incisors after subchronic dosing and elongation, whitening and abrasion of the upper and lower incisors as well as abnormal amelogenesis (formation of tooth enamel) of the upper incisor after longer-term dosing. It should be noted that the molecule contains fluorine. Although there are no specific studies on the release of fluoride from the molecule, these dental and bone effects would be consistent with the presence of free fluoride. The NOAEL for elongation of incisors in the 90-day study was 5000 ppm (equal to 300 mg/kg bw per day). In the 2-year study conducted at higher doses, the dental effects occurred at the LOAEL of 5000 ppm (equal to 187 mg/kg bw per day), and the NOAEL was 50 ppm (equal to 1.83 mg/kg bw per day). Thickening and hyperplasia of the parietal bone were observed in rats only after chronic dosing at the highest dose tested, 10 000 ppm (equal to 386 mg/kg bw per day). In the 2-year rat study conducted at lower doses, no dental or bone effects were observed at the highest dose tested (64 mg/kg bw per day). An overall NOAEL for dental and bone abnormalities and liver effects from the two long-term rat studies combined is 64 mg/kg bw per day.

The dog was the most sensitive species following short-term dosing. The NOAEL in the 90-day study was 200 ppm (equal to 5.33 mg/kg bw per day), based on liver effects (increased serum levels of triglycerides and alkaline phosphatase, absolute and relative weights and incidence of centrilobular hepatocellular hypertrophy), as well as mucous stool (observed after repeated dosing), decreased absolute and relative prostate weights and slight to moderate prostate acinar cell atrophy. The NOAEL in the 1-year dog study was also 200 ppm (equal to 4.62 mg/kg bw per day), based on liver effects (increased serum level of alkaline phosphatase, incidence of liver enlargement and incidence of centrilobular hepatocellular hypertrophy) at the LOAEL of 1000 ppm (equal to 23.5 mg/kg bw per day). In contrast, the NOAEL in the 90-day mouse study was 1600 ppm (equal to 214 mg/kg bw per day), and the NOAEL in the 90-day rat study was 1000 ppm (equal to 61.8 mg/kg bw per day), in both cases based on liver effects.

Two carcinogenicity studies each were conducted in the rat and the mouse due to inadequate dosing in the initial studies. In the first mouse study, animals were dosed at 0, 15.1, 60.1 or 241 mg/kg bw per day (average concentrations in the diet were 0, 143, 564 and 2285 ppm), and no adverse effects were observed at any dose. In the second mouse carcinogenicity study, the animals were dosed at 0, 2250 or 4500 ppm (equal to 0, 242 and 482 mg/kg bw per day), and these doses were still not sufficient to produce adverse effects in females. In males, liver effects (increased incidence of fatty change) were observed at the highest dose. However, based on a weight of evidence evaluation, the study was considered acceptable for the assessment of carcinogenicity in mice. In the first rat study, animals were dosed at 0, 4, 16 or 64 mg/kg bw per day (approximately 0, 112, 449 and 1786 ppm). Testicular interstitial cell tumours were increased in all dose groups compared with controls (1/80, 10/80, 10/80 and 11/78, respectively); however, this was not considered to be treatment related, as it was not dose related, the incidence in the control group in this study was very low compared with historical control data for the laboratory and the strain, and an increase in the tumours was not observed in the second study at higher doses (5/50, 2/50, 4/50 and 1/50 at 0, 1.83, 187 and 386 mg/kg bw per day, respectively). Furthermore, special studies were conducted to examine testicular effects in the rat. These studies showed that etoxazole did not affect the proliferative activity of testicular interstitial cells after 4 or 13 weeks of dosing, nor did it have a significant impact on circulating levels of male reproductive hormones, the histology of the testis or epididymides or spermatogenesis after 13 weeks of dosing. An increased incidence of pancreatic islet cell adenomas was observed in the females at 64 mg/kg bw per day; however, this was not considered to be treatment related in the absence of an increase in carcinomas in the same study or an increase in adenomas at higher doses in the second study. Therefore, no adverse effects were observed at any dose. In the second rat study, the animals were dosed at 0, 50, 5000 or 10 000 ppm (equal to 0, 1.83, 187 and 386 mg/kg bw per day), and no increase in tumours was observed. Overall, there was no evidence of carcinogenicity in either the rat or the mouse when the results from all of these studies are considered.

Etoxazole was adequately tested for genotoxicity in vitro and in vivo in a range of assays. Several negative results were obtained in a battery of in vitro and in vivo genotoxicity studies. A positive response was obtained at cytotoxic doses in a study with human lymphocytes. In the mouse lymphoma assay with metabolic activation, a weakly positive response occurred at a dose approaching cytotoxic doses, and an inconclusive result was found without metabolic activation.

The Meeting concluded that etoxazole was unlikely to be genotoxic.

On the basis of the absence of treatment-related carcinogenicity in rodents and the lack of genotoxicity, the Meeting concluded that etoxazole is unlikely to pose a carcinogenic risk to humans.

No effects on reproduction were noted in a multigeneration reproduction study in the rat. However, there was an increase in mortality of the offspring during early lactation in both generations at 2000 ppm (equal to 139 mg/kg bw per day), the highest dose tested. Increases in the number of pup deaths as well as the number of litters with pup deaths were observed. Furthermore, at this dose, the viability index on lactation day 4 was below historical control values. Effects in parental animals at

the high dose were limited to non-adverse changes in organ weights (increased absolute and relative liver weights in males and increased relative weight in adrenal gland in females, with no corresponding histopathology noted in these tissues) in the first generation and slight hepatotoxicity in males (increased absolute and relative liver weights, slight centrilobular hepatocellular fatty change in two males) of the second generation. The NOAEL for parental and offspring toxicity was 400 ppm (equal to 28.2 mg/kg bw per day), and the NOAEL for reproductive toxicity was 2000 ppm (equal to 139 mg/kg bw per day), the highest dose tested.

No developmental toxicity was observed when pregnant rats were dosed up to 1000 mg/kg bw per day over the period of major organogenesis. Slight reductions in body weight and feed consumption were observed in maternal animals at this dose. The NOAEL for maternal toxicity was 200 mg/kg bw per day, and the NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested. In rabbits, the fetal and litter incidences of skeletal variations were increased following prenatal exposure to etoxazole at 1000 mg/kg bw per day, in the presence of maternal toxicity (i.e. liver enlargement as well as body weight reduction). The NOAEL for maternal and developmental toxicity was 200 mg/kg bw per day.

The Meeting concluded that etoxazole induced developmental toxicity only in the presence of maternal toxicity and that it was not teratogenic.

The clinical observations of the repeated-dose studies did not reveal any evidence of neurotoxicity. In addition, a functional observational battery, which included an assessment of motor activity, grip strength and sensorimotor reaction to stimuli, conducted at 1 year in the 2-year study in rats, yielded negative results for neurotoxicity.

There were no reports of adverse health effects in manufacturing plant personnel or in operators and workers exposed to etoxazole formulations during their use. Also, there was no evidence to support any findings in relation to poisoning with etoxazole.

The Meeting concluded that the existing database on etoxazole was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg bw on the basis of an overall NOAEL of 5.33 mg/kg bw per day in the 90-day and 1-year studies in dogs for liver effects (e.g. increases in serum levels of alkaline phosphatase and triglycerides, absolute and relative liver weights and incidence of centrilobular hepatocyte hypertrophy). A safety factor of 100 was applied.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for etoxazole in view of its low acute toxicity, the absence of relevant developmental toxicity in rats and rabbits that could have occurred as a consequence of an acute exposure, and the absence of any other toxicological effect that would be elicited by a single dose.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	2250 ppm, equal to 242 mg/kg bw per day	4500 ppm, equal to 482 mg/kg bw per day
Rat	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	1786 ppm, equal to 64 mg/kg bw per day	5000 ppm, equal to 187 mg/kg bw per day
		Carcinogenicity	10 000 ppm, equal to 386 mg/kg bw per day[c]	—

Species	Study	Effect	NOAEL	LOAEL
	Two-generation study of reproductive toxicity[a]	Parental toxicity	400 ppm, equal to 28.2 mg/kg bw per day	2000 ppm, equal to 139 mg/kg bw per day[c]
		Offspring toxicity	400 ppm, equal to 28.2 mg/kg bw per day	2000 ppm, equal to 139 mg/kg bw per day[c]
		Reproductive toxicity	2000 ppm, equal to 139 mg/kg bw per day[c]	—
	Developmental toxicity[d]	Maternal toxicity	200 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	1000 mg/kg bw per day[c]	—
Rabbit	Developmental toxicity[d]	Maternal toxicity	200 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	200 mg/kg bw per day	1000 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity[a,b]	Toxicity	200 ppm, equal to 5.33 mg/kg bw per day	1000 ppm, equal to 23.5 mg/kg bw per day

[a] Dietary administration.
[b] Two or more studies combined.
[c] Highest dose tested.
[d] Gavage administration.

Estimate of acceptable daily intake for humans

0–0.05 mg/kg bw

Estimate of acute reference dose

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to etoxazole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; approximately 60%
Distribution	Wide; highest concentrations in liver
Potential for accumulation	None
Rate and extent of excretion	Largely complete within 48 h; primarily via faeces (77–88%, bile 30–54%) and to a lesser extent via urine (8–17%)
Metabolism in animals	Extensive; mainly by hydroxylation of the 4,5-dihydrooxazole ring followed by cleavage of the molecule and hydroxylation of the *tert*-butyl side-chain
Toxicologically significant compounds in animals, plants and the environment	Parent compound

Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 1.09 mg/l (highest attainable concentration)

Rabbit, dermal irritation	Not irritating
Rabbit, eye irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman test)

Short-term studies of toxicity

Target/critical effect	Increased absolute and relative liver weights, clinical chemistry changes, centrilobular hepatocyte hypertrophy, prostate atrophy
Lowest relevant oral NOAEL	5.33 mg/kg bw per day (13-week study in dogs)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data

Long-term studies of toxicity and carcinogenicity

Target/critical effect	Increased absolute and relative liver weights, clinical chemistry changes, histopathological changes in liver, dental and bone abnormalities
Lowest relevant NOAEL	64 mg/kg bw per day (2-year study in rats)
Carcinogenicity	Not carcinogenic in rats or mice

Genotoxicity

Unlikely to be genotoxic

Reproductive toxicity

Reproduction target/critical effect	No effect on fertility at highest dose tested; slight decrease in viability of pups and pup body weight at parentally toxic dose
Lowest relevant reproductive NOAEL	28.2 mg/kg bw per day for offspring toxicity in two-generation reproduction study in rats
Developmental target/critical effect	Increased incidence of skeletal variations at maternally toxic dose
Lowest relevant developmental NOAEL	200 mg/kg bw per day in rabbits

Neurotoxicity/delayed neurotoxicity

No evidence of neurotoxicity

Other toxicological studies

Special studies on testicular function in rats revealed no effect on proliferative activity of interstitial cells, changes in circulating male hormones or histopathology

Medical data

No data

Summary

	Value	Study	Safety factor
ADI	0–0.05 mg/kg bw	Dog, 90-day and 1-year study	100
ARfD	Unnecessary		

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FLUBENDIAMIDE

First draft prepared by
G. Wolterink[1] and A. Moretto[2]

[1] Centre for Substances and Integrated Risk Assessment, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[2] Department of Environmental and Occupational Health, University of Milan, International Centre for Pesticides and Health Risk Prevention, Luigi Sacco Hospital, Milan, Italy

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Explanation

Flubendiamide is the International Organization for Standardization (ISO)–approved name for 3-iodo-*N*′-(2-mesyl-1,1-dimethylethyl)-*N*-{4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-*o*-tolyl}-phthalamide (International Union of Pure and Applied Chemistry [IUPAC]), which has the Chemical Abstracts Service (CAS) No. 272451-65-7. Flubendiamide is an insecticide that operates by a highly specific biochemical mode of action. It is a ryanodine receptor agonist, which activates ryanodine-sensitive intracellular calcium release channels in neuromuscular junctions, leading to an overstimulation of these cells. Flubendiamide does not bind to mammalian type 1, 2 and 3 ryanodine receptors.

Flubendiamide is being evaluated for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) at the request of the Codex Committee on Pesticide Residues (CCPR).

All critical studies complied with good laboratory practice (GLP).

Evaluation for acceptable daily intake

The structure of flubendiamide is shown in Figure 1.

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

Groups of male and female Fischer F344/DuCrj rats were treated with single oral (gavage) doses of [^{14}C]flubendiamide (radiochemical purity > 98%), labelled either in the phthalic acid ring or in the aniline ring, diluted with non-labelled flubendiamide (purity 99.6%; lot No. 1FH0013P), suspended in aqueous vehicle (containing 2% carboxymethylcellulose with 0.4% Tween 80).

The experimental designs are presented in Table 1. Metabolites were analysed in urine, faeces plus gastrointestinal contents and liver (see section 1.2). Statements of adherence to quality assurance (QA) and GLP were included.

Total recovery of radiolabel in all experiments was greater than 94%. Flubendiamide at 2 mg/kg body weight (bw) was relatively slowly and incompletely absorbed after oral administration, with peak plasma concentrations of 0.233 and 0.196 µg equiv./g and time to peak plasma concentrations (T_{max} values) of 12 and 6 hours in males and females, respectively. Plasma half-lives were 12.6 and 37.6 hours in males and females, respectively. At 200 mg/kg bw, plasma concentrations were only about 2–4 times higher than at 2 mg/kg bw, indicating that absorption is almost saturated at the high dose.

At 2 mg/kg bw in males and females, 1.7% and 0.4% of radiolabel were excreted in urine, respectively, with 96.2% and 91.4% being excreted in faeces over a 168-hour sampling period. Urinary excretion at 200 mg/kg bw was even less (~0.1% in both sexes), whereas at this dose, 93.6% and 99.6% were excreted in faeces of males and females, respectively. Faecal excretion was rapid, with 77%/58% (males/females) and 90%/99% (males/females) being excreted within 24 hours after administration of the low and high doses, respectively. No significant excretion in expired air was observed. Radiolabel was widely distributed over tissues and organs, including bone marrow. At 9 hours after dosing of 2 mg/kg bw, highest radiolabel concentrations were found in the gastrointestinal tract, liver, adrenals, fat and kidneys, with higher levels in males than in females. Excretion in females was slower than in males, resulting in higher tissue levels in females than in males at 24 and 168 hours. Although excretion was slower in females than in males, tissue levels in both sexes had markedly and time-dependently decreased at 24 and 168 hours. Tissue levels were higher at 200 mg/kg bw,

Figure 1. Structure of flubendiamide

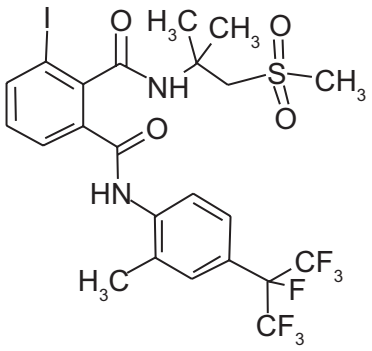


Table 1. Experimental designs

Position of label	Dose (mg/kg bw)	Number and sex of animals	Samples (h post-dosing)
Phthalic acid ring	2	4M, 4F	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
Phthalic acid ring	200	4M, 4F	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
Phthalic acid ring	2	4M, 4F	Organs/tissues: 9
Phthalic acid ring	2	4M, 4F	Organs/tissues: 24
Phthalic acid ring	2	4M, 4F	Excreta: 24, 48, 72, 96, 120, 144, 168 Expired air: 24 (2 selected animals) Organs/tissues: 168
Phthalic acid ring	200	4M, 4F	Organs/tissues: 9
Phthalic acid ring	200	4M, 4F	Organs/tissues: 24
Phthalic acid ring	200	4M, 4F	Excreta: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
Aniline ring	2	2M	Excreta: 24 Expired air: 24
Aniline ring	2	2F	Excreta: 24, 48 Expired air: 24, 48
Aniline ring	2	4M, 4F	Excreta: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168

From Motoba (2004a)
bw, body weight; F, females; M, males

but far less than the 100-fold dosing difference, again indicating saturated absorption. Relative tissue distribution at 200 mg/kg bw per day was similar to that observed after 2 mg/kg bw per day. After 168 hours, less than 1% and less than 5% of administered radiolabel were recovered from the carcasses of males and females, respectively. Tissue distribution and excretion were almost identical between aniline ring–labelled and phthalic acid ring–labelled groups (Motoba, 2004a).

The oral absorption and biliary excretion of [^{14}C]flubendiamide (radiochemical purity > 98%), labelled in the phthalic acid ring, administered by gavage at 2 mg/kg bw, were studied in male and female Fischer F344/DuCrj rats. The labelled substance was diluted with non-labelled flubendiamide (purity 99.6%) and suspended in aqueous vehicle (containing 2% carboxymethylcellulose with 0.4%

Table 2. Excretion and distribution of phthalic acid ring–labelled flubendiamide in bile duct–cannulated rats administered 2 mg/kg bw

	Radioactivity (% of administered dose) at 48 h	
	Males (n = 3)	Females (n = 6)
Bile	11.1	3.3
Urine	0.8	0.2
Faeces	12.8	11.0
Gastrointestinal tract contents	60.4	50.6
Gastrointestinal tract	2.5	2.4
Liver	3.3	5.5
Carcass	5.9	22.8
Total oral absorption	23.5	34.1
Total recovery	96.6	95.7

From Motoba (2004b)

Tween 80). The levels of radiolabel were determined in bile, faeces and urine, collected over 48 hours, and in gastrointestinal tract and liver, obtained after 48 hours. Furthermore, the metabolites in bile, faeces and gastrointestinal contents (see section 1.2) were analysed. Statements of adherence to QA and GLP were included.

The percentages of radioactivity recovered in the various compartments and excreta are presented in Table 2.

The data indicate that absorption is relatively slow and incomplete, and excretion occurs mainly via bile (Motoba, 2004b).

In a third study, Fischer F344/DuCrj rats (four of each sex per group) received [^{14}C]flubendiamide (radiochemical purity > 98.2%), labelled in the phthalic acid ring, administered by gavage at 2 mg/kg bw per day for 14 days. The vehicle was an aqueous solution containing sodium carboxymethylcellulose and 0.4% Tween 80. Blood samples were obtained 24 hours after the 6th and 13th doses and at termination. Urine and faeces were collected every 24 hours until 168 hours after the last dosing. Groups of four animals each were sacrificed 9, 24 and 168 hours after the last dose, respectively. At termination, the animals were necropsied and gastrointestinal contents and major organs, tissues and residual carcass were sampled for radioactivity measurement. Statements of adherence to QA and GLP were included.

Blood and plasma radioactivity levels at days 6, 13 and 14 were similar to each other and comparable to those observed after a single administration (Motoba, 2004a), with the highest concentration observed at 9 hours after the last dosing in both sexes. At 168 hours after the last dosing, radioactivity was hardly detectable in blood and plasma. Highest tissue levels of radiolabel were observed in liver, kidneys, adrenals, gastrointestinal tract and fat. Radioactivity levels were generally higher in tissue of females. In males, tissue levels after repeated dosing were comparable to those observed after a single dose (Motoba, 2004a); they peaked at 9 hours and were markedly decreased at 24 and 168 hours, indicating no potential for accumulation in males. In females, however, after repeated dosing, tissue levels at 24 hours were comparable to those at 9 hours, and significant levels of radiolabel were still found at 168 hours. Compared with the tissue levels after a single dose, after 14 days of dosing, tissue levels in females were considerably higher (e.g. 6–13 times higher at 24 hours after the last dose), indicating a considerable potential for accumulation in females.

In both sexes, radiolabel was excreted almost completely in faeces. Less than 0.5% of the administered dose was excreted in urine, and 0.02% and 0.42% of the total administered dose remained in the carcass 168 hours after the last dose in males and females, respectively (Motoba, 2004c).

Table 3. Metabolites of phthalic acid ring–labelled or aniline ring–labelled flubendiamide[a] in excreta and liver of rats after administration of single oral doses of 2 or 200 mg/kg bw

	Dose (mg/kg bw)	Radioactivity (% of administered dose)					
		Males			Females		
		Urine[b]	Faeces[b]	Liver[c]	Urine	Faeces	Liver
Flubendiamide	2	0.01	15.4	—	0.09	65.8	—
		0.04	*30.5*	*0.08*	*0.21*	*65.7*	*8.64*
	200	0.04	89.1	—	< 0.01	97.8	—
Flubendiamide-benzyl alcohol	2	0.45	37.3	—	0.05	5.4	—
		0.42	*30.8*	*0.22*	*0.05*	*5.7*	*0.25*
	200	0.01	0.2	—	< 0.01	ND	—
Flubendiamide-benzaldehyde	2	0.01	0.44	—	ND	ND	—
		0.03	*0.31*	*0.19*	*ND*	*ND*	*ND*
	200	ND	0.3	—	ND	0.24	—
Flubendiamide-benzoic acid	2	0.03	16.4	—	0.01	ND	—
		0.05	*14.9*	—	*0.01*	*0.13*	—
	200	ND	ND	—	ND	ND	—
Sum of others	2	0.84	12.7	—	0.08	4.7	—
		0.78	*9.5*	*1.4*	*0.11*	*4.1*	*0.67*
	200	0.02	0.27	—	0.04	0.31	—
Non-extractable	2	0.28	12.54	—	0.07	5.01	—
		0.2	*6.64*	*0.42*	*0.08*	*3.93*	*0.18*
	200	0.03	0.14	—	0.02	0.16	—
Total	2	1.61	94.8	—	0.30	81.0	—
		1.52	*92.6*	*2.4*	*0.47*	*79.6*	*9.74*
	200	0.09	89.9	—	0.06	98.5	—

From Motoba (2004a)
ND, not detected
[a] Results of aniline ring label shown in italics.
[b] For the phthalic acid ring–labelled 2 mg/kg bw group, excreta obtained until 72 h after dosing were analysed, and until 24 h for the 200 mg/kg bw group. For the aniline ring–labelled 2 mg/kg bw group, excreta obtained until 24 h after dosing were analysed.
[c] Liver was analysed 24 h after dosing.

1.2 Biotransformation

Rats

In the study of Motoba (2004a), metabolites were analysed in urine, faeces plus gastrointestinal contents and liver. The study design is described under section 1.1. Statements of adherence to QA and GLP were included.

The levels of the major metabolites in excreta and liver are presented in Table 3.

Apart from the metabolites in Table 3, hydroxyflubendiamide was also detected, but not quantified. Other non-identified metabolites occurred at levels below 1% of the administered dose.

In males at 2 mg/kg bw, unchanged flubendiamide was one of the main compounds in faeces, together with flubendiamide-benzyl alcohol and flubendiamide-benzoic acid. In females at 2 mg/kg bw, faeces and liver contained mostly unchanged flubendiamide. This may indicate slower metabolism in females. At 200 mg/kg bw, faeces of both sexes contained mainly unchanged flubendiamide. No significant difference in metabolite profiles was found between aniline ring–labelled and phthalic

acid ring–labelled groups. Small but significant amounts of NNI-0001-iodophthalimide (A-14) were detected in the fat extract of both male and female rats (3.3% and 1.0% of total radioactivity in the fat, respectively). The detection of NNI-0001-iodophthalimide (A-14) implied the hydrolysis of the amide bond between the phthalic acid ring and the thioalkylamine moiety (Motoba, 2004a).

The oral absorption and biliary excretion of [^{14}C]flubendiamide (radiochemical purity > 98%), labelled in the phthalic acid ring, administered by gavage at 2 mg/kg bw, were studied in male and female Fischer F344/DuCrj rats. The labelled substance was diluted with non-labelled flubendiamide (purity 99.6%) and suspended in aqueous vehicle (containing 2% carboxymethylcellulose with 0.4% Tween 80). The levels of radiolabel were determined in bile, faeces and urine, collected over 48 hours, and in gastrointestinal tract and liver, obtained after 48 hours. Furthermore, the metabolites in bile, faeces and gastrointestinal contents were analysed. Statements of adherence to QA and GLP were included.

The levels of the major metabolites in bile, faeces and gastrointestinal contents are presented in Table 4.

The metabolic profile indicates that metabolism occurs mainly by oxidation of the methyl groups, followed by glucuronidation of the hydroxyl moiety, in particular in males, and direct glutathione conjugation of the phthalic acid ring, particularly in females (Motoba, 2004b).

The proposed metabolic pathway, based on the studies of Motoba (2004a,b), is depicted in Figure 2.

The metabolism following repeated oral administration of flubendiamide was studied by Motoba (2004c) (see study description under section 1.1). In this study, metabolites in faeces, urine and fat were analysed by thin-layer chromatography and high-performance liquid chromatography (HPLC). Statements of adherence to QA and GLP were included.

In faeces of males and females, 82.2% and 91.3%, respectively, of the total administered dose were unchanged flubendiamide. Flubendiamide-benzyl alcohol represented 7.2% and 2.2% of radiolabel in faeces of males and females, respectively. In faeces of males, 2.8% of radiolabel represented flubendiamide-benzoic acid. Other metabolites in faeces were present at levels below 1.6%. The metabolic profile indicates that metabolism occurs mainly by oxidation of the methyl group of the aniline ring. In fat of female rats, almost all radiolabel represented flubendiamide, whereas in males, flubendiamide (53%), flubendiamide-benzyl alcohol (13%), flubendiamide-benzaldehyde (14%) and others (14%) were found. Additionally, in both sexes, the metabolite flubendiamide-iodophthalimide was found in low quantities (3.3% and 1%, respectively). The data indicate that there are no qualitative differences in metabolism between single and repeated dosing of flubendiamide and that the rate of metabolism is slower in female rats (Motoba, 2004c).

The sex difference in metabolism of [^{14}C]flubendiamide (radiochemical purity > 98%) labelled in the phthalic acid ring was investigated in vitro using hepatic microsomes obtained from Fischer F344/DuCrj rats, CD-1 mice, Beagle dogs and humans. Statements of adherence to QA and GLP were included.

It was found that all microsome preparations caused hydroxylation of flubendiamide to flubendiamide-benzyl alcohol as a first step of metabolism, except for the microsomes of female rats. Immunological inhibition tests indicated that the hydroxylation in the rat may be mediated by cytochrome P450 (CYP) isoforms that are immunologically indistinguishable from CYP2C11. In human microsomes, hydroxylation of flubendiamide to flubendiamide-benzyl alcohol was predominantly mediated by CYP3A4. The study authors reported that in humans, CYP3A4 is the most abundant, versatile and important isoform for metabolism of xenobiotics and that it is known not to have sex differences or genetic polymorphisms; therefore, no sex-dependent or individual differences in metabolism of flubendiamide are expected in humans (Motoba, 2004d).

Table 4. Metabolites of phthalic acid ring–labelled flubendiamide in bile duct–cannulated rats following a single oral dose of 2 mg/kg bw

	Radioactivity (% of administered dose)					
	Males (n = 3)			Females (n = 6)		
	Bile	Faeces	Gastrointestinal contents	Bile	Faeces	Gastrointestinal contents
Flubendiamide	ND	12.0	56.3	ND	10.7	49.7
Flubendiamide-benzyl alcohol	1.3	0.6	3.4	0.1	0.1	0.6
Flubendiamide-benzoic acid	2.3	ND	ND	ND	ND	ND
Flubendiamide cyclic acetal	1.8	ND	ND	0.2	ND	ND
Flubendiamide carboxy-benzylalcohol	0.3	ND	ND	0.2	ND	ND
Flubendiamide benzylalcohol glucuronide	0.9	ND	ND	0.1	ND	ND
Flubendiamide hydroxy-benzylalcohol glucuronide	1.4	ND	ND	ND	ND	ND
Flubendiamide glutathione conjugate	1.2	ND	ND	0.8	ND	ND
Flubendiamide–cysteinyl-glycine conjugate	0.3	ND	ND	0.5	ND	ND
Flubendiamide–cysteine conjugate	0.3	ND	ND	1.4	ND	ND
Sum of other minor unknowns	1.3	—	0.1	—	—	—
Non-extractable	NA	0.2	0.5	NA	0.2	0.3
Total	11.1	12.8	60.4	3.3	11.0	50.6

From Motoba (2004b)
NA, not applicable; ND, not detected

Male and female Fischer F344/DuCrj rats and Crlj CDI (ICR) mice (n = 3–4 per group) received daily oral doses of flubendiamide (purity > 96.7%) of 200 mg/kg bw for 1, 7 or 14 days, after which the levels of flubendiamide and its metabolite flubendiamide-iodophthalimide in plasma, liver and fat were measured using HPLC. Statements of adherence to QA and GLP were included.

Plasma levels of flubendiamide were low in all animals, with the highest levels (1.4 mg/l) being observed in female rats. Levels of flubendiamide were high in liver (19–27 mg/kg) and fat (47–68 mg/kg) of female rats at days 1, 7 and 14, with the levels after 7 and 14 doses being about 1.5 times higher than those after a single dose. In male rats and in both sexes of mice, levels of flubendiamide in liver and fat were much lower (6–39 times lower) than in female rats. The levels of the metabolite flubendiamide-iodophthalimide in plasma and liver of rats and mice and in fat of mice were generally close to or below the limit of quantification (0.1 mg/l or mg/kg). Fat of male and female rats contained between 2.8 and 3.7 mg/kg of this metabolite after 7 or 14 doses (Motoba, 2005).

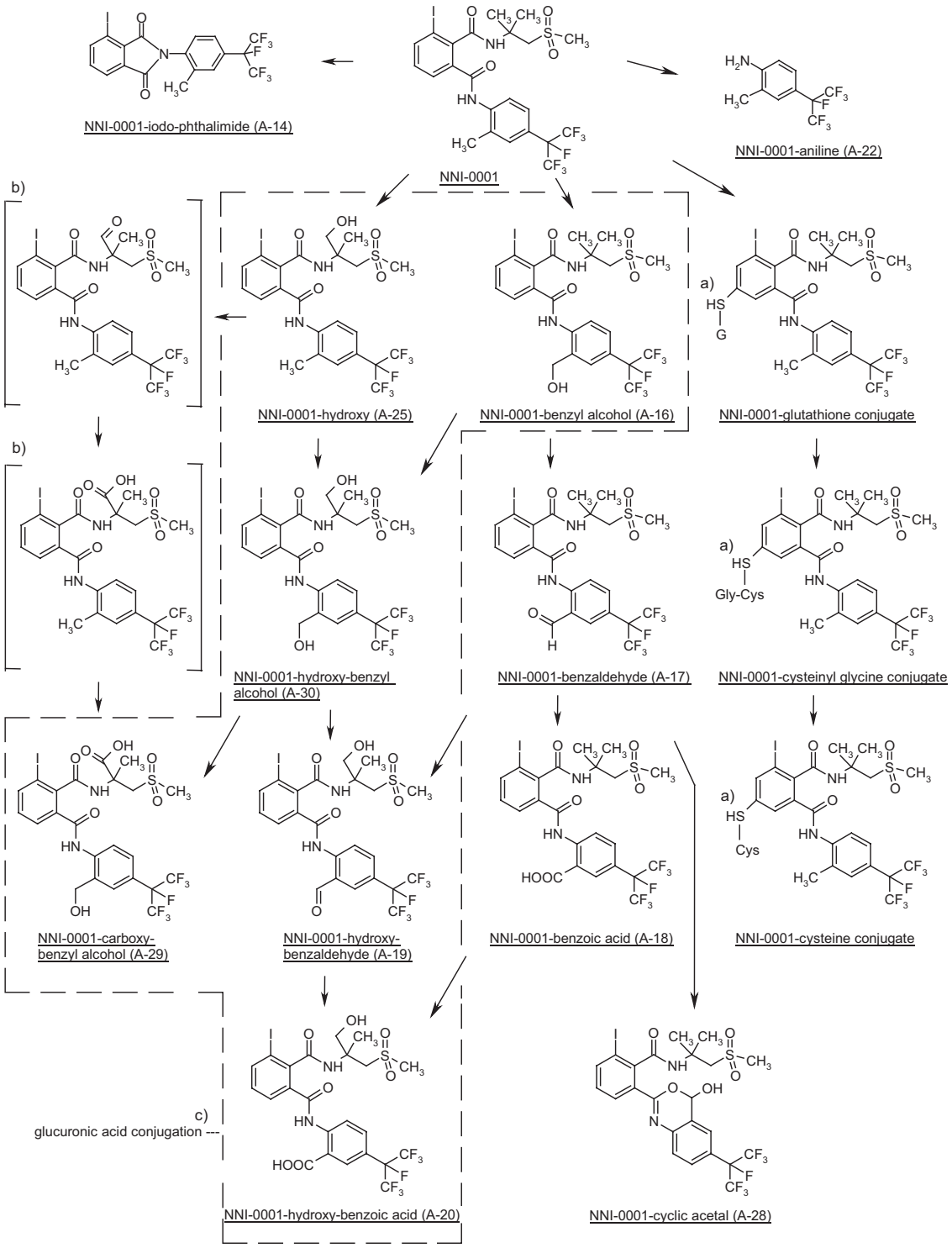
2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with flubendiamide are summarized in Table 5. No substance-related clinical signs were observed in the oral and dermal acute toxicity studies. During and immediately after the 4-hour exposure period in the inhalation study, increased respiratory rate, wet fur, hunched posture, piloerection and red/brown staining around the eyes were observed. These clinical signs are considered to be related to the exposure and restraining procedure. Apart from one

Figure 2. Proposed metabolic pathway of flubendiamide (company code NNI-0001) in the rat, summarizing the results of all metabolism studies



Notes:

a) The position of the glutathione conjugation (gs-substituent at c-5 of the phthalic acid ring) was not clearly identified and may be different.

b) The two structures in parentheses represent proposed intermediates.

c) The frame around the group of six hydroxy compounds indicates additional glucuronic acid conjugates.

Table 5. Results of studies of acute toxicity with flubendiamide

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50} (mg/kg bw); LC_{50} (mg/l)	Reference[a]
Rat	Sprague-Dawley (Slc:SD)	Male/ female	Oral	Aqueous methylcellulose	97.8[b]	> 2000	Horiuchi (2003a)[c]
Rat	Sprague-Dawley (Slc:SD)	Male/ female	Dermal	Aqueous methylcellulose	97.8[b]	> 2000	Horiuchi (2003b)[d]
Rat	Sprague-Dawley Crl:CD(SD) IGS BR	Male/ female	Inhalation	PEG 200	96.7[e]	> 0.0685	Wesson (2004)[f]

LC_{50}, median lethal concentration; LD_{50}, median lethal dose; PEG, polyethylene glycol

[a] All studies include statements of adherence to GLP and QA.

[b] Lot No. 1FH0018P.

[c] Performed according to Organisation for Economic Co-operation and Development (OECD) guideline 401.

[d] Performed according to OECD guideline 402.

[e] Lot No. 1FH0019M. A 25% flubendiamide/75% PEG (weight per weight [w/w]) formulation was used. The maximum achievable concentration was 0.0685 mg/l. Mass median aerodynamic diameter (MMAD) 4.18 µm (geometric standard deviation [GSD] 2.74), inhalable fraction ~48%.

[f] Performed according to OECD guideline 403, nose-only exposure.

animal showing red/brown staining around the eyes 1 hour after exposure, no clinical signs following inhalation of flubendiamide were observed in the 14-day observation period.

(b) Dermal irritation

In a dermal irritation study, performed according to Organisation for Economic Co-operation and Development (OECD) guideline 404, three male Japanese White rabbits were dermally exposed for 4 hours under semiocclusive conditions to 0.5 g flubendiamide (purity 97.8%; lot No. 1FH0018P; pulverized crystal) moistened with deionized water. Dermal irritation was scored according to the Draize system at 1, 24, 48 and 72 hours post–patch removal. Statements of adherence to QA and GLP were included.

No dermal irritation was observed at any point during the study (Horiuchi, 2004a).

(c) Ocular irritation

In an eye irritation study, performed according to OECD guideline 405, 0.1 ml (approximately 66 mg) of flubendiamide (purity 97.8%; lot No. 1FH0018P; pulverized crystal) was instilled into the conjunctival sac of the left eye of three male Japanese White rabbits. The untreated left eye served as a control. An additional group of three male rabbits was similarly treated, and their eyes were washed 30 seconds after instillation of the test material. Eye irritation was scored at 1, 24, 48 and 72 hours post-instillation according to Draize. Statements of adherence to QA and GLP were included.

No corneal opacity or chemosis of the conjunctiva was noted. Iritis (score 1) was observed only at 1 hour in one animal in both the washed and the non-washed group. In the non-washed group, conjunctival redness (score of 1) was noted in all eyes at 1 and 24 hours. Discharge (score of 1 and 2) was noted in all eyes at 1 hour. No signs of irritation were observed at 48 and 72 hours. In the washed group, the scores for conjunctival discharge were reduced (all eyes score 1) and the duration of conjunctival redness was shortened in two animals. It is concluded that flubendiamide is not an eye irritant (Horiuchi, 2004b).

(d) Dermal sensitization

In a dermal sensitization study using the Magnusson & Kligman maximization test, in accordance with OECD guideline 406, flubendiamide (purity 97.8%; lot No. 1FH0018P; pulverized and suspended in propylene glycol) was tested in 20 female Hartley guinea-pigs. The control group consisted of 10

animals. In the first induction phase, the animals of the treatment group received three intradermal injections on both sides of the neck. Each injection consisted of 0.05 ml of a suspension containing a) 1% flubendiamide in liquid paraffin, b) 50% volume per volume (v/v) mixture of Complete Freund's Adjuvant in purified water or c) 1% flubendiamide in a 50% (v/v) mixture of Complete Freund's Adjuvant. Control animals were similarly treated with vehicle only. One week later, this was followed by a topical application (48 hours) under occlusion (Elastopore) of 0.3 ml of a solution containing 50% (weight per volume [w/v]) flubendiamide in propylene glycol. The animals were pretreated with sodium lauryl sulfate 24 hours prior to the topical applications. In the challenge phase, 21 days after study initiation, the animals received topical applications of 0.15 ml of the challenge solution containing 50% (w/v) flubendiamide in propylene glycol for 24 hours. At 24 and 48 hours post–patch removal, the degree of dermal irritation was scored according to the Magnusson & Kligman grading scale. 2,4-Dinitrochlorobenzene was used as a positive control. Statements of adherence to QA and GLP were included.

At challenge, the highest grade of dermal reaction in the control group to both vehicle and flubendiamide challenge was 1 (in 5/10 and 4/10 animals, respectively). In the flubendiamide test group, the highest scores to a vehicle and flubendiamide challenge were 2, both observed in the same female. The highest score to vehicle and flubendiamide in the other animals was 1 (in 12/20 and 7/20 animals, respectively). The 2,4-dinitrochlorobenzene group showed a 100% positive reaction. It is concluded that flubendiamide is not a dermal sensitizer under the conditions of the maximization test (Horiuchi, 2004c).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day range-finding feeding study, performed in accordance with OECD guideline 407, flubendiamide (purity 97.7%; lot No. 0FH0009P) was administered to ICR (Crj:CD-l) SPF mice (six of each sex per dose) at a dose level of 0, 20, 200, 2000 or 20 000 parts per million (ppm), equal to 0, 2.7, 26.9, 265 and 2678 mg/kg bw per day for males and 0, 2.9, 30.0, 299 and 3024 mg/kg bw per day for females. Animals were checked daily for clinical signs and mortality, and a detailed clinical examination was performed weekly. Body weight and group mean feed consumption were measured weekly. Haematology and clinical chemistry were performed at termination after 4 weeks of treatment. Also at termination, gross examinations were performed on all animals, and selected organs were weighed. Livers of all animals were histologically examined. Statements of adherence to QA and GLP were included.

No treatment-related mortalities or clinical signs of toxicity were observed. Body weight gain, feed consumption and haematological parameters in treated animals were comparable to those of controls. A small (30%) but statistically significant increase in alkaline phosphatase activity was noted in high-dose females. Small increases in relative liver weight (9–10%) were observed in high-dose animals, reaching statistical significance in males only. Necropsy showed dark colouring of the liver in two males at the high dose and in one male at 2000 ppm. Histology showed increased incidences of fatty changes in centrilobular hepatocytes in males at 2000 ppm and in both sexes at the high dose. Centrilobular hepatocyte hypertrophy was observed in both sexes at doses of 2000 ppm and above.

The no-observed-adverse-effect level (NOAEL) was 200 ppm, equal to 26.9 mg/kg bw per day, based on dark-coloured livers, fatty changes in centrilobular hepatocytes and centrilobular hepatocyte hypertrophy at 2000 ppm (Takeuchi, 2001).

In a 13-week feeding study in ICR (Crj:CD-1) mice (10 of each sex per dose), the toxicity of flubendiamide (purity 98.5%; lot No. 0FH0010P) was investigated. The study was performed in

accordance with OECD guideline 408. Flubendiamide was administered in the diet at 0, 50, 100, 1000 or 10 000 ppm, equal to 0, 6.0, 11.9, 123 and 1214 mg/kg bw per day for males and 0, 7.1, 14.7, 145 and 1424 mg/kg bw per day for females. Animals were observed at least once daily for clinical signs and mortality. Detailed clinical examination was performed weekly. Body weight and group mean feed consumption were measured weekly. Haematology and clinical chemistry were performed at week 13 of treatment. After 13 weeks of treatment, the animals were killed and necropsied, and selected organs were weighed. An extensive range of organs and tissues of control and high-dose animals was histologically examined. Liver, thyroid and gross lesions of animals of the low- and middle-dose groups were also histologically examined. Statements of adherence to QA and GLP were included.

No treatment-related effects on mortality, clinical observations, body weight, feed consumption or haematology were observed. Bilirubin levels were increased by 19% in females of the high-dose group. Absolute (up to 16%) and relative liver weights (up to 22%) were increased in females at 1000 ppm and higher, as was relative liver weight (13%) in high-dose males. Relative ovary weight was increased (37%) in high-dose females. At the high dose, four males and one female showed dark-coloured liver (not observed in controls). The incidences of centrilobular hepatocyte hypertrophy and fatty change were increased at 1000 and 10 000 ppm in both sexes. In the ovaries, no microscopic lesions were noted.

The NOAEL was 100 ppm, equal to 11.9 mg/kg bw per day, based on centrilobular hepatocyte hypertrophy and fatty change in both sexes and increased liver weight in females at 1000 ppm, equal to 123 mg/kg bw per day (Takeuchi, 2002).

Rats

In a 28-day feeding study, performed in accordance with OECD guideline 407, flubendiamide (purity 97.7%; lot No. 0FH0009P) was administered in the diet to Fischer (F344/DuCrj) rats (six of each sex per dose) at 0, 20, 50, 200, 2000 or 20 000 ppm, equal to 0, 1.5, 3.9, 15.1, 152 and 1575 mg/kg bw per day for males and 0, 1.6, 4.2, 16.1, 156 and 1605 mg/kg bw per day for females. Animals were observed at least once daily for clinical signs and mortality. Detailed clinical examination was performed weekly. Body weight and group mean feed consumption were measured weekly. Haematology, clinical chemistry and urinalysis were performed at termination after 4 weeks of treatment. Also at termination, gross examinations were performed on all animals, and selected organs were weighed. An extensive range of organs and tissues of all animals was histologically examined. Statements of adherence to QA and GLP were included.

No mortalities or clinical signs of toxicity were observed. Body weight gain and feed consumption in treated animals were comparable to those of controls. Haematology revealed statistically significant but small decreases (≤ 3%) in mean corpuscular volume (MCV) at doses of 2000 ppm and higher in females and at 20 000 ppm in males and an increase in platelet count (12%) in the high-dose males. As these effects were small, they are considered not toxicologically relevant. Clinical chemistry showed small (≤ 19%) but statistically significant reductions in alkaline phosphatase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. However, reductions in levels of these enzymes are generally not considered to be adverse. γ-Glutamyl transpeptidase (GGT) levels were statistically significantly increased (doubled) in females at 200 ppm and higher. Total protein, globulin, inorganic phosphorus and potassium levels were slightly increased (≤ 16%) in females at 2000 ppm and/or 20 000 ppm, and glucose levels were increased by 10% in males at 2000 ppm and higher. Cholesterol levels were slightly increased (up to 15%) in all treated males except for the high dose. Decreases in cholesterol (up to 28%), triglyceride (up to 20%) and plasma cholinesterase (up to 43%) levels were observed in females at 2000 ppm and higher. Relative liver weights were increased by 8% and 14% in males at 2000 and 20 000 ppm, respectively, and by 9%, 33% and 45% in females at 200, 2000 and 20 000 ppm, respectively. The absolute liver weights were also increased in females

at these doses. Histological examination revealed periportal fat storage in hepatocytes in females at 200 ppm and above and in two males at the high dose. In females at 2000 ppm and above, hepatocyte hypertrophy was observed. Hypertrophy of follicular cells in the thyroid was observed in high-dose males and in females at 2000 ppm and higher. Urinalysis showed no treatment-related effects.

The NOAEL was 50 ppm, equal to 4.2 mg/kg bw per day, based on fatty changes in periportal hepatocytes, increases in plasma GGT and increases in absolute and relative liver weights in females (Enomoto, 2001).

The toxicity of flubendiamide (purity 97.7%; lot No. 0FH0009P) was investigated in a 13-week feeding study, performed in accordance with OECD guideline 408. Flubendiamide was administered to Fischer (F344/DuCrj) rats (10 of each sex per dose) at a dietary concentration of 0, 20, 50, 200, 2000 or 20 000 ppm, equal to 0, 1.2, 2.9, 11.4, 116 and 1192 mg/kg bw per day for males and 0, 1.3, 3.3, 13.1, 128 and 1320 mg/kg bw per day for females. In order to study the reversibility of effects, additional groups of rats (10 of each sex per dose) received flubendiamide at 0 or 20 000 ppm for 13 weeks and were observed for an additional 4 weeks without treatment. Animals were observed at least once daily for clinical signs and mortality. Detailed clinical examination was performed weekly. The animals were subjected to a functional observational battery (FOB) during week 11 and week 4 of the recovery period. Body weight and group mean feed consumption were measured weekly. Haematology, clinical chemistry, urinalysis and ophthalmoscopy were performed at week 13 of treatment and week 4 of the recovery period. At termination of treatment in week 13 or at the end of the 4-week recovery period, gross examinations were performed on all animals, and selected organs were weighed. An extensive range of organs and tissues of all animals was histologically examined. Statements of adherence to QA and GLP were included.

No treatment-related effects on mortalities or general clinical observations were observed. Slight increases in rearing activity were observed in females of the high-dose group during weeks 7, 8 and 11. However, as this finding was not observed in similarly treated females of the satellite group and was not accompanied by other behavioural parameters or histological changes in the nervous system, it is considered not toxicologically significant. Body weight gain, feed consumption and ophthalmology in treated animals were comparable to those of controls. Haematology revealed statistically significant decreases in MCV at 20 000 ppm in males (−2%) and in females at 200 (−2%), 2000 (−3%) and 20 000 ppm (−6%). In females of the 2000 and 20 000 ppm groups, haematocrit (6–7%) and haemoglobin levels (5%) were decreased, and a reduction in mean corpuscular haemoglobin (5%) was found in females at 20 000 ppm. Platelet counts were increased (7–16%) in both sexes at doses of 2000 ppm and higher. The changes in haematological parameters were only partially reversible after 4 weeks of recovery, and they were not accompanied by histopathological changes in bone marrow or spleen. Clinical chemistry showed a reduction in alkaline phosphatase, plasma cholinesterase, triglycerides and total bilirubin levels and a 3-fold increase in GGT activity in females at 2000 ppm or higher. ALT and total bile acid levels were decreased in females at 20 000 ppm. Increases in total protein (5–7%) and albumin levels (6%) were observed in both sexes at 20 000 ppm. Globulin levels were increased (9%) in females at the high dose. Cholesterol was increased (up to 25%) in males at doses of 50 ppm and higher, but decreased by 24% in females at the high dose. A slight increase in potassium levels (up to 14%, not dose dependent) was observed in females at doses of 2000 ppm and higher. Most of the biochemical effects were reversible during the recovery phase. No treatment-related effects on urinalysis were observed. The incidences of dark-coloured and enlarged livers were increased in both sexes at the high dose and in females at 2000 ppm. These effects were no longer observed after 4 weeks of recovery. Absolute and relative liver weights were increased in males at the high dose (relative weight 11%) and in females at doses of 200 ppm and higher (relative weight 11–47%). Absolute and relative kidney weights were increased (up to 13%) in females at 2000 ppm and higher. Absolute and relative weights of the adrenals and ovaries and

relative weight of the heart were also statistically significantly increased in high-dose females. After 4 weeks of recovery, the absolute and relative kidney and ovary weights were still increased, whereas the other organ weight changes were less prominent. Other effects on organ weights were small and not always dose dependent and are considered not toxicologically significant. Hepatocyte hypertrophy was observed in females at 2000 ppm and higher. In the liver, increased incidences in periportal fatty changes and thyroid follicular cell hypertrophy were found in high-dose males and in females at 200 ppm and higher. Most effects observed at the end of the treatment period at 20 000 ppm were partially or fully reversible within the 4-week recovery period.

The NOAEL was 50 ppm, equal to 3.3 mg/kg bw per day, based on increases in absolute and relative liver weights, increased incidences of periportal fatty changes and follicular cell hypertrophy of the thyroid observed in females at 200 ppm, equal to 13.1 mg/kg bw per day (Enomoto, 2003).

In a 1-year dietary study in rats, performed in accordance with OECD guideline 452, the effects of flubendiamide (purity 97.8%; lot No. 1FH0018P) were investigated. Flubendiamide was administered to male and female Fischer (F344/DuCrj) rats (25 of each sex per dose) at 0, 20, 50, 2000 or 20 000 ppm, equal to 0, 0.78, 2.0, 79 and 822 mg/kg bw per day for males and 0, 0.96, 2.4, 98 and 998 mg/kg bw per day for females. Clinical signs and mortality were recorded daily. A detailed clinical examination was performed weekly. Special neurobehavioural examinations (FOB and motor activity test) were performed on 10 animals of each sex per dose during week 49 of treatment. Body weight and feed consumption were recorded weekly from week 1 to week 13 and once every 4 weeks thereafter. Ophthalmological examinations were carried out pretreatment and during week 52. Haematology, clinical chemistry and urinalysis were performed during weeks 13, 26 and 51/52. Brain cholinesterase activity and selected organ weights were measured in 10 animals of each sex per dose at week 52. At termination, all animals were necropsied. Histological examination of an extensive range of organs was performed on all animals of the control and high-dose groups and on all animals found dead during the treatment period. Furthermore, liver, kidneys, thyroids and all gross lesions from the 20, 50 and 2000 ppm groups were histologically examined. Statements of adherence to QA and GLP were included.

Incidences of mortality and clinical signs were not affected by treatment. High-dose females showed increased rearing, mainly between weeks 6 and 15. No toxicologically relevant changes in body weight gain were observed. In high-dose males and females, small but statistically significant increases (104–107% of control) in feed consumption were found. Ophthalmoscopy and urinalysis showed no treatment-related changes. In high-dose males and in females of the 2000 and 20 000 ppm groups, statistically significant decreases in haematocrit (up to 9%), haemoglobin (up to 9%), erythrocyte count (up to 4%), MCV (up to 6%) and mean corpuscular haemoglobin (MCH) (up to 6%) were found. These decreases generally were observed at all time points (weeks 13, 26 and 52). In both sexes of the 2000 and 20 000 ppm groups, increases in platelet counts (up to 10%) and reticulocyte counts (up to 64%) were occasionally observed. Mean corpuscular haemoglobin concentration (MCHC) was occasionally slightly increased (up to 3%) in high-dose males and in females at 2000 and 20 000 ppm. In females at 50 ppm, sporadic slight but statistically significant decreases in haematocrit (−3%), MCV (1–2%) and MCH (2%) were observed. These findings are considered not adverse. Bone marrow cytology at week 52 showed a slight decrease in erythroblast count. These haematological changes indicate that flubendiamide induces microcytic anaemia and possibly reactive haematopoiesis. A decrease in segmented neutrophil counts was detected in both sexes at 20 000 ppm. In males at doses of 2000 ppm and higher, slight increases in prothrombin time (up to 11%) and activated partial thromboplastin time (up to 5%) were found. Reductions in AST, ALT and bilirubin levels in males and/or females at doses of 2000 ppm and higher were considered not toxicologically relevant. In both sexes at 2000 ppm and above, GGT levels were increased (up to 300% of controls). In high-dose females, increased levels of total protein, albumin and potassium levels

were observed. Decreases in cholesterol (up to 23%), triglyceride (up to 63%), plasma cholinesterase (up to 34%), total bile acid (up to 53%) and bilirubin levels (up to 43%) and increases in inorganic phosphorus (up to 31%) and erythrocyte cholinesterase (up to 16%) levels were found in females at doses of 2000 ppm and higher. Other changes in haematology and clinical chemistry parameters were considered incidental, as they occurred sporadically, were marginal or were not dose related. Brain cholinesterase was not affected by treatment. Absolute and relative liver weights were increased (up to 41%) in both sexes at doses of 2000 ppm and above. Females at these doses also exhibited increased weights of kidneys (up to 13%), adrenals (up to 14%), heart (up to 10%) and ovaries (up to 17%), whereas spleen weights were reduced by 10%. At the high dose, absolute and relative thyroid weights were increased in both sexes (up to 26%), reaching statistical significance in males only. Females at 2000 and 20 000 ppm showed high incidences of dark-coloured and enlarged livers. Histopathology showed high incidences of periportal fatty changes, hypertrophy and foci of cellular alteration (basophilic cell type) in females at doses of 2000 ppm and higher and increased incidences of follicular cell hypertrophy of the thyroid in both sexes at doses of 2000 ppm and higher. No treatment-related effects on the incidences of neoplastic lesions were observed.

The study demonstrated toxicologically relevant changes in haematology, clinical chemistry, organ weights, and liver and thyroid histology in males and/or females at doses of 2000 ppm or higher. Based on these effects, the NOAEL was 50 ppm, equal to 2.0 mg/kg bw per day (Enomoto, 2004a).

Dogs

In a 28-day oral range-finding study, flubendiamide (purity 98.5%; lot No. 0FH0010P) was administered to Beagle dogs (one animal of each sex per dose) in feed at a dose of 0, 40, 400, 4000 or 40 000 ppm, equal to 0, 1.1, 10.7, 101.1 and 1111 mg/kg bw per day in males and 0, 1.1, 12.0, 120 and 1180 mg/kg bw per day in females. The animals were observed daily for mortality and general condition, and detailed clinical examinations were conducted pre-study and then weekly thereafter. Body weight was measured weekly, and feed consumption was measured daily. All animals were subjected to ophthalmoscopy, urinalysis, haematology and clinical chemistry prior to study start and at week 2 (except ophthalmoscopy) and week 4. After 4 weeks, the dogs were killed and subjected to gross examinations, and selected organs were weighed. Liver, kidneys and adrenals were evaluated microscopically.

No treatment-related effects on survival, body weight, feed consumption, ophthalmology, haematology, urinalysis, gross pathology or histopathology were observed. Slightly increased incidences of loose stool, observed in the male and female dogs at 40 000 ppm, are probably not treatment related, as loose stool was also observed pretreatment. Detailed clinical examination showed no treatment-related effects. The activity of alkaline phosphatase was increased (up to 5 times) in both sexes at 4000 and 40 000 ppm at weeks 2 and 4. At 400 ppm, the alkaline phosphatase activity in the female was 3-fold higher than in the control female. At 40 000 ppm, relative liver weights of the male and female dogs were 35% and 9% higher than those of their respective controls (Kuwahara, 2001).

In a 90-day study, Beagle dogs (four of each sex per dose) were fed on a diet containing flubendiamide (purity 98.5%; lot No. 0FH0010P) at 0, 100, 2000 or 40 000 ppm, equal to 0, 2.6, 53 and 1076 mg/kg bw per day in males and 0, 2.8, 60 and 1135 mg/kg bw per day in females. The study was performed in accordance with OECD guideline 409. Mortality, clinical signs and feed consumption were recorded daily. Weekly, a detailed clinical examination, including motor activity, open-field and social behaviour, was performed, and body weights were recorded. Ophthalmoscopy was performed prior to the start of the study and prior to termination. Blood and urine samples were collected pretest and at weeks 4, 8 and 13 for haematology, coagulation, clinical chemistry and urinalysis. At termination, the dogs were killed and subjected to gross examinations. Selected organs were weighed. An extensive range of organs was examined histologically. Statements of adherence to QA and GLP were included.

Table 6. Effects of flubendiamide on clinical chemistry parameters

Parameter	Week	% of control values					
		Males			Females		
		Dietary concentration (ppm)					
		100	2000	40 000	100	2000	40 000
Alkaline phosphatase	4	87	171	363*	104	495	741*
	8	86	240	508*	118	771*	1151*
	13	92	292	713*	121	992*	1584*
Triglyceride	4	105	114	112	104	125	133*
	8	98	125	118	114	142*	163*
	13	94	111	125	107	136*	158*

From Kuwahara (2003)
* $P < 0.05$

An increased incidence of loose stool was observed in both sexes at the high dose and in females at 2000 ppm. Other clinical signs were considered not to be treatment related. The terminal body weight of high-dose males was 10% lower than in controls. A small reduction in body weight gain in mid-dose males was due to a markedly lower weight gain in one animal. Feed consumption was not affected by treatment. Ophthalmoscopy and urinalysis showed no treatment-related changes. Haematology revealed, in both sexes of the middle- and high-dose groups, a shortening of the activated partial thromboplastin time (up to 83% of control) at weeks 4 (not in 2000 ppm females), 8 and 13. In high-dose males, increases in haemoglobin (weeks 8 and 13, up to 16%) and red blood cells (weeks 8 and 13, up to 18%) were observed. Effects on clinical chemistry parameters, expressed as a percentage of control values, are presented in Table 6.

The marked increases in alkaline phosphatase levels were not accompanied by macroscopic or histopathological signs of liver (biliary tract) lesions. Also, no changes in osteoblasts (alkaline phosphatase–producing cells), thyroid or parathyroid (organs that regulate osteoblast proliferation) were observed. In dogs, alkaline phosphatase may be induced by corticoids, and increased adrenal weights and hypertrophy of cortical cells were observed in mid- and high-dose animals. However, no changes in other steroid-producing organs or in the pituitary were observed. Accordingly, the source of the increased plasma alkaline phosphatase levels is not clear.

Significant decreases in cholesterol levels were observed in high-dose males at weeks 4 and 8, but less so at week 13. As the changes were within historical control levels and as one control male had cholesterol levels above the historical control range, the effects on cholesterol levels are considered not to be treatment related.

Absolute and relative adrenal weights were increased (not statistically significantly) in all treated males (relative weights by 18%, 24% and 39%, respectively) and mid- and high-dose females (by 27% and 18%, respectively). No macroscopic changes were observed. Histopathological examination revealed cortical hypertrophy of cells in the zona glomerulosa and zona fasciculata of the adrenals in two of four males and one of four females at the high dose and in two of four females of the 2000 ppm group. Other histopathological changes were considered not treatment related.

The NOAEL was 100 ppm, equal to 2.6 mg/kg bw per day, based on a shortening of the activated partial thromboplastin time, increased alkaline phosphatase levels, increased adrenal weights and adrenal cortical hypertrophy observed at 2000 ppm, equal to 53 mg/kg bw per day (Kuwahara, 2003).

Flubendiamide (purity 96.7%; lot No. 1FH0019M) was administered in feed to Beagle dogs (four of each sex per dose) at a dose level of 0, 100, 1500 or 20 000 ppm (equal to 0. 2.2, 35 and

484 mg/kg bw per day for males and 0, 2.5, 38 and 533 mg/kg bw per day for females) for a period of 52 weeks. The animals were subjected to general clinical observations and checked for mortality on a daily basis. Detailed clinical examinations were performed weekly. Individual body weights and feed consumption were recorded weekly for the first 13 weeks and every 4 weeks thereafter. Ophthalmology, urinalysis, haematology and blood biochemistry were performed before initiation of treatment and at weeks 13, 26, 39 (except for ophthalmology) and 52 of treatment. After 52 weeks of treatment, the animals were killed and necropsied, selected organ weights were recorded and an extensive range of organs was examined histologically. Statements of adherence to QA and GLP were included.

No mortality was observed. Flubendiamide had no effect on clinical signs, feed intake, ophthalmoscopy or urinalysis. A marginally reduced body weight gain (10–11%) was found at 20 000 ppm in both sexes. Throughout the treatment period, a shortening of activated partial thromboplastin time was found in both sexes at 1500 ppm (6–17%, most prominent in females) and 20 000 ppm (13–21%). An increase in platelet count was observed in high-dose females at weeks 39 (57%) and 52 (38%). Other haematological findings were considered not to be treatment related. Increases in the serum levels of alkaline phosphatase (8- to 27-fold, throughout the treatment period) and ALT (2- to 3-fold, at weeks 39 and 52) were measured at the high dose in both sexes. Increases (7- to 11-fold) in alkaline phosphatase levels were observed in both sexes at 1500 ppm, but reached statistical significance only in females. High-dose males showed decreases in albumin and in albumin to globulin ratio. The significance of slight (1.2–1.4%) but statistically significant reductions in sodium levels in males at 1500 and 20 000 ppm at weeks 13 and 26 is questionable. Triglyceride levels were increased (2-fold maximally), but not dose-dependently, in mid- and high-dose males. In high-dose females, increases in GGT and triglyceride levels and a decrease in glucose level were recorded.

Absolute (12–23%) and relative weights (26–37%) of the liver were increased in both sexes at 20 000 ppm. At 1500 ppm, relative liver weights were increased by 14% in males and females. One high-dose female had an enlarged and dark-coloured liver. Brown pigment deposition in the Kupffer cells of the liver was observed in two males and one female at 20 000 ppm.

The NOAEL was 100 ppm, equal to 2.2 mg/kg bw per day, based on increased alkaline phosphatase, shortening of activated partial thromboplastin time and increased relative liver weights in both sexes and a possible decrease in sodium concentration at 1500 ppm (Kuwahara, 2004).

(b) Dermal application

In a 1-month dermal study, performed in accordance with OECD guideline 410, flubendiamide (purity 97.1%; lot No. 1FH0019M) was applied to the shaved, intact dorsal skin of Fischer F344/NHsd rats (10 of each sex per dose). The rats were exposed to the test substance under semiocclusion for 6 hours per day, 5 days per week, for 3 weeks, followed by 8 days of consecutive treatment. Exposure doses were 0, 10, 100 or 1000 mg/kg bw per day. The test substance was moistened with tap water. Clinical signs were recorded daily, and body weight, feed consumption and water consumption were measured weekly. Open-field behaviour was assessed weekly. Ophthalmological examinations were performed on all animals prior to testing and on control and high-dose animals at the end of treatment. At termination, blood and urine were collected for clinical chemistry, haematology and urinalysis, the animals were necropsied and selected organs were weighed and microscopically examined. Statements of adherence to QA and GLP were included.

No flubendiamide-related effects on survival, open-field behaviour, clinical observations, body weight gain, water and feed consumption or ophthalmology were observed. In females at 1000 mg/kg bw per day, slight decreases in MCV (3%) and haematocrit (5%) were noted. A decrease (15%) in AST levels in high-dose females is not considered to be toxicologically relevant. Other occasionally observed differences in haematology, clinical chemistry and urinalysis were small and generally not dose dependent. At the high dose, absolute and relative liver weights were increased in males (relative

weight 11%) and females (relative weight 16%). Histological examination showed a slight increase in incidence and/or severity of elevated fat-positive reaction in the periportal zone of the livers in both sexes at the high dose and a slightly higher incidence of follicular cell hypertrophy of the thyroid gland in high-dose females. The finding of a decrease in absolute and relative uterus weights (~20%) in high-dose females had no microscopic correlate and is considered to be incidental. Repeated dermal administration of flubendiamide induced no local skin effects.

The NOAEL for systemic effects was established at 100 mg/kg bw per day, based on thyroid follicular cell hypertrophy and a possibly marginal decrease in haematocrit and MCV in females and a slightly elevated fat-positive reaction in periportal hepatocytes in both sexes at 1000 mg/kg bw per day (Kroetlinger, 2004).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month carcinogenicity feeding study, performed in accordance with OECD guideline 451, flubendiamide (purity 96.7%; lot No. 1FH0019M) was administered to SPF ICR (Crj:CD-1) mice (52 of each sex per dose) at 0, 50, 1000 or 10 000 ppm, equal to 0, 4.9, 94 and 988 mg/kg bw per day in males and 0, 4.4, 93 and 937 mg/kg bw per day in females. During the treatment period, all animals were checked daily for mortality and general clinical signs. Detailed clinical observations were carried out weekly. Body weights and feed consumption were monitored weekly for the first 13 weeks and every 4 weeks thereafter. Haematological examinations were performed on all surviving animals after 78 weeks of treatment. At the end of the treatment period, the animals were killed and necropsied. Selected organ weights for 10 animals of each sex per dose were measured. An extensive range of organs and tissues of high-dose and control animals and of animals of the low- and mid-dose groups that were killed in extremis was examined histologically. In addition, in low- and mid-dose animals, liver, kidneys, thyroids (both sexes), pituitary (females only) and gross lesions were examined histologically. Statements of adherence to QA and GLP were included.

No treatment-related effects on mortality, general clinical observations, body weights or feed consumption were observed. Haematological examinations showed a statistically significant decrease in eosinophil count in high-dose males. Increases in absolute and relative liver weights (37–64%) and thyroid weights (175–176%) in both sexes and adrenal weights (32–44%) in males were observed at the high dose. Liver weights were also increased (by 23–30%) in both sexes at the middle dose. At necropsy, both sexes showed increased incidences of dark-coloured liver and enlargement of the thyroid. In addition, treated males showed an increased incidence of masses in the liver. Histology showed higher incidences of centrilobular hypertrophy and centrilobular microvesicular fatty change of hepatocytes (both sexes) and diffuse microvesicular and macrovesicular fatty change (females only) in the liver at 1000 and 10 000 ppm. In addition, high-dose females showed a periportal fatty change, whereas in males at this dose, higher incidences of foci of cellular alteration were found. In the thyroid, hypertrophy with hydropic change and increased large-sized follicles were observed in both sexes at doses of 1000 ppm and higher. In addition, incidences of altered colloid (both sexes) and follicular cell hyperplasia (females only) were increased at the high dose. Females at 10 000 ppm also showed a slightly, but not statistically significantly, higher incidence of anterior cell hyperplasia of the pituitary. No substance-related effect on tumour incidence was noted.

The NOAEL was 50 ppm, equal to 4.4 mg/kg bw per day, based on increased liver weight, centrilobular hypertrophy, centrilobular microvesicular fatty change, enlarged thyroid, increased thyroid weights, increased incidence of thyroid follicular cell hypertrophy and hydropic change and increased large size follicles in both sexes, diffuse microvesicular and macrovesicular fatty change in females and discoloration of the liver in males at 1000 ppm, equal to 93 mg/kg bw per day (Takeuchi, 2004).

Rats

In a 2-year dietary carcinogenicity study, performed in accordance with OECD guideline 451, flubendiamide (purity 97.8%, lot No. 1FH0018P, up to weeks 75–76; purity 96.7%, lot No. 1FH0019M, from weeks 75–76 to week 105) was administered to Fischer (F344/DuCrj) rats (50 of each sex per dose) at doses of 0, 50, 1000 or 20 000 ppm, equal to 0, 1.7, 34 and 705 mg/kg bw per day in males and 0, 2.2, 44 and 912 mg/kg bw per day in females. The animals were observed daily for mortality, morbidity and clinical signs. Detailed clinical examinations were conducted weekly. Body weight and feed consumption were measured weekly for the first 13 weeks and once every 4 weeks thereafter. Blood samples collected after termination were used for haematology. All animals were necropsied. In 10 rats of each sex per dose, selected organs were weighed. A wide range of tissues collected from the control and high-dose groups and from low- and mid-dose rats killed in extremis or found dead was evaluated microscopically. Liver, kidneys, thyroid, spleen and bone marrow of all animals were examined histologically. Statements of adherence to QA and GLP were included.

Mortality was not affected by treatment. Females at doses of 1000 ppm and higher showed increased incidences of hair loss. Body weights of high-dose females were increased during weeks 2–5 and decreased to about 90% from control during weeks 76–104. Feed consumption of high-dose animals was on average 105% of that of controls from week 9 onward. Eosinophil counts were significantly decreased in males at doses of 1000 ppm and above. Neutrophil counts were decreased in females at 50 and 1000 ppm, but not at 20 000 ppm.

Analysis of organ weights (excluding organs with neoplastic and non-neoplastic diseases) showed increased absolute (17–37%) and relative (16–45%) liver weights in males and females at doses of 1000 ppm and higher. Increases in absolute (33–53%) and relative (25–68%) thyroid weights were noted in high-dose males and females. Relative kidney weights were increased in mid-dose (12%) and high-dose (19%) females, whereas absolute kidney weights (11%) and relative adrenal weights (19%) were also increased in high-dose females. Increases in absolute and relative ovary (25% and 35%, respectively) and testes weights (48% and 40%, respectively) were found at the high dose. Necropsy revealed increased incidences of dark-coloured spleens and liver spots, accentuated lobular pattern, coarse surface and masses in high-dose males and dark-coloured and enlarged livers in mid- and high-dose females.

Periportal fatty change of hepatocytes was noted in males and females at doses of 1000 ppm and higher. Diffuse fatty change and diffuse hypertrophy of hepatocytes were also found in females at these doses. Most of the periportal and diffuse fatty change of hepatocytes was microvesicular (observed as small lipid droplets in the hepatocytes), but some lesions also contained macrovesicles (large lipid droplets). Some of the masses in the liver observed at necropsy in males at 20 000 ppm corresponded histopathologically to foci of cellular alterations or were due to leukaemia, and only a few were diagnosed as hepatocellular tumours. Chronic nephropathy was observed in all dose groups, including controls, with a dose-related increase in incidence at the middle and high doses. However, chronic progressive nephropathy is a kidney alteration that occurs spontaneously in ageing rats and is not considered relevant for humans (Hard & Khan, 2004). Follicular cell hypertrophy of the thyroid was noted in high-dose males and in mid- and high-dose females. Lymphocytic folliculitis, characterized by mononuclear cell infiltration around hair bulbs in some or many follicles, was detected in female skin at 1000 ppm and above.

No treatment-related effect on tumour incidence was found.

The NOAEL was 50 ppm, equal to 1.7 mg/kg bw per day, based on increased liver weights and periportal fatty change (both sexes), increased incidence of hair loss associated with folliculitis, dark-coloured and enlarged livers, hepatocyte hypertrophy, diffuse fatty change and diffuse hypertrophy, increased kidney weight and increased incidences of thyroidal follicular cell hypertrophy in females and decreased eosinophil count in males at 100 ppm, equal to 34 mg/kg bw per day (Enomoto, 2004b).

Table 7. Results of studies on the genotoxicity of flubendiamide

End-point	Test object	Concentration	Purity (%)	Results	Reference[a]
In vitro					
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, *Escherichia coli* WP2 uvrA	3.9–313 µg/plate (−S9) 62–5000 µg/plate (+S9)	97.8	Negative	Inagaki (2002)[b]
Gene mutation	V79, HGPRT test	7.5–240 µg/ml (±S9)	96.6–97.3	Negative	Herbold (2003)[c]
Chromosomal aberrations	Chinese hamster lung cells	550–2200 µg/ml (±S9, 6 h) 125–500 µg/ml (−S9, 40 h) 300–1200 µg/ml (+S9, 20 h)	97.8	All negative	Miyahana (2004)[d]
In vivo					
Micronucleus formation	Mouse bone marrow (males/females)	Gavage dose of 500, 1000 or 2000 mg/kg bw	97.8	Negative	Miyahana (2003)[e]
Micronucleus formation	Male mouse bone marrow	Two intraperitoneal injections of 1000, 2000 or 4000 mg/kg bw separated by 24 h	98.9	Negative	Herbold (2005)[f]

S9, 9000 × *g* rat liver supernatant

[a] Positive and negative (solvent) controls were included in all studies. In all studies, statements of adherence to GLP and QA were included.

[b] Lot No. 1FH0018P. Performed in accordance with OECD guideline 471. –S9: precipitation at ≥ 104 µg/plate. +S9: precipitation at ≥ 556 µg/plate. At ≥ 1670 µg/plate, precipitation made judgement of cytotoxicity impossible.

[c] Lot No. 1FH0019M. Precipitation at ≥ 160 µg/ml. Performed in accordance with OECD guideline 476. Cytotoxicity was observed at high doses.

[d] Lot No. 1FH0018P. Performed in accordance with OECD guideline 473.

[e] Lot No. 1FH0018P. Performed in accordance with OECD guideline 474. As no clinical signs of toxicity and no changes in the polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) ratio were observed at any dose, it is not clear whether the bone marrow was sufficiently exposed in this study. However, in the toxicokinetic study of Motoba (2004a), radiolabel was detected in bone marrow, and low concentrations of parent compound were detected in urine following single oral administration of flubendiamide at a dose of 2 or 200 mg/kg bw, suggesting that the bone marrow will have been exposed to flubendiamide.

[f] Lot No. 3FH0032M. Performed in accordance with OECD guideline 474. Clinical signs of toxicity at all doses. Changed PCE/NCE ratio at doses of ≥ 2000 mg/kg bw.

2.4 Genotoxicity

Flubendiamide was tested for genotoxicity in a range of OECD guideline–compliant assays. No evidence for genotoxicity was observed in any test.

The results of the genotoxicity tests are summarized in Table 7. It is concluded that flubendiamide is unlikely to be genotoxic.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

A preliminary study of reproductive toxicity with flubendiamide (purity 98.5%; lot No. OFH0010P) was performed in groups of Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (eight of each sex per dose). The animals received diets containing flubendiamide at 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 1.2, 11.7, 118 and 1196 mg/kg bw per day in males and 0, 2.2, 22, 224 and 2214 mg/kg bw per day in females) during a premating period of 3 weeks and subsequent breeding periods until weaning of the first generation (F_1) pups. The rats were observed daily for mortality and

clinical signs, and detailed clinical observations were performed at least once weekly. Body weights and feed consumption were recorded on a weekly basis and included gestation days (GDs) 0, 7, 14 and 21 and postnatal days (PNDs) 0, 7, 14 and 21. In parental (P) animals, estrous cycle, mating performance, fertility, duration of gestation, gestation indices and number of implantation sites were recorded. The parental animals were necropsied, and livers were weighed. In the offspring, mortality, clinical signs, number of live and dead pups, sex ratio, viability index and body weights at PNDs 0, 4, 7, 14 and 21 were recorded. Litter size was reduced to eight pups on PND 4. All pups, killed at PND 4 (culling) or at PNDs 22–24, were grossly examined. Statements of adherence to QA and GLP were included.

In the parental rats, no mortality and no treatment-related clinical signs were observed. At 2000 and 20 000 ppm, body weight gains of females were increased during gestation. Feed consumption was not affected. No toxicologically relevant effects on estrous cycle, mating index, fertility index, gestation index, gestation duration or number of implantation sites were seen. At 20 000 ppm, almost all parental females showed liver enlargement and dark-coloured liver at necropsy, and the absolute (+46%) and relative liver weights (+37%) were statistically significantly higher than those of controls. In the 2000 ppm group, liver enlargement and dark-coloured livers were found in about half the females. Both absolute (+25%) and relative liver weights (+19%) of the females in this group were higher than those of controls, although no statistically significant differences were observed. No gross abnormalities were found in males. In the offspring, no treatment-related effects on mortality, clinical signs, number of pups delivered, sex ratio or viability index were found. At PND 21, pup weights at 20 000 ppm were decreased (91% of control, statistically significant in females only). Necropsy of the pups revealed no macroscopic alterations.

The NOAEL for parental toxicity was 200 ppm, equal to 22 mg/kg bw per day, based on enlarged and darkened livers in females at 2000 ppm.

The NOAEL for reproductive toxicity was 20 000 ppm, equal to 1196 mg/kg bw per day, the highest dose tested.

The NOAEL for offspring toxicity was 2000 ppm, equal to 118 mg/kg bw per day, based on reduced body weight gain in female pups at PND 21 at 20 000 ppm (Hojo, 2001).

In a two-generation study of reproductive toxicity, performed in accordance with OECD guideline 416, Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (24 of each sex per dose) were given diets containing flubendiamide (purity 96.7%; lot No. 1FH0019M) at a concentration of 0, 20, 50, 2000 or 20 000 ppm, equal to 0, 1.3, 3.3, 131 and 1307 mg/kg bw per day for P generation males, 0, 1,6, 4.0, 159 and 1577 mg/kg bw per day for P generation females, 0, 1.6, 4.1, 162 and 1636 mg/kg bw per day for F_1 generation males and 0, 1.8, 4.6, 176 and 1808 mg/kg bw per day for F_1 generation females, respectively. Treatment started 10 weeks before mating. Litters were culled to eight pups at PND 4. The rats were observed daily for clinical signs, and detailed clinical observations were performed at least once weekly. Body weights and feed consumption were recorded on a weekly basis and included GDs 0, 7, 14 and 21 and PNDs 0, 7, 14 and 21. In P and F_1 parental animals, estrous cycle, mating performance, fertility, duration of gestation, gestation indices, sperm parameters and number of implantation sites were recorded. Sexual maturation (preputial separation and vaginal opening) in F_1 pups selected for breeding a second generation (F_2) and anogenital distance on PND 4 in F_2 pups were recorded. All the P and F_1 parental animals were necropsied, and selected organs were weighed. In the control and high-dose parental animals, reproductive organs and liver, thyroid, kidneys and spleen were examined histologically. Histology was also performed on pituitaries, thyroid, liver and ovaries of all P and F_1 parental animals of the 20, 50 and 2000 ppm groups and on adrenals and kidneys of P females at 20, 50 and 2000 ppm. In the offspring, mortality, clinical signs, number of live and dead pups, sex ratio, viability index and body weights at lactation days (LDs) 0, 4, 7, 14 and 21 were recorded. All pups were grossly examined. In selected pups, a range of organs

was weighed. In selected F_1 and F_2 pups of the control and high-dose groups, thyroid, thymus, spleen, liver and uterus were examined histologically. In addition, thyroid and liver of F_1 and F_2 weanlings of the 20, 50 and 2000 ppm groups were examined histologically. Statements of adherence to QA and GLP were included.

One P generation female in the 2000 ppm group and one P generation female and two F_1 generation females in the 20 000 ppm group died during delivery. No treatment-related clinical signs were observed. Incidental changes in body weights are considered not treatment related. In F_1 parental males at 20 000 ppm, a slight, but statistically significant, reduction (11%) in sperm head counts per testis was found. Other small effects on reproductive parameters are considered incidental and not treatment related. The parental P and F_1 females showed increased incidences of enlarged and dark-coloured livers at 2000 and 20 000 ppm. Absolute and relative liver weights were increased up to 47% at 2000 ppm and up to 63% at 20 000 ppm compared with controls. Enlargement and a brown coloration of the thyroids were found in both sexes at the high dose. Brown coloration of the thyroid was also observed in females at 2000 ppm. Absolute and relative thyroid weights were increased up to 36% at 2000 ppm in females and up to 84% at 20 000 ppm compared with controls.

In females mainly of the P generation, increases in relative kidney (up to 15%) and uterus weights (up to 22%) were found at 2000 ppm and above, and increases in relative adrenal (11%) and ovary weights (21%) at 20 000 ppm. Relative spleen weight was decreased (by 12–19%) in P and F_1 generation females at 2000 ppm and above; relative pituitary weight was decreased (by 12–18%) in F_1 generation males and females at 2000 ppm and above.

In high-dose males, increases in periportal fatty change of hepatocytes (P and F_1), diffuse hypertrophy of hepatocytes (F_1), deposition of brown pigment in the hepatic portal area (F_1, also at 2000 ppm), hypertrophy of thyroid follicular cells (P and F_1, also at 2000 ppm) and a hydropic degeneration of basophilic cells of the pituitary in three animals (P and F_1) were seen. In females, increases in periportal fatty change of hepatocytes, diffuse hypertrophy of hepatocytes and deposition of brown pigment in the hepatic portal area (P and F_1 at 2000 and 20 000 ppm), increased multinucleated hepatocytes (P at 20 000 ppm), proliferation of the bile duct (P and F_1 at 20 000 ppm), hypertrophy of thyroid follicular cells (P and F_1 at 2000 and 20 000 ppm), hydropic degeneration of the basophilic cells of the pituitary (P and F_1 at 20 000 ppm), vacuolation of interstitial cells in the ovaries (P at 2000 and 20 000 ppm, F_1 at 20 000 ppm), diffuse hypertrophy of adrenal cortical cells (P at 20 000 ppm) and a tubular basophilic change and urinary casts in the kidneys (P at 2000 and 20 000 ppm) were observed. Synechia (adherence of the anterior epithelium of the iris to the posterior epithelium of the cornea) was noted in one 20 000 ppm F_1 female, which may be a treatment-related sequela of eyeball effects that also occurred postnatally in some weanlings (see below).

Examination of the offspring showed a lower pup body weight (91–92% of control) in both sexes on PND 21 in 20 000 ppm F_1 and F_2 litters. During PNDs 15–21, an enlargement of the eyeball (buphthalmos) was noted in 16 out of 180 F_1 pups at 2000 ppm (6/23 litters) and in 3 out of 143 pups at 20 000 ppm (2/19 litters). In the F_2 generation, 4 pups out of 130 were affected in the 2000 ppm group (2/18 litters) and 5 pups out of 157 (5/20 litters) in the 20 000 ppm group. Occasional slightly lower viability indices at 2000 and 20 000 ppm were considered incidental. At 2000 ppm and higher, F_1 and F_2 weanlings or pups found dead during PNDs 5–21 showed increased incidences of dark-coloured livers, increases in absolute and relative liver weights and decreases in absolute and relative spleen and thymus weights. Other effects on organ weights observed at doses of 2000 ppm and higher were considered not compound related in view of the poor dose–response relationship and lack of confirmation in an additional one-generation study (Hojo, 2004b).

Histological examination of the offspring showed diffuse fatty change of hepatocytes, diffuse hypertrophy of hepatocytes, deposition of brown pigment in the hepatic portal area, proliferation of the bile duct and hypertrophy of thyroid follicular cells in 2000 and 20 000 ppm F_1 and F_2 weanlings of both sexes. Histology of the enlarged eyeballs demonstrated synechia in all of these eyes, hydropic

degeneration of the basal layers of the corneal epithelium in most of these eyes and haemorrhage, keratitis, iritis and cataract in some of these eyes.

In the F_1 males of the 2000 and 20 000 ppm groups, completion of balano-preputial separation (at PND 43.7) was significantly delayed compared with controls (at PND 41.3), with mean body weights on the completion day of balano-preputial separation being statistically significantly higher (5.3–7.2%) than control. Based on the occurrence of other signs of toxicity, the delay in balano-preputial separation is considered to reflect general toxicity rather than a specific effect on the development of the prepuce. A small delay in balano-preputial separation (at PND 42.5) observed in the 50 ppm F_1 parental males was not considered toxicologically relevant, as it was within the historical control range (40.9–43.4 days) and it could not be confirmed in an additional one-generation study (Hojo, 2004b). No significant effect was noted in the sexual development (days of age at completion of vaginal opening) of the F_1 parental females or on anogenital distance on PND 4 in F_2 pups.

The overall NOAEL for parental toxicity was 50 ppm, equal to 3.3 mg/kg bw per day, based on increased relative liver weight and absolute thyroid weights (P males), brown pigment deposition in the portal area of the liver and decreased absolute and relative pituitary weights (F_1 males), increased incidence of thyroid follicular cell hypertrophy (P and F_1 males), enlarged livers, dark-coloured livers, increased liver weight, hepatocyte hypertrophy, hepatic periportal fatty change, hepatic brown pigment deposition, increased thyroid weight, thyroid follicular cell hypertrophy, increased kidney weights, decreased spleen weight (P and F_1 females), renal tubular basophilic change and urinary casts, vacuolation of ovarian interstitial cells, increased uterus weight (P females) and decreased pituitary weight (F_1 females) at 2000 ppm, equal to 131 mg/kg bw per day.

The overall NOAEL for reproductive toxicity was 2000 ppm, equal to 162 mg/kg bw per day, based on significantly lower sperm head counts in the testes of F_1 parental males, observed at 20 000 ppm, equal to 1636 mg/kg bw per day.

The overall NOAEL for offspring toxicity was 50 ppm, equal to 3.3 mg/kg bw per day, based on dark-coloured livers and increased liver weight, hepatocyte hypertrophy, diffuse fatty change, brown pigment deposition, proliferation of bile duct, thyroid follicular cell hypertrophy, decreased spleen and thymus weights, delayed balano-preputial separation, enlargement of eyeballs with synechia, hydropic degeneration of the basal layers of the corneal epithelium, haemorrhage, keratitis, iritis and cataract formation in F_1 and F_2 offspring at 2000 ppm, equal to 131 mg/kg bw per day (Hojo, 2004a).

In the two-generation study of reproductive toxicity by Hojo (2004a), effects on, among others, the eyes and balano-preputial separation were observed. In order to further investigate these findings, a one-generation study of reproductive toxicity was performed. Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (24 of each sex per dose) were given diets containing flubendiamide (purity 96.7%; lot No. 1FH0019M) at concentrations of 0, 50, 200, 2000 or 20 000 ppm, equal to 0, 3.3, 13, 127 and 1287 mg/kg bw per day for P males and 0, 3.9, 15, 149 and 1490 mg/kg bw per day for P females. Treatment started 10 weeks before mating. Litters were culled to eight pups at PND 4. The rats were observed daily for clinical signs, and detailed clinical observations were performed at least once weekly. Body weights and feed consumption were recorded on a weekly basis and included GDs 0, 7, 14 and 20 and PNDs 0, 7, 14 and 21. In P parental animals, estrous cycle, mating performance, fertility, duration of gestation, gestation indices and number of implantation sites were recorded. In F_1 parental animals, sexual maturation (preputial separation and vaginal opening) was assessed. All the P and F_1 parental animals were necropsied, and selected organs were weighed. In the offspring, mortality, clinical signs, number of live and dead pups, sex ratio, viability index and body weights at LDs 0, 4, 7, 14 and 21 were recorded. Anogenital distance was measured at PND 4. All pups were grossly examined. In one weanling of each sex per litter, a range of organs was weighed. Histopathological examinations of all ocular lesions and normal eyes in a subset of weanlings of the 0, 200, 2000 and 20 000 ppm groups were performed. Statements of adherence to QA and GLP were included.

Two P generation females in the 20 000 ppm group died during delivery. Other effects on fertility were not observed. Body weights were not affected by treatment. In parental animals, enlargement of eyeballs was found during clinical examinations in one F_1 generation male of the control group, one F_1 generation female of the 2000 ppm group and one F_1 generation male and one F_1 generation female of the 20 000 ppm group. Similar to the two-generation study, in the 2000 and 20 000 ppm groups, the completion of the balano-preputial separation was statistically significantly delayed (42.2–43.1 versus 41 days), with the mean body weight on the day of completion of preputial separation in the 20 000 ppm group being statistically significantly higher (7.7%) than control. In contrast to the two-generation study, however, in the 20 000 ppm group, the age and the mean body weight at completion of vaginal opening were statistically significantly increased. However, as no effect on vaginal opening was observed in the study of Hojo (2004a), the present finding was considered incidental. Dark-coloured livers were noted in P generation females at 200 ppm and above and in F_1 males of the parental generation at 20 000 ppm. In addition, enlarged livers were found in P generation females at 2000 and 20 000 ppm and in F_1 generation females at 20 000 ppm. Brownish coloration of thyroids was noted in P and F_1 parental male animals mainly at 20 000 ppm and in P generation females at 2000 ppm and above. Slightly increased incidences of enlarged thyroids were seen only in males at 20 000 ppm. In P and F_1 generation animals, treatment-related increases in absolute and relative liver weights were observed in male adults at 20 000 ppm (11–16%) and in females at 2000 ppm (24–40%) and 20 000 ppm (43–53%). Thyroid weights were increased (17–28%) in P generation females at 2000 and 20 000 ppm and in F_1 generation females at 20 000 ppm. In females mainly of the F_1 generation, increases in absolute and relative kidney weights (9–16%) were noted at 200 ppm and above. The relative uterus weight was marginally increased (17%) in P generation adults at 20 000 ppm, and absolute and relative ovary weights were increased (16–21%) in F_1 generation animals at 2000 and 20 000 ppm. Absolute and relative weights of the pituitary were decreased (9–24%) in F_1 generation males and females at 2000 ppm and above. The statistically significantly lower mean relative pituitary weight (8%) in F_1 generation female adults at 200 ppm was not considered treatment related. In contrast to the two-generation study, relative adrenal and spleen weights were not decreased, suggesting that the differences from control are possibly due to the inherent variability and not a true effect of treatment. The same is assumed for the statistically significantly lower relative weight of the seminal vesicles (10%) at 2000 ppm and above, which was observed in the present supplemental study only. The slight decrease in relative testes weight (7%) in F_1 generation males at 20 000 ppm only is considered due to chance variation, as no such change was observed either in the main study or in other rat feeding studies. The statistically significant increase in the absolute ovary weights without a statistically significant increase in relative weight at 200 ppm and above is considered to be due to the increased body weight rather than to a direct effect on the organ itself.

The litter parameters determined at birth were not affected by treatment. In the offspring, a statistically significant decrease in body weight was noted at 20 000 ppm on day 21 in both sexes. During PNDs 5–21, clinical and gross examinations revealed enlargement of the eyeballs in pups at 2000 ppm (in 7/191 pups) and 20 000 ppm (in 24/154 pups). The enlarged eyes were confirmed to have various histological changes, including synechia, haemorrhage, keratitis, iritis, cataract, hydropic degeneration of the basal layer of the corneal epithelium and/or corneal epithelial vacuolation. Gross examination revealed dark-coloured livers, increased absolute and relative liver weights and decreases in absolute and relative spleen weights at 2000 ppm and above. In F_1 generation female weanlings, there were also statistically significant decreases in absolute and relative weights of the thymus at 2000 ppm and above.

The NOAEL for parental toxicity was 50 ppm, equal to 3.9 mg/kg bw per day, based on dark-coloured livers in P generation females and increased kidney weights in F_1 generation females at 200 ppm, equal to 15 mg/kg bw per day.

The NOAEL for reproductive toxicity was 20 000 ppm, equal to premating doses of 1287 mg/kg bw per day, the highest dose tested. The increase in time to balano-preputial separation is considered

treatment related and due to systemic toxicity and not a specific effect on the development of the prepuce. Therefore, the delay in balano-preputial separation is not used as a measure of reproductive function.

The NOAEL for offspring toxicity was 200 ppm, equal to 15 mg/kg bw per day (i.e. the maternal compound intake), based on dark-coloured livers and increased liver weight, decreased spleen and thymus weights, delayed balano-preputial separation, enlargement of eyeballs, synechia, haemorrhage, keratitis, iritis, cataract, hydropic degeneration of the basal layer of the corneal epithelium and/or corneal epithelial vacuolation at 2000 ppm, equal to 149 mg/kg bw per day (Hojo, 2004b; Takeuchi, 2005).

(b) Developmental toxicity

Rats

In a preliminary developmental toxicity study, flubendiamide (purity 98.5%; lot No. OFH0010P) in 1% aqueous carboxymethylcellulose was administered by gavage to time-mated female Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (seven per dose) on GDs 6–19 at a dose of 0, 20, 100 or 1000 mg/kg bw per day. Animals were examined daily for clinical signs. Body weight and feed consumption were recorded on GDs 9, 12, 15, 18 and 20. At termination on GD 20, the gravid uterus, live fetuses and placentas were weighed, and the number of live and dead fetuses, fetal resorptions, sex, external alterations, intrauterine location and identification number were recorded. Approximately half of the fetuses from each litter were examined for visceral abnormalities. All remaining live fetuses from the control and high-dose groups were examined for skeletal alterations. Dams were necropsied. Statements of adherence to QA and GLP were included.

No test substance–related effects on maternal test parameters were found. In the low- and mid-dose groups, but not in the high-dose group, increased incidences of left umbilical artery were found. In high-dose fetuses, increased incidences of supernumerary ribs were observed. Other fetal parameters were not affected by treatment (Aoyama, 2002).

In the main developmental toxicity study, performed in accordance with OECD guideline 414, flubendiamide (purity 96.7%; lot No. 1FH0019M) in 1% aqueous carboxymethylcellulose was administered by gavage to time-mated female Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (24 per dose) on GDs 6–19 at a dose of 0, 10, 100 or 1000 mg/kg bw per day. Animals were examined daily for clinical signs. Body weight and feed consumption were recorded on GDs 0, 6, 9, 12, 15, 18 and 20. At termination on GD 20, the gravid uterus, live fetuses and placentas were weighed, and number of live and dead fetuses, fetal resorptions, sex, external alterations, intrauterine location and identification number were recorded. Approximately half of the fetuses from each litter were examined for visceral abnormalities. All remaining live fetuses were examined for skeletal alterations. Dams were necropsied, and livers were weighed. Statements of adherence to QA and GLP were included.

Dose-related increases in absolute and relative liver weights were measured in dams at 100 (8%) and 1000 mg/kg bw per day (15–16%). No other treatment-related effects on maternal test parameters were found. Treatment with flubendiamide did not influence any of the fetal parameters, including the incidences of supernumerary ribs.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on the increases in absolute and relative liver weights at 1000 mg/kg bw per day.

The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested. No evidence for a teratogenic effect of flubendiamide was observed (Aoyama, 2003).

Rabbits

In a preliminary developmental toxicity study, flubendiamide (purity 98.5%; lot No. OFH0010P) in 1% aqueous methylcellulose was administered by gavage to pregnant Japanese White (Kbl:JW)

rabbits (six per dose) on GDs 6–27 at a dose of 0, 30, 100, 300 or 1000 mg/kg bw per day. Animals were examined daily for clinical signs. Body weight and feed consumption were recorded on GDs 0, 3 (feed consumption only), 6, 9, 12, 15, 18, 21, 24, 27 and 28. At termination on GD 28, dams were necropsied, fetal resorptions were recorded, live fetuses and placentas were weighed, and number of live and dead fetuses, fetal resorptions, sex, external alterations and intrauterine location were recorded. Statements of adherence to QA and GLP were included.

No treatment-related effects on maternal parameters were observed. At 300 mg/kg bw per day, one fetus with mandibular and maxillar microagnathia and short tail and one fetus with omphalocele were observed (Takahashi, 2001).

In the main developmental toxicity study, performed in accordance with OECD guideline 414, flubendiamide (purity 96.7%; lot No. 1FH0019M) in 1% aqueous carboxymethylcellulose was administered by gavage to pregnant Japanese White (Kbl:JW) rabbits (25 per dose) on GDs 6–27 at a dose of 0, 20, 100 or 1000 mg/kg bw per day. Animals were examined daily for clinical signs. Body weight and feed consumption were recorded on GDs 0, 3 (feed consumption only), 6, 9, 12, 15, 18, 21, 24, 27 and 28. At termination on GD 28, dams were necropsied, numbers of live and dead fetuses and fetal resorptions were recorded, live fetuses and placentas were weighed, and number of live fetuses, sex, external alterations and intrauterine location were recorded. All fetuses were examined for thoracic and abdominal visceral abnormalities. In approximately half of the fetuses from each litter, the heads were examined for visceral abnormalities. The carcasses of all fetuses and the heads of about half of the fetuses were examined for skeletal alterations. Statements of adherence to QA and GLP were included.

In high-dose dams, increased incidences of loose stool were noted. From GD 24 to GD 28, there was a tendency towards a decrease in body weight gain, and on GDs 27–28, feed consumption was decreased in high-dose females. In fetuses, occasionally visceral and/or skeletal variations and malformations were observed in all groups, including controls. The total number of skeletal malformations was increased at 100 and 1000 mg/kg bw per day, but it was not dose related and was statistically significant only at 100 mg/kg bw per day. However, for each individual type of malformation and variation, no statistically significant difference was noted in any of the incidences of external, visceral and skeletal malformations and variations between the control group and any of the treated groups.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased incidences of loose stool, reduced feed consumption on GDs 27–28 and a tendency towards reduced body weight gain during the latter part of gestation.

The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested. No evidence for a teratogenic effect of flubendiamide was observed (Takahashi, 2002).

2.6 Special studies

(a) Neurotoxicity studies

In an acute neurotoxicity study, performed in accordance with OECD guideline 424, flubendiamide (purity 97.0%; lot No. 1FH0019M) was administered in 0.5% methylcellulose/0.4% Tween 80 by gavage to Fischer CDF(F-344)CrlBR rats (12 of each sex per dose) at a dose of 0, 209, 731 or 2213 mg/kg bw for both males and females. Detailed physical examinations for clinical signs of toxicity were carried out and recorded once each day for 14 days following dosing. Animals were weighed weekly, as a component of the FOB, which was performed 1 week prior to treatment and 8 hours, 7 days and 14 days after dosing. At day 14 or 15 after dosing, the animals were killed and necropsied. Brains were weighed, and parts of the central and peripheral nervous systems, selected

muscle tissue of control and high-dose animals and any gross lesions were examined histologically. Statements of adherence to QA and GLP were included.

No adverse compound-related effects on any of the investigated parameters were observed in either sex. The NOAEL is 2213 mg/kg bw, the highest dose tested (Gilmore & Lake, 2003).

In a developmental neurotoxicity study, performed in accordance with United States Environmental Protection Agency OPPTS guideline 870.6300, groups of time-mated female Wistar Han Crl:Wi (Glx/Brl/Han) IGSBR rats (30 per dose) received diets containing flubendiamide (purity 97.3%; lot. No. 1FH0019M) at 0, 120, 1200 or 12 000 ppm (equal to 0, 9.9, 100 and 980 mg/kg bw per day) from GD 7 to PND 21. On PND 4, litters were culled to eight pups (four of each sex when possible). Pups were weaned on PND 21 and allowed to grow to adulthood. Offspring were assigned to one of four subsets (at least 10 animals of each sex per dose) for testing of 1) motor activity, 2) auditory startle, 3) passive avoidance, water maze and FOB and 4) histological and morphometric analysis of the brain at PND 21. At least 10 animals of each sex per dose were selected for ophthalmology at PNDs 50–60 and histology of neural and muscle tissue at about PND 75. In addition, brains of 10 animals of each sex per dose were weighed at about PND 75. All dams were examined daily for clinical signs and subjected to an FOB on GD 13 and GD 20. Ten dams per dose were tested in the FOB on LD 11 and LD 21. Body weight and feed consumption were measured weekly. At termination on LD 21 in 10 dams per dose, liver and thyroid were weighed and examined histologically.

Live pups were examined daily for clinical signs up to LD 21 and were counted and weighed on LDs 0, 4, 11, 17 and 21. After weaning, detailed examinations for clinical signs and body weight measurements were performed on a weekly basis. The age of onset of balano-preputial separation and vaginal patency was recorded. Subsets of offspring were subjected to the following tests: 1) FOB on PNDs 4, 11, 21, 35, 45 and 60, 2) motor activity on PNDs 13, 17, 21 and 60, 3) auditory startle on PNDs 22 and 60, 4) passive avoidance (PNDs 22 and 29) and water maze (PND 60). At termination on PND 21 or PND 75, the offspring were necropsied, and nervous and muscle tissues from subsets of animals were examined histologically. Statements of adherence to QA and GLP were included.

In dams, increased absolute and relative liver weights were observed at 1200 ppm (absolute 26%, relative 20%) and 12 000 ppm (absolute 34%, relative 28 %). No other treatment-related effects were observed. Enlarged eyeballs were observed at 1200 ppm (1/124 pups, 1/21 litters) and at 20 000 ppm (22/131 pups, 9/23 litters). Enlarged eyeball was first observed at PND 15 and also occurred after cessation of treatment (e.g. an enlarged eyeball was first evident in one animal as late as PND 48). Also, dose-dependent increased incidences of exophthalmos and general ocular opacities and decreases in preweaning body weight gain were observed in the offspring at 1200 and 12 000 ppm. In addition, a delayed balano-preputial separation was found in both groups. In addition, at 12 000 ppm, dark red eyes and dilated or slightly constricted pupils and a delayed vaginal patency were observed, and body weights remained decreased compared with controls throughout the study duration. No other treatment-related effects on offspring were found.

The NOAEL for maternal toxicity was 120 ppm, equal to 9.9 mg/kg bw per day, based on increases in absolute and relative liver weights at 1200 ppm, equal to 100 mg/kg bw per day.

The NOAEL for offspring toxicity was 120 ppm, equal to 9.9 mg/kg bw per day, based on effects on the eye, decreased preweaning body weight and delayed balano-preputial separation at 1200 ppm, equal to 100 mg/kg bw per day (Sheets, Gilmore & Hoss, 2006).

(b) Perinatal ocular toxicity studies

In the reproductive toxicity studies and developmental neurotoxicity study in rats, effects on the eye were observed. It was hypothesized that the effects on the developing eyes in rat studies may be a rat-specific phenomenon due to the fact that female rats are poor metabolizers of flubendiamide. Whereas microsomes from mice, dogs and humans (both sexes) and male rats efficiently caused

hydroxylation of flubendiamide to flubendiamide-benzyl alcohol as a first step of metabolism, female rats lack this capacity (see section 1). Therefore, the effects of flubendiamide on the development of the eye were further studied in mice.

In a pilot study, the effects of flubendiamide (purity 97.1%; lot No. 1FH0019M) on perinatal ocular development in mice were investigated. Pregnant CD-1 mice (n = 7) received flubendiamide in the diet from GD 6 to PND 21. At PND 4, litters were culled to eight pups. After weaning, pups were raised until PND 42. The initial dietary concentration during gestation of 4500 ppm, equal to 1101 mg/kg bw per day, was reduced on PND 3 to 2250 ppm, equal to 1395 mg/kg bw per day, in order to adjust for the increased feed intake during lactation. A group of seven pregnant mice served as a control group.

No treatment-related findings on appearance, behaviour, mortality, feed and water intake or excretions of the maternal animals were observed. The pups of the dose group showed no treatment-related findings on appearance, behaviour, mortality or body weight. Eye lesions were not noted (Langewische, 2005).

Following the pilot study, the effects of flubendiamide (purity 97.1%; lot No. 1FH0019M) on perinatal ocular development in mice were further investigated. Pregnant CD-1 mice (n = 28) received flubendiamide in the diet from GD 6 to PND 21. At PND 4, litters were culled to eight pups. After weaning, pups were raised until PND 42. The initial dietary concentration of 4500 ppm, equal to 931 mg/kg bw per day, was reduced on PND 3 to 2000 ppm, equal to 1133 mg/kg bw per day, in order to adjust for the increased feed intake during lactation. A group of 25 pregnant mice served as a control group. In the dams, clinical signs, body weight, feed consumption and excretory products were assessed daily during treatment. In addition, water intake (weekly), fertility index, gestation index and rearing index, duration of gestation, course of birth and lactation behaviour were recorded. In pups, appearance (with special focus on eye lesions), general behaviour and mortality were checked daily. Body weights were measured on PNDs 0, 4, 7, 14, 21 and 42. Statements of adherence to QA and GLP were included.

No effects of flubendiamide on appearance, behaviour, mortality, body weight, water intake or urinary and faecal excretion were found. Maternal feed consumption was slightly decreased at several time points. Fertility index was slightly reduced, but gestation and rearing indices were unaffected. Flubendiamide induced a slightly lower pup weight between PND 17 and PND 21, which was attributed to the decreased feed intake in the dams. No other treatment-related effects, including effects on eye development, were noted in the pups. As no macroscopic lesions of the eye were noted, histological examination of the eyes was not performed (Langewische, 2006).

(c) In vitro studies

Flubendiamide, an iodobenzene derivative, induced follicular cell hypertrophy and an increase in thyroid weight in a number of rat studies at high doses. As certain iodobenzene derivatives have been reported to inhibit iodothyronine deiodinase type I (ID-I), the interaction of flubendiamide with iodothyronine deiodinase, derived from rat liver, was investigated in vitro in a study from the public literature. Enzymatic release of radiolabelled iodide from the phenolic ring of the radiolabelled 3,3′,5′-L-triiodothyronine was taken as a measure of ID-I enzymatic activity.

Flubendiamide did not affect ID-I activity at concentrations up to 100 µmol/l, the maximally testable concentration (Freyberger, 2003).

(d) Mechanistic studies

A study was performed to investigate the effect of flubendiamide on thyroid-related hormones and metabolizing enzymes in the liver. Groups of female Fischer (F344/DuCrj) rats (10 per dose)

were treated with flubendiamide (purity 96.7%; lot No. 1FH0019M) at a dietary concentration of 0, 1000 or 10 000 ppm, equal to 0, 83 and 812 mg/kg bw per day, for 7 or 28 days. Clinical signs were recorded daily, and body weight and feed consumption were calculated on a weekly basis. In one group, blood was sampled on days −1, 3 and 14 and at termination on day 28; in the second group, blood was sampled on days −1 and 1 and at termination on day 7. At termination, the animals were necropsied, and liver, thyroid and pituitary were weighed. Serum levels of total serum thyroxine (T_4), circulating total triiodothyronine (T_3) and thyroid stimulating hormone (TSH) were analysed. On days 7 and 28, uridine diphosphate-glucuronosyltransferase (UDPGT) activity, 7-ethoxyresorufin *O*-deethylase (EROD) activity (CYP1A2), 7-pentoxyresorufin *O*-dealkylase (PROD) activity (CYP2B) and CYP content were measured in liver. Tissue samples of liver, thyroid and pituitary were investigated histopathologically in all animals. Statements of adherence to QA and GLP were included.

No toxicologically relevant effects on clinical signs, body weight, feed consumption or macroscopy were seen. At days 7 and 28, absolute (21–62%) and relative liver weights (18–54%) were dose- and time-dependently increased at 1000 and 10 000 ppm. At day 28, increases in absolute (33%) and relative thyroid weights (27%) were found at 10 000 ppm. Activities of UDPGT (31–97%), EROD (30–53%) and CYP (36–57%) in liver were increased at days 7 and 28 in both dose groups, although no clear dose dependency was observed. Changes in PROD levels in treated groups compared with control values ranged from −12% to +17%, without dose dependency. Microsomal protein level was reduced (11%) at day 7 in the high-dose group. On days 14 and 28, TSH concentrations were significantly increased at 1000 ppm (by 19% and 36%, respectively) and 10 000 ppm (by 30% and 64%, respectively). Also at these doses, slight, but statistically significant, T_3 increases (14–28%) were noted on days 7, 14 and 28. Small increases (8–34%) in T_4 levels were found at all time points at both doses. Histological examination showed hepatocyte hypertrophy and periportal vacuolation of hepatocytes and follicular cell hypertrophy in the thyroid at both doses. The incidences and severity of these observations increased with dose and treatment duration. At day 7, increased mitotic figures of hepatocytes were observed at both doses.

The study indicates that flubendiamide induces UDPGT and EROD activities and an increase in CYP content. Flubendiamide also increases serum TSH levels and thyroid weight and induces thyroid follicular cell hypertrophy (Amanuma, 2005).

(e) Immunotoxicity studies

In a 28-day immunotoxicity feeding study, performed in accordance with OECD guideline 407, flubendiamide (purity 97.2%; lot No. 1FH0019M) was administered to Wistar (HsdCpb:WU) rats (10 of each sex per dose) at 0, 40, 400 or 4000 ppm, equal to 0, 3.3, 34 and 336 mg/kg bw per day for males and 0, 4.0, 39 and 359 mg/kg bw per day for females. Animals were checked daily for mortality and morbidity and weekly for clinical signs. Body weight and feed consumption were recorded weekly. On days 21/22, blood was sampled for haematology and clinical chemistry. On day 23, animals were injected intravenously with 0.2 ml of 1×10^9 sheep red blood cells (SRBC) per millilitre. On day 28, SRBC-specific immunoglobulin M (IgM) levels in blood were measured. At day 28, each animal was examined grossly, and the adrenals, thymus, liver, spleen and thyroid were weighed. At necropsy, spleens were removed and examined for plaque-forming cell response, and the cell counts in the spleen were determined, subpopulations of the spleen cells were analysed by FACScan and the antibody (IgG, IgM and IgA) titres in the sera were determined. Statements of adherence to QA and GLP were included.

No treatment-related effects on clinical signs, body weight, gross pathology, organ weight or SRBC-specific antibody (IgM) response were observed. In females at 400 and 4000 ppm, a slight reduction (8–14%) in feed consumption was observed. Decreased erythrocyte counts (6%) were detected at 4000 ppm in females. The haemoglobin concentrations and the haematocrit values were slightly decreased (maximally 7%) at 400 ppm and above in females. The finding of a slightly shortened prothrombin time in all treatment groups in males was considered incidental, as they were within

the historical control range, no effects were found in females and no such effects were found in the subchronic rat study (Enomoto, 2003). Decreases in ALT and AST activities at doses of 400 ppm and higher were noted in both sexes. However, reductions in levels of these enzymes are generally not considered to be adverse. Necropsy showed an increased incidence of pale kidney in high-dose males. Absolute and relative liver weights were increased in mid-dose (18% and 23%, respectively) and high-dose females (50% and 46%, respectively). Slight increases (5–8%) in absolute and relative liver weights were observed in high-dose males. No substance-induced effects were observed on splenic cell counts or on the size distribution of spleen cells. A statistically significant decrease (32%) in the antibody titre of IgA at 4000 ppm was observed in females. No other toxicologically relevant effects on the antibody titre of IgA, IgM or IgG or on plaque-forming cells were induced by flubendiamide. A decrease in CD45 total (13% in males, 9% in females) and CD45 high positive cells (26% in males, 29% in females) and consequently an increase in CD45 low positive spleen cells (33% in males, 24% in females) were observed at 4000 ppm. Other observed changes were within the historical control range and were considered not toxicologically relevant. The changes in IgA and CD45 lymphocytes are interpreted as secondary changes due to the liver toxicity observed at 400 ppm and above.

The NOAEL for immunotoxicity was 400 ppm, equal to 34 mg/kg bw per day, based on a decrease in CD45 lymphocytes in both sexes and a decrease in IgA antibody titres in females at 4000 ppm, equal to 336 mg/kg bw per day, which are considered secondary changes due to liver toxicity.

The NOAEL for other effects was 40 ppm, equal to 4 mg/kg bw per day, based on decreases in food intake, haemoglobin and haematocrit and increases in liver weights at 400 ppm, equal to 34 mg/kg bw per day (Kroetlinger & Vohr, 2005).

(f) Studies with metabolites

Acute toxicity and genotoxicity studies with the metabolites flubendiamide-des-iodo and flubendiamide-3-OH were available.

(i) Acute toxicity

The results of studies of acute toxicity with metabolites of flubendiamide are summarized in Table 8.

(ii) Genotoxicity

The results of genotoxicity studies with metabolites of flubendiamide are summarized in Table 9.

3. Observations in humans

Occupational medical surveillance of workers exposed to flubendiamide has not revealed any adverse effects (Steffens, 2009).

Comments

Biochemical aspects

After administration of a single oral dose (2 mg/kg bw) of [^{14}C]flubendiamide to rats, about 23–34% of the radiolabel was absorbed. Peak plasma concentrations were reached within 6–12 hours. Plasma half-lives were 12.6 and 37.6 hours in males and females, respectively. The radiolabel was widely distributed, with highest concentrations in liver, adrenals, fat and kidneys. Male rats showed slightly higher peak organ and tissue levels than females, but also had a more rapid clearance of residues. After a single high oral dose of 200 mg/kg bw, plasma and tissue levels in rats were only slightly higher than after 2 mg/kg bw, indicating saturated absorption. Repeated oral dosing with 2 mg/kg

Table 8. Results of studies of acute toxicity with flubendiamide metabolites

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference[a]
Rat	Sprague-Dawley (Slc:SD)	Female	Oral	Flubendiamide-des-iodo[b]	100	> 2000	Horiuchi (2004d)[c]
Rat	Sprague-Dawley (Slc:SD)	Female	Oral	Flubendiamide-3-OH[d]	97.5	> 2000	Horiuchi (2004e)[c]

[a] Statements of adherence to GLP and QA were included in both studies.
[b] Lot No. 1FH0103S. No substance-related clinical signs were observed.
[c] Performed in accordance with OECD guideline 423.
[d] Lot No. 3FH0203S. Soiled fur was observed in one animal from 30 min to 3 h after administration and also in four animals at 6 h. Loose stool was observed in one animal at 30 min and in six animals at 6 h.

Table 9. Results of studies on the genotoxicity of metabolites of flubendiamide

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference[a]
In vitro						
Flubendiamide-des-iodo[b]	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA	1.7–1250 µg/plate (−S9) 6.9–5000 µg/plate (+S9)	100	Negative	Inagaki (2004a)
Flubendiamide-3-OH[c]	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA	*S. typhimurium.* strains: 3.9–313 µg/plate (±S9) *E. coli*: 6.9–5000 µg/plate (±S9)	97.5	Negative	Inagaki (2004b)

[a] Positive and negative (solvent) controls were included in all studies. In all studies, statements of adherence to GLP and QA were included.
[b] Performed in accordance with OECD guideline 471. Lot No. 1FH0103S. Precipitation at ≥ 139 µg/plate (−S9) and ≥ 556 µg/plate (+S9). At 1250 (−S9) and 5000 (+S9) µg/plate, precipitation made judgement of cytotoxicity impossible.
[c] Performed in accordance with OECD guideline 471. Lot No. 3FH0203S. Slight cytotoxicity at 313 µg/plate (±S9) with *Salmonella* strains. Precipitation at ≥ 556 (−S9) and ≥ 1670 µg/plate (+S9).

bw for 14 days did not affect metabolism and excretion in rats; whereas residue levels in males were similar to those observed after a single dose of 2 mg/kg bw, residue levels in females were considerably higher, indicating a considerable potential for accumulation in female rats. Excretion occurred predominantly through bile and faeces. Urinary excretion was 1.7% and 0.4% of the 2 mg/kg bw dose in males and females, respectively.

In mice, male rats (but not female rats), dogs and humans, flubendiamide can be readily metabolized by oxidation of the methyl groups linked to the aniline ring and at the alkyl bridge between the amide and sulfonyl functions, resulting in the corresponding alcohol, aldehyde and benzoic acid derivatives, followed by formation of glucuronide conjugates of hydroxylated metabolites. As female rats have very limited capability to oxidize these methyl groups, in these animals, flubendiamide is metabolized by direct conjugation with glutathione, leading to further amino acid conjugates with cysteine and glycine. This metabolic pathway is less efficient than the oxidation route, leading to a less rapid excretion of flubendiamide in female rats. Small but significant amounts of flubendiamide-iodophthalimide were detected in the fat extract of both male and female rats, implying the hydrolysis of the amide bond between the phthalic acid ring and the thioalkylamine moiety.

The observed sex difference in clearance and metabolism in rats (both are slower in females) was further investigated. Liver microsomes from mice (both sexes), male rats, dogs (both sexes) and humans (both sexes) efficiently caused hydroxylation of flubendiamide to flubendiamide-benzyl alcohol as a first step of metabolism. Female rat microsomes, however, have very limited capability to oxidize the methyl moieties.

Toxicological data

The acute toxicity of flubendiamide is low in rats (oral and dermal LD_{50} > 2000 mg/kg bw; inhalation LC_{50} > 0.0685 mg/l, maximum achievable concentration). Flubendiamide is not irritating to the skin and eyes of rabbits and is not a skin sensitizer (Magnusson & Kligman test in guinea-pigs).

In repeated-dose studies with rodents, the most sensitive target was the liver, followed by the thyroid and the red blood cell system (rats only). In general, female rats were more sensitive than males to the effects of flubendiamide. This is likely due to the fact that female rats are poor metabolizers of flubendiamide.

A 28-day mechanistic study in female rats showed that flubendiamide induces liver enzyme activities (UDPGT and EROD) and increases in CYP content in liver and serum TSH levels. The mechanistic study was not adequate to elucidate the mode of action of thyroid activation in rodents.

The repeated-dose toxicity of flubendiamide was investigated in mice (28-day and 13-week studies), rats (28-day, 3-month and 1-year studies) and dogs (3-month and 1-year studies). The overall NOAEL from the 28-day and 13-week studies in mice was 200 ppm (equal to 26.9 mg/kg bw per day), based on dark-coloured liver, fatty changes in centrilobular hepatocytes and hepatocyte hypertrophy at 1000 ppm (equal to 123 mg/kg bw per day).

In the 28-day, 3-month and 1-year oral feeding studies with flubendiamide in rats, the lowest NOAEL was 50 ppm (equal to 2.0 mg/kg bw per day), based on liver effects (increased liver weights, dark-coloured and enlarged livers, periportal fatty changes, hepatocyte hypertrophy and foci of cellular alterations [basophilic cell type], clinical chemistry changes), haematological effects (decreased haematocrit, haemoglobin, erythrocyte count, MCV and MCH, indicative of microcytic anaemia and possibly reactive haematopoiesis) and thyroid effects (follicular cell hypertrophy) observed at 2000 ppm (equal to 79 mg/kg bw per day), observed in the 1-year study. Similar effects had been observed in the 28-day and 3-month studies in rats at 200 ppm (equal to 13–15 mg/kg bw per day). After cessation of treatment in the 3-month rat study, liver, thyroid and red blood cell effects were partially or fully reversible after a 4-week recovery period.

In repeated-dose studies in dogs, the most sensitive targets were the liver, blood and adrenals. In a 3-month and a 1-year study in dogs, the NOAEL was 100 ppm, equal to 2.6 and 2.2 mg/kg bw per day, respectively, based on increased alkaline phosphatase levels and shortened activated prothrombin time (both studies), increased adrenal weights and adrenal cortical hypertrophy (3-month study) and increased liver weights (1-year study) observed at 2000 and 1500 ppm, equal to 53 and 35 mg/kg bw per day, respectively.

In an 18-month feeding study in mice, the NOAEL was 50 ppm (equal to 4.4 mg/kg bw per day), based on increased liver weight, centrilobular hypertrophy, centrilobular microvesicular fatty change, enlarged thyroid, increased thyroid weights, increased incidence of thyroid follicular cell hypertrophy and hydropic change and increased large size follicles in both sexes, diffuse microvesicular and macrovesicular fatty change in the liver of females and discoloration of the liver in males observed at 1000 ppm (equal to 93 mg/kg bw per day). No effect of flubendiamide on tumour incidence was found.

In a 2-year feeding study in rats, the NOAEL was 50 ppm (equal to 1.7 mg/kg bw per day), based on increased liver weights and periportal fatty change (both sexes), increased incidence of hair loss, dark-coloured and enlarged livers, hepatocyte hypertrophy, increased kidney weight and increased incidence of thyroidal follicular cell hypertrophy in females and decreased eosinophil count in males observed at 1000 ppm (equal to 34 mg/kg bw per day). No treatment-related effect on tumour incidence was found.

The Meeting concluded that flubendiamide is not carcinogenic in rodents.

Flubendiamide was tested for genotoxicity in an adequate range of in vitro and in vivo studies. No evidence for genotoxicity was observed in any test.

The Meeting concluded that flubendiamide is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that flubendiamide is unlikely to pose a carcinogenic risk to humans.

One-generation and two-generation studies of reproductive toxicity in rats were available. The overall NOAEL for parental toxicity was 50 ppm (equal to 3.9 mg/kg bw per day), based on dark-coloured livers in parental females and increased liver weights in F_1 females observed in a one-generation study of reproductive toxicity at 200 ppm (equal to 15 mg/kg bw per day). No reproductive toxicity was seen at 2000 ppm (equal to 162 mg/kg bw per day), the highest relevant dose tested. The overall NOAEL for offspring toxicity (combined data from the one- and two-generation studies of reproductive toxicity) was 200 ppm, equal to 15 mg/kg bw per day (i.e. the maternal compound intake), based on dark-coloured livers and increased liver weight, decreased spleen and thymus weights, delayed balano-preputial separation, enlargement of eyeballs, synechia, haemorrhage, keratitis, iritis, cataract, hydropic degeneration of basal layer of the corneal epithelium and/or corneal epithelial vacuolation at 2000 ppm (equal to 131–149 mg/kg bw per day).

No effects on the eye were observed in special perinatal ocular toxicity studies in female mice in which the animals received flubendiamide at doses up to and including 1395 mg/kg bw per day.

In a developmental toxicity study in rats, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on the small increases in absolute and relative liver weights at 1000 mg/kg bw per day. The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rabbits, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased incidences of loose stools, reduced feed consumption on GDs 27–28 and a tendency to a reduced body weight gain during the latter part of gestation. The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested.

No evidence for a teratogenic effect of flubendiamide was observed in rats or rabbits.

The Meeting concluded that it could not exclude the possibility that flubendiamide induces eye anomalies due to exposure during gestation or early postnatal life or the possibility that the effects on the developing eye are the result of a single exposure to flubendiamide.

In an oral (gavage) study of acute neurotoxicity in rats in which flubendiamide was given by gavage, the NOAEL was 2213 mg/kg bw, the highest dose tested.

In a dietary developmental neurotoxicity study in rats, no neurotoxic effects were observed at doses up to 12 000 ppm (equal to 980 mg/kg bw per day), the highest dose tested. The NOAEL for maternal toxicity was 120 ppm (equal to 9.9 mg/kg bw per day), based on increases in absolute and relative liver weights at 1200 ppm (equal to 100 mg/kg bw per day). The NOAEL for offspring toxicity was 120 ppm (equal to 9.9 mg/kg bw per day), based on effects on the eye (increased incidences of enlarged eyeballs and general ocular opacities), decreased preweaning body weight and delayed balano-preputial separation at 1200 ppm (equal to 100 mg/kg bw per day).

It is noted that in the developmental neurotoxicity study and in the studies of reproductive toxicity, effects on the eye were observed, whereas in developmental toxicity studies in rats and rabbits, no effects on the eye were found. This suggests that the effects on the development of the eyes occur after birth, although it cannot be excluded that the initial lesion occurs during gestation.

In a 28-day dietary immunotoxicity study in rats, the NOAEL for immunotoxicity was 400 ppm (equal to 34 mg/kg bw per day), based on a decrease in CD45 lymphocytes in both sexes and a decrease in IgA antibody titres in females at 4000 ppm (equal to 336 mg/kg bw per day). These effects are considered secondary changes due to liver toxicity. The NOAEL was 40 ppm (equal to 4 mg/kg bw per day), based on decreases in feed intake, haemoglobin and haematocrit and increases in liver weights at 400 ppm (equal to 34 mg/kg bw per day).

Occupational medical surveillance of workers exposed to flubendiamide has not revealed any adverse effects.

In studies of acute oral toxicity, the flubendiamide metabolites flubendiamide-des-iodo and flubendiamide-3-OH had LD_{50}s of greater than 2000 mg/kg bw. These metabolites gave negative results in a test for reverse mutation in bacteria.

The Meeting concluded that the existing database on flubendiamide is sufficient to characterize the potential hazards to fetuses, infants and children.

The Meeting noted that new studies are being performed to better characterize the risk to humans of the effects of flubendiamide on the developing eye observed in rats.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for flubendiamide of 0–0.02 mg/kg bw on the basis of a NOAEL of 50 ppm (equal to 1.7 mg/kg bw per day), based on effects on the liver (both sexes), kidney, thyroid and hair loss (females) and decreased eosinophil count (males) observed in a 2-year feeding study in rats, and on the basis of a NOAEL of 100 ppm (equal to 2.2 mg/kg bw per day), based on increased alkaline phosphatase levels, shortened activated partial thromboplastin time and increased liver weights observed in a 1-year study in dogs. A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) of 0.2 mg/kg bw, based on an overall NOAEL of 15 mg/kg bw per day for effects on the developing eye observed in one- and two-generation reproductive toxicity studies and a developmental neurotoxicity study in rats. A safety factor of 100 was applied.

Although the eye effects became apparent after birth, it is not clear whether the initial lesion occurs during gestation or postnatally. It cannot be excluded that the effects on eye development are the result of a single prenatal or postnatal exposure to flubendiamide.

Levels relevant for risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 4.4 mg/kg bw per day	1000 ppm, equal to 93 mg/kg bw per day
		Carcinogenicity	10 000 ppm, equal to 937 mg/kg bw per day[b]	—
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 1.7 mg/kg bw per day	1000 ppm, equal to 34 mg/kg bw per day
		Carcinogenicity	20 000 ppm, equal to 705 mg/kg bw per day[b]	—
	One- and two-generation studies of reproductive toxicity[a]	Parental toxicity	50 ppm, equal to 3.9 mg/kg bw per day[c]	200 ppm, equal to 15 mg/kg bw per day[c]
		Offspring toxicity	200 ppm, equal to 15 mg/kg bw per day[c]	2000 ppm, equal to 131 mg/kg bw per day[c]
		Reproductive toxicity	2000 ppm, equal to 162 mg/kg bw per day	20 000 ppm, equal to 1636 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	100 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	1000 mg/kg bw per day[b]	—
	Acute neurotoxicity study[d]	Neurotoxicity	2213 mg/kg bw per day[b]	—
	Developmental neurotoxicity study[a]	Maternal toxicity	120 ppm, equal to 9.9 mg/kg bw per day	1200 ppm, equal to 100 mg/kg bw per day
		Offspring toxicity	120 ppm, equal to 9.9 mg/kg bw per day	1200 ppm, equal to 100 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[d]	Maternal toxicity	100 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	1000 mg/kg bw per day[b]	—
Dog	One-year study of toxicity[a]	Toxicity	100 ppm, equal to 2.2 mg/kg bw per day	1500 ppm, equal to 35 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Two or more studies combined.
[d] Gavage administration.

Estimate of acceptable daily intake for humans

0–0.02 mg/kg bw

Estimate of acute reference dose

0.2 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Data from ongoing studies on the effect of flubendiamide on the developing eye, and results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to flubendiamide

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of absorption	Relatively slow, incomplete oral absorption (23–34% at 2 mg/kg bw)
Distribution	Extensive (rats)
Potential for accumulation	At 2 mg/kg bw, low in both sexes; at 200 mg/kg bw, low in males and moderate in females (rats)
Rate and extent of excretion	Plasma half-lives: males, 12.6 h; females, 37.6 h At 2 mg/kg bw: 1.7% and 0.4% in urine of males and females, respectively (rats)
Metabolism in animals	Extensive, by oxidation of the methyl groups linked to the aniline ring and at the alkyl bridge between amide and sulfonyl functions in mice, male rats, dogs and humans. As female rats have very limited capability to oxidize these methyl groups, they metabolize flubendiamide by direct conjugation of flubendiamide with glutathione.
Toxicologically significant compounds	Flubendiamide

Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 0.0685 mg/l (highest achievable concentration)
Rabbit, dermal irritation	Not an irritant
Rabbit, ocular irritation	Not an irritant
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman test)

Short-term studies of toxicity	
Target/critical effect	Liver (rat, dog), thyroid (rat), red blood cell system (rat), adrenals (dog)
Lowest relevant oral NOAEL	2 mg/kg bw per day (rat), 2.2 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	100 mg/kg bw per day (rat)
Lowest relevant inhalatory NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver, thyroid, kidney, skin (rat)
Lowest relevant NOAEL	1.7 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic (mouse, rat)
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	None
Lowest relevant reproductive NOAEL	162 mg/kg bw per day, highest relevant dose tested (rat)
Developmental target	Eye effects in reproductive toxicity studies and developmental neurotoxicity studies (rat)
Lowest relevant developmental NOAEL	15 mg/kg bw per day (rat)
Neurotoxicity/delayed neurotoxicity	
Neurotoxicity	No neurotoxic effects
Developmental neurotoxicity	No neurotoxic effects
Other toxicological studies	
Immunotoxicity	No immunotoxic effects
Medical data	
	Occupational medical surveillance of workers exposed to flubendiamide has not revealed any adverse effects

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year study in rat, one-year study in dog	100
ARfD	0.2 mg/kg bw	One- and two-generation studies of reproductive toxicity, developmental neurotoxicity study in rat	100

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FLUOPYRAM

First draft prepared by
Rudolf Pfeil[1] and Alan Boobis[2]

[1] Toxicology of Pesticides and Biocides, Federal Institute for Risk Assessment, Berlin, Germany
[2] Experimental Medicine & Toxicology, Division of Investigative Science, Faculty of Medicine, Imperial College London, London, England

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Explanation

Fluopyram is the International Organization for Standardization (ISO)–approved common name for *N*-(2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl)-2-(trifluoromethyl)benzamide (International Union of Pure and Applied Chemistry [IUPAC]) (Chemical Abstracts Service [CAS] No. 658066-35-4), a novel broad-spectrum fungicide from the pyridinyl-ethyl-benzamide class.

Fluopyram acts by inhibiting the enzyme succinate dehydrogenase (SDH, so-called complex II in the mitochondrial respiratory chain), which is a functional part of the tricarboxylic acid cycle, linked to mitochondrial electron transport. SDH consists of four subunits (A, B, C and D), and fluopyram, like a number of SDH inhibitors, acts by blocking the enzyme binding site for ubiquinone, which is formed by the subunits B, C and D. Fluopyram has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All pivotal studies were certified as complying with good laboratory practice (GLP) or an approved quality assurance programme.

Evaluation for acceptable daily intake

Unless otherwise stated, the studies evaluated in this monograph were performed by laboratories that were certified for GLP and complied with the relevant Organisation for Economic Co-operation and Development (OECD) test guidelines or similar guidelines of the European Union or United States Environmental Protection Agency. As these guidelines specify the clinical pathology tests normally performed and the tissues normally examined, only significant exceptions to these guidelines are reported here, to avoid repetitive listing of study parameters.

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution, metabolism and elimination (ADME) of [phenyl-UL-^{14}C]-fluopyram (chemical purity of non-radiolabelled substance 94.7%; chemical purity of radiolabelled substance > 99%; radiochemical purity > 98%) were investigated in male and female Wistar HsdCpb:WU rats (Klempner, 2008a). The study design is summarized in Table 1.

The rats received [phenyl-UL-^{14}C]fluopyram by oral gavage as a suspension in 0.5% aqueous tragacanth; the dosing volume was 1 ml (group 1) or 2 ml (groups 2–6). The rats were sacrificed 7 days after dosing (groups 2–6) or 2 days after dosing in the bile fistulation experiment (group 1). The body weights of the rats of groups 2–6 were determined at administration and sacrifice and of group 1 at sacrifice, because they were operated on for the bile fistulation experiment. Two animals of group 1 died during the second night after administration, and one other animal of group 1 had no bile flow and was sacrificed prior to the end of the test. These three animals were excluded from the test and from the evaluation. The total radioactivity, which included the test item and metabolites, was determined in plasma samples, bile, urine and faeces, as well as in organs and tissues at sacrifice. The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting. Small organs or tissues (adrenal glands, thyroid, ovaries, perirenal fat and uterus) were solubilized by means of a tissue solubilizer, and radioactivity was measured by liquid scintillation counting. All other solid samples were combusted in an oxygen atmosphere and radioassayed by liquid scintillation counting. The distribution of the test substance from the central compartment to the different organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. The toxicokinetic parameters were calculated with the software TOPFIT version 2.0 and by using a three-compartment disposition model. The metabolism was investigated by radio–high-performance liquid chromatography (HPLC) and spectroscopic methods in selected bile and urine samples and faecal extracts.

The absorption of [phenyl-UL-^{14}C]fluopyram from the gastrointestinal tract of male and female rats was rapid in all test groups. The absorption commenced immediately after oral dosing, as

Table 1. Study design for ADME study with [phenyl-UL-^{14}C]fluopyram in rats

Group	No./sex	Dose regimen	Sample collection schedule
1 (BF)	5 males	5 mg/kg bw, single oral low dose, bile fistulation	Bile, urine and faeces at various times after administration
			Blood, gastrointestinal tract, skin and carcass: at sacrifice, 48 h after administration
2 (LD)	4 males	5 mg/kg bw, single oral low dose	Plasma, urine and faeces at various times after administration
3 (LD)	4 females	5 mg/kg bw, single oral low dose	
4 (HD)	4 males	250 mg/kg bw, single oral high dose	Blood, organs and tissues, gastrointestinal tract, skin and carcass: at sacrifice, 168 h after administration
5 (HD)	4 females	250 mg/kg bw, single oral high dose	
6 (PT)	4 males	5 mg/kg bw, single oral low dose, 14-day pretreatment	

From Klempner (2008a)
BF, bile fistulation; bw, body weight; HD, high dose; LD, low dose; PT, 14-day pretreatment

shown by the plasma curves and the values calculated for the absorption half-lives (0.1–0.5 hour). The absorption rate of [phenyl-UL-^{14}C]fluopyram was calculated from bile, urine and body excluding gastrointestinal tract and accounted for approximately 93% of the total recovered radioactivity for male rats (group 1), leading to the conclusion that the administered dose was absorbed completely and systemically bioavailable (Table 2).

The maximum plasma concentration of radioactivity (C_{max}) after a single oral low dose (5 mg/kg body weight [bw]) was reached approximately 24 and 8 hours after dosing (T_{max}) in male and female rats, respectively; after a single high dose (250 mg/kg bw), the maximum plasma concentration of radioactivity was reached at approximately 48 hours after dosing in males and females, respectively. In male rats after the repeated low dose (5 mg/kg bw), the maximum plasma concentration of radioactivity was reached significantly more rapidly, approximately 0.7 hour after dosing (Table 3).

The maximum equivalent concentrations (C_{max}) were in a similar range for male and female rats of the low-dose groups as well as for pretreated low-dose male rats (1.5–2.2 µg/g). The maximum equivalent concentrations (C_{max}) of the high-dose tests were considerably higher but also comparable for male and female rats (60–63 µg/g).

The plasma curves showed fast absorption in all low-dose groups with a moderate decline to approximately 5–8% of the maximum concentration from T_{max} to 168 hours. The plasma curves of the high-dose groups showed slightly slower absorption followed by a long and broad maximum with a slower decline. The maximum concentration from T_{max} to 168 hours in the high-dose groups declined to 11% in males and to 32% in females. The plasma curves of the high-dose groups showed slightly slower absorption followed by a long and broad maximum with a slower decline.

The areas under the curve ($AUC_{0-\infty}$) indicated a slightly higher systemic exposure for females (148 µg·h/g) than for males (107 µg·h/g) of the low-dose groups. The difference in the AUC values was also observed in the high-dose groups, amounting to 7060 µg·h/g for females and 5680 µg·h/g for males. The AUCs were proportional to the dose. The mean residence time (MRT_{tot}) of fluopyram-related radioactivity was moderate for both sexes, ranging from 52 to 84 hours.

At termination, 168 hours post-administration, males and females had excreted 90.6–99.3% of the administered dose via urine and faeces (Table 2). Excretion of the total radioactivity was slightly faster in the low-dose than in the high-dose groups, but still ongoing 168 hours post-administration. The radioactivity in the body ranged from 2% to 6% of the administered dose. The ratio of the renal and faecal excretion in females of the low-dose group was comparable (45% or 47% via urine or faeces, respectively), whereas in all other groups, slightly more radioactivity was excreted via faeces (53–64%) than via urine.

Table 2. Recovery of radioactivity in rats after administration of [phenyl-UL-^{14}C]fluopyram

	Recovery of radioactivity (% of administered dose)					
	1 (BF/M)	2 (LD/M)	3 (LD/F)	4 (HD/M)	5 (HD/F)	6 (PT/M)
	Dose (mg/kg bw)					
	5	5	5	250	250	5
Urine	7.29	38.25	45.27	35.70	35.53	35.14
Bile	78.54	n.a.	n.a.	n.a.	n.a.	n.a.
Faeces	3.70	53.06	46.58	63.60	57.12	55.45
Total excreted	89.53	91.31	91.85	99.30	92.65	90.59
Skin	1.34	0.47	0.76	0.55	0.68	0.30
Sum organs/tissues	6.38	2.85	4.73	2.00	2.71	1.91
Body without gastrointestinal tract	7.72	3.32	5.50	2.54	3.39	2.20
Gastrointestinal tract	3.17	0.24	0.42	0.32	0.32	0.18
Total in body	10.88	3.56	5.91	2.86	3.72	2.38
Balance	100.4	94.9	97.8	102.2	96.4	93.0

From Klempner (2008a)
BF, bile fistulation; bw, body weight; F, female; HD, high dose; LD, low dose; M, male; n.a., not analysed; PT, 14-day pretreatment

Table 3. Distribution and plasma kinetics in rats after administration of [phenyl-UL-^{14}C]-fluopyram

	2 (LD/M)	3 (LD/F)	4 (HD/M)	5 (HD/F)	6 (PT/M)
	Dose (mg/kg bw)				
	5	5	250	250	5
T_{max} measured (h)	24.0	8.0	48.0	48.0	0.7
T_{max} calculated (h)	15.0	11.2	34.5	41.9	0.8
C_{max} measured (µg/g)	1.45	2.11	59.94	63.09	1.44
C_{max} calculated (µg/g)	1.54	2.16	60.9	62.2	1.54
$t_{½}$ abs (h)	0.1	0.4	0.5	0.5	0.5
$t_{½}$ elim 1 (h)	3.9	16.2	4.8	4.8	4.6
$t_{½}$ elim 2 (h)	30.9	53.0	23.6	29.0	36.8
$AUC_{0-\infty}$ (µg·h/g)	107	148	5680	7060	80
MRT_{tot} (h)	51.8	65.2	68.8	83.6	53.3
MRT_{abs} (h)	11.7	8.6	34.8	41.8	0.3
MRT_{disp} (h)	40.0	56.7	34.0	41.9	53.1

From Klempner (2008a)
$AUC_{0-\infty}$, area under the plasma radioactivity concentration–time curve; F, female; HD, high dose; LD, low dose; M, male; MRT_{abs}, mean residence time of the absorption; MRT_{disp}, mean residence time of the disposition; MRT_{tot}, mean residence time of the total radioactivity; PT, 14-day pretreatment; $t_{½}$ abs, absorption half-life; $t_{½}$ elim, elimination half-life

Bile duct–cannulated male rats showed a total excretion of 89.5% of the administered dose. The main radioactivity, 78.5% of the administered dose, was eliminated via the bile, and a further 7.3% was eliminated via urine. Sixty-one per cent of the administered dose was excreted via the bile within the first 24 hours post-administration.

The radioactive residues in the organs and tissues determined at sacrifice showed that the radioactivity in the gastrointestinal tract amounted to 0.2–0.4% of the administered dose. The radioactivity in skin was slightly higher and amounted to 0.3–0.8% of the administered dose. The highest amounts were detected in the liver (0.4–1.0% of the administered dose). The highest residue concentrations (Table 4) were detected in liver (0.6–1.2 µg/g in low-dose groups and 16–21 µg/g in high-dose groups) and in kidney (0.5–1.1 µg/g in low-dose groups and about 16 µg/g in high-dose groups). Higher residues were also detected in carcass (0.2–0.3 µg/g in low-dose groups and 5.4–7.8 µg/g in high-dose groups) and in erythrocytes (about 0.2 µg/g in low-dose groups and about 10 µg/g in high-dose groups), ovaries (0.7 µg/g in low-dose group and 11.2 µg/g in high-dose group) and the adrenal glands (0.1–0.9 µg/g in low-dose groups and 10–13 µg/g in high-dose groups). The concentrations in the other organs and tissues were lower (< 0.33 µg/g in low-dose groups and < 10 µg/g in high-dose groups).

The absorption, distribution, metabolism and elimination of [pyridyl-2,6-^{14}C]fluopyram (chemical purity of non-radiolabelled substance > 99%; chemical purity of radiolabelled substance > 99%; radiochemical purity > 99%) were investigated in male and female Wistar HsdCpb:WU rats (Klempner, 2008b). The study design is summarized in Table 5. The methods employed were essentially comparable to the methods described for the study of Klempner (2008a).

The absorption of [pyridyl-2,6-^{14}C]fluopyram from the gastrointestinal tract of male and female rats was rapid in all test groups. The absorption commenced immediately after oral dosing as shown by the plasma curves and the values calculated for the absorption half-lives (0.3–0.4 hour). The absorption rate of [pyridyl-2,6-^{14}C]fluopyram was calculated from bile, urine and body excluding gastrointestinal tract and accounted for 97.7% of the total recovered radioactivity for male rats from test group 1, leading to the conclusion that the administered dose was absorbed virtually completely and became systemically bioavailable (Table 6).

The maximum plasma concentration of radioactivity (C_{max}) was reached 0.7 hour after dosing (T_{max}) in male rats and 3.0 hours after dosing in female rats (Table 7). The maximum equivalent concentrations (C_{max}) amounted to 1.8 and 1.5 µg/g for males and females, respectively. The plasma concentration in both sexes declined to about 1% of the maximum concentration within 120 hours post-administration, indicating that no retention of the compound-related residues in the body of the animals took place. The plasma curves of both sexes showed a very fast elimination phase at the beginning of the test, followed by a slower terminal elimination phase. The level of the plasma curve was slightly higher for females than for males. The plasma curves in both sexes revealed oscillation in the declining part of the curve during the elimination phase, indicating enterohepatic circulation between small intestine and liver. The values for the initial elimination phase were about 11 hours for males and 10 hours for females, followed by a slower terminal elimination phase of about 56 and 73 hours for males and females, respectively. The area under the curve ($AUC_{0-\infty}$) indicated a slightly higher systemic exposure for females (37 µg·h/g) than for males (22 µg·h/g). The mean residence time (MRT_{tot}) of fluopyram-related radioactivity was short for both sexes, ranging from about 29 to 33 hours.

Excretion was almost completed 72 hours after administration. At this time, both sexes had excreted more than 98% of the administered dose via urine and faeces. Sex differences were observed in the ratio of renal to faecal excretion. Excretion in males was slightly higher by the faecal than by the renal route (53% and 45%, respectively). In females, in contrast, renal excretion was higher than faecal excretion (60% and 39%, respectively). However, the total excretion via urine and faeces was comparable for the individual sampling intervals in both sexes (Table 6). Bile duct–cannulated male rats showed a total excretion of 99.5% of the administered dose. The main portion of radioactivity (i.e. 86.8% of the administered dose) was eliminated via bile, and a further 10.4% via urine. About 86% of the administered dose was excreted via the bile within the first 24 hours post-administration. The high biliary excretion and low renal excretion in the bile fistulation experiment (test group 1)

Table 4. Equivalent concentrations in organs and tissues of rats after administration of [phenyl-UL-^{14}C]fluopyram

	Equivalent concentration (µg/g)					
	1 (BF/M)	2 (LD/M)	3 (LD/F)	4 (HD/M)	5 (HD/F)	6 (PT/M)
	Dose (mg/kg bw)					
	5	5	5	250	250	5
	Time after final dosing (h)					
	48	168	168	168	168	168
Erythrocytes	0.406	0.169	0.242	10.190	10.130	0.155
Plasma	n.a.	0.098	0.189	6.306	9.287	0.082
Spleen	n.a.	0.163	0.277	7.202	9.424	0.140
Liver	n.a.	0.725	1.221	15.810	20.620	0.580
Kidney	n.a.	0.726	1.078	15.720	15.520	0.532
Perirenal fat	n.a.	0.010	0.096	2.661	3.653	0.034
Adrenal gland	n.a.	0.069	0.919	10.240	13.360	0.337
Testis	n.a.	0.138	n.a.	6.309	n.a.	0.103
Ovary	n.a.	n.a.	0.667	n.a.	11.210	n.a.
Uterus	n.a.	n.a.	0.159	n.a.	7.389	n.a.
Skeletal muscle	n.a.	0.130	0.253	5.439	7.780	0.068
Bone femur	n.a.	0.054	0.101	3.690	3.865	0.037
Heart	n.a.	0.188	0.328	6.725	9.025	0.098
Lung	n.a.	0.135	0.238	6.966	9.110	0.104
Brain	n.a.	0.110	0.217	6.132	8.308	0.083
Thyroid gland	n.a.	0.047	0.297	7.336	9.859	0.124
Skin	0.308	0.092	0.171	5.087	6.997	0.058
Carcass	0.527	0.153	0.298	5.354	7.832	0.085
Gastrointestinal tract	1.699	0.096	0.200	5.615	7.800	0.095

From Klempner (2008a)
BF, bile fistulation; F, female; HD, high dose; LD, low dose; M, male; PT, 14-day pretreatment; n.a., not analysed

Table 5. Study design for ADME study with [pyridyl-2,6-^{14}C]fluopyram in rats

Group	No./sex	Dose regimen	Sample collection schedule
1 (BF)	6 males	5 mg/kg bw, single oral low dose, bile fistulation	Bile, urine and faeces at various times after administration Blood, gastrointestinal tract, skin and carcass: at sacrifice, 48 h after administration
2 (LD)	4 males	5 mg/kg bw, single oral low dose	Plasma, urine and faeces at various times after administration Blood, organs and tissues, gastrointestinal tract, skin and carcass: at sacrifice, 168 h after administration
3 (LD)	4 females	5 mg/kg bw, single oral low dose	Plasma, urine and faeces at various times after administration Blood, organs and tissues, gastrointestinal tract, skin and carcass: at sacrifice, 168 h after administration

From Klempner (2008b)
BF, bile fistulation; LD, low dose

Table 6. Recovery of radioactivity in rats after administration of [pyridyl-2,6-^{14}C]fluopyram

	Recovery of radioactivity (% of administered dose)		
	1 (BF/M)	2 (LD/M)	3 (LD/F)
	Dose level (mg/kg bw)		
	5	5	5
Urine	10.40	45.44	60.44
Bile	86.81	n.a.	n.a.
Faeces	2.30	53.04	39.49
Total excreted	99.51	98.48	99.93
Skin	0.061	0.049	0.043
Sum organs/tissues	0.393	0.293	0.263
Body without gastrointestinal tract	0.454	0.342	0.306
Gastrointestinal tract	0.047	0.020	0.026
Total in body	0.501	0.361	0.332
Balance	100.0	98.84	100.3

From Klempner (2008b)
BF, bile fistulation; F, female; LD, low dose; M, male; n.a., not analysed

Table 7. Distribution and plasma kinetics in rats after administration of [pyridyl-2,6-^{14}C]-fluopyram

	2 (LD/M)	3 (LD/F)
	Dose (mg/kg bw)	
	5	5
T_{max} measured (h)	0.7	3.0
T_{max} calculated (h)	0.7	3.3
C_{max} measured (µg/g)	1.78	1.50
C_{max} calculated (µg/g)	1.79	1.43
$t_{½}$ abs (h)	0.3	0.4
$t_{½}$ elim 1 (h)	11.2	9.8
$t_{½}$ elim 2 (h)	55.9	72.9
$AUC_{0-\infty}$ (µg·h/g)	22	37
MRT_{tot} (h)	32.9	29.1
MRT_{abs} (h)	0.6	7.3
MRT_{disp} (h)	32.3	21.8

From Klempner (2008b)
$AUC_{0-\infty}$, area under the plasma radioactivity concentration–time curve; F, female; HD, high dose; LD, low dose; M, male; MRT_{abs}, mean residence time of the absorption; MRT_{disp}, mean residence time of the disposition; MRT_{tot}, mean residence time of the total radioactivity; PT, 14-day pretreatment; $t_{½}$ abs, absorption half-life; $t_{½}$ elim, elimination half-life

compared with the high renal excretion in the experiment without bile fistulation (test group 2) were an indication of significant enterohepatic circulation.

The radiolabelled residues in the organs and tissues of the animals were determined at sacrifice, 168 hours after the oral administration. The residues in most of the organs and tissues of male and female rats were low (< 0.1% of the dose). Negligible amounts of radioactivity were found in

Table 8. Equivalent concentrations in organs and tissues of rats after administration of [pyridyl-2,6-^{14}C]fluopyram

	Equivalent concentration (µg/g)		
	1 (BF/M)	2 (LD/M)	3 (LD/F)
	Dose (mg/kg bw)		
	5	5	5
	Time after dosing (h)		
	48	168	168
Erythrocytes	0.029	0.100	0.077
Plasma	0.026	0.008	0.007
Spleen	n.a.	n.s.	0.021
Liver	n.a.	0.115	0.113
Kidney	n.a.	0.048	0.049
Perirenal fat	n.a.	0.010	0.031
Adrenal gland	n.a.	0.019	0.021
Testis	n.a.	0.008	n.a.
Ovary	n.a.	n.a.	0.017
Uterus	n.a.	n.a.	0.013
Skeletal muscle	n.a.	0.009	0.007
Bone femur	n.a.	0.008	0.006
Heart	n.a.	0.016	0.012
Lung	n.a.	0.022	0.019
Brain	n.a.	0.007	0.004
Thyroid gland	n.a.	0.022	0.021
Skin	0.015	0.010	0.009
Carcass	0.037	0.011	0.010
Gastrointestinal tract	n.a.	0.007	0.012

From Klempner (2008b)
BF, bile fistulation; F, female; LD, low dose; M, male; n.a., not analysed; n.s., no sample

the skin (0.04–0.06% of the dose). The radioactivity in the body (including gastrointestinal tract) amounted to 0.3–0.5% of the administered dose and thus was much lower than with the other label. The highest residue concentrations (Table 8) were detected in erythrocytes (0.1 µg/g), as well as in liver (0.12 µg/g) and in kidney (0.05 µg/g). The concentrations in the other organs and tissues were low and ranged from 0.004 to 0.02 µg/g. A slight sex difference was observed for the residues in perirenal fat, where females showed about 3 times higher residues than males (0.01 and 0.03 µg/g, respectively) (Klempner, 2008b).

The distribution of [phenyl-UL-^{14}C]fluopyram (chemical purity of non-radiolabelled substance 99.8%; chemical purity of radiolabelled substance > 99%; radiochemical purity > 98%) was investigated in eight male and eight female Wistar HsdCpb:WU rats by quantitative whole-body autoradiography using the radioluminography technique. The data were obtained over a testing period of 7 days following a single oral administration of [phenyl-UL-^{14}C]fluopyram at a dose level of 3 mg/kg bw. One rat each was taken for sectioning at 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing. The excretion of radioactivity via urine, faeces and expired air was also investigated. In addition, one control animal of each sex that received non-radiolabelled fluopyram was terminated at 4 hours after dosing for detection of possible chemographic effects.

Fluopyram was readily absorbed from the gastrointestinal tract, distributed among almost all organs and tissues investigated and excreted constantly over the whole experimental period. Excretion via faeces (65% and 53% for males and females, respectively) was higher than excretion via urine (32% and 41% for males and females, respectively). Less than 0.1% of the administered dose was expired as $^{14}CO_2$ or other volatiles during a sampling period of 48 hours.

In male rats, the maximum equivalent concentrations were reached for nearly all organs and tissues during the first day after administration. Within this period, the respective values remained at a comparably high level. For most organs of the central compartment (e.g. liver, kidney) and the peripheral tissues fat, muscle, some glands (e.g. adrenal, thyroid, Harderian) and nasal mucosa, maximum equivalent concentrations were higher than in blood at the times of T_{max} and $T_{168\,h}$, suggesting a rapid clearance from blood and distribution to organs and tissues of the animals. Owing to the relatively high values at the terminal time, a delayed depletion of test compound–related radioactivity from the organs and tissues is assumed.

In female rats, the maximum equivalent concentrations were reached for most organs and tissues during the first day post-dosing as well. The respective values remained at a comparably high level within this period. Only for the nasal mucosa and glandula preputialis did the maximum concentrations peak after 48 hours. For most organs of the central compartment (e.g. liver, kidney) and the peripheral tissues fat, muscle, some glands (e.g. adrenal, thyroid, preputialis, Harderian), ovary and nasal mucosa, maximum equivalent concentrations were higher than in blood at the times of T_{max} and for most of them also at $T_{168\,h}$. Similarly to the males, the absorbed radioactivity is rapidly cleared from blood and distributed to organs and tissues of the animals. The higher values for the organs at $T_{168\,h}$ indicate a still ongoing degradation (liver) and excretion (kidney) at a high level and additionally a delayed depletion of test compound–related radioactivity from the other organs.

From peak values, a continuous but slow decline of radioactivity concentrations was observed for all organs and tissues during the whole testing period. Seven days after administration, quantifiable radioactive residues were still measured in all of them except the pineal body, the only one that had reached the limit of quantification. This indicated that distribution, metabolism and elimination of radioactivity from all these organs and tissues as well as excretion via urine and faeces were still ongoing processes. Therefore, retention of fluopyram-related radioactivity in any of the organs and tissues investigated can be excluded (Koester, 2008a).

The distribution of [pyridyl-2,6-^{14}C]fluopyram (chemical purity of non-radiolabelled substance 99.8%; chemical purity of radiolabelled substance ≥ 99%; radiochemical purity > 98%) was investigated in eight male and eight female Wistar HsdCpb:WU rats by quantitative whole-body autoradiography using the radioluminography technique. The data were obtained over a testing period of 7 days following a single oral administration of [pyridyl-2,6-^{14}C]fluopyram at a dose level of 3 mg/kg bw in males and 4.5 mg/kg bw in females. One rat each was taken for sectioning at 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing. The excretion of radioactivity via urine, faeces and expired air was also investigated. In addition, one control animal of each sex that received non-radiolabelled fluopyram was terminated at 4 hours after dosing for detection of possible chemographic effects.

Fluopyram was readily absorbed from the gastrointestinal tract and distributed among almost all organs and tissues investigated. The excretion via urine and faeces was nearly completed after 72 hours. The male rats showed slightly higher faecal (54%) and lower urinary (51%) excretion. For female rats, the urinary excretion was slightly higher (50%) than the faecal excretion (43%). Less than 1% of the administered radioactivity was expired as $^{14}CO_2$ or other volatiles during a sampling period of 48 hours.

In male rats, the maximum equivalent concentrations (C_{max}) were reached for all organs and tissues at 1 hour after administration. For most organs of the central compartment (e.g. liver, kidney)

and the peripheral tissues fat, some glands (e.g. adrenal, thyroid, Harderian) and nasal mucosa, maximum equivalent concentrations were higher than in blood at T_{max} and for liver also at $T_{168\,h}$, suggesting a rapid clearance from blood and distribution to organs and tissues of the animals. For the high value in liver at $T_{168\,h}$, a still ongoing degradation at a high level is assumed.

In female rats, the maximum equivalent concentrations (C_{max}) were reached for most organs and tissues during the first hour post-dosing as well, except for the kidney and perirenal fat, which peaked after 4 hours. For most organs of the central compartment (e.g. liver, kidney) and the peripheral tissues fat, brain, some glands (e.g. adrenal, thyroid, preputialis, Harderian), ovary and nasal mucosa, maximum equivalent concentrations were higher than in blood at T_{max} and for liver, nasal mucosa, Harderian gland and glandula preputialis also at $T_{168\,h}$. Similarly to the males, the absorbed radioactivity is rapidly cleared from blood and distributed to organs and tissues of the animals. The higher values for the organs and tissues at $T_{168\,h}$ indicate a still ongoing degradation at a high level in the liver and additionally a delayed depletion of test compound–related radioactivity from the other organs.

From peak values, a continuous but slow decline of radioactivity concentrations was observed for all organs and tissues during the whole testing period. Seven days after administration, quantifiable radioactive residues were still measured in most of them. This indicated that distribution, metabolism and elimination of radioactivity from all these organs and tissues as well as excretion via urine and faeces were still ongoing processes. Therefore, a retention of fluopyram-related radioactivity in any of the organs and tissues investigated can be excluded (Koester, 2008b).

The depletion of radioactive residues of [pyridyl-2,6-^{14}C]fluopyram (chemical purity > 99%; radiochemical purity > 99%) from organs and tissues (plasma, liver, kidney and perirenal fat), excretion in urine and metabolism were investigated in Wistar HsdCpb:WU rats. Three groups of four male and four female rats each were administered by oral gavage a single dose of fluopyram in 0.5% aqueous tragacanth at a nominal dose level of 5 mg/kg bw. The animals were sacrificed 1, 4 and 24 hours after dosing. The total radioactivity, which included the unchanged test item and metabolites, was determined in the excreted urine samples over the sampling times (0–1 hour, 0–4 hours and 0–24 hours) as well as in plasma, liver, kidney and perirenal fat at sacrifice. The metabolism was investigated in urine and plasma samples and in extracts from liver, kidney and fat using HPLC and thin-layer chromatography with radiodetection.

The overall recovery accounted for 97.2–98.8% of the administered dose in male rats (groups 1–3) and for 97.8–99.7% in female rats (groups 4–6). The urinary excretion started immediately after administration and increased to 28.7% and 43.1% of the administered dose after 24 hours in male and female rats, respectively (Table 9).

The highest equivalent concentrations (i.e. total radioactive residues) were detected in the organs and tissues as well as in the combined gastrointestinal tract plus faeces at 1 hour after administration (Table 10). The distribution of radioactivity within the central compartments of the body (e.g. blood, liver and kidney) was fast and showed a distinct preference for the liver and, to a lesser extent, the kidney. All values decreased to significantly lower values by the end of the test. It is expected that residual amounts in these samples are subject to further elimination from the body, and retention of test item–related radioactivity in any of the organs and tissues investigated can therefore be excluded. In contrast to the male rats, the residue concentrations of organs and tissues from female rats were higher at nearly all points in time, which was particularly noticeable for the perirenal fat values (factor of 4.3 at 24 hours) (Koester & Klempner, 2008).

A comparison of the major toxicokinetic parameters from single oral dose studies with [pyridyl-2,6-^{14}C]fluopyram and [phenyl-UL-^{14}C]fluopyram was discussed by Neumann (2009). The maximum plasma concentration (C_{max}) was reached more rapidly after administration of the pyridyl-labelled fluopyram (Table 11), and elimination from the plasma was completed within the test period (> 99%

Table 9. Recovery of radioactivity in rats after administration of [pyridyl-2,6-^{14}C]fluopyram at a dose of 5 mg/kg bw

	Recovery of radioactivity (% of administered dose)					
	1 (M)	2 (M)	3 (M)	4 (F)	5 (F)	6 (F)
	Sacrifice time (h)					
	1	4	24	1	4	24
Urine	1.44	4.81	28.69	1.90	6.74	43.06
Plasma	0.32	0.21	0.09	0.40	0.26	0.11
Carcass	13.82	6.02	2.71	29.16	27.09	6.12
Kidneys	0.40	0.29	0.12	0.57	0.42	0.12
Liver	4.57	3.08	1.71	5.52	4.00	1.23
Skin	4.72	1.51	0.76	17.65	10.29	1.44
Perirenal fat	0.66	0.29	0.06	1.09	1.50	0.31
Body without gastrointestinal tract	24.50	11.40	5.46	54.40	43.56	9.32
Gastrointestinal tract + faeces	71.23	82.61	64.20	42.76	47.52	47.36
Total body residues	95.72	94.01	69.66	97.16	91.08	56.68
Total recovery	97.16	98.81	98.35	99.06	97.82	99.74

From Koester & Klempner (2008)
F, female; M, male

Table 10. Equivalent concentrations in organs and tissues of rats after administration of [pyridyl-2,6-^{14}C]fluopyram at a dose of 5 mg/kg bw

	Equivalent concentration (µg/g)					
	1 (M)	2 (M)	3 (M)	4 (F)	5 (F)	6 (F)
	Sacrifice time (h)					
	1	4	24	1	4	24
Plasma	1.27	0.69	0.34	1.70	1.26	0.41
Carcass	1.18	0.50	0.22	2.98	2.66	0.53
Kidneys	2.97	1.86	0.81	5.06	3.98	0.96
Liver	7.22	4.98	1.83	8.67	6.01	1.54
Skin	1.0	0.29	0.15	4.60	2.56	0.33
Perirenal fat	7.26	2.74	0.57	11.49	13.15	2.51
Body without gastrointestinal tract	1.40	0.62	0.29	3.71	2.84	0.54
Gastrointestinal tract + faeces	47.53	58.81	20.82	34.26	37.13	18.29
Total body	5.03	4.74	3.19	6.10	5.48	2.85

From Koester & Klempner (2008)
F, female; M, male

eliminated in 168 hours). The radioactivity was distributed among all tissues with both labels. This was confirmed by the results of the two whole-body autoradiography studies. Tissue concentrations were higher after application of the phenyl label. However, all residues decreased significantly until the end of the study, albeit more slowly than following application of the pyridyl label. The different biokinetic behaviour of both radiolabels was obviously due to the metabolism of fluopyram, particularly cleavage of the molecule and the formation of label-specific metabolites. The active substance

Table 11. Comparison of major toxicokinetic parameters from single-dose studies with [pyridyl-2,6-^{14}C]fluopyram and [phenyl-UL-^{14}C]fluopyram

	Males			Females		
	Pyridyl	Phenyl	Phenyl	Pyridyl	Phenyl	Phenyl
	Dose (mg/kg bw)					
	5	5	250	5	5	250
T_{max} (h)	0.7	24.0	48.0	3.0	8.0	48.0
C_{max} (µg/ml)	1.78	1.45	59.94	1.5	2.11	63.09
$AUC_{0-\infty}$ (mg·h/l)	22	107	5680	37	148	7060
$t_{½\ abs}$ (h)	0.3	0.1	0.5	0.4	0.4	0.5
$t_{½\ elim\ initial}$ (h)	11.2	3.9	4.8	9.8	16.2	4.8
$t_{½\ elim\ terminal}$ (h)	55.9	30.9	23.6	72.9	53.0	29.0

From Klempner (2008a,b)
$AUC_{0-\infty}$, area under the plasma radioactivity concentration–time curve; $t_{½}$ abs, absorption half-life; $t_{½}$ elim, elimination half-life

was extensively metabolized; unchanged parent compound was found only in low percentages of the applied dose. The nature and amounts of common metabolites were in good agreement in all rat studies and both labels and thus did not cause the differences. Additionally, molecular cleavage ranged from 35% to 48% of the administered dose (percentage from ADME studies, low dose), leading to complementary, label-specific metabolites. These metabolites resulted in the different biokinetic parameters of the two labels.

The cumulative urinary excretion data of the low-dose ADME experiments (Table 12) clearly showed different behaviour between the two radiolabels. For the phenyl label, a slower onset and delayed urinary excretion were observed, compared with the pyridyl label. It can therefore be concluded that the phenyl label–specific metabolites had a longer retention time in the rat's body. This was consistent with the results of the quantitative whole-body autoradiography studies.

From these findings, it can be concluded that the early phase of biokinetics was similar for both radiolabels and mainly reflected the behaviour of the primary metabolites, which still contained the whole molecular moiety. Later on, the cleavage products were dominating the biokinetic behaviour of the radioactive residues. After the cleavage, absorption and distribution in the body of the rat, the pyridyl label–specific metabolites were obviously excreted more rapidly than the phenyl label–specific metabolites (i.e. mainly fluopyram-benzamide). This different behaviour can be explained by the more polar nature of the metabolites from the pyridyl part of the molecule. Consequently, the application of phenyl-labelled fluopyram resulted in higher T_{max} and AUC values for the radioactive residues compared with the application of the pyridyl label (Neumann, 2009).

1.2 Biotransformation

For the investigation of the metabolism of [phenyl-UL-^{14}C]fluopyram in the toxicokinetics study of Klempner (2008a) described above, bile and urine samples as well as extracts of faeces were analysed and quantified by radio-HPLC. Faecal samples were conventionally extracted with different mixtures of acetonitrile/water under neutral, acidic and alkaline conditions. The extraction efficiency of the faecal samples (groups 2–6) was high and accounted for about 90–97% of the radioactivity present in the samples.

The identification rates in all matrices were high and accounted for 72.7–86.5% of the administered dose. The parent compound was of minor importance in low-dose tests and represented only

Table 12. Cumulative urinary excretion of radioactivity after administration of [pyridyl-2,6-^{14}C]-fluopyram and [phenyl-UL-^{14}C]fluopyram to male rats at 5 mg/kg bw

Time after administration (h)	Cumulative urinary excretion of radioactivity (% of administered dose)			
	Males		Females	
	Pyridyl	Phenyl	Pyridyl	Phenyl
4	5.46	1.02	6.25	1.43
8	12.16	2.69	16.68	3.00
12	17.96	5.15	—	—
24	35.44	15.67	46.73	16.64
48	43.77	25.27	57.34	28.62
72	44.91	30.53	59.28	34.89
96	45.22	33.87	59.82	39.30
120	45.33	35.87	60.10	41.89
144	45.39	37.26	60.31	43.82
168	45.44	38.25	60.44	45.27

From Klempner (2008a,b)

0.4–1.2% of the administered dose in the faeces of groups 2, 3 and 6. In high-dose groups, the parent compound amounted to 10.5% of the administered dose in male rats and 16.7% of the administered dose in female rats, respectively. Fluopyram was extensively metabolized in male and female rats; 29 metabolites were identified, and a further 34 metabolites were characterized by their chromatographic behaviour. The main and most prominent identified metabolites are summarized in Table 13.

Molecular cleavage in the range of about 35–43% of the administered dose was observed with the phenyl label in both sexes of all low- and high-dose tests represented by numerous label-specific metabolites. The amount of such label-specific metabolites in the bile fistulation test was distinctively lower and amounted to 5.2% of the administered dose. Three metabolite groups with the intact molecular core structure (two isomers of each of the glucuronic acid conjugates fluopyram-7-OH-GA, fluopyram-8-OH-GA and fluopyram-enol-GA) were the major metabolites in bile, which were likely formed during the first pass of the parent compound in the liver. Following enterohepatic circulation, the conjugates were cleaved in the small intestine and their aglycones partly excreted via faeces. The aglycones were also partly reabsorbed and further metabolized by cleavage of the molecule. These metabolites were mainly excreted via urine and mainly observed in the tests running for 168 hours. Only label-specific metabolites (fluopyram-benzoic acid, fluopyram-benzamide and its derivatives) were excreted in urine during the later sampling intervals. The common metabolites, such as the conjugates of the previously hydroxylated test item, had been excreted within the earlier sampling intervals.

For the investigation of the metabolism of [pyridyl-2,6-^{14}C]fluopyram in the toxicokinetics study of Klempner (2008b) described above, bile and urine samples as well as extracts of faeces were analysed and quantified by radio-HPLC. Faecal samples were conventionally extracted with different mixtures of acetonitrile/water under neutral, acidic and alkaline conditions. The extraction efficiency of the faecal samples (groups 2 and 3) was high and accounted for more than 90% of the radioactivity present in the samples.

The identification rates in all matrices accounted for 80–83% of the administered dose. Fluopyram was extensively metabolized in male and female rats; 25 metabolites were identified, and

Table 13. Summary of urinary, biliary and faecal metabolites in rats after administration of [phenyl-UL-^{14}C]fluopyram

	% of administered dose					
	1 (BF/M)	2 (LD/M)	3 (LD/F)	4 (HD/M)	5 (HD/F)	6 (PT/M)
	Dose (mg/kg bw)					
	5	5	5	250	250	5
Fluopyram	—	0.80	1.16	10.52	16.70	0.41
-benzamide-OH-GA, isomer 1	—	1.08	1.75	0.32	0.61	1.11
-benzoyl-serine	0.21	0.39	0.44	0.20	0.06	0.37
-benzamide-SA, isomer 1	—	1.50	1.49	1.01	0.71	1.51
-benzamide-*N*,*O*-GA	0.60	2.04	1.96	3.72	2.95	2.21
-benzoic acid	0.74	4.39	6.40	6.65	5.09	5.43
-benzamide-OH-GA, isomer 2	—	0.11	0.41	0.18	0.22	0.27
-benzamide-OH-GA, isomer 3	0.12	1.81	3.12	0.00	0.08	0.00
-hydroxy-benzamide	—	0.22	0.00	0.00	0.00	0.00
-benzamide-SA, isomer 2	—	0.57	0.93	0.74	0.69	0.69
-benzamide-cysteine	0.07	1.65	1.99	1.28	1.03	1.52
-BA-methyl-sulfone	—	4.17	2.94	1.69	1.47	3.31
-BA-methyl-sulfoxide	—	0.54	0.45	0.00	0.13	0.51
-benzamide	3.50	16.18	21.52	23.89	24.49	24.08
-methoxy-di-OH-GA	0.39	0.29	0.18	0.15	0.00	0.00
-7-OH-phenol-GA	2.93	4.02	3.33	0.93	1.33	3.20
-7-OH-GA, isomer 1	20.20	1.88	1.53	2.16	2.34	2.00
-7-OH-GA, isomer 2	2.61	0.14	0.32	0.44	0.31	0.00
-7-OH-hydroxy-phenol-SA	0.06	0.25	0.24	0.05	0.20	0.00
-di-OH-GA	1.55	0.04	0.24	0.62	0.80	0.00
-enol-GA, isomer 1	0.59	0.00	0.00	0.00	0.00	0.00
-7-OH-phenol-SA	0.66	2.76	0.24	0.62	0.15	1.27
-phenol-GA	1.55	0.18	0.26	0.00	0.00	0.00
-8-OH-GA, isomer 1	3.79	0.04	0.57	0.24	0.84	0.24
-8-OH-GA, isomer 2	16.59	0.00	1.33	0.00	3.37	0.71
-enol-GA, isomer 2	19.95	0.89	0.25	1.91	0.59	1.23
-7-OH-phenol	0.90	11.36	3.55	1.82	0.90	7.84
-7-OH-methyl-sulfone	—	0.40	0.89	1.18	0.78	0.72
-7-hydroxy	0.28	10.30	7.46	15.82	8.07	14.33
-8-hydroxy	—	6.02	7.67	10.37	11.25	4.06
Total identified	**77.28**	**74.02**	**72.65**	**86.53**	**85.17**	**77.00**
Total characterized	8.55	10.27	12.23	5.71	2.52	8.10
Number of characterized metabolites	13	22	22	13	9	11
Total excreted	**89.53**	**91.31**	**91.85**	**99.30**	**92.65**	**90.59**
Faeces (post-extraction solids)	—	4.36	4.46	2.29	1.60	3.08
Faeces not analysed	—	2.66	2.51	4.77	3.36	2.41
Total analysed	**85.83**	**84.29**	**84.88**	**92.24**	**87.69**	**85.10**

From Klempner (2008a)
BA, benzamide; BF, bile fistulation; F, female; GA, glucuronic acid; HD, high dose; LD, low dose; M, male; OH, hydroxy; PT, 14-day pretreatment; SA, sulfate

a further 25 metabolites were detected and characterized by their chromatographic behaviour. The parent compound was of minor importance at this low dose level and represented only 1.4–1.9% of the administered dose in faeces. Molecular cleavage in the range of 42–48% of the administered dose was observed in both sexes, as proven by the abundance of numerous label-specific metabolites. The amount of such label-specific metabolites in the bile fistulation test was distinctly lower and amounted to 12% of the administered dose only. Three metabolite groups (two isomers of each of the glucuronic acid conjugates fluopyram-7-OH-GA, fluopyram-8-OH-GA and fluopyram-enol-GA) were the major metabolites in bile. Following enterohepatic circulation, the conjugates were cleaved and their aglycones partly excreted via faeces or further metabolized by cleavage of the molecule. These latter metabolites (label-specific cleavage products) were mainly excreted via urine.

A sex difference in the ratio of label-specific metabolites was observed, as the amount of fluopyram-pyridyl-acetic acid was about 3 times higher, and the amounts of fluopyram-7-hydroxy and fluopyram-7-OH-phenol 2 times lower, in female rats than in male rats. Fluopyram-ethyl-diol metabolites were about 4 times higher in male rats than in female rats. It is assumed that the proportions of the three metabolite groups with the intact molecular core structure (the glucuronic acid conjugates fluopyram-7-OH-GA, fluopyram-8-OH-GA and fluopyram-enol-GA) formed during the first-pass effect of the parent compound in the liver were different in males and females. Following enterohepatic circulation, these metabolites were degraded, and different label-specific metabolites were obtained. The amount and the type of the label-specific metabolites were in line with the differences in the excretion pattern in males and females. The identified metabolites are summarized in Table 14.

For the investigation of the metabolism of [pyridyl-2,6-^{14}C]fluopyram in the study of Koester & Klempner (2008) described above, urine and plasma samples and extracts from liver, kidney and fat were analysed using HPLC and thin-layer chromatography with radiodetection. Fluopyram was extensively metabolized, and more than 20 metabolites were identified. Molecular cleavage occurred at least in a range of 23–34% of the administered dose in both sexes, represented by numerous label-specific metabolites. In the various samples, sex-specific differences in the ratio of pyridyl label–specific and common metabolites that still contained the intact molecular structure were observed. The metabolic transformation of the parent compound was generally more pronounced in male rats than in female rats (Table 15).

The metabolism of [pyridyl-2,6-^{14}C]fluopyram in male and female rats was principally oxidative and took place mainly at the ethylene bridge of the molecule. The metabolic transformations detected were hydroxylation of the ethyl linking group of the parent compound forming fluopyram-7-hydroxy and fluopyram-8-hydroxy metabolites and one dihydroxylated metabolite. Hydroxylation of the phenyl ring of fluopyram led to fluopyram-phenol and fluopyram-7-OH-phenol. Hydrolytic cleavage led to fluopyram-pyridyl-hydroxyethyl and fluopyram-pyridyl-carboxylic acid. Subsequent oxidation of fluopyram-pyridyl-hydroxyethyl led to mainly fluopyram-pyridyl-acetic acid and, to a lesser extent, fluopyram-ethyl-diol and fluopyram-hydroxy-pyridyl-acetic acid. Elimination of water from compounds hydroxylated in the ethylene bridge afforded the fluopyram-*Z*-olefine and fluopyram-*E*-olefine (*E*-olefine and *Z*-olefine can isomerize into each other). Several hydroxylated metabolites were conjugated with glucuronic acid and, to a lesser extent, with sulfate (Koester & Klempner, 2008).

The major metabolic pathways of fluopyram in rats derived from both radiolabels are shown in Figure 1.

Table 14. Summary of urinary, biliary and faecal metabolites in rats after administration of [pyridyl-2,6-^{14}C]fluopyram at a dose of 5 mg/kg bw

	% of administered dose		
	1 (BF/M)	2 (LD/M)	3 (LD/F)
Fluopyram (AE C656948)	—	1.41	1.85
-pyridyl-carboxylic acid	0.67	4.99	0.81
-hydroxy-pyridyl-acetic acid	1.34	5.25	1.56
-ethyl-diol-GA, isomer 1	0.35	2.30	0.81
-ethyl-diol-GA, isomer 2	0.07	1.20	0.36
-ethyl-diol-GA, isomer 3	3.72	10.69	2.71
-pyridyl-acetic acid	4.99	12.28	37.82
-hydroxyethyl-GA	0.42	3.74	2.93
-ethyl-diol	0.76	1.59	0.59
-methoxy-di-OH-GA	0.60	0.16	0.41
-pyridyl-methyl-cysteine	—	0.12	—
-7-OH-phenol-GA	5.17	2.30	3.85
-7-OH-GA, isomer 1	23.99	1.50	1.82
-7-OH-GA, isomer 2	3.33	0.10	0.32
-7-OH-hydroxy-phenol-SA	0.26	0.06	0.12
-di-OH-GA	1.15	0.48	0.78
-enol-GA, isomer 1	0.28	0.88	0.26
-7-OH-phenol-SA	0.07	0.43	0.16
-phenol-GA	1.09	0.10	0.97
-8-OH-GA, isomer 1	3.03	0.18	0.16
-8-OH-GA, isomer 2	13.79	—	1.34
-enol-GA, isomer 2	16.46	1.24	0.23
-7-OH-phenol	1.28	9.34	3.92
-7-OH-methyl-sulfone	0.05	0.79	0.88
-7-hydroxy	0.23	15.70	7.51
-8-hydroxy	—	5.68	8.13
Total identified	**83.11**	**82.50**	**80.30**
Total characterized	10.75	9.47	12.59
Number of characterized metabolites	21	21	25
Total excreted	**99.51**	**98.48**	**99.93**
Radioactivity in urine not analysed	0.11	0.53	1.16
Faeces (post-extraction solids)	—	3.90	3.51
Faeces not analysed	2.30	2.08	2.37
Total analysed	**97.10**	**91.97**	**92.89**

From Klempner (2008b)
BF, bile fistulation; F, female; GA, glucuronic acid; LD, low dose; M, male; OH, hydroxy; SA, sulfate

Table 15. Comparison of the metabolic profiles in urine, plasma and tissues of rats after administration of [pyridyl-2,6-^{14}C]fluopyram at a dose of 5 mg/kg bw

	% of administered dose					
	1 (M)	2 (M)	3 (M)	4 (F)	5 (F)	6 (F)
	Sacrifice time (h)					
	1	4	24	1	4	24
Urine						
Sum pyridyl label–specific metabolites	1.115	3.702	22.706	1.631	5.574	33.712
Sum common metabolites	0.211	0.687	4.161	0.202	0.572	6.361
Unchanged parent compound	0.024	—	—	0.026	0.019	0.027
Plasma						
Sum pyridyl label–specific metabolites	0.026	0.048	0.029	0.036	0.026	—
Sum common metabolites	0.233	0.127	0.026	0.080	0.070	0.055
Unchanged parent compound	0.058	0.008	—	0.281	0.148	0.012
Liver						
Sum pyridyl label–specific metabolites	0.190	0.208	0.380	0.124	0.117	0.062
Sum common metabolites	2.604	1.618	0.443	1.541	1.421	0.415
Unchanged parent compound	0.815	0.075	—	3.385	1.885	0.075
Kidney						
Sum pyridyl label–specific metabolites	0.147	0.169	0.075	0.159	0.156	0.047
Sum common metabolites	0.170	0.085	0.024	0.081	0.069	0.035
Unchanged parent compound	0.062	0.009	—	0.314	0.170	0.007
Perirenal fat						
Sum pyridyl label–specific metabolites	0.006	0.006	0.021	—	—	—
Sum common metabolites	0.269	0.129	0.034	0.098	0.140	0.063
Unchanged parent compound	0.363	0.139	0.001	0.944	1.336	0.203

From Koester & Klempner (2008)
F, female; M, male

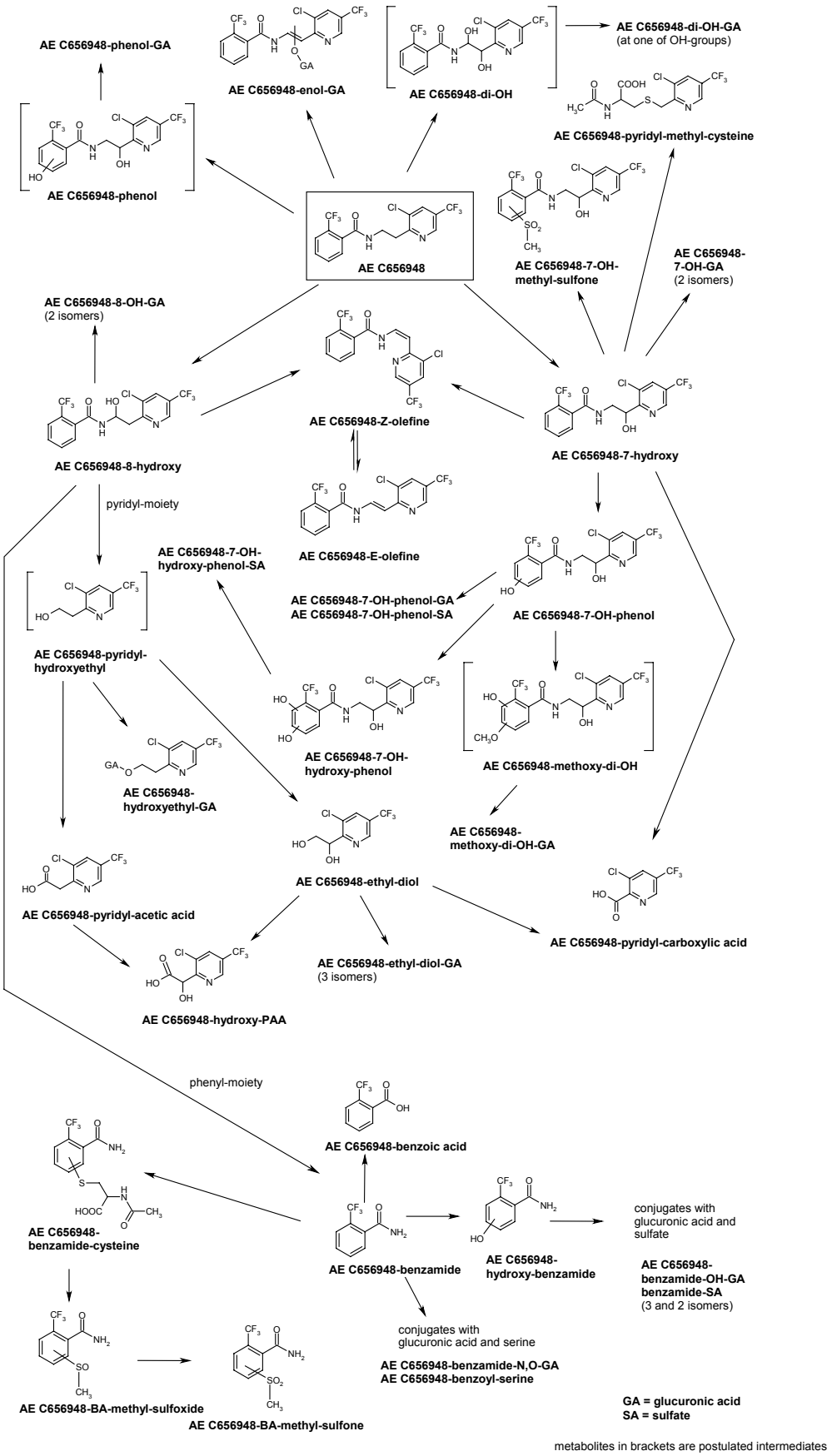
2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In an acute oral toxicity study conducted according to OECD test guideline 423 (acute toxic class method), three female (HsdCpb:WU) rats received fluopyram (purity 94.7%) in demineralized water with 2% Cremophor EL at a single dose of 2000 mg/kg bw by gavage at a volume of 10 ml/kg bw. The rats were monitored daily for mortality and clinical signs for a 2-week period, and body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were killed, necropsied and examined for gross pathological changes. Because no deaths and no clinical signs occurred in the first group, the procedure was repeated with the same limit dose level. No mortalities occurred at 2000 mg/kg bw, the only dose tested. No clinical signs were observed with the possible exception of an increased water intake observed in three of three animals (first phase) from day 2 to day 6. However, this finding was not confirmed in the second group (0/3) and thus is considered equivocal. No

Figure 1. Proposed metabolic pathway of fluopyram in rats



abnormalities were observed at gross necropsy. The oral LD_{50} was greater than 2000 mg/kg bw for male and female rats. According to the Globally Harmonised System for the classification of chemicals, the median lethal dose (LD_{50}) cut-off of fluopyram was greater than or equal to 5000 mg/kg bw (category 5/unclassified) (Eiben, 2005a).

In an acute dermal toxicity study conducted according to OECD test guideline 402, groups of five (HsdCpb:WU) rats of each sex were administered fluopyram (purity 94.7%) at a single dose of 2000 mg/kg bw. The pure solid test substance was transferred to a wet gauze layer (6.0 cm × 5.0 cm) coated with airtight Leukoflex®. The gauze strip was placed on the rat's back and secured in place using adhesive stretch tape and additionally covered with a rat jacket. After 24 hours, the dressing was removed and the area rinsed with tepid water using soap followed by gentle patting of the area to dry it. The rats were observed for clinical signs and mortality for at least 14 days, and weight was checked weekly. On day 15, surviving animals were terminated, necropsied and examined for gross pathological changes. No clinical signs were observed and no mortalities occurred at 2000 mg/kg bw, the only dose tested. No abnormalities were observed at gross necropsy. The dermal LD_{50} was greater than 2000 mg/kg bw for male and female rats (Eiben, 2005b).

In an acute inhalation toxicity study conducted according to OECD test guideline 403, groups of five (HsdCpb:WU) rats of each sex were exposed (nose only) to a dust aerosol of fluopyram (purity 94.7%) at a limit concentration of 5.1 mg/l for 4 hours. The observation period lasted for 14 days. On day 15, all animals were terminated, necropsied and examined for gross pathological changes. Measurements of particle size distribution showed a mass median aerodynamic diameter (MMAD) of 5.6 µm (geometric standard deviation ± 2.0 µm), and 19% of the total particulate had an aerosol mass less than 3 µm. No mortality occurred at the tested concentration. All rats tolerated the exposure with some evidence of reversible signs. Clinical signs in both sexes exposed to fluopyram included bradypnoea, laboured breathing patterns, reduced motility, piloerection, ungroomed hair-coat and limpness. A battery of reflex measurements was made on the first post-exposure day. Reflexes tested were normal in all males, whereas one female showed reduced tonus and vertical grip strength together with an impaired righting response. Rectal temperature was lowered in both sexes after treatment. All clinical signs were fully reversible within 1 day in male rats and within 5 days in females. The mean body weights revealed no toxicologically significant changes throughout the study period. No abnormalities were observed at gross necropsy. Under the conditions of the study, the median lethal concentration (LC_{50}) for male and female rats after dust inhalation was greater than 5.1 mg/l (Folkerts, 2006).

The acute oral, dermal and inhalation toxicity of fluopyram is summarized in Table 16.

(b) Dermal and ocular irritation and dermal sensitization

In a study of skin irritation potential conducted according to OECD test guideline 404, 0.5 g of fluopyram (purity 94.7%) moistened with distilled water was applied to shorn dorsal skin of three female New Zealand White (HsdIf:NZW) rabbits under a semioccluded dressing for 4 hours. Skin reactions were scored at 1, 24, 48 and 72 hours post-treatment. No signs of systemic toxicity or mortality were observed during the study period. No signs of erythema or oedema were observed during the conduct of the study. The individual mean scores of the 24-, 48- and 72-hour observations for erythema and oedema were 0 and 0, respectively, for each of three animals. It was concluded that fluopyram is non-irritating to rabbit skin (Schuengel, 2005a).

In a study of eye irritation potential conducted according to OECD test guideline 405, 0.1 g of fluopyram (purity 94.7%) was placed into the conjunctival sac of one eye of each of three female New

Table 16. Summary of acute oral, dermal and inhalation toxicity of fluopyram

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/l)	Reference
Rat	HsdCpb:WU	F	Oral	94.7	> 2000	Eiben (2005a)
Rat	HsdCpb:WU	M & F	Dermal	94.7	> 2000	Eiben (2005b)
Rat	HsdCpb:WU	M & F	Inhalation	94.7	> 5.1	Folkerts (2006)

F, female; M, male

Zealand White (Crl:KBL(NZW)BR) rabbits. Ocular lesions were scored at 1, 24, 48 and 72 hours post-instillation. No signs of systemic toxicity were observed during the study period. No signs of corneal opacity or iris effects were observed in any of the test animals during the study. Slight conjunctival irritation was present in all test animals after 1 hour and in two animals after 24 hours, but had disappeared after 48 hours. The individual average scores for the 24-, 48- and 72-hour observation periods for conjunctival redness were 0.3, 0.0 and 0.3, respectively, for all three test animals. On the basis of this study, fluopyram is only minimally irritating and is not considered irritating to the eyes according to the classification criteria (Schuengel, 2005b).

In a study of skin sensitization potential conducted according to OECD test guideline 429 (local lymph node assay), fluopyram (purity 94.7%) was topically applied to the dorsal surface of each ear of female (CBA/J) mice. Groups of five mice per dose were dosed once daily for 3 consecutive days with 25 µl of a formulation of fluopyram at a concentration of 0.5%, 1.0%, 2.5% or 5% in dimethylformamide. These dose levels were chosen on the basis of preliminary results showing that concentrations of 10%, 25%, 50% and 100% caused excessive cellular toxicity. Two additional groups received the vehicle (dimethylformamide) or α-hexylcinnamaldehyde (25%) to serve as negative and positive controls, respectively. Animals were checked for mortality and clinical signs at least daily during the study. In particular, the site of application was examined for signs of local irritation. Individual body weights were measured at study start and at scheduled termination. No mortality or clinical signs were observed during the study. In particular, no cutaneous reactions were observed at the application site. Body weight changes were comparable between control and treated groups. Hence, there were no confounding effects of irritation or toxicity. Negative responses were observed at all dose levels of fluopyram. The results of the positive control demonstrated the validity of the assay. On the basis of this study, fluopyram did not show any sensitization potential and does not warrant classification and labelling as a skin sensitizer (Repetto-Larsay, 2006).

Studies on the irritation and skin sensitization potential of fluopyram are summarized in Table 17.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a range-finding study, groups of five male and five female C57BL/6J mice were given diets containing fluopyram (purity 99.4%) at a concentration of 0, 150, 1000 or 5000 ppm, equal to 0, 24.7, 162 and 747 mg/kg bw per day in males and 0, 31.1, 197 and 954 mg/kg bw per day in females, for 28 days. Feed consumption and body weight data were recorded, as well as organ weights and pathology of selected organs. Blood samples for clinical chemistry analysis were taken at termination.

Table 17. Summary of irritation and skin sensitization potential of fluopyram

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
Rabbit	HsdIf:NZW	F	Skin irritation	94.7	Not irritating	Schuengel (2005a)
Rabbit	Crl:KBL(NZW)BR	F	Eye irritation	94.7	Not irritating	Schuengel (2005b)
Mouse	CBA/J	F	Skin sensitization (LLNA)	94.7	Not sensitizing	Repetto-Larsay (2006)

F, female; LLNA, local lymph node assay

All males and three of five females at 5000 ppm showed severe clinical signs, comprising reduced motor activity, hunched posture, piloerection, wasted appearance and/or coldness to touch in both sexes, together with laboured respiration in three of five males and distended abdomen in two of three females. These animals were therefore humanely terminated between study days 17 and 27, on the same day or a few days after the signs were first apparent. No mortalities occurred in the other dose groups. One of the surviving females had a distended abdomen between study days 8 and 10. A loss of body weight accompanied these signs, as did reduced feed intake. There were no clinical signs in either the 150 or 1000 ppm dose groups. The two surviving females at 5000 ppm had elevated total cholesterol (+218%) and total protein (+16%) concentrations and alanine aminotransferase (ALT) activities (+484%). At 1000 ppm, ALT activity was increased in males (+359%, not statistically significant), and mean albumin concentration was lower in females (−12%, $P < 0.05$).

In the decedent animals at 5000 ppm, treatment-related effects were seen in the adrenal glands, liver, lungs, spleen, thymus and thyroid gland. Hypertrophy, vacuolation and degeneration/necrosis of the zona fasciculata were seen in the adrenal glands in all animals, together with perivascular and intra-alveolar haemorrhage and degeneration/inflammation of pulmonary veins in the lungs and erythroid extramedullary haematopoiesis in the spleen. Focal haemorrhage was seen in the thyroid gland in three of five males, and decreased cellularity of the cortex and focal haemorrhage were seen in the thymus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes (mainly centrilobular), hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis and single-cell hepatocellular necrosis were seen in all animals, and centrilobular degeneration/necrosis was seen in one of five males.

In animals surviving to termination, effects of treatment with fluopyram were seen in the liver in both sexes and in the adrenal glands in females only. Hypertrophy of the zona fasciculata was seen in the adrenal glands in the two surviving females at 5000 ppm and in three of five females at 1000 ppm. Hypertrophy of centrilobular hepatocytes was seen in the liver of both females at 5000 ppm, in all animals at 1000 ppm and in all males and two of five females at 150 ppm, with evidence of a dose–response relationship. Single-cell hepatocellular necrosis was seen in one of two females dosed at 5000 ppm and in all males dosed at 1000 ppm. Focal necrosis was noted in two of two females dosed at 5000 ppm and in three of five males and two of five females dosed at 1000 ppm. Hepatocellular eosinophilia and bile duct/oval cell hyperplasia were noted in the two surviving females at 5000 ppm and in one of five females at 1000 ppm (Table 18).

The NOAEL was 150 ppm, equal to 24.7 mg/kg bw per day, based on effects in liver (hepatocellular necrosis) and adrenals (hypertrophy of the zona fasciculata) at 1000 ppm, equal to 162 mg/kg bw per day, and above (Kennel, 2004b).

In a study of toxicity conducted according to OECD test guideline 408, groups of 10 male and 10 female C57BL/6J mice were given diets containing fluopyram (purity 99.0%) at a concentration of 0, 30, 150 or 1000 ppm, equal to 0, 5.4, 26.6 and 188 mg/kg bw per day in males and 0, 6.8, 32.0 and 216 mg/kg bw per day in females, for 90 days. Study animals were checked daily for mortality and clinical signs. Clinical examinations were performed pretest and once weekly. Body weights and feed

Table 18. Summary of selected findings in a 28-day oral toxicity range-finding study in mice

	Males				Females			
	Dietary concentration (ppm)							
	0	150	1000	5000	0	150	1000	5000
Number examined	5	5	5	0[a]	5	5	5	2[a]
Liver weight, absolute (g)	0.80	0.94	1.13**	—	0.68	0.79	0.94**	1.66
Liver weight, relative (% of body weight)	4.05	4.90	5.91**	—	4.50	5.28*	5.71**	10.44
Liver: hepatocellular hypertrophy, centrilobular								
- Minimal	0	3	0	—	0	2	1	0
- Slight	0	2	0	—	0	0	4	0
- Moderate	0	0	5	—	0	0	0	2
- Total	0	5	5	—	0	2	5	2
Liver: hepatocellular necrosis, single cell								
- Minimal	0	0	5	—	0	0	0	1
Liver: hepatocellular necrosis, focal								
- Minimal	0	0	3	—	0	0	2	0
- Slight	0	0	0	—	0	0	0	2
- Total	0	0	3	—	0	0	2	2
Liver: hepatocellular eosinophilia								
- Minimal	0	0	0	—	0	0	1	2
Liver: hyperplasia of bile ducts/oval cells								
- Minimal	0	0	0	—	0	0	1	2

From Kennel (2004b)
* $P < 0.05$; ** $P < 0.01$
[a] 0/2 survivals in males/females, respectively.

consumption measurements were obtained pretest and weekly during the treatment period. Blood samples were collected for clinical chemistry analysis at termination (10 of each sex per group). After at least 90 days of treatment, animals were terminated, selected organs were weighed and organ to body weight and organ to brain weight ratios were calculated. Complete macroscopic examinations and histopathological evaluation of tissues were conducted on all animals. Analyses conducted during the treatment period confirmed that the dietary levels of fluopyram were appropriate for each concentration of the test substance administered.

No treatment-related mortality or clinical signs of toxicity occurred at any dose level. The body weight gain of animals was unaffected by treatment in any dose group. Feed consumption was slightly increased in males at 1000 ppm, but unaffected by treatment at 150 or 30 ppm or in females.

Clinical chemistry assessment revealed at 1000 ppm a higher ALT activity and a slightly lower albumin concentration in both sexes, together with a lower total cholesterol concentration, a higher alkaline phosphatase activity and a tendency towards higher aspartate aminotransferase (AST) activity in males. These changes were all statistically significant.

At necropsy, absolute and relative liver weights were increased at 1000 ppm by 34–38% in males and by 38–45% in females. In addition, in males, mean absolute and relative adrenal gland weights were increased by 87–92%. At macroscopic examination, enlarged and dark livers were observed in both sexes. Microscopic examination revealed treatment-related changes in the liver and adrenal glands in both sexes. In the liver at 1000 ppm, minimal to moderate hypertrophy of centrilobular hepatocytes was observed in all animals, together with a greater incidence of minimal focal

Table 19. Summary of selected findings in a 90-day oral toxicity study in mice

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	1000	0	30	150	1000
Number of animals examined	9	9	10	10	10	10	10	10
Liver weight, absolute (g)	0.91	0.94	1.04	1.24**	0.75	0.84*	0.96**	1.09**
Liver weight, relative (% of body weight)	3.97	4.06	4.59**	5.50**	4.10	4.57*	5.12**	5.68**
Liver: hepatocellular hypertrophy, centrilobular								
- Minimal	0	0	3	0	0	0	5	1
- Slight	0	0	7	0	0	0	0	8
- Moderate	0	0	0	10	0	0	0	1
- Total	0	0	10	10	0	0	5	10
Liver: hepatocellular necrosis, focal								
- Minimal	0	0	0	3	1	1	0	3
- Slight	0	0	0	0	0	0	0	3
- Total	0	0	0	3	1	1	0	6
Adrenals: cortical ceroid pigment								
- Minimal	5	3	4	0	0	0	0	0
- Slight	1	1	0	0	0	0	0	0
- Total	6	4	4	0	0	0	0	0
Adrenals: cortical vacuolation								
- Minimal	0	0	0	0	3	1	2	9
- Slight	0	0	0	0	0	0	0	1
- Total	0	0	0	0	3	1	2	10

From Kennel (2005b)
* $P < 0.05$; ** $P < 0.01$

necrosis in 3 of 10 males and minimal to slight focal necrosis in 6 of 10 females. In the adrenal glands at 1000 ppm, a lower incidence of ceroid pigment was noted in males, whereas a greater incidence of minimal to slight cortical vacuolation was observed in females, compared with controls.

At 150 ppm, absolute and relative liver weights were increased by 9–16% in males and by 25–28% in females, in association with minimal to slight hypertrophy of centrilobular hepatocytes noted in all males and in 5 of 10 females at microscopic examination (Table 19).

The NOAEL was 150 ppm, equal to 26.6 mg/kg bw per day, based on effects in liver (hepatocellular necrosis) and adrenals (cortical vacuolation) at 1000 ppm, equal to 188 mg/kg bw per day. The changes in the liver observed at 150 ppm were considered to be adaptive and not toxicologically relevant to humans (Kennel, 2005b).

Rats

In a range-finding study, groups of five male and five female Wistar Rj: WI (IOPS HAN) rats were given diets containing fluopyram (purity 98.6%) at a concentration of 0, 50, 400 or 3200 ppm, equal to 0, 4.0, 31.0 and 254 mg/kg bw per day in males and 0, 4.6, 36.1 and 263 mg/kg bw per day in females, for 4 weeks. Animals were observed daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and feed consumption were recorded weekly. Blood samples were taken before final necropsy for haematology and clinical chemistry determinations. At study termination, all animals were necropsied, selected organs were weighed and a range of tissues

Table 20. Summary of selected findings in a 28-day oral toxicity range-finding study in rats

	Males				Females			
	Dietary concentration (ppm)							
	0	50	400	3200	0	50	400	3200
Liver weight, absolute (g)	11.13	11.18	12.45	17.11**	5.84	6.24	6.76	9.64**
Liver weight, relative (% of body weight)	2.86	2.81	3.06	4.40**	2.67	2.77	3.07	4.60**
Total CYP (nmol/mg protein)	1.19	1.24	1.43	1.63	0.85	0.89	0.98	1.20
BROD (pmol/mg protein per minute)	8.90	9.86	71	171.82	2.79	4.32	26.27	86.55
EROD (pmol/mg protein per minute)	59.24	46.80	66.78	79.23	52.16	54.4	68.57	87.90
PROD (pmol/mg protein per minute)	6.56	4.68	29.54	68.31	2.81	3.49	12.92	45.68

From Kennel (2004a)
BROD, 7-benzyloxyresorufin *O*-debenzylase; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-dependentylase; ** $P < 0.01$

was taken, fixed and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparations in order to determine the cytochrome P450 (CYP) enzyme profile.

No treatment-related mortality or clinical signs of toxicity occurred at any dose level. Body weight gain was reduced at 3200 ppm by between 12% and 29% during study weeks 1 and 3 in both sexes and also during study week 4 in females. The overall mean cumulative body weight gain was comparable to the control value in males, but reduced by 14% in females. Feed consumption was reduced at 3200 ppm throughout the study by between 4% and 10% in females.

Clinical chemistry assessment at 3200 ppm revealed higher mean total cholesterol and triglyceride concentrations together with slightly lower AST and alkaline phosphatase activities in both sexes, lower glucose concentrations in males and higher total protein and albumin concentrations in females. All changes, unless otherwise indicated, were statistically significant.

Absolute and relative liver weights were increased in both sexes by 54–74% at 3200 ppm and by 7–21% at 400 ppm (Table 20). In males only, absolute and relative kidney weights were increased by 18–21% at 3200 ppm and by 15–20% at 400 ppm, whereas absolute and relative thyroid gland weights were increased by 43–46% at 3200 ppm. At macroscopic examination, enlarged and dark livers were observed in almost all males and females at 3200 ppm, together with pale kidneys in all males. At 400 ppm, pale kidneys were observed in three of five males, a dark liver was observed in one of five males and an enlarged liver was seen in one of five females.

The microscopic examination at 3200 ppm revealed treatment-related changes in the liver, thyroid gland and kidney in both sexes and in the pituitary gland in males only. Minimal to moderate hypertrophy of centrilobular hepatocytes was present in all animals. Minimal to slight diffuse hypertrophy of follicular cells was seen in the thyroid gland in three of five males and two of five females, together with depletion of colloid in two of five males. Minimal diffuse hypertrophy of basophils was noted in the pituitary gland in three of five males. Hyaline droplet nephropathy related to the accumulation of α_{2u}-globulin in the proximal tubules was noted in all males and in one of five females. However, this finding was considered not toxicologically relevant to humans, as α_{2u}-globulin is present only in trace amounts in humans. At 400 ppm, minimal hypertrophy of centrilobular hepatocytes was noted in all males and in one of five females.

Assessment of hepatic enzyme activities at 3200 ppm showed a slight increase in total CYP content and a marked induction (up to 30- and 15-fold, respectively) in 7-benzyloxyresorufin *O*-debenzylase (BROD; CYP3A) and 7-pentoxyresorufin *O*-dependentylase (PROD; CYP2B) activities in both sexes. At 400 ppm, there was a slight increase in total CYP content and a moderate increase (up to 8- and 4-fold, respectively) in BROD and PROD activities in both sexes (Table 20).

The NOAEL was 400 ppm, equal to 31.0 mg/kg bw per day, based on effects in liver (increased weight, hepatocellular hypertrophy, enzyme induction) and thyroid (hypertrophy of follicular cells, colloid depletion) at 3200 ppm, equal to 254 mg/kg bw per day. The changes in the liver at 400 ppm were considered to be adaptive and not toxicologically relevant to humans (Kennel, 2004a).

In a study of toxicity conducted according to OECD test guideline 408, groups of 10 male and 10 female Wistar Rj: WI (IOPS HAN) rats were given diets containing fluopyram (purity 99.0%) at a concentration of 0, 50, 200, 1000 or 3200 ppm, equal to 0, 3.06, 12.5, 60.5 and 204 mg/kg bw per day in males and 0, 3.63, 14.6, 70.1 and 230 mg/kg bw per day in females, for at least 90 days. An additional 10 males and 10 females fed either 0 or 3200 ppm of test diet for 90 days were maintained for 1 month after withdrawing the test diets to examine the reversibility of any effects seen.

Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals (except for animals of the recovery groups) were subjected to a neurotoxicity assessment (motor activity, sensory reactivity and grip strength) during weeks 11–12 of the study. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals of the control and high-dose groups during week 13. Urine samples were collected overnight on the week before scheduled necropsy on selected animals. Before scheduled necropsy, a blood sample was collected on selected animals for haematology and clinical chemistry determinations. In addition, a blood sample was collected from selected animals for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analysis during weeks 3 and 13 of the study and on days 29–30 of the recovery phase. All animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

No treatment-related mortality or clinical signs of toxicity occurred at any dose level. One male at 1000 ppm and one male at 50 ppm were prematurely terminated for humane reasons, because of severe clinical signs, with no established relationship to treatment. There was evidence that the latter animal had experienced accidental trauma.

Body weight was decreased by 4–6% in males and by 4–8% in females throughout the course of the study at 3200 ppm, and overall body weight gain was reduced by 9% in males and by 17% in females in this dose group. At 1000 ppm, a slight decrease of 15% in body weight gain per day was noted in females during the first week of treatment. Feed consumption in females was slightly decreased, by 5–12%, on days 29–90 at 3200 ppm and was slightly decreased, by between 4% and 9%, on most occasions at 1000 ppm. After 1 month of recovery in the high-dose group, body weight was still reduced by 7% and 6% in males and females, respectively. Feed consumption was similar to the control values in both sexes.

The neurotoxicity assessment and the ophthalmological examination revealed no treatment-related effects in either sex.

At the haematology evaluation at 3200 ppm, prothrombin time was increased in males, whereas platelet and reticulocyte (absolute and percentage) counts were increased in females. In addition, slightly lower haemoglobin concentrations were noted in both sexes, in association with lower haematocrit in males and lower mean corpuscular volume and mean corpuscular haemoglobin in females. After 1 month of recovery, only a partial reversibility was observed, as haemoglobin concentration was still reduced by 4% and 3% in males and females, respectively.

Clinical chemistry assessment at 3200 ppm revealed an increase in concentrations of total cholesterol, γ-glutamyl transferase (GGT), total protein, globulin, inorganic phosphorus and calcium in both sexes, creatinine and urea in males, and triglycerides in females, whereas a decrease was noted in levels of total bilirubin and chloride in both sexes, glucose levels in males and alkaline

phosphatase activity and albumin to globulin ratio in females. After 1 month of recovery, only partial reversibility was observed in total cholesterol and globulin concentrations and albumin to globulin ratio changes in females. At 1000 ppm, there was an increase in total cholesterol levels in both sexes, an increase in creatinine, inorganic phosphorus and calcium concentrations in males and a decrease in total bilirubin levels in both sexes and chloride levels in males.

Urinalysis at 3200 ppm revealed an increased incidence and severity of cellular casts in males, compared with controls. After 1 month of recovery, cellular casts were still observed in males, but with a lower incidence and severity than at the end of the dosing phase. A treatment-related increase in the incidence and severity of cellular casts in urine of males was also seen at 1000, 200 and 50 ppm.

At week 3, hormonal assessment at 3200 ppm revealed an increase in TSH level in both sexes (+63% and +71% in males and females, respectively), together with an increase in T_3 and T_4 levels in females (+24% and +54%, respectively); at week 13, only increases in TSH and T_3 levels were noted in males (+88% and +40%, respectively). At 1000 ppm, TSH level was increased by 54% (not statistically significant) and T_4 level was significantly increased by 43% in males at week 13. Dose-dependent increases in T_3, T_4 and TSH levels were observed in both sexes at most time points and for most parameters, but in general, the changes were statistically significant only at the highest dose and were only marginal at the lower two dose levels of 200 and 50 ppm (Table 21). After 1 month of recovery, all changes detected during the study phase were reversed.

At necropsy at 3200 ppm, absolute and relative liver weights were increased by 53–74% and absolute and relative thyroid gland weights were slightly increased by 10–22% in both sexes. In addition, absolute and relative kidney weights were increased by 28–36% in males. The macroscopic examination showed enlarged and dark liver and/or prominent lobulation of the liver in both sexes, together with enlarged and/or pale kidneys in males. After 1 month of recovery, absolute and relative kidney weights were still increased in males, and enlarged kidneys were noted in 2 of 10 males. At 1000 ppm, absolute and relative liver weights were increased by 20–27% in both sexes. In addition, in males, absolute and relative kidney weights were increased by 25–32%. Enlarged liver and prominent lobulation of the liver were noted in both sexes, together with dark liver and enlarged and/or pale kidneys in males.

Treatment-related microscopic changes were seen in the liver and thyroid gland in both sexes and in the kidney in males. In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed at 200 ppm and above in males and at 1000 ppm and above in females, together with minimal to moderate periportal to midzonal hepatocellular macrovacuolation in females at 200 ppm and above. The hepatocellular hypertrophy in two males at 200 ppm was of minimal severity and thus considered to be an adaptive change.

In the thyroid gland, minimal to slight diffuse hypertrophy of follicular cells was seen at 3200 ppm in 8 of 10 males and in 1 of 10 females, at 1000 ppm in 4 of 10 males and in 2 of 10 females, and at 200 ppm in 1 of 10 males.

After the recovery period, liver and thyroid were comparable between the high-dose and control groups, indicating that the changes noted after 90 days of treatment were reversible in these organs.

In the kidney of males, the incidence and severity of hyaline droplet nephropathy were increased at 50 ppm and above, whereas the incidence of hyaline casts was increased at 1000 ppm and above. After 1 month of recovery at 3200 ppm, hyaline droplet nephropathy and hyaline casts persisted in some animals.

The NOAEL was 200 ppm, equal to 12.5 mg/kg bw per day, based on effects in the liver (hepatocellular hypertrophy and vacuolation) at 1000 ppm, equal to 60.5 mg/kg bw per day, and above. The hyaline droplet nephropathy (and associated effects) observed in male rats from all treated groups is considered to be non-relevant to humans, as this specific nephropathy is due to an accumulation

Table 21. Summary of selected findings in a 90-day oral toxicity study in rats

	Males					Females				
	Dietary concentration (ppm)									
	0	50	200	1000	3200	0	50	200	1000	3200
Total cholesterol (mmol/l)										
- Week 13	1.87	1.73	2.23	2.72*	2.95**	1.88	2.13	2.11	2.79**	3.78**
T_3 (nmol/l)										
- Week 3	1.33	1.37	1.31	1.50	1.51	1.22	1.41	1.21	1.37	1.51*
- Week 13	1.14	1.27	1.33	1.39	1.60**	1.46	1.45	1.44	1.60	1.73
T_4 (nmol/l)										
- Week 3	61.9	57.8	59.1	65.8	55.4	34.0	45.2*	36.7	44.5	52.3**
- Week 13	48.4	53.5	51.7	69.4**	58.2	34.1	38.3	44.8	47.1	38.6
TSH (ng/ml)										
- Week 3	6.32	6.58	5.71	7.35	10.3**	3.51	3.70	4.13	4.14	5.99**
- Week 13	4.44	6.00	5.76	6.82	8.35**	4.16	3.62	3.73	3.94	5.42
Liver weight										
- Absolute (g)	10.9	10.8	12.0	13.6**	16.8**	5.74	5.94	6.40	7.09**	9.09**
- Relative (% of body weight)	2.28	2.28	2.40	2.74**	3.67**	2.12	2.24	2.37	2.70**	3.70**
Kidney weight										
- Absolute (g)	2.81	2.89	3.10	3.65**	3.60**	1.65	1.74	1.73	1.73	1.70
- Relative (% of body weight)	0.59	0.61	0.62	0.73**	0.79**	0.61	0.66	0.64	0.66	0.69**
Thyroid weight										
- Absolute (mg)	27.2	22.4	26.9	28.2	29.9	18.5	20.3	16.4	18.4	20.6
- Relative (% of body weight, ×1000)	5.68	4.71	4.96	5.65	6.57	6.89	7.67	6.10	6.96	8.41*
No. examined microscopically	*10*	*9*	*10*	*9*	*10*	*10*	*10*	*10*	*10*	*10*
Liver: centrilobular hepatocellular hypertrophy, diffuse										
- Minimal	0	0	2	5	0	0	0	0	5	3
- Slight	0	0	0	4	2	0	0	0	2	5
- Moderate	0	0	0	0	8	0	0	0	0	2
- Total	0	0	2	9	10	0	0	0	7	10
Liver: periportal to midzonal hepatocellular macrovacuolation, focal/multifocal										
- Minimal	0	0	0	0	0	0	0	3	6	3
- Slight	0	0	0	0	0	0	0	0	0	1
- Moderate	0	0	0	0	0	0	0	0	0	1
- Total	0	0	0	0	0	0	0	3	6	5
Kidney: hyaline droplets, proximal tubules										
- Minimal	1	3	7	0	0	0	0	0	0	0
- Slight	0	0	3	0	0	0	0	0	0	0
- Moderate	0	0	0	9	1	0	0	0	0	0
- Marked	0	0	0	0	9	0	0	0	0	0
- Total	1	3	10	9	10	0	0	0	0	0
Kidney: basophilic tubules, focal/multifocal										
- Minimal	2	1	3	3	1	0	0	0	0	0
- Slight	0	1	0	6	8	0	0	0	0	0

Table 21 (continued)

	Males					Females				
	Dietary concentration (ppm)									
	0	50	200	1000	3200	0	50	200	1000	3200
- Moderate	0	1	0	0	1	0	0	0	0	0
- Total	2	3	3	9	10	0	0	0	0	0
Kidney: granular casts, medulla										
- Minimal	0	0	1	2	6	0	0	0	0	0
- Slight	0	0	0	6	2	0	0	0	0	0
- Moderate	0	0	0	0	1	0	0	0	0	0
- Total	0	0	1	8	9	0	0	0	0	0
Kidney: hyaline casts, focal/multifocal										
- Minimal	1	0	0	4	6	0	1	1	0	2
- Slight	1	0	0	0	0	0	0	0	0	0
- Total	2	0	0	4	6	0	1	1	0	2
Thyroid: follicular cell hypertrophy, diffuse										
- Minimal	0	0	1	3	5	0	0	0	2	1
- Slight	0	0	0	1	3	0	0	0	0	0
- Total	0	0	1	4	8	0	0	0	2	1

From Kennel (2005a)
* $P < 0.05$; ** $P < 0.01$

of α_{2u}-globulin in the proximal tubules, a protein that is found only in trace amounts in humans. The changes in the thyroid (follicular cell hypertrophy) were attributable to the apparent susceptibility of rats to thyroid hormone imbalance and were therefore not considered relevant to humans (Kennel, 2005a).

Dogs

In a range-finding study, groups of two male and two female Beagle dogs were administered fluopyram (purity 99.0%) by oral gavage at dose levels of 0, 30, 150 and 750 mg/kg bw per day for 28 days. Mortality, general behavioural changes and feed consumption were recorded daily throughout the study, whereas body weight was measured weekly and prior to necropsy. Additionally, a detailed clinical examination was performed weekly prior to treatment and during the treatment period. During the acclimatization phase and at the end of treatment, ophthalmology, haematology, clinical chemistry analysis and urinalysis were performed. All animals were subjected to a detailed necropsy. Selected organs were weighed, and a range of tissues was taken and processed for histopathological examination.

There were no mortalities throughout the study. At 750 mg/kg bw per day, the only treatment-related clinical sign consisted of liquid faeces noted on a few occasions throughout the study in one of two males and on two occasions in one of two females. Body weight parameters and feed consumption were comparable to those of the controls. Ophthalmological examination revealed no treatment-related abnormalities. Haematology assessment revealed low erythrocyte count, low haemoglobin and low haematocrit in the two males, relative to their individual pretreatment values. Clinical chemistry evaluation revealed low albumin concentration, low albumin to globulin ratio and high alkaline phosphatase activity in one of two males and one of two females, relative to their pretreatment values. In addition, high GGT activity and high triglyceride concentration were observed in one of two females. Urinalysis showed no relevant changes. At termination, mean body weights were

comparable to control values in both sexes. Absolute and relative liver weights were higher in both sexes, when compared with the control values. In association with this, enlarged livers were noted at gross observation in one of two males and one of two females. Histological examination revealed treatment-related changes in the liver only. Minimal to slight centrilobular to panlobular hepatocellular hypertrophy was observed for all animals, together with minimal to slight eosinophilic inclusion bodies for one of two males and for both females.

At 150 and 30 mg/kg bw per day, the only finding was liquid faeces noted on one or two occasions compared with none in the controls.

The NOAEL was 150 mg/kg bw per day, based on treatment-related clinical signs (liquid faeces) and evidence of liver toxicity at 750 mg/kg bw per day (Kennel, 2004c).

In a study of toxicity conducted according to OECD test guideline 409, groups of four male and four female Beagle dogs were given diets containing fluopyram (purity 94.6%) at a concentration of 0, 800, 5000 or 20 000 ppm (10 000 ppm from day 15 onwards), equal to 0, 28.5, 171 and 332 mg/kg bw per day in males and 0, 32.9, 184 and 337 mg/kg bw per day in females, for 90 days. Each animal was checked for clinical signs and mortality twice daily or once daily on weekends and public holidays during the acclimatization phase and throughout the study. Feed consumption was recorded daily throughout the study, and body weight was measured weekly and prior to necropsy. Additionally, a detailed physical examination was performed approximately weekly prior to treatment and during the treatment period. Ophthalmological examination was performed during the acclimatization phase and at the end of the study. Haematology and clinical chemistry analyses and urinalysis were performed once during the acclimatization phase, on week 7 or 8 and at the end of the study. All animals were subjected to a detailed necropsy. Selected organs were weighed, and a range of tissues was taken and processed for histopathological examination.

There were no mortalities throughout the study. Clinical signs were noted at the top dose and were limited to wasted appearance in one male between days 14 and 19 and in two females between days 56 and 90 and between days 14 and 90, respectively.

A body weight loss of 0.9 kg was noted in both sexes during the first 2 weeks of treatment at 20 000 ppm. This was accompanied by a reduced feed consumption in animals of these groups, which was attributed to a lack of palatability of the test substance in the diet. The dietary level of the high-dose group was therefore reduced to 10 000 ppm from day 15 onwards, after which the difference in mean body weight gain was maintained in comparison with controls on most occasions. Overall, body weight losses of 0.8 kg and 1.1 kg were noted in males and females, respectively, compared with a body weight gain of 1.0 kg in controls, which resulted in an 18% and 29% reduction in final body weight for males and females, respectively, compared with controls. At 5000 ppm, mean absolute weight gains were slightly reduced in both sexes compared with controls. Overall, body weight gains of 0.6 kg and 0.2 kg were noted for males and females, respectively, compared with 1.0 kg in both male and female control groups. This corresponded to 7% and 11% reductions in final body weight in males and females, respectively, compared with controls. At 800 ppm, body weight parameters were comparable to those of controls in males, whereas there was a slight reduction in body weight gain in females. At the end of the study, mean female body weight was 8% lower than in controls. In all cases, effects on body weight parameters were concomitant with a lower feed consumption of comparable magnitude (Table 22).

Feed consumption was markedly reduced at 20 000 ppm in both sexes (by 25–53% and 53–58% in males and females, respectively) during the first 2 weeks of treatment compared with controls. Thereafter, at 10 000 ppm, feed consumption was still reduced by 9–38% in males and 28–53% in females. To a lesser extent, feed consumption was also reduced at 5000 ppm in both sexes (overall, 7% and 22% reduction in males and females, respectively, compared with controls). In addition, feed consumption was still slightly reduced in females at 800 ppm (9% in comparison with controls). The

Table 22. Summary of selected findings in a 90-day oral toxicity study in dogs

	Males				Females			
	Dietary concentration (ppm)							
	0	800	5000	10 000/20 000[a]	0	800	5000	10 000/20 000[a]
Body weight (kg)								
- Day 1	7.4	7.4	7.2	7.7	5.5	5.6	5.6	5.7
- Day 8	7.5	7.6	7.3	7.4	5.7	5.7	5.6	5.3
- Day 15	7.6	7.8	7.4	6.8	5.7	5.7	5.5	4.8
- Day 90	8.4	8.4	7.8	6.9	6.5	6.0	5.8	4.6
Feed consumption (g/day)								
- Week 1	641	672	548	478*	638	525	490	299**
- Week 2	629	698	659	296**	626	507	467	260**
- Week 3	667	715	654	581	662	553	536	323**
- Weeks 1–13	701	722	653	528	676	612	527	368
AP (IU/l)								
- Week 8	97	129	325*	461**	111	190	299	375**
- Week 13	93	139	372*	555**	111	190	355	383*
GGT (IU/l)								
- Week 8	1	2	3	10**	2	2	4	10*
- Week 13	2	3	3	13**	2	3	5	14**
ALT (IU/l)								
- Week 8	32	27	34	115	33	28	33	25
- Week 13	31	28	38	215	30	28	46	45
Liver weight								
- Absolute (g)	280.9	351.9	448.0**	410.3**	228.7	295.8	308.2*	307.8*
- Relative (% of body weight)	3.32	4.18	5.61**	5.91**	3.55	4.95	5.30*	6.57**
Liver: hepatocellular hypertrophy, diffuse								
- Minimal	0	0	3	1	0	0	3	4
- Slight	0	0	1	3	0	0	1	0
- Total	0/4	0/4	4/4	4/4	0/4	0/4	4/4	4/4
Liver: hepatocellular intracytoplasmic eosinophilic droplets, multifocal								
- Minimal	0	1	1	3	0	1	1	2
- Slight	0	0	2	1	0	0	2	0
- Moderate	0	0	0	0	0	0	1	0
- Total	0/4	1/4	3/4	4/4	0/4	1/4	4/4	2/4
Liver: hepatocellular single-cell necrosis, focal/multifocal								
- Minimal	0	0	2	3	0	0	0	0
- Moderate	0	0	0	0	0	0	1	0
- Total	0/4	0/4	2/4	3/4	0/4	0/4	1/4	0/4
Thymus: decreased size of cortex, involution								
- Minimal	4	1	1	0	4	3	2	0
- Slight	0	2	3	2	0	0	2	1
- Moderate	0	0	0	1	0	0	0	2
- Marked	0	1	0	1	0	0	0	0
- Total	4	4	4	4	4/4	3/4	4/4	3/4

From Kennel (2006a)

AP, alkaline phosphatase; IU, international units; * $P < 0.05$; ** $P < 0.01$

[a] Dose levels were lowered at the beginning of week 3.

decrease in feed consumption was attributed to a lack of palatability of the test substance when incorporated into the diet. Hence, lower dietary concentrations were associated with better feed intake, and the use of commercial dog food moistened with the diet to improve the palatability of the diet and to stimulate the appetite of the dogs resulted in an improved feed intake. No information on feed conversion efficiency was provided for this study.

Ophthalmological examination revealed no treatment-related abnormalities. Haematology assessment revealed higher mean platelet counts at 20 000/10 000 ppm throughout the study, although the effect was less pronounced at the end of the study (week 13) than at week 8. In addition, lower erythrocyte counts, haemoglobin concentrations and haematocrit values were observed in females.

Clinical chemistry findings at 20 000/10 000 ppm included higher alkaline phosphatase and GGT activities in both sexes, whereas AST and ALT activities were higher only in males compared with controls. In addition, lower total bilirubin, mean albumin (and, as a consequence, albumin to globulin ratio) and mean total protein levels were observed in both sexes. To a lesser extent, the same parameters were also affected at 5000 ppm, whereas no adverse effects were noted at 800 ppm in either sex (Table 22). Urinalysis showed no treatment-related changes.

Absolute and relative liver weights were higher in both sexes at 20 000/10 000 ppm and 5000 ppm compared with controls. In addition, in females, absolute and relative thymus weights were lower in comparison with controls. These changes were associated with gross and histopathological changes. At macroscopic examination, enlarged liver was noted in both sexes in two of four and one of four animals at the high and intermediate doses, respectively, and generalized atrophy of the thymus was noted in all females at the high dose.

At microscopic examination, minimal to slight hepatocellular hypertrophy and intracytoplasmic eosinophilic droplets were observed in all animals of both sexes at both 20 000/10 000 and 5000 ppm (Table 22). In addition, at these dose levels, hepatocellular single-cell necrosis was observed in males. In the thymus, a slightly higher severity of thymic involution was observed in both sexes compared with controls. In addition, in females at the high dose, disturbance of the estrous cycle was observed, as only an anestrous phase was seen. However, these two latter effects (thymus and estrous cycle) were likely a secondary effect of the decrease in feed consumption and body weight or may even have been incidental.

The NOAEL was 800 ppm, equal to 28.5 mg/kg bw per day, based on evidence of liver toxicity (increased liver weight, histopathological findings including necrosis, related clinical chemistry changes) at 5000 ppm, equal to 171 mg/kg bw per day, and above (Kennel, 2006a).

In a study of toxicity conducted according to OECD test guideline 452, groups of four male and four female Beagle dogs were given diets containing fluopyram (purity 94.6%) at a concentration of 0, 100, 400 or 2000 ppm, equal to 0, 3.0, 13.2 and 67.7 mg/kg bw per day in males and 0, 3.8, 14.4 and 66.1 mg/kg bw per day in females, for 1 year. Each animal was checked for clinical signs and mortality twice daily or once daily on weekends and public holidays during the acclimatization phase and throughout the study. Feed consumption was recorded daily throughout the study, and body weight was measured weekly and prior to necropsy. Additionally, a detailed physical examination was performed approximately weekly prior to treatment and during the treatment period. Ophthalmological examination was performed during the acclimatization phase and at the end of the study. Haematology and clinical chemistry analyses were performed once during the acclimatization phase, at months 3 and 6 and at the end of the study. Urinalysis was performed once during the acclimatization phase, at months 4 and 6 and at the end of the study. All animals were subjected to a detailed necropsy. Selected organs were weighed, and a range of tissues was taken and processed for histopathological examination.

The treatment with fluopyram induced no mortalities, treatment-related clinical signs or changes at the physical and ophthalmological examinations.

There was a body weight loss of 0.2 and 0.1 kg in males and females, respectively, at 2000 ppm during the first week of treatment. Thereafter, body weight gains were comparable with those of controls in both sexes. The initial decrease in body weight gain was associated with lower feed consumption in both sexes over this period. At 400 and 100 ppm, body weight and body weight gain were not affected by the treatment in either sex.

Feed consumption was reduced by 30% and 24% in males and females, respectively, at 2000 ppm during the first week of treatment. This initial effect was most likely due to lack of palatability of the test compound, as already observed in a previous study in dogs. Overall feed consumption was comparable to that of controls in males, whereas this parameter remained slightly lower in females throughout the study, resulting in an overall 10% reduction.

Haematology evaluation and urinalysis showed no treatment-related changes. Clinical chemistry evaluation at 2000 ppm showed a higher mean alkaline phosphatase activity in males and females throughout the study. In addition, one treated male had a higher GGT activity at month 12, in comparison with the controls and with its own pretest value. The slightly elevated alkaline phosphatase activity seen in males at 400 ppm was judged not to be toxicologically relevant, as there was no other finding at this dose. No effect was seen at 100 ppm.

The only statistically significant change in organ weight was a higher mean absolute thyroid gland weight and mean thyroid gland to brain weight ratio in females at 2000 ppm. However, this change was considered not to be adverse, as there was no associated histopathological effect. No treatment-related changes were observed at the macroscopic examination. At the microscopic examination, the liver was characterized by a minimal diffuse centrilobular hepatocellular hypertrophy in three of four males at 2000 ppm. This was the only treatment-related finding (Table 23). A minimal diffuse hypertrophy of the follicular cells was observed in the thyroid gland of two of four and one of four males at 2000 and 100 ppm, respectively. In the absence of a dose–response relationship, this minor change was judged to be incidental.

The NOAEL was 400 ppm, equal to 13.2 mg/kg bw per day, based on evidence of liver toxicity (hepatocellular hypertrophy, increase in alkaline phosphatase activity) at 2000 ppm, equal to 66.1 mg/kg bw per day (Kennel, 2007).

(b) Dermal application

Rats

In a study of dermal toxicity conducted in compliance with OECD test guideline 410, groups of 10 male and 10 female Wistar Hanover CRL: WI [GLX/BRL/HAN] IGS BR rats were administered fluopyram (purity 99.0%) dermally for 28 days (6 hours per day, 5 days per week, semiocclusive dressing) at a dose level of 0, 100, 300 or 1000 mg/kg bw per day. The test substance was applied to a commercially available adhesive bandage that had been moistened with 1 ml of deionized water. During the study, the animals were evaluated for the effects of the test compound on body weight, feed consumption, clinical signs, eyes, clinical chemistry and haematology. Gross necropsy evaluations were performed on all animals. Histopathological evaluation of selected tissues was conducted on the control and high-dose groups and for tissues from the low- and mid-dose groups, when findings were observed in tissues from the high-dose group.

There were no mortalities, no treatment-related clinical observations and no ophthalmic findings at any dose level. No effects on body weights or on feed consumption were observed.

Haematology and clinical chemistry analyses at 1000 mg/kg bw per day revealed a statistically significant increase in prothrombin time for males (19.1 seconds versus 16.3 seconds in controls) and a statistically significant increase in total cholesterol level for females (59 mg/dl versus 40 mg/dl in controls).

Table 23. Summary of selected findings in a 1-year oral toxicity study in dogs

	Males				Females			
	Dietary concentration (ppm)							
	0	100	400	2000	0	100	400	2000
AP (IU/l)								
- Pretest	111	142	119	119	121	134	135	145
- Month 3	120	107	162	256*	119	123	129	229**
- Month 6	154	108	215	341	147	134	134	334
- Month 12	117	81	176	299	140	135	161	285
Liver weight								
- Absolute (g)	329.1	312.7	345.2	368.2	297.0	288.6	294.3	316.4
- Relative (% of body weight)	3.80	3.06	3.68	4.38	3.72	3.70	3.75	4.21
Liver: centrilobular hepatocellular hypertrophy, diffuse								
- Minimal	0/4	0/4	0/4	3/4	0/4	0/4	0/4	0/4
Thyroid: follicular epithelium hypertrophy, diffuse								
- Minimal	0/4	1/4	0/4	2/4	0/4	0/4	0/4	0/4

From Kennel (2007)
AP, alkaline phosphatase; IU, international units; * $P < 0.05$; ** $P < 0.01$

Liver weights at 1000 mg/kg bw per day were statistically significantly increased in males (relative weight) and females (absolute and relative weights). These findings were further substantiated by the presence of a minimal to mild degree of hepatocellular hypertrophy (centrilobular and midzonal) in all males and females at 1000 mg/kg bw per day. All other microscopic observations were considered to be incidental and/or background and not related to treatment.

The NOAEL for general systemic toxicity was 300 mg/kg bw per day, based on clinical chemistry changes (increased prothrombin time, increased cholesterol level) and liver effects (hepatocellular hypertrophy) at 1000 mg/kg bw per day. There were no substance-related signs of local irritation up to 1000 mg/kg bw per day, the highest dose tested (Eigenberg, 2007).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity conducted according to OECD test guideline 451, groups of 60 male and 60 female C57BL/6J mice were given diets containing fluopyram (purity 94.5%) at a concentration of 0, 30, 150 or 750 ppm, equal to 0, 4.2, 20.9 and 105 mg/kg bw per day in males and 0, 5.3, 26.8 and 129 mg/kg bw per day in females, for up to 18 months. An interim kill was performed after 52 weeks of treatment on 10 males and 10 females from each group. Mortality and clinical signs were checked daily. Additionally, detailed physical examinations including palpation for masses were performed weekly throughout treatment. Body weight and feed consumption were measured weekly for the first 13 weeks of the study, then monthly thereafter. Haematology determinations were performed at approximately 12 and 18 months from designated animals. Where possible, blood smears were prepared from moribund animals just before termination. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final termination. Designated tissues were fixed and examined microscopically.

There was no treatment-related effect on mortality, clinical signs or feed consumption at any dose level tested. Body weight at 750 ppm in males was comparable to that of controls from week 1 to week 26. Between weeks 30 and 58 of the study, body weight was reduced by up to 5% and cumulative body weight gain by up to 13% over this period from the start of treatment. The effect was statistically significant at most time points during this period. Thereafter, body weight and cumulative body weight gain were comparable to those of controls until the end of the study. Body weight parameters were not affected in females at 750 ppm or in either sex at 150 or 30 ppm.

Haematological evaluation showed slightly, but statistically significantly, higher platelet counts at 750 ppm in males at month 13 (+25%) and month 19 (+22%).

At the interim kill after 12 months, absolute and relative liver weights at 750 and 150 ppm were statistically significantly increased by 11–25% in males and by 17–30% in females, whereas no effect was seen at 30 ppm. Absolute and relative kidney weights at 750 ppm were statistically significantly lower in both sexes, whereas no effect was seen at 150 or 30 ppm. At the microscopic examination of the thyroid gland, follicular cell hyperplasia was seen in 2 of 10 and 2 of 9 males at 750 and 150 ppm, respectively.

At the termination of the study, absolute and relative liver weights at 750 and 150 ppm were statistically significantly increased by 14–31% in males and by 13–36% in females. In addition, absolute and/or relative kidney weights at 750 ppm were statistically significantly lower, by 7–9% in males and by 5–6% in females. The macroscopic examination revealed enlarged liver at 750 ppm in 3 of 42 males and 30 of 38 females, and dark liver was noted in 14 of 42 males and 4 of 38 females. These findings were associated with relevant histopathological findings (Table 24).

At microscopic examination, treatment-related changes were seen in the liver, kidney and thyroid gland. The only neoplastic change was a statistically significantly greater incidence of follicular cell adenoma of the thyroid gland in males (7/50), compared with the concurrent control group (1/50) and the historical controls. Historical control incidences for thyroid follicular cell adenoma from 10 studies conducted at the same laboratory were 2/492 (0.4%, range 0–2%) for male mice and 12/492 (2.4%, range 0–6%) for female mice (van Goethem, Wason & Mileson, 2009).

Non-neoplastic changes were seen in the liver, kidney and thyroid gland. In the liver, a higher incidence of eosinophilic foci of altered hepatocytes was observed in females at 750 ppm. Centrilobular to panlobular hypertrophy was observed in both sexes, with a dose-related effect at 750 and 150 ppm. Hepatocellular cholestasis was noted in males at 750 ppm. Higher incidences of hepatocellular single-cell degeneration/necrosis were noted in males at 750 and 150 ppm, together with interstitial mixed-cell infiltrate, eosinophilic inclusion bodies and multinucleated hepatocytes in males at 750 ppm only. At 750 ppm, there was a markedly lower incidence of mainly centrilobular hepatocellular vacuolation with concomitant minimal to moderate hepatocellular hypertrophy in males. At 30 ppm, there was a higher incidence of mainly centrilobular hepatocellular vacuolation in males. However, in the absence of an effect at the higher dose level of 150 ppm, this finding was considered to have occurred by chance at 30 ppm. In the kidney, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaline casts and interstitial mononuclear cell infiltrate, together with a higher incidence of glomerular congestion/haemorrhages, associated with higher severity of amyloid deposition (mainly observed within glomerular interstitium), were noted in females at 750 ppm. In the thyroid gland, a markedly higher incidence of follicular cell hyperplasia was noted in both sexes at 750 ppm and in males at 150 ppm (Table 24).

The NOAEL for oncogenicity in the mouse was 150 ppm, equal to 20.9 mg/kg bw per day, based on an increased incidence of follicular cell adenoma in the thyroid gland in males at 750 ppm, equal to 105 mg/kg bw per day. The NOAEL for non-neoplastic changes was 30 ppm, equal to 4.2 mg/kg bw per day, based on liver toxicity (hepatocellular single-cell degeneration/necrosis) and thyroid gland changes (follicular cell hyperplasia) in males at 150 ppm and above (Wason, 2007).

Table 24. Summary of selected findings in an 18-month carcinogenicity study in mice: terminal sacrifice

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	750	0	30	150	750
Liver weight								
- Absolute (g)	1.17	1.26*	1.34**	1.49**	1.26	1.31	1.45**	1.70**
- Relative (% of body weight)	4.23	4.50**	4.83**	5.53**	5.24	5.36	5.92**	7.14**
Kidney weight								
- Absolute (g)	0.542	0.557	0.556	0.491**	0.432	0.447	0.435	0.408
- Relative (% of body weight)	1.95	1.99	2.01	1.82**	1.80	1.83	1.78	1.71**
Liver								
No. examined	*49*	*49*	*49*	*50*	*48*	*50*	*50*	*50*
Eosinophilic foci	1	0	0	2	0	1	2	5*
Hepatocellular hypertrophy	0	0	38**	50**	0	0	18**	26**
Hepatocellular cholestasis	0	0	2	31**	0	0	0	0
Hepatocellular degeneration/necrosis	1	2	7*	40**	1	1	0	2
Interstitial mixed-cell infiltrate	18	16	19	40**	10	11	12	8
Eosinophilic inclusion bodies	2	3	5	19**	0	0	0	0
Multinucleated hepatocytes	3	1	4	27**	1	0	0	0
Hepatocellular vacuolation	27	38*	27	3**	5	10	1	1
Kidney								
No. examined	*50*	*50*	*50*	*50*	*48*	*50*	*50*	*50*
Cortical basophilic tubules	36	35	33	22**	19	14	21	34**
Glomerular congestion	0	0	0	1	2	2	3	27**
Amyloid deposition	32	20	23	29	43	40	40	41
Hyaline casts	3	1	3	1	0	2	1	11**
Interstitial mononuclear cell infiltrate	32	29	29	27	42	38	39	42
Thyroid								
No. examined	*50*	*50*	*50*	*50*	*48*	*50*	*50*	*50*
Follicular cell hyperplasia								
- Total	4	6	21**	32**	17	8*	19	33**
- Minimal	0	2	10	18	11	4	10	10
- Slight	3	2	5	6	5	2	5	12
- Moderate	1	2	2	5	1	2	3	6
- Marked	0	0	3	3	0	0	1	4
- Severe	0	0	1	0	0	0	0	1
Follicular cell adenoma	1	1	3	7*	3	1	3	1

From Wason (2007)
* $P < 0.05$; ** $P < 0.01$

Rats

In a combined study of chronic toxicity and carcinogenicity conducted according to OECD test guideline 453, groups of 70 male and 70 female Wistar Rj: WI (IOPS HAN) rats were given diets containing fluopyram (purity 94.5%) at a concentration of 0, 30, 150 or 750 ppm (reduced to

375 ppm from week 85 onwards due to the high mortality rate in this dose group over the first year) for males, equal to 0, 1.2, 6.0 and 29 mg/kg bw per day, and at a concentration of 0, 30, 150 and 1500 ppm for females, equal to 0, 1.68, 8.6 and 89 mg/kg bw per day, for up to 24 months. An interim kill of 10 males and 10 females from each group was performed after 52 weeks of treatment. Mortality and clinical signs were checked daily. Detailed physical examinations including palpation for masses were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Feed consumption was recorded twice weekly for the first 6 weeks of the study, then weekly up to week 13 and approximately every 4 weeks thereafter. Ophthalmological examinations were performed on all animals during acclimatization and after 12 and 24 months. Haematology, clinical chemistry determinations and urinalysis were performed during months 3, 6, 12, 18 and 24 on selected animals. All surviving animals allocated to the chronic and carcinogenicity phases were subjected to necropsy after a minimum of 52 weeks or 104 weeks of treatment, respectively. Selected organs were weighed, and designated tissues were sampled and examined microscopically. Decedent animals were subjected to necropsy, and designated tissues were sampled and examined microscopically.

During the first year of treatment, there were no treatment-related clinical signs. During the second year of treatment, a higher incidence of hair loss and wasted appearance was noted in high-dose females (1500 ppm), whereas in high-dose males (750/375 ppm), a slightly higher incidence of the usual signs associated with morbidity (prostration, general pallor and soiled anogenital region) was noted, reflecting the higher mortality observed in this group. No treatment-related clinical signs were noted in the mid- and low-dose groups.

Within the first year in high-dose males, 11 of 70 animals were found dead or were terminated prematurely for humane reasons, compared with 6 of 70 in the control group (Table 25). The main clinical signs in these early decedent males consisted of soiled fur or anogenital region (3/11) and focal swelling (2/11), together with usual signs associated with morbidity (limited use of hindlimbs, reduced motor activity, general pallor, wasted appearance). No effect on mortality was noted in females. After 2 years of treatment, analysis of the survival rates showed that the mortality incidence was statistically significantly increased in high-dose males and in low-dose females. The higher mortality in males was considered to be treatment related, as the trend test was statistically significant; in low-dose females, in contrast, it was considered to be incidental and not related to treatment, as the trend test was not statistically significant. No clear factor contributing to the death of these animals could be established.

Body weights or body weight gains in high-dose females were essentially comparable to those of controls throughout the first 3 months of treatment. Thereafter, cumulative body weight gain was lower than in the control group between weeks 14 and 26 (−29%), weeks 26 and 54 (−15%) and weeks 54 and 79 (−59%), whereas body weight was lower by 3%, 5%, 14% and 12% at weeks 26, 54, 79 and 102 when compared with the controls. In high-dose males, body weights or body weight gains were essentially comparable to those of the controls throughout the study. At the mid- and low-dose levels (150 and 30 ppm), body weight or body weight gain was unaffected by the treatment in both sexes. Feed consumption was similar to that of the controls throughout the study in both sexes and at all dose levels, with the only exception being a slight reduction by up to 7% in high-dose males between study days 18 and 39.

At the ophthalmological assessment, an abnormal colour (pale) of the retinal fundus was observed in 4 of 67 high-dose females, compared with 0 of 69 controls. No treatment-related ophthalmological findings were noted in males or in mid- and low-dose females at the end of the first year of treatment. At the end of the second year of treatment, higher incidences of small retinal vessels, abnormal colour (pale) of the retinal fundus and hyperreflectivity in the retina were noted in high-dose females. In mid- and/or high-dose males, higher incidences of corneal opacity, oedema of the cornea, nuclear opacity of lens, small retinal vessels and abnormal colour (pale) of the retinal fundus

Table 25. Summary of selected findings in a 24-month combined toxicity/carcinogenicity study in rats

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	750/375[a]	0	30	150	1500
Mortality, week 52								
- *N*[b]	6/70	3/70	2/70	11/70	1/70	2/70	5/70	3/70
- (%)	(8.6)	(4.3)	(2.9)	(15.7)	(1.4)	(2.9)	(7.1)	(4.3)
Mortality, week 104								
- *N*[c]	37/60	44/60	34/60	49/60	19/60	32/60	25/60	30/60
- (%)	(61.7)	(73.3)	(56.7)	(81.7*)	(31.7)	(53.3*)	(41.7)	(50.0)
Body weight (g), day 708	672	639	686	627	425	452	425	374*
Body weight gain (g), days 1–708	453	421	470	410	269	296	269	216**
Retinal fundus: pale								
- Week 52	0/65	0/68	1/68	0/59	0/69	1/68	0/66	4/67
- Week 104	2/25	1/21	2/31	6/14	3/43	3/31	2/37	15/32
Urine: cellular casts								
- Month 3	1/19	2/19	10/20	17/19	0/18	0/20	0/20	0/19
- Month 6	0/20	0/19	7/20	19/20	0/20	0/20	0/18	0/18

From Kennel (2008)
* $P < 0.05$; ** $P < 0.01$
[a] Dietary concentration was lowered to 375 ppm from week 85 onwards.
[b] Animals allocated to the chronic toxicity and carcinogenicity study phases.
[c] Animals allocated to the carcinogenicity study phase.

were noted. No treatment-related ophthalmological findings were observed in mid-dose females or at the low dose in either sex at the end of the second year of treatment.

Haematological analysis showed a significant tendency towards lower erythrocyte parameters in high-dose females throughout the study and in high-dose males at most time points, whereas platelet count was significantly higher in high-dose males and females at month 6.

Clinical chemistry evaluation in high-dose females showed slightly higher total cholesterol concentrations throughout the study, whereas higher triglyceride concentrations were observed at months 3 and 6 (+36%), and slightly lower mean glucose concentrations were noted at months 6, 12 and 18 (−9%, −13% and −16%, respectively).

Abnormal colour of urine was noted in high-dose females at month 6 (red colour in 9/18 animals), month 12 (orange to dark orange colour in 14/20 animals) and month 18 (orange colour in 6/8 animals). In high- and mid-dose males, a dose-related increase in the incidence and severity of cellular casts was observed at months 3 and 6, whereas this finding was noted in only 1 of 20 high-dose males at month 12 and not observed any longer at months 18 and 24. No treatment-related findings were noted at the urinalysis at the intermediate dose level in females or at the low dose level in either sex.

At the interim termination after 12 months, body weight in high-dose females was 10% lower than that of the controls but was unaffected at all dose levels tested in males and at the intermediate and low dose levels in females (Table 26). Also in the high-dose groups, the following increases in organ weights were noted: absolute and relative liver weights in males (17–18%) and females (39–54%), absolute and relative kidney weights in males (28%), relative kidney weight in females (22%) and absolute and relative thyroid gland weights in females (23–38%). This last effect was associated with follicular cell hypertrophy at microscopic examination.

Table 26. Summary of selected morphological findings in a 24-month combined toxicity/carcinogenicity study in rats: interim sacrifice (after 52 weeks)

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	750[a]	0	30	150	1500
Body weight (g)	655	659	662	653	346	338	339	313
Liver weight								
- Absolute (g)	12.21	12.70	12.43	14.31*	7.51	7.37	7.67	10.44**
- Relative (% of body weight)	1.86	1.93	1.88	2.19**	2.17	2.19	2.26	3.35**
Kidney weight								
- Absolute (g)	3.51	3.64	3.78	4.48**	2.39	2.40	2.48	2.64
- Relative (% of body weight)	0.54	0.55	0.58	0.69**	0.70	0.71	0.73	0.85**
Thyroid weight								
- Absolute (mg)	27.2	28.0	24.4	29.5	20.1	19.6	21.3	24.8
- Relative (% of body weight, ×1000)	4.2	4.3	3.8	4.5	5.8	5.7	6.3	8.0**
Liver								
No. examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Eosinophilic foci	2	1	2	5	0	0	0	3
Hepatocellular hypertrophy	0	0	3	10	0	0	0	10
Hepatocellular macrovacuolation	0	5	6	9	0	0	0	7
Hepatocellular vacuolation	0	5	0	2	2	2	2	4
Hepatocellular single-cell necrosis	0	0	0	0	0	0	0	2
Increased no. of mitoses	0	0	0	0	2	0	1	6
Kidney								
No. examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Chronic progressive nephropathy	0	0	3	2	0	0	0	0
Intratubular brown pigment	0	3	1	0	1	2	1	5
Hyaline droplets, proximal tubules	0	1	8	10	0	0	0	0
Basophilic tubules, bilateral	3	4	0	6	2	0	0	2
Hyaline casts	2	1	0	1	1	0	1	4
Thyroid								
No. examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Follicular cell hyperplasia, total	0	0	2	4	0	0	0	5
Colloid alteration	4	5	4	7	1	0	0	4

From Kennel (2008)

* $P < 0.05$; ** $P < 0.01$

[a] Dietary concentration was lowered to 375 ppm from week 85 onwards.

Gross pathological findings in high-dose females were enlarged liver in 9 of 10 animals, dark liver in 8 of 10 animals and prominent lobulation of the liver in 4 of 10 animals, compared with zero incidence in the controls. In high-dose males, enlarged liver was found in 1 of 10 animals and prominent lobulation of the liver in 3 of 10 animals, compared with zero incidence in the controls. Dark kidneys were found in 8 of 10 high-dose females, compared with zero incidence in the controls, whereas pale kidneys, enlarged kidneys or irregular surface of the kidneys was found in some high-dose males, compared with zero incidence in the controls.

In the liver of high-dose females, a higher incidence of altered hepatocytes (eosinophilic foci), focal/multifocal hepatocellular vacuolation, increased number of mitoses, hepatocellular single-cell necrosis, hepatocellular brown pigments, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. In high-dose males, higher incidences of altered hepatocytes (eosinophilic foci), centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. Hepatocellular vacuolation or macrovacuolation noted in mid- and low-dose males was considered not to be adverse, as it was not associated with any degenerative change in the liver. No treatment-related changes were noted in the liver at the intermediate and low doses in females.

In the kidney of high-dose females, higher incidences of tubular golden/brown pigments and hyaline casts were noted. In high- and mid-dose males, chronic progressive nephropathy was observed, together with a higher incidence of hyaline droplets. No treatment-related changes were noted in mid-dose females or in either sex at the low dose.

In the thyroid gland of high-dose females and males, follicular cell hypertrophy was noted, together with a higher incidence and severity of colloid alteration. In the male mid-dose group, only follicular cell hypertrophy was observed. No treatment-related changes were noted in the thyroid gland in mid-dose females or in either sex at the low dose.

At the termination of the study after 24 months, body weight in the high-dose groups was 11% lower in females and 7% lower in males, compared with the controls, and was unaffected at the intermediate and low doses in both sexes (Table 27). In high-dose females, absolute and relative liver weights were 39–56% higher than those of the controls and were associated with a higher incidence of enlarged liver, dark liver, prominent lobulation, and red and white foci on the liver at macroscopic observation, compared with the controls. Also, liver nodules/masses (5/60 females, compared with zero incidence in the controls) were noted and associated with liver cell carcinoma or adenoma noted at microscopic examination, the increase in carcinomas not reaching statistical significance. In high-dose males, absolute and relative liver weights were 5–12% higher than those of the controls. At macroscopic observation, higher incidences of enlarged liver and white foci on the liver were found, together with higher incidences of enlarged kidney and irregular surface of the kidney, compared with the controls.

At microscopic examination, a higher incidence of tumours (adenoma and carcinoma) in the liver was noted in high-dose females (11/59), in comparison with the concurrent controls (2/60) and the historical controls (11/585, mean 1.9%, range 0–5%) from 10 studies conducted at the same laboratory (van Goethem, Wason & Mileson, 2009). These findings were associated with non-neoplastic/preneoplastic changes and were seen at a dose causing marked hepatocellular toxicity. There was no evidence of a treatment-related increased incidence of tumours of any type in any other organ.

Non-neoplastic histological findings in the liver of high-dose females included proliferative changes such as altered hepatocytes (eosinophilic foci), clear cell foci, multinucleated hepatocytes with anisocaryosis or an increased number of mitoses and were associated with metabolic morphological changes: centrilobular to panlobular hypertrophy, focal/multifocal hepatocellular vacuolation, centrilobular to midzonal hepatocellular macrovacuolation, brown pigments in Kupffer cells or hepatocellular brown pigments. Also, degenerative changes (hepatocellular single-cell necrosis) and minimal to slight extramedullary haematopoiesis were observed. In high-dose males, a higher incidence of altered hepatocytes (eosinophilic foci), centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were noted. In mid-dose males, only eosinophilic foci and centrilobular to panlobular hypertrophy were observed, whereas no treatment-related changes in the liver were noted in mid-dose females or in either sex at the low dose.

In the kidney, marked degenerative changes were noted at the high dose in both sexes and in mid-dose males. Specific findings (tubular hyperplasia, tubular dilatation or renal cysts) were judged to be associated with chronic nephropathy. In addition, increased incidences of tubular golden/brown

Table 27. Summary of selected morphological findings in a 24-month combined toxicity/ carcinogenicity study in rats: terminal sacrifice (after 104 weeks)

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	750[a]	0	30	150	1500
Body weight (g)	617	577	637	576	400	422	393	358
Liver weight								
- Absolute (g)	12.56	11.09*	14.02*	13.24	9.46	9.69	9.89	13.16**
- Relative (% of body weight)	2.06	1.94	2.22	2.32	2.37	2.31	2.52	3.70**
Kidney weight								
- Absolute (g)	4.52	4.17	4.86	5.08	2.95	3.15	3.12	3.12
- Relative (% of body weight)	0.75	0.73	0.77	0.90	0.58	0.77	0.81	0.89*
Thyroid weight								
- Absolute (mg)	29.5	29.7	30.4	30.3	23.8	24.5	25.1	23.4
- Relative (% of body weight, ×1000)	4.92	5.20	4.79	5.28	6.06	5.96	6.45	6.65*
Liver								
No. examined	*60*	*60*	*60*	*58*	*60*	*60*	*60*	*59*
Hepatocellular hypertrophy	1	1	14**	30**	0	0	0	48**
Clear cell foci	10	8	7	16	1	4	4	11**
Eosinophilic foci	16	24	31*	28**	29	26	30	48**
Hepatocellular vacuolation	10	7	16	7	6	14**	9	22**
Increased no. of mitoses	0	0	0	0	6	1	5	33**
Multinucleated hepatocytes	1	0	1	0	4	2	6	38**
Hepatocellular single-cell necrosis	2	0	2	1	0	4	1	37**
Hepatocellular brown pigment	0	0	0	0	1	1	2	24**
Kupffer cells with brown pigment	7	2	10	7	12	10	11	32**
Hepatocellular macrovacuolation	0	0	2	10**	0	0	0	11**
Extramedullary haematopoiesis	18	10	11	16	19	25	24	33**
Hepatocellular adenoma	2	1	2	1	2	2	0	9*
Hepatocellular carcinoma	0	0	0	0	0	0	2	3
Hepatocellular adenoma/carcinoma	2	1	2	1	2	2	2	11**[b]
Kidney								
No. examined	*60*	*60*	*60*	*60*	*60*	*60*	*59*	*60*
Chronic progressive nephropathy	31	37	42	52**	20	24	23	42**
Tubular hypertrophy	9	11	24**	19**	9	16	13	12
Tubular hyperplasia	5	2	8	11*	2	5	4	5
Collecting duct hyperplasia	5	2	5	9	2	2	0	8
Intratubular golden/brown pigment	4	3	4	8	32	19	24	57**
Hyaline droplets, proximal tubules	3	2	1	10	2	2	1	2
Cortical tubular dilatation	10	5	15	28**	4	5	7	16**
Medullary tubular dilatation	10	15	22*	40**	13	8	12	22*
Renal cysts	7	3	6	13*	2	2	3	3
Thyroid								
No. examined	*58*	*59*	*57*	*54*	*60*	*60*	*60*	*58*

Table 27 (continued)

	Males				Females			
	Dietary concentration (ppm)							
	0	30	150	750[a]	0	30	150	1500
Follicular cell hypertrophy	1	0	4	7**	0	0	1	12**
Follicular cell hyperplasia	1	0	1	3	2	2	1	4
Colloid alteration	28	15	32	39**	7	7	16*	36**
Eye								
No. examined	*60*	*60*	*60*	*59*	*60*	*60*	*60*	*60*
Retinal atrophy, bilateral	0	0	1	0	0	0	1	27**
Peripheral retinal atrophy, bilateral	1	0	0	1	3	1	1	7
Lenticular degeneration, focal	2	1	1	2	3	3	4	9*

From Kennel (2008)

* $P < 0.05$; ** $P < 0.01$

[a] Dietary concentration was lowered to 375 ppm from week 85 onwards.

[b] One animal had both adenoma and carcinoma.

pigments and collecting duct hyperplasia were noted in high-dose females, higher incidences of tubular hypertrophy, collecting duct hyperplasia and hyaline droplets were noted in high-dose males and a higher incidence of tubular hypertrophy was noted in mid-dose males. There were no treatment-related changes in the kidney in mid-dose females or in either sex at the low dose.

In the thyroid gland, in the high-dose groups, follicular cell hypertrophy together with a higher incidence of colloid alteration and a slightly higher incidence of follicular cell hyperplasia were noted. At the intermediate dose, follicular cell hypertrophy was observed in males and a higher incidence of colloid alteration was noted in females. There were no treatment-related changes in the thyroid at the low dose in either sex.

In the eye of high-dose females, bilateral retinal atrophy was observed, together with higher incidences of lens degeneration and peripheral bilateral retinal atrophy. Retinal atrophy was characterized by degeneration of the outer plexiform layer, outer nuclear layer and rods/cones lamina. No treatment-related changes in the eye were noted in males or in mid- and low-dose females.

In the testis, a higher incidence of arteritis/periarteritis was noted in high- and mid-dose males (15/59 and 18/60, respectively, versus 8/60 in controls, $P \leq 0.05$). This vascular change was isolated (not found in sensitive tissues such as aorta, mesenteric arteries) and is most likely explained by secondary hypertensive changes due to increased severity and incidence of chronic nephropathy. Therefore, this change was considered not to be a direct effect of the treatment.

In the stomach of high-dose males, higher incidences of regenerative non-glandular hyperplasia: focal/multifocal (10/58 versus 6/58 in controls), non-glandular erosion: focal/multifocal (7/58 versus 3/58 in controls) and submucosal oedema (10/58 versus 4/58 in controls, $P \leq 0.05$) were noted. These minor changes were mainly observed in animals found prematurely dead and were attributed to secondary stress due to morbidity. Therefore, their increased incidence was explained by the increased mortality rate in the male high-dose group.

The NOAEL for oncogenicity in the rat was 150 ppm, equal to 8.6 mg/kg bw per day, based on an increased incidence of liver cell tumours (adenoma and carcinoma) in females at 1500 ppm, equal to 89 mg/kg bw per day. The NOAEL for non-neoplastic changes was 30 ppm, equal to 1.2 mg/kg bw per day, based on increased incidences of findings in the liver (hepatocellular hypertrophy and eosinophilic foci in males) at 150 ppm and above. The hyaline droplet nephropathy observed in male rats was considered not to be relevant to humans, as this effect is due to an accumulation of α_{2u}-globulin

in the proximal tubules, a protein that is found only in trace amounts in humans. The changes in the thyroid (follicular cell hypertrophy, colloid alteration) at 150 ppm and above were attributable to the apparent susceptibility of rats to thyroid hormone imbalance and were therefore not considered relevant to humans (Kennel, 2008).

2.4 Genotoxicity

In a reverse gene mutation assay in bacteria conducted according to OECD test guideline 471, *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to fluopyram (purity 94.7%), using dimethyl sulfoxide (DMSO) as solvent, in the presence and absence of S9 metabolic activation in two independent sets of experiments. For the initial plate incorporation test using doses up to and including 5000 µg/plate, three plates were used for each strain, condition and dose. Vehicle and positive controls were included in each experiment. The independent repeat was performed with preincubation for 20 minutes at 37 °C. Other conditions remained unchanged. Doses up to and including 5000 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged, and no inhibition of growth was observed. Substance precipitation occurred at the dose 1581 µg/plate and above. Nevertheless, evaluation was possible up to and including 5000 µg/plate.

No evidence for mutagenic activity of fluopyram was seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls induced the appropriate responses in the corresponding strains. Therefore, fluopyram was considered to be not mutagenic in the bacterial strains tested, in either the presence or absence of metabolic activation (Wirnitzer, 2006; Herbold, 2009a,b).

A second reverse gene mutation assay was conducted in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, because the impurity profile of the technical material was slightly different from that of the test material used in the initial test. This assay was also performed according to OECD test guideline 471. Bacteria were exposed to fluopyram (purity 95.7%), using DMSO as solvent, in the presence and absence of S9 metabolic activation in two independent sets of experiments. For the initial plate incorporation test using doses up to and including 5000 µg/plate, three plates were used for each strain, condition and dose. Vehicle and positive controls were included in each experiment. The independent repeat was performed with preincubation for 20 minutes at 37 °C using doses up to and including 1581 µg/tube. Other conditions remained unchanged. Doses up to and including 158 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged, and no inhibition of growth was observed. At higher doses (≥ 500 µg/plate), the substance had a strain-specific bacteriotoxic effect. Therefore, only the doses up to and including 1581 µg/plate were used for assessment.

No evidence of mutagenic activity of fluopyram was seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls induced the appropriate responses in the corresponding strains. Therefore, fluopyram was considered to be not mutagenic in the bacterial strains tested, in either the presence or absence of metabolic activation (Herbold, 2008, 2009c).

In an in vitro mammalian cell gene mutation test conducted according to OECD test guideline 476, fluopyram (purity 94.7%) dissolved in DMSO was tested for its ability to induce forward mutations at the HPRT locus in Chinese hamster V79 cells. Two independent sets of experiments were conducted in the presence and absence of S9 metabolic activation. Based on the results of a preliminary cytotoxicity assay, concentrations of 4–256 µg/ml were used in the main study both with and without metabolic activation, and the same concentrations were used for the independent

repeats. Only without S9 mix, cytotoxic effects were observed at 256 µg/ml. However, precipitation was observed under both activation conditions, starting at 128 µg/ml. Ethyl methanesulfonate and dimethylbenzanthracene served as positive controls in the experiments without and with metabolic activation, respectively. The cells were treated for 5 hours in both experiments, without and with metabolic activation. After this, the incubation media were replaced by culture medium, and the cells were incubated for about 1 week for expression of mutant cells. This was followed by incubation of cells for 1 week in selection medium containing 6-thioguanine.

Neither in the initial nor in the confirmatory studies was any increase in the mutant frequency observed. In contrast to this, the positive control substances ethyl methanesulfonate and dimethylbenzanthracene resulted in a marked increase in mutant frequency. Based on the results of the study, fluopyram was considered to be not mutagenic in the V79/HPRT forward mutation assay, in either the presence or absence of metabolic activation (Herbold, 2006).

In an in vitro mammalian chromosomal aberration test conducted according to OECD test guideline 473, the clastogenic potential of fluopyram (purity 94.7%) dissolved in DMSO was tested in Chinese hamster V79 cells. Based on the results of preliminary assays (cytotoxicity at ≥ 120 µg/ml without S9 mix and at ≥ 180 µg/ml with S9 mix, precipitation at ≥ 120 µg/ml), concentrations of 60, 120 and 180 µg/ml were used with and without metabolic activation in an experiment with 4 hours of treatment and harvest at 18 hours (60–180 µg/ml) or 30 hours (180 µg/ml). In addition, with 18 hours of treatment without S9 mix, concentrations of 60, 120 and 180 µg/ml were chosen. Vehicle (DMSO) and positive controls (cyclophosphamide and mitomycin C for the test with and without metabolic activation, respectively) were included to demonstrate the sensitivity of the test system. Prior to cell harvest, Colcemid was added to arrest cells in metaphase. After slide preparation and staining of the cells, 200 metaphases per dose and treatment condition were analysed for chromosomal aberrations.

None of the cultures treated with fluopyram in the absence or in the presence of S9 mix showed any biologically relevant or statistically significant increase in the numbers of aberrant metaphases. The positive controls mitomycin C and cyclophosphamide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the S9 mix used. Based on the results of this test, fluopyram was considered not to be clastogenic for mammalian cells in vitro, in either the presence or absence of metabolic activation (Nern, 2005).

In a mammalian erythrocyte micronucleus test conducted according to OECD test guideline 474, groups of five male Hsd/Win: NMRI mice received two intraperitoneal doses (24 hours apart) of fluopyram (purity 94.7%) at 0 (0.5% aqueous Cremophor emulsion), 250, 500 or 1000 mg/kg bw in a volume of 10 ml/kg bw. The vehicle served as negative control, and cyclophosphamide (1× 20 mg/kg bw) as positive control. The animals were terminated 24 hours after the last administration, the bone marrow of the two femora was prepared, and 2000 polychromatic erythrocytes were evaluated per animal and investigated for micronuclei. The number of normochromatic erythrocytes with and without micronuclei occurring per 2000 polychromatic erythrocytes was also recorded.

A dose-related increase in clinical signs was observed in fluopyram-treated mice, starting at 250 mg/kg bw: apathy, semi-anaesthetized state, roughened fur, loss of weight, sternal recumbency, spasm, periodic stretching of the body and difficulty breathing. There was no substance-induced mortality. No signs were recorded for the control groups, and no animals died in these groups.

Two intraperitoneal doses of fluopyram up to and including 1000 mg/kg bw did not lead to any increase in the number of polychromatic erythrocytes with micronuclei. A slight inhibition of erythropoiesis, determined from the ratio of polychromatic to normochromatic erythrocytes, indicated that the test substance did reach the bone marrow. The positive control caused a clear increase in the

Table 28. Summary of genotoxicity studies with fluopyram

End-point	Test object	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*S. typhimurium* (TA98, TA100, TA102, TA1535, TA1537)	±S9 mix: 0, 16, 50, 158, 500, 1581, 5000 µg/plate	94.7	Negative	Wirnitzer (2006)
Reverse mutation	*S. typhimurium* (TA98, TA100, TA102, TA1535, TA1537)	±S9 mix: 0, 5, 16, 50, 158, 500, 1581, 5000 µg/plate	95.7	Negative	Herbold (2008)
Gene mutation, HPRT locus	Chinese hamster V79 cells	±S9 mix: 0, 4, 8, 16, 32, 64, 128, 256 µg/ml	94.7	Negative	Herbold (2006)
Chromosomal aberration	Chinese hamster V79 cells	±S9 mix: 0, 60, 120, 180 µg/ml	94.7	Negative	Nern (2005)
In vivo					
Micronucleus induction	Male Hsd/Win: NMRI mice bone marrow erythroblasts	0, 250, 500 and 1000 mg/kg bw; twice (24 h apart); intraperitoneal administration	94.7	Negative	Herbold (2005)

S9, 9000 × *g* rat liver supernatant

number of polychromatic erythrocytes with micronuclei, thus demonstrating the sensitivity of the test system. Based on the results of this test, fluopyram was considered not to be clastogenic in vivo in mice (Herbold, 2005).

The genotoxicity studies conducted with fluopyram are summarized in Table 28.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a two-generation reproductive toxicity study conducted according to OECD test guideline 416, groups of 30 male and 30 female Wistar Han Crl: WI(HAN) rats were given diets containing fluopyram (purity 94.7%) at a concentration of 0, 40, 220 or 1200 ppm during the premating (10 weeks), mating (14 days) and gestation periods (approximately 22 days); during the lactation period (21 days), the concentration of the test substance in the diet was adjusted down by 50%. The doses were selected based upon the results from a previous range-finding study (Milius, 2008). F_1 pups were maintained after weaning for approximately 6 weeks prior to initiation of the second generation. The mean test substance intakes during the premating period were equal to 0, 2.6–2.7, 13.9–15.1 and 82.4–83.1 mg/kg bw per day in males (F_1 and P generations, respectively) and 0, 3.1–3.2, 16.8–17.6 and 95.6–96.3 mg/kg bw per day in females (F_1 and P generations, respectively). During the lactation period, intakes were equal to 0, 3.1–3.3, 15.9–18.1 and 92.5–103.2 mg/kg bw per day (F_1 and P generations, respectively). Parental animals were observed for clinical signs and mortality, body weight, feed consumption, estrous cyclicity, and haematology and clinical chemistry parameters. The size of each litter was adjusted on lactation day 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (e.g. three females and five males). No adjustments were made for litters of fewer than eight pups. On lactation day 21, a sufficient number of F_1 pups of each sex per litter was maintained to produce the next generation. F_1 pups not selected to become parents of the next generation and all F_2 pups were terminated and examined macroscopically, and their organs were weighed. One pup of each

sex per litter for each generation had tissues collected and evaluated for histopathological changes, particularly as they related to the organs of the reproductive system. Terminal studies on parental animals included gross findings at necropsy and histopathological examinations of selected tissues. For males, sperm was collected from one testes and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, the morphology, number and motility of sperm from the distal portion of the vas deferens were evaluated.

There were no substance-related mortalities or clinical observations at any dietary level tested in either generation. There were no substance-related findings on body weight or feed consumption in P and F_1 generation males. In females at 1200 ppm, a decline in body weight and/or body weight gain was noted during the premating period and during gestation in the P generation, whereas body weight and feed consumption were increased during gestation in the F_1 generation (Table 29).

Assessment of reproductive function including sperm parameters and estrous cycle number or length revealed no treatment-related effects. Also, reproductive performance was not affected for any parameter (e.g. mating, fertility or gestation indices, days to insemination, gestation length or the median number of implants) in either generation at any dietary level tested.

Haematological changes were noted at 1200 ppm in P and/or F_1 generation females (decreased haemoglobin and/or haematocrit) and in F_1 generation females (increased white blood cell and monocyte absolute cell counts).

Clinical chemistry findings were observed at 1200 ppm in P generation males (increased creatinine, total protein and albumin levels), in F_1 generation males (increased urea nitrogen and total protein levels) and F_1 generation females (increased cholesterol levels).

Terminal studies on parental animals showed that test substance–related organ weight changes were limited to the kidneys, liver and spleen. Kidney weights (absolute and relative) were increased at 1200 ppm in P and F_1 generation males. Liver weights (absolute and relative) were increased at 1200 ppm in P and F_1 generation males and females. Spleen weights were decreased at 1200 ppm in P generation females (absolute) and F_1 generation females (absolute and relative) and at 220 ppm in F_1 generation females (relative). The decrease in spleen weights in females at 1200 and 220 ppm was not associated with any corresponding histopathological findings. However, as spleen weights were also affected in F_1 and F_2 pups, this finding may be treatment related.

Treatment-related histopathological findings on parental animals were noted at 1200 ppm and included effects on the kidneys (increased incidence of protein droplet nephropathy and lymphocytic infiltration) in males and on the liver (increased incidence of centrilobular hypertrophy) in males and females. There was no evidence of test substance–related changes in the kidneys or liver at 220 ppm.

There were no substance-related effects on viability or clinical signs of the pups in either generation at any dietary level tested. Pup body weights at birth were comparable between the treated groups and the control group. For F_1 pups at 1200 ppm, body weight gain was reduced for both males (statistically significant decline of 7.8%) and females (non-statistically significant decline of 6.2%) from days 7 to 14 of lactation. For F_2 pups at 1200 ppm, non-statistically significant declines in body weight (6.1% less than control) by day 4 were observed, with statistically significant declines (by 8.1%) in body weight observed by day 21. Overall, for F_2 pups, body weight gain throughout lactation declined by 8.6%, compared with controls. No effects on body weight or body weight gain were observed in either generation at 220 or 40 ppm (Table 30).

A slight delay in preputial separation in the F_1 males at 1200 ppm (42.5 days versus 41.0 days in controls) was observed. Although statistically significant, this finding was well within the laboratory's historical control range (40.7–44.0 days) and was considered to be secondary to the decline in body weight gain observed during lactation. There were no findings on preputial separation at any other dietary level tested. There was no effect observed on vaginal patency at any dietary level tested. Anogenital distance was measured on lactation day 0 for the F_2 pups, with no effects noted.

Table 29. Summary of selected findings for P and F_1 generation rats

	Males				Females			
	Dietary concentration (ppm)							
	0	40	220	1200	0	40	220	1200
P generation								
Body weight gain, premating period[a] (g)	196.3	210.9	207.1	195.8	72.9	64.1	66.8	58.3
Body weight, gestation day 0 (g)	—	—	—	—	247.4	239.3	243.1	232.7**
Body weight gain, gestation period (g)	—	—	—	—	102.3	94.4	100.6	103.4
Body weight, terminal (g)	452.1	461.3	463.4	454.5	278.9	268.9	278.5	266.0
Liver weight, absolute (g)	15.31	15.65	15.63	18.70*	12.29	11.15*	12.31	13.49*
Liver weight, relative (% of body weight)	3.38	3.39	3.37	4.12*	4.39	4.14	4.42	5.08*
Spleen weight, absolute (g)	0.71	0.71	0.71	0.76	0.59	0.58	0.57	0.52*
Spleen weight, relative (% of body weight)	0.16	0.15	0.15	0.17	0.21	0.22	0.21	0.20
Liver: hepatocellular hypertrophy	0/30	0/0	0/30	29*/30	0/30	0/0	0/30	13*/30
Kidney: protein droplet nephropathy	0/30	0/0	0/30	30*/30	0/30	0/0	0/1	0/30
Sperm motility								
- % motile	89.8	89.5	89.8	89.9	—	—	—	—
- % progressive	64.0	62.7	64.6	63.3	—	—	—	—
Sperm counts (sperm/g)								
- Testis	38.2	N/A	N/A	34.92	—	—	—	—
- Epididymis	240.5	N/A	N/A	219.7	—	—	—	—
Sperm morphology (no.)								
- Normal	198.7	N/A	N/A	197.3	—	—	—	—
- Abnormal	0.9	N/A	N/A	2.0	—	—	—	—
- Detached head	0.3	N/A	N/A	0.8	—	—	—	—
F_1 generation								
Body weight gain, premating period[a] (g)	193.7	186.5	191.5	189.2	62.4	66.7	61.3	56.3
Body weight, gestation day 0 (g)	—	—	—	—	240.2	247.5	241.5	231.2
Body weight gain, gestation period (g)	—	—	—	—	89.1	90.8	85.3	100.9*
Body weight, terminal (g)	469.2	458.8	473.3	464.8	272.2	274.1	280.0	271.7
Liver weight, absolute (g)	15.19	14.96	15.71	19.24*	12.38	13.22	13.28	14.50*
Liver weight, relative (% of body weight)	3.23	3.26	3.32	4.14*	4.53	4.82	4.72	5.32*
Spleen weight, absolute (g)	0.71	0.70	0.73	0.74	0.61	0.57	0.56	0.52*
Spleen weight, relative (% of body weight)	0.15	0.15	0.15	0.16	0.22	0.21	0.20*	0.19*
Liver: hepatocellular hypertrophy	0/30	0/0	0/30	30*/30	0/30	0/0	0/30	7*/30
Kidney: protein droplet nephropathy	0/30	0/0	0/30	29*/30	0/30	0/0	0/2	0/30
Sperm motility								
- % motile	87.8	86.4	87.0	87.2	—	—	—	—
- % progressive	60.4	61.1	61.8	61.9	—	—	—	—
Sperm counts								
- Testis (sperm/g)	28.3	N/A	N/A	29.1	—	—	—	—
- Epididymis (sperm/g)	189.4	N/A	N/A	161.9	—	—	—	—
Sperm morphology (no.)								
- Normal	197.0	N/A	N/A	195.3	—	—	—	—

Table 29 (continued)

	Males				Females			
	Dietary concentration (ppm)							
	0	40	220	1200	0	40	220	1200
- Abnormal	2.0	N/A	N/A	4.2	—	—	—	—
- Detached head	1.0	N/A	N/A	0.5	—	—	—	—

From Milius & Bommegowda (2008)
N/A, not assessed; * $P < 0.05$; ** $P < 0.01$
[a] Weeks 1–15 or 1–14 for males (P or F_1, respectively), weeks 1–10 for females.

Table 30. Summary of selected findings for reproductive performance and litter parameters

	Dietary concentration (ppm)			
	0	40	220	1200
P generation – F_1 litter				
Number mated	30	29	30	28
Number of animals delivered	30	25	28	26
Number of animals with implantations	30	25	29	26
Mating index	100.0	96.7	100.0	93.3
Fertility index	100.0	86.2	96.7	92.9
Gestation index	100.0	100.0	96.6	100.0
Days to insemination	3.0	2.4	2.7	2.3
Gestation length (days)	21.9	21.8	21.8	21.8
Total number of implantation sites (mean)	377 (12.6)	274 (11.0)	323 (11.1)	304 (11.7)
Total number born	354	257	303	295
Number stillborn	1	1	0	1
Sex ratio day 0 (% male)	53.2	54.0	46.0	44.6
Mean litter size	11.8	10.3	10.8	11.3
Birth index	93.9	91.8	89.6	96.8
Live birth index	99.7	99.7	100.0	99.7
Viability index	99.7	96.0	99.3	97.8
Lactation index	98.8	99.5	99.6	99.5
Body weight, lactation days 21, males/females (g)	50.2/48.0	49.0/46.0	49.7/48.2	47.6/45.9
Preputial separation/vaginal opening (days)	41.0/34.8	41.8/36.1	41.5/34.4	42.5**/34.7
F_1 generation – F_2 litter				
Number mated	29	30	30	30
Number of animals delivered	27	27	27	28
Number of animals with implantations	27	27	27	29
Mating index	96.7	100.0	100.0	100.0
Fertility index	93.1	90.0	90.0	96.7
Gestation index	100.0	100.0	100.0	96.6
Days to insemination	2.9	3.0	2.3	2.8
Gestation length (days)	21.8	21.7	21.6	21.5
Total number of implantation sites (mean)	301 (11.1)	311 (11.5)	297 (11.0)	323 (11.1)
Total number born	287	299	289	303

Table 30 (continued)

	Dietary concentration (ppm)			
	0	40	220	1200
Number stillborn	4	0	1	3
Sex ratio day 0 (% male)	47.4	49.7	49.1	44.1
Mean litter size	10.6	11.1	10.7	10.8
Birth index	95.0	96.3	97.1	91.0
Live birth index	98.7	100.0	99.7	99.2
Viability index	99.6	98.2	98.1	98.3
Lactation index	98.6	94.9	98.8	99.6
Body weight, lactation days 21, males/females (g)	50.3/48.2	49.6/47.8	47.8/45.6	46.1**/44.6*
Spleen weight, absolute, males/females (mg)	233/233	238/235	225/219	205*/196**
Spleen weight, relative, males/females (% of body weight)	0.46/0.48	0.48/0.49	0.47/0.48	0.44/0.44*
Thymus weight, absolute, males/females (mg)	226/233	215/216	213/215	194**/194**
Thymus weight, relative, males/females (% of body weight)	0.45/0.49	0.44/0.45*	0.44/0.47	0.42/0.44**

From Milius & Bommegowda (2008)
* $P < 0.05$; ** $P < 0.01$

There were no treatment-related changes in organ weights in F_1 pups, whereas in F_2 pups at 1200 ppm, decreased spleen and thymus weights (absolute and relative) were noted in both males and females.

The NOAEL for effects on fertility was 1200 ppm, equal to 82.4 mg/kg bw per day in males and 95.6 mg/kg bw per day in females, the highest dose tested.

The NOAEL for parental toxicity was 220 ppm, equal to 13.9 mg/kg bw per day in males and 16.8 mg/kg bw per day in females, based on decreased body weight and/or body weight gain in females and liver toxicity in males and females at 1200 ppm.

The NOAEL for offspring toxicity was 220 ppm, equal to 13.9 mg/kg bw per day in males and 16.8 mg/kg bw per day in females, based on decreased body weight gain and decreased spleen and thymus weights at 1200 ppm (Milius & Bommegowda, 2008).

(b) Developmental toxicity

Rats

In a prenatal developmental toxicity study conducted according to OECD test guideline 414, groups of 23 time-mated female Sprague-Dawley Crl:CD(SD) rats received fluopyram (purity 94.6%) by oral gavage at a dose level of 0, 30, 150 or 450 mg/kg bw per day from gestation day (GD) 6 to GD 20. The test substance was administered as a suspension in an aqueous solution of 0.5% methylcellulose 400 at a dose volume of 10 ml/kg bw. Clinical observations were recorded daily. Maternal body weights were recorded for all females on GDs 0, 6, 8, 10, 12, 14, 16, 18 and 21. Feed consumption was also measured for all females during the intervals GDs 1–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18 and 18–21. At scheduled termination, on GD 21, the dams were subjected to a macroscopic examination of the visceral organs, the gravid uterine weight was recorded and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). In addition, the liver was weighed at scheduled termination of all pregnant females. The liver from all females on study was retained in 10% neutral buffered formalin, and a subsequent histopathological examination was performed. Fetuses were removed from the uteri, counted, weighed

and sexed. Live fetuses were examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained for skeletal examination of bone and cartilage.

Up to and including the highest dose level tested (450 mg/kg bw per day), there were no mortalities and no treatment-related clinical signs during the course of the study. Significant reductions in body weight gain and feed consumption were observed at 450 and 150 mg/kg bw per day during the treatment period or on GDs 6–14, respectively, whereas a slight and transient effect (GDs 6–8) on body weight gain and feed consumption was noted at 30 mg/kg bw per day. Corrected body weight gains at 450, 150 and 30 mg/kg bw per day were reduced by 37%, 23% and 14%, respectively.

At necropsy, maternal liver weights at 450 and 150 mg/kg bw per day were increased by 40% and 15%, respectively, and 4 of 23 females at 450 mg/kg bw per day had enlarged livers. At the histopathological examination of the liver, diffuse centrilobular hepatocellular hypertrophy was observed in all females at 450 mg/kg bw per day and in 20 of 23 females at 150 mg/kg bw per day (Table 31).

There were no substance-related effects on gestational parameters up to and including the highest dose level. Conception rate, mean number of corpora lutea, total implantations, resorptions and live fetuses, fetal sex ratio and the values calculated for the preimplantation and the postimplantation losses were unaffected by treatment.

Fetal body weight was 5% lower at 450 mg/kg bw per day and was unaffected at 150 and 30 mg/kg bw per day. At the external fetal examination, no treatment-related malformations or variations were observed, and the incidence of runt fetuses was similar to that of the controls.

At the visceral fetal examination, there were no treatment-related malformations. The incidence of the variations "thymic remnant present" and "ureter convoluted and/or dilated" at 450 mg/kg bw per day was higher than in the concurrent controls at both the fetal and litter levels, whereas no treatment-related effects were noted at 150 or 30 mg/kg bw per day. The incidences at 450 mg/kg bw per day were also outside the in-house historical control range at both the fetal and litter levels for the former finding and at the fetal level only for the latter finding (Table 31).

At the skeletal examination, there were no treatment-related malformations. The incidences of the variations "at least one thoracic centrum split/split cartilage" and "at least one thoracic centrum dumbbell and/or bipartite/normal cartilage" at 450 mg/kg bw per day were higher than in the concurrent controls at both the fetal and litter levels, whereas no treatment-related effects were noted at 150 or 30 mg/kg bw per day. The incidences of both findings at 450 mg/kg bw per day were also outside the in-house historical control range at both the fetal and litter levels.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on decreased body weight gain and feed consumption, increased liver weights and hepatocellular hypertrophy at 150 mg/kg bw per day and above.

The NOAEL for prenatal developmental toxicity was 150 mg/kg bw per day, based on lower fetal body weights and an increased incidence of visceral and skeletal variations at 450 mg/kg bw per day (Wason, 2008).

Rabbits

In a prenatal developmental toxicity study conducted according to OECD test guideline 414, groups of 23 time-mated female New Zealand White Crl:KBL (NZW) rabbits received fluopyram (purity 94.6%) by oral gavage at a dose level of 0, 10, 25 or 75 mg/kg bw per day from GD 6 to GD 28. The test substance was administered as a suspension in an aqueous solution of 0.5% methylcellulose 400 at a dose volume of 4 ml/kg bw. The doses were selected based upon the results from a previous range-finding study (Kennel, 2004d). Maternal body weights were recorded for all surviving females on GDs 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Feed consumption was measured

Table 31. Summary of selected findings in a prenatal developmental toxicity study in rats

	Dose (mg/kg bw per day)				Historical controls
	0	30	150	450	
No. of mated/pregnant females	23/22	23/22	23/22	23/22	
Body weight (g)					
- GD 6	307.0	304.7	302.6	302.8	
- GD 21	435.0	427.7	423.5	410.7	
Body weight change (g)					
- GDs 6–8	6.8	4.7	−0.4**	0**	
- GDs 6–14	36.0	32.0	22.9**	16.9**	
- GDs 6–18	79.3	74.7	70.6	58.4**	
- Corrected for uterine weight	59.4	51.2	45.8	37.6**	
Feed consumption (g/day)					
- GDs 6–8	25.5	22.9**	20.9**	21.6**	
- GDs 8–10	25.7	23.8	21.9**	22.3**	
- GDs 10–12	26.5	25.1	22.7**	22.8**	
- GDs 12–14	27.3	26.4	24.7*	23.1**	
Liver weight (g)	13.95	14.06	16.05**	19.47**	
Hepatocellular hypertrophy					
- Total	0	1	20	23	
- Minimal + slight	0	0	18	1	
- Moderate + marked	0	0	2	22	
No. of live fetuses/litters	303/22	307/22	322/22	308/22	
Fetal weight (g)					
- Males	5.67	5.59	5.54	5.37*	
- Females	5.40	5.38	5.25	5.13*	
Thymus remnant present					
- Fetal incidence	6/146	7/147	14/155	21/149	2/153–11/175
- (mean % of fetuses affected)	(3.9)	(4.6)	(9.2)	(14.5)	(1.3–6.0)
- Litter incidence	5/22	5/22	8/22	10/22	1/19–8/24
- (% of litters affected)	(22.7)	(22.7)	(36.4)	(45.5)	(5.3–33.3)
Ureter convoluted or dilated					
- Fetal incidence	46/146	57/147	72/155	88/149	22/153–78/175
- (mean % of fetuses affected)	(33.2)	(36.9)	(46.2)	(58.6)	(20.5–45.1)
- Litter incidence	17/22	17/22	20/22	20/22	17/25–23/24
- (% of litters affected)	(77.3)	(77.3)	(90.9)	(90.9)	(68.0–95.8)
Thoracic centrum split					
- Fetal incidence[a]	0/157	1/160	0/167	4/159	0/189–1/155
- (mean % of fetuses affected)	(0.0)	(0.6)	(0.0)	(2.6)	(0.0–0.6)
- Litter incidence[a]	0/22	1/22	0/22	4/22	0/25–1/21
- (% of litters affected)	(0.0)	(4.5)	(0.0)	(18.2)	(0.0–4.8)
Thoracic centrum dumbbell					
- Fetal incidence[b]	3/157	9/160	12/167	29/159	1/144–12/176
- (mean % of fetuses affected)	(1.8)	(5.3)	(7.1)	(20.8)	(0.7–7.2)

Table 31 (continued)

	Dose (mg/kg bw per day)				Historical controls
	0	30	150	450	
- Litter incidence[b]	2/22	7/22	9/22	14/22	1/19–9/23
- (% of litters affected)	(9.1)	(31.8)	(40.9)	(63.6)	(5.3–39.1)

From Wason (2008)
* $P < 0.05$; ** $P < 0.01$
[a] "At least one thoracic centrum split/split cartilage".
[b] "At least one thoracic centrum dumbbell and/or bipartite/normal cartilage".

for all surviving females during regular intervals between GD 3 and GD 29. Clinical observations were recorded daily. At scheduled termination, on GD 29, the gravid uterine weight and the number of ribs were recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). The liver was retained from all females and was weighed only from pregnant females surviving to scheduled termination. Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of approximately half of the fetuses from each litter were immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sexed. Fetuses were eviscerated, skinned, fixed in absolute ethanol and stained for skeletal examination of bone and cartilage.

One female in the control group was terminated due to accidental trauma on GD 15, and one female at 75 mg/kg bw per day died on GD 21. Both deaths were attributable to a gavage error. In addition, one female at 25 mg/kg bw per day that showed a severe fracture of the right hindlimb due to accidental trauma, in association with massive subcutaneous haemorrhaging and a distal epiphyseal femoral disjunction, was killed for humane reasons on GD 23.

There were no treatment-related clinical signs in any group, and there were no abortions throughout the study.

Body weight gain at 75 mg/kg bw per day was reduced on GDs 14–18 and between GD 18 and GD 22. Thereafter, body weight gain was similar to that of the controls, resulting in an overall body weight gain between GD 6 and GD 29 of 0.20 kg compared with 0.31 kg for the controls, although the difference was not statistically significant. Body weight change at 25 and 10 mg/kg bw per day was comparable with that of the controls. Maternal corrected body weight change (i.e. maternal body weight change independent of the uterine weight) was more pronounced at 75 mg/kg bw per day (−0.25 kg) compared with the controls (−0.17 kg), whereas no effect was noted at 25 and 10 mg/kg bw per day. Feed consumption at 75 mg/kg bw per day was reduced by between 22% and 34% ($P \leq 0.01$) for all intervals over GDs 14–26, in comparison with controls, whereas no effect was seen at 25 and 10 mg/kg bw per day (Table 32).

At necropsy of dams, there were no treatment-related macroscopic findings or liver weight effects.

There were no substance-related effects on gestational parameters up to and including the highest dose level. Conception rate, mean number of corpora lutea, total implantations, resorptions and live fetuses, fetal sex ratio and the values calculated for the preimplantation and the postimplantation losses were unaffected by treatment.

Fetal body weights for the combined sexes and for the individual sexes were 11% lower than those of the controls. There was no effect on fetal body weight at 25 and 10 mg/kg bw per day.

The external examination revealed that the number of "runts" or small fetuses (body weight < 28.0 g), classed as a variation, was increased at 75 mg/kg bw per day; the percentage of fetuses classified as small was 12.5%, and the percentage of litters affected was 47.6%, compared with 3.0%

Table 32. Summary of selected findings from a prenatal developmental toxicity study in rabbits

	Dose level (mg/kg bw per day)			
	0	10	25	75
No. of females mated	23	23	23	23
Body weight, GD 6 (kg)	3.51	3.53	3.48	3.46
Body weight change (kg)				
- GDs 6–18	0.21	0.24	0.18	0.12**
- GDs 6–22	0.28	0.30	0.23	0.15**
- GDs 6–26	0.31	0.32	0.28	0.17**
- Corrected for uterine weight	−0.17	−0.16	−0.17	−0.25
Feed consumption (g/day)				
- GDs 14–18	157.6	171.4	154.8	123.6**
- GDs 18–22	176.4	177.1	159.9	115.9**
- GDs 22–26	131.7	118.2	125.7	93.3**
No. of litters with live fetuses	21	22	21	21
No. of live fetuses	173	197	171	187
No. of live fetuses per litter	8.2	9.0	8.1	8.9
Fetal body weights (g)				
- Males + females	39.0	38.1	39.1	34.7**
- Males	39.6	39.1	39.4	35.2**
- Females	38.4	37.1	38.9	34.3*
"Runts"[a]				
- No. of fetuses (%)	6 (3.0)	10 (4.1)	5 (2.1)	25 (12.5)
- No. of litters (%)	5 (23.8)	5 (22.7)	4 (19.0)	10 (47.6)

From Kennel (2006b)

* $P < 0.05$; ** $P < 0.01$

[a] Runts defined as fetuses with a body weight of < 28.0 g.

and 23.8%, respectively, in the control group. At 25 and 10 mg/kg bw per day, the percentage of small fetuses and percentage of litters affected were very similar to the control values (Table 32).

At the visceral examination, there were two fetuses from separate litters with "gall bladder absent" at 75 mg/kg bw per day, compared with no instance of this malformation in the current control group. However, considering the low incidence of this finding and the fact that it had already been observed at similar incidences in previous studies conducted in the same strain (Wason, 2009), it was considered not to be treatment related. All other malformations and variations occurred as isolated findings or at a similar frequency across the dose groups, including the controls, and were considered to have occurred by chance.

No treatment-related effects on malformations or variations at the skeletal examination were evident.

The NOAEL for maternal toxicity was 25 mg/kg bw per day, based on reduced body weight gains and feed consumption at 75 mg/kg bw per day.

The NOAEL for prenatal developmental toxicity was 25 mg/kg bw per day, based on reduced fetal body weights and an increased number of small fetuses ("runts") at 75 mg/kg bw per day (Kennel, 2006b).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study conducted in accordance with OECD test guideline 424, groups of 12 male and 12 female Wistar HAN CRL:WI (HAN) rats received fluopyram (purity 94.7%) by oral gavage at a dose level of 0, 125, 500 or 2000 mg/kg bw. The test substance was administered in 2% (volume per volume) Cremophor EL in deionized water at a dose volume of 10 ml/kg bw. As there were compound-related effects on measures of motor and locomotor activities at 125 mg/kg bw in females, a follow-up study was conducted under the same conditions at nominal doses of 0, 25, 50 and 100 mg/kg bw to establish a NOAEL in females. Based on analytical results, the actual doses for the initial study were 0, 126, 498 and 1840 mg/kg bw for males and females, and for the follow-up study, the actual doses were 0, 25, 51 and 100 mg/kg bw for females. All animals (12 of each sex per dose) were used for neurobehavioural evaluation, with 6 of each sex per dose from the initial study used for histopathology. For the initial study, observations for moribundity and mortality were performed at least once daily (unless otherwise noted), detailed clinical observations for each animal were performed daily throughout the study and body weight measurements (measured weekly as a component of the functional observational battery [FOB]) were performed. Also, an FOB and automated measurements of activity (figure-eight maze) were conducted during the week prior to treatment and on days 0 (day of treatment, at the time of peak effect), 7 and 14. Lastly, all animals from the initial study were subjected to a gross necropsy. Selected animals (six of each sex per dose) were perfused, the brain was weighed and skeletal muscle, peripheral nerves, eyes (with optic nerves) and tissues from the central nervous system were examined microscopically for lesions. Observations and measurements included in the follow-up study consisted of observations for moribundity and mortality performed at least once daily (unless otherwise noted), detailed clinical observations for each animal performed daily throughout the study and body weight measurements (measured weekly as a component of the FOB). Also, an FOB and automated measurements of activity (figure-eight maze) were conducted during the week prior to treatment and on day 0 (day of treatment, at the time of peak effect). Animals from the follow-up study were terminated once all signs of toxicity were no longer evident. It should be noted that animals from the follow-up study were treated in the same way as animals from the initial study for consistency. The NOAEL for clinical observations, FOB and body weight changes had already been established in the initial study.

There were no treatment-related deaths at any dose level in either sex. One control female from the follow-up study was found dead on day 1 of the study. Treatment-related clinical signs were limited to urine stain in four males at 2000 mg/kg bw (initial study). This finding was first evident on day 0 and generally resolved 2–5 days after treatment. There were no compound-related clinical signs evident in males at lower dose levels or in females at any dose level (initial study and follow-up study).

Body weight was not affected by treatment at any dose level in either sex (initial study and follow-up study).

There were no FOB findings in males at any dose level. In females, treatment-related FOB findings were limited to a statistically significant decrease in body temperature at 2000 and 500 mg/kg bw (i.e. 36.9 °C and 37.4 °C, respectively, versus 37.9 °C for controls). Also, there were statistically significant differences from control involving ease of removal from the home cage in high-dose females, with a similar (non-statistically significant) trend in mid-dose females (i.e. a lower incidence of animals that vocalized compared with those that did not: three mid-dose animals and one high-dose animal versus six controls). There were no treatment-related FOB findings in females from the follow-up study.

Motor and locomotor activities in males were statistically significantly reduced on day 0 at 2000 mg/kg bw (71% and 73%, respectively) and at 500 mg/kg bw (51% and 49%, respectively); all

Table 33. Summary of selected findings in an acute neurotoxicity study in rats

	Females (follow-up study)				Females (initial study)				Males (initial study)			
	Dose (mg/kg bw)											
	0	25	50	100	0	125	500	2000	0	125	500	2000
Motor activity (total activity counts for session)												
Pretreatment	504	451	457	429	435	490	405	530	493	579	432	464
Day 0	539	423	430	335	521	385*	247*	148*	480	454	237*	139*
Day 7	nd	nd	nd	nd	480	484	426	573	496	518	429	398
Locomotor activity (total activity counts for session)												
Pretreatment	322	286	300	256	284	330	251	347	325	415	299	292
Day 0	353	302	289	218	362	251*	151*	82*	335	322	171*	92*
Day 7	nd	nd	nd	nd	311	333	261	348	323	363	295	251

From Gilmore & Hoss (2007)
nd, not determined; * $P < 0.05$

effects resolved by day 7. In females, motor and locomotor activities were statistically significantly reduced on day 0 at 2000 mg/kg bw (72% and 77%, respectively), at 500 mg/kg bw (53% and 58%, respectively) and at 125 mg/kg bw (26% and 31%, respectively); all effects resolved by day 7. In females at 100 mg/kg bw (follow-up study), motor and locomotor activities were non-statistically significantly reduced (38% each) on day 0 (Table 33).

There were no gross lesions at termination in males or females in the initial study at any dose level that might have been treatment related. In addition, there were no effects on terminal body weight in any group or on absolute or relative brain weight in perfused animals at any level of exposure.

Microscopic evaluation did not reveal compound-related histological lesions in the nervous system in high-dose males or females in the initial study. Therefore, it was not necessary to perform necropsy or histopathology on animals in the follow-up study.

Under the conditions of this study, the NOAEL for acute neurotoxicity in male and female rats was 125 and 50 mg/kg bw, respectively, based on decreased motor and locomotor activities at 500 or 100 mg/kg bw and above (Gilmore & Hoss, 2007).

In a subchronic neurotoxicity study conducted in accordance with OECD test guideline 424, groups of 12 male and 12 female Wistar HAN CRL:WI (HAN) rats were given diets containing fluopyram (purity 94.7%) at a concentration of 0, 100, 500 or 2500 ppm, equal to 0, 6.69, 33.2 and 164.2 mg/kg bw per day in males and 0, 8.05, 41.2 and 197.1 mg/kg bw per day in females, for 13 weeks. For neurobehavioural evaluation, 10–12 rats of each sex per dietary level were used, with histopathology performed on selected tissues from 6 rats of each sex from the control and high-dose groups. Body weight and feed consumption determinations, as well as detailed clinical observations, were conducted weekly throughout the study. Observations for moribundity and mortality were performed at least once daily. Automated measurements of activity (figure-eight maze) and an FOB were conducted the week prior to treatment and during weeks 2, 4, 8 and 13. Ophthalmological examinations were conducted on all animals prior to dosing and then again on all study animals during week 12. All animals placed on study were subjected to a gross necropsy. For selected animals, the brain was weighed in order to calculate the brain to body weight ratio, and skeletal muscle, peripheral nerves, eyes (with optic nerves) and tissues from the central nervous system were also examined microscopically for lesions. On the day of termination, known target organs (liver, kidney and thyroid) were collected and weighed, and haematological and serum chemistry evaluations were performed on surviving non-perfused animals (4–6 of each sex per dietary level) for reference to verify that a sufficiently high dose had been tested and for comparison with other studies at these dietary levels.

There were no treatment-related deaths or clinical signs at any dose in either sex.

Body weight at 2500 ppm was slightly (non-statistically significantly) decreased (6–7%) in males on days 35 through 56. In addition, there was a trend for a slight decrease (maximum 5%) in body weight on days 21–28 and again on day 63, continuing until study termination. In females at 2500 ppm, body weight was statistically significantly reduced (7–12%) on day 21 and on days 42 through study termination. Lastly, body weight was non-statistically significantly reduced (5–6%) on days 28–35 in high-dose females. Body weight was not affected by treatment at the lower dietary levels in either sex. Total body weight gain at 2500 ppm was statistically significantly decreased (26%) in females and slightly (non-statistically significantly) reduced (10%) in males. Total body weight gain was not different from controls in low- and mid-dose males and females (Table 34).

Feed consumption at 2500 ppm was statistically significantly decreased (15%) in males on day 21 and non-statistically significantly decreased (6–8%) on days 35–49. In females at 2500 ppm, feed consumption was statistically significantly decreased (13–24%) beginning on day 21 and continuing for all remaining weeks measured. In addition, feed consumption was statistically significantly decreased (7–12%) in females at 500 ppm on days 21–42, days 63–70 and again on day 91. Feed consumption was not affected by treatment in mid-dose males or in low-dose males or females.

Haemoglobin and haematocrit levels in females at 2500 ppm were significantly reduced (−10% and −9%, respectively), whereas no relevant changes were noted at lower dose levels or in males. Clinical chemistry analysis at 2500 ppm showed significantly decreased glucose levels and significantly increased total protein and cholesterol levels in both sexes and significantly increased triglyceride levels in females.

There were no compound-related effects evident by clinical observations or FOB evaluation at any dietary level in either sex.

For motor activity, the pretreatment values for the dose groups averaged from 12% to 18% higher than for animals assigned to the control group for males and from 13% lower to 8% higher than controls for females. For locomotor activity, the pretreatment values for the dose groups averaged from 5% to 14% higher than controls for males and from 12% lower to 10% higher than controls for females. These results show that differences of approximately ±20% are within the range of normal variability in this laboratory for groups of 10–12 rats of each sex per dietary level and therefore are not biologically significant. This large variation must be taken into account in interpreting the data. Thus, it is concluded that, for the overall 60-minute test session, motor and locomotor activities were not affected by treatment at any dietary level in either sex. There were intergroup differences, but these were small (i.e. in the pretreatment range), not dose related or not consistently seen throughout the study. Furthermore, these differences did not achieve statistical significance (Table 34).

At necropsy, statistically significant increases were noted at 2500 ppm for liver weights (absolute and relative) in both sexes and for kidney weights (absolute and relative) in males. Thyroid weights (absolute and relative) were increased in males at 500 ppm by 17–24% and at 2500 ppm by 32–38%, but the differences were not statistically significant.

There were no treatment-related findings in neural and/or non-neural tissues from perfusion-fixed high-dose males or females related to administration of the test substance. Tissues from animals at lower dose levels were therefore not examined. Liver, kidney and thyroid tissues were not examined microscopically, because the microscopic findings that were associated with the measured changes in tissue weight had been established in other studies.

Under the conditions of this study, the NOAEL for neurotoxicity was 2500 ppm, equal to 164.2 mg/kg bw per day in males and 197.1 mg/kg bw per day in females (the highest doses tested, respectively).

The NOAEL for general toxicity was 500 ppm, equal to 33.2 mg/kg bw per day in males and 41.2 mg/kg bw per day in females, based on decreases in body weight, body weight gain and feed

Table 34. Summary of selected findings in a subchronic neurotoxicity study in rats

	Males				Females			
	Dietary concentration (ppm)							
	0	100	500	2500	0	100	500	2500
Body weight (g)								
- Day 0	244.3	244.4	245.6	247.2	156.0	158.3	152.4	155.8
- Day 28	347.8	344.5	347.3	331.1	205.6	206.7	199.9	194.7*
- Day 56	398.5	391.4	400.0	373.5	226.4	224.9	219.9	208.9*
- Day 91	432.9	428.7	437.8	416.2	245.8	243.2	239.1	223.2*
Body weight gain, days 0–91 (g)	188.6	184.3	192.3	169.0	89.3	85.1	86.8	66.5*
Food consumption (g/day)								
- Day 21	23.7	24.2	23.2	20.1*	18.2	16.1	16.1	14.6*
- Day 42	24.0	23.7	23.6	22.5	18.5	17.1	16.5*	14.7*
- Day 91	21.4	22.1	21.9	21.8	17.9	16.6	16.0*	14.8*
Haemoglobin (g/dl)	17.5	17.2	16.8	16.6	17.0	16.8	16.6	15.3*
Haematocrit (%)	49.0	48.5	45.9*	46.2	46.2	46.8	45.3	42.0*
MCV (μm^3)	52.7	50.4*	50.7*	49.5*	56.3	54.2*	52.8*	49.0*
Total protein (g/dl)	6.7	6.8	6.9	7.2*	6.6	7.0	7.0	7.2*
Glucose (mg/dl)	110	117	109	94*	103	104	99	88*
Cholesterol (mg/dl)	57	58	110	82*	51	50	65	86*
Triglyceride (mg/dl)	97	56	172	62	30	32	31	57*
Motor activity[a]								
- Pretreatment	542	+12%	+18%	+15%	824	+1%	−13%	+8%
- Week 2	547	+23%	+19%	+28%	882	−19%	−8%	+4%
- Week 4	558	+20%	+5%	−1%	759	−7%	−9%	+3%
- Week 8	507	+16%	−1%	+3%	798	0%	−14%	−13%
- Week 13	414	+8%	+9%	+19%	641	+0.5%	+5%	+3%
Locomotor activity[a]								
- Pretreatment	332	+5%	+14%	+14%	388	+10%	−12%	+10%
- Week 2	290	+28%	+21%	+25%	419	−12%	−8%	−4%
- Week 4	276	+25%	+13%	−1%	361	−9%	−17%	−7%
- Week 8	220	+35%	+11%	+13%	360	+4%	−16%	−24%
- Week 13	177	+11%	+20%	+29%	286	+3%	+5%	−9%
Liver weight								
- Absolute (g)	14.94	15.97	18.18	21.36*	7.61	7.60	8.50	10.73*
- Relative (% of body weight)	3.75	4.08	4.35*	5.70*	3.37	3.46	4.05*	5.57*
Kidney weight								
- Absolute (g)	2.82	2.84	3.57	3.36*	1.74	1.78	1.69	1.66
- Relative (% of body weight)	0.71	0.73	0.85	0.90*	0.77	0.81	0.80	0.86
Thyroid weight								
- Absolute (mg)	25	26	31	33	20	20	19	23
- Relative (% of body weight, ×1000)	6.3	6.7	7.4	8.7	9.1	9.1	9.0	12.0

From Gilmore (2008)

* $P < 0.05$

[a] Control group: total activity counts; dose groups: % difference from control.

consumption (both sexes), increased liver weights (both sexes) and increased kidney weights (males) at 2500 ppm (Gilmore, 2008).

(b) Mechanistic studies

(i) Rat liver tumours

A mechanistic study was conducted to examine the effects of fluopyram on the rat liver and to clarify its mode of action (MOA), especially in view of the liver tumours observed in female rats. Fluopyram (purity 94.7%) was administered to a group of 15 female Wistar Rj:WI (IOPS HAN) rats in the diet at a concentration of 3000 ppm (equal to 193 mg/kg bw per day) for 7 days. A similarly constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and feed consumption were recorded at the end of the 7-day treatment period. Hepatic cell proliferation was assessed by administration of 5-bromo-2′-deoxyuridine (BrdU, an analogue of thymidine) to all animals in the drinking-water for 7 days before sacrifice. Water consumption was measured during the BrdU administration period. All animals were subjected to necropsy. Brain and liver were weighed. Selected portions of the liver were fixed for conventional histopathological examination and cell proliferation measurement. The remaining portions of the livers of 10 females from each group were homogenized for microsomal preparations in order to determine the total CYP content and specific CYP isoenzyme profiles, including activities of 7-ethoxyresorufin *O*-deethylase (EROD; CYP1A2), 7-pentoxyresorufin *O*-dependentylase (PROD; CYP2B) and 7-benzyloxyresorufin *O*-debenzylase (BROD; CYP3A), whereas phase II activities were determined by measuring uridine diphosphate-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate.

Fluopyram at 3000 ppm in the diet had no effect on body weight parameters. There was no evidence of a treatment-related effect on clinical signs, feed consumption or water consumption. Mean absolute and relative liver weights were increased by between 40% and 43% when compared with the controls ($P < 0.01$). This increase was associated with macroscopic findings (enlarged and dark livers) in 13 of 15 females compared with 0 of 15 and 1 of 15 in the controls, respectively. At the histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A markedly decreased incidence of periportal vacuolation was also noted (Table 35).

Assessment of cell proliferation in the liver revealed a 4 times higher mean BrdU labelling index in both the perilobular and centrilobular areas of the hepatic lobule in treated animals, compared with the controls.

Assessment of total CYP content and microsomal proteins revealed a slight increase in total CYP content and in mean EROD activity, a moderate increase in mean PROD and UDPGT activities and a marked increase in BROD activity (all statistically significantly different from the control group; $P < 0.01$).

These data showed that fluopyram, when administered for 7 days at a dose level of 193 mg/kg bw per day, had the ability to induce xenobiotic metabolizing enzymes in the rat liver. For the individual enzymes, the extent of induction was very different, ranging from minimal (total CYP) to marked (BROD). Pathological examination confirmed hypertrophy and cell proliferation in the liver (Blanck, 2008a).

In a parallel mechanistic study conducted to evaluate the liver changes caused by phenobarbital, a known liver enzyme inducer, a group of 15 female Wistar Rj:WI (IOPS HAN) rats received phenobarbital sodium salt (purity 99.6%) orally by gavage at a dose level of 80 mg/kg bw per day for 7 days. The test substance was administered in 0.5% aqueous solution of methylcellulose 400 at a dosing volume of 10 ml/kg bw. A similarly constituted group received the vehicle only and acted as a control group.

Table 35. Summary of selected findings from the rat liver tumour mechanistic study[a]

	Dietary concentration (ppm)	
	0	3000
Liver weight, absolute (g)	5.61	7.86** (+40%)
Liver weight, relative (% of body weight)	2.53	3.63** (+43%)
Hepatocellular hypertrophy, diffuse	0/15	15/15
Hepatocellular vacuolation, diffuse	11/15	1/15
BrdU-positive cells in the centrilobular zone	44.54	179.68** (+303%)
BrdU-positive cells in the periportal zone	28.55	112.94** (+296%)
Overall BrdU-positive cells	36.54	146.31** (+300%)
Total CYP (nmol/mg protein)	0.91	1.23** (+35%)
EROD (pmol/mg protein per minute)	47.99	103.18** (+115%)
PROD (pmol/mg protein per minute)	6.65	28.55** (+329%)
BROD (pmol/mg protein per minute)	6.39	74.51** (+1066%)
UDPGT (nmol/mg protein per minute)	6.42	30.69** (+378%)

From Blanck (2008a)
** $P < 0.01$
[a] % change from control shown in parentheses.

Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and feed consumption were recorded at the end of the 7-day treatment period. Hepatic cell proliferation was assessed following administration of BrdU to all animals in the drinking-water for 7 days before sacrifice by determining the BrdU labelling index. Water consumption was measured during the BrdU administration period. All animals, except animals found dead during the study, were subjected to necropsy. Brain and liver were weighed. Selected portions of the liver were fixed for conventional histopathological examination and cell proliferation measurement. The remaining portions of the liver of 10 females from each group were homogenized for microsomal preparations in order to determine total CYP content and CYP isoenzyme and UDPGT activities.

Administration of phenobarbital at 80 mg/kg bw per day for 7 days caused a reduced motor activity in all animals tested. One animal was found dead on study day 5. There was a slight effect on body weight, with an overall mean absolute body weight gain of 0 g, compared with 7 g in the control group.

At necropsy, there was no relevant change in mean terminal body weights when compared with controls. Mean absolute and relative liver weights were increased by between 19% and 22%, compared with the controls ($P < 0.01$). At macroscopic examination, dark liver was found in 5 of 14 females and enlarged liver in 3 of 14 females, compared with no cases in the controls. At histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A decreased incidence of periportal hepatocellular vacuolation was also noted (Table 36).

Assessment of cell proliferation in the liver revealed a 2–3 times higher mean BrdU labelling index in the perilobular area and in the centrilobular area in the treated animals compared with the controls.

Assessment of total CYP content and hepatic enzyme activities revealed a slight increase in total CYP content and in UDPGT activities, a moderate increase in PROD activity and a marked increase in BROD activity (all statistically significantly different from the control group; $P < 0.01$), whereas EROD activity was increased only minimally (Table 36).

Table 36. Summary of selected findings in a parallel mechanistic study in rats using phenobarbital[a]

	Dose (mg/kg bw per day)	
	0	80
Liver weight, absolute (g)	5.55	6.63 (+19%)
Liver weight, relative (% of body weight)	2.47	3.02 (+22%)
Hepatocellular hypertrophy, diffuse	0/15	14/14
Hepatocellular vacuolation, diffuse	7/15	3/14
Hepatocellular necrotic foci	0/15	4/14
BrdU-positive cells in the centrilobular zone	21.73	55.21** (+154%)
BrdU-positive cells in the periportal zone	16.70	33.19** (+99%)
Overall BrdU-positive cells	19.22	44.20** (+130%)
Total CYP (nmol/mg protein)	0.95 ± 0.20	1.49** (+57%)
EROD (pmol/mg protein per minute)	38.25 ± 6.42	47.56* (+24%)
PROD (pmol/mg protein per minute)	4.89 ± 0.61	26.36** (+439%)
BROD (pmol/mg protein per minute)	4.91 ± 0.70	94.43** (+1823%)
UDPGT (nmol/mg protein per minute)	6.99 ± 0.52	13.47** (+93%)

From Blanck (2008b)
* $P < 0.05$; ** $P < 0.01$
[a] % change from control shown in parentheses.

These data confirmed the well-known ability of phenobarbital to induce the activities of hepatic enzymes such as PROD, BROD and UDPGT and to cause liver cell hypertrophy and proliferation. However, the magnitude of effects was different from that seen with fluopyram (Blanck, 2008a), especially when EROD activity was concerned. Furthermore, the effect of phenobarbital on hepatic cell proliferation was much more prominent in the centrilobular region where proliferation by fluopyram equally affected the centrilobular and the periportal parts of the liver (Blanck, 2008b).

(ii) Mouse thyroid tumours

A mechanistic study was conducted to examine the effects of fluopyram on the mouse liver and on pituitary and thyroid hormone levels and to clarify its MOA, especially in view of the thyroid tumours observed in male mice. Fluopyram (purity 94.7%) was administered to two groups of 15 male C57BL/6J mice in the diet for 3 or 14 days at a concentration of 2000 ppm, equal to 308 and 314 mg/kg bw per day, respectively. Two similarly constituted groups of 15 males received untreated diet and acted as control groups. Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed weekly. On study day 4 and study day 15, before necropsy, blood samples were taken for measurement of hormone levels (T_3, T_4 and TSH). The liver and the brain were weighed, and the liver and the thyroid gland were sampled for microscopic examination. In addition, hepatic CYP isoenzyme activities and UDPGT activity (using 4-nitrophenol as substrate) were also measured.

There were no mortalities and no treatment-related findings in terms of clinical signs or body weight parameters during the course of the study.

After 3 days of exposure at 2000 ppm, mean T_3 level was not changed, whereas mean T_4 level was decreased (−30%) and mean TSH level was increased (+18%), compared with untreated controls (Table 37). Mean absolute and relative liver weights were increased by approximately 60% when compared with control animals. At macroscopic observation, enlarged liver was found in all treated animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy

Table 37. Summary of selected findings from mouse thyroid tumour mechanistic study[a]

	3-day exposure		14-day exposure	
	Dietary concentration (ppm)			
	0	2000	0	2000
T_3 (nmol/l)	1.62	1.64 (+1%)	1.45	1.52 (+5%)
T_4 (nmol/l)	43.7	30.7** (−30%)	38.1	27.7** (−27%)
TSH (ng/ml)	3.81	4.48** (+18%)	3.81	4.09* (+7%)
Liver weight, absolute (g)	1.24	1.97** (+59%)	1.25	1.99** (+59%)
Liver weight, relative (% of body weight)	5.40	8.71** (+61%)	5.23	8.42** (+61%)
Hepatocellular hypertrophy	0/5	5/5	0/5	5/5
Increased number of mitoses	0/5	5/5	0/5	0/5
Hepatocellular single-cell necrosis	0/5	1/5	0/5	4/5
CYP (nmol/mg protein)	1.08	2.33** (+116%)	1.26	2.15* (+71%)
EROD (pmol/mg protein per minute)	90.25	302.52** (+235%)	99.05	262.24** (+165%)
PROD (pmol/mg protein per minute)	4.93	143.42** (+2890%)	4.19	94.80** (+2163%)
BROD (pmol/mg protein per minute)	12.99	1145.28** (+8717%)	12.83	1175.30** (+9061%)
UDPGT (nmol/mg protein per minute)	16.04	15.36	17.09	14.32** (−16%)

From Rouquie (2008a)
* $P < 0.05$; ** $P < 0.01$
[a] % change from control shown in parentheses.

and an increased number of mitoses were seen in all examined livers from treated animals. Moreover, hepatocellular single-cell necrosis was observed in one out of five livers examined from treated animals. No significant microscopic change was observed in the thyroid gland.

Total CYP content was highly increased (+116%) by the treatment. EROD activities were marginally increased (+235%), whereas PROD and BROD activities were markedly increased (+2809% and +8717%, respectively) when compared with controls. No significant changes were observed in UDPGT activity.

After 14 days of exposure at 2000 ppm, mean T_3 level was not changed, whereas mean T_4 level was decreased (−27%) and mean TSH level was increased (+7%) when compared with controls (Table 37). Mean absolute and relative liver weights were increased by approximately 60% when compared with control animals. At macroscopic observation, enlarged liver was found in 13 of 15 treated animals compared with no instances in the controls; moreover, dark liver was observed in 14 of 15 treated animals compared with 1 of 15 control animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in all examined livers from treated animals. Moreover, hepatocellular single-cell necrosis was observed in four out of five livers examined from treated animals. No significant microscopic change was observed in the thyroid gland.

Total CYP content was moderately increased (+71%) by the treatment. EROD activities were marginally increased (+165%), whereas PROD and BROD activities were markedly increased (+2163% and +9061%, respectively) when compared with controls. No relevant changes were observed in UDPGT activity.

In conclusion, fluopyram induced total CYP, PROD, BROD and EROD activities in the mouse liver after 3 and/or 14 days of dietary administration at a concentration of 2000 ppm (equal to 308–314 mg/kg bw per day), whereas no increase in UDPGT activity was observed. Hepatotoxicity became apparent by organ weight increase and concomitant histological lesions. Fluopyram administration also resulted in a disturbance of thyroid hormone balance by causing a decrease in T_4 levels and a

concomitant increase in TSH levels. However, there was no histological evidence of toxic effects on the thyroid, probably due to the relatively short exposure period (Rouquie, 2008a).

In a parallel mechanistic study conducted to evaluate the liver effects and pituitary and thyroid hormone changes caused by phenobarbital, two groups of 15 male C57BL/6J mice received phenobarbital sodium salt (purity 99.6%) orally by gavage at a dose level of 80 mg/kg bw per day for 3 or 14 days. Phenobarbital acted as a reference compound known to induce an increase in T_4 clearance in the mouse through induction of T_4 glucuronidation. The test substance was administered in 0.5% aqueous solution of methylcellulose at a dose volume of 10 ml/kg bw. A similarly constituted group received the vehicle only and acted as a control group. Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed weekly. Before necropsy, blood samples were taken for thyroid and pituitary gland hormone analyses (T_3, T_4 and TSH). At final sacrifice times, liver and brain were weighed and liver and thyroid gland sampled for the assessment of morphological changes. In addition, hepatic CYP isoenzyme activities and UDPGT activity (using 4-nitrophenol as substrate) were assessed.

There were no mortalities and no treatment-related findings in terms of clinical signs during the course of the study.

After 3 days of exposure, at terminal sacrifice, mean T_3 and T_4 levels were decreased (−10% and −27%, respectively) and mean TSH level was not affected when compared with controls (Table 38). At necropsy, mean absolute and relative liver weights were increased by 4–11% when compared with controls. At macroscopic observation, enlarged liver was found in 1 of 15 treated animals; moreover, dark liver was observed in 6 of 15 treated animals, compared with no instances in the controls. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in four of five examined livers from treated animals and an increased number of mitoses in three of five livers from treated animals.

Total CYP content was highly increased (+146%) by the treatment. EROD activity was marginally increased (+297%), whereas PROD and BROD activities were markedly increased (+1381% and +4930%, respectively) when compared with controls. No significant change was observed in UDPGT activity.

After 14 days of exposure at terminal sacrifice, mean T_3 level was not changed, whereas mean T_4 level was decreased (−19%) and mean TSH level was increased (+9%) when compared with controls (Table 38). At necropsy, mean absolute and relative liver weights were increased by 21–23% when compared with controls. At macroscopic observation, enlarged liver was found in 12 of 15 treated animals compared with 1 of 15 controls; moreover, dark liver was observed in 4 of 15 treated animals compared with no instances in controls. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in all examined livers from treated animals.

Total CYP content was slightly increased (+36%) by the treatment. EROD activity was slightly increased (+375%), whereas PROD and BROD activities were markedly increased (+1345% and 2844%, respectively) when compared with controls. No significant change was observed in UDPGT activities.

In conclusion, this study demonstrated that phenobarbital administration at a dose level of 80 mg/kg bw per day in the C57BL/6J mouse for 3 and 14 days induced hepatic total CYP, PROD and BROD activities and a modification of the pituitary and thyroid hormone levels (Rouquie, 2008b).

In a mechanistic study (non-GLP compliant) conducted to determine the potential effects of fluopyram on the expression of a selection of genes involved in the metabolism of xenobiotics in the mouse liver, fluopyram (purity 94.7%) and phenobarbital sodium salt (purity 99.6%; suspended in a 0.5% aqueous solution of methylcellulose) were administered daily to two groups of 10 male

Table 38. Summary of selected findings in a parallel mechanistic study in mice using phenobarbital[a]

	3-day exposure		14-day exposure	
	Dose (mg/kg bw per day)			
	0	80	0	80
T_3 (nmol/l)	1.72	1.54* (−10%)	1.61	1.57 (−2%)
T_4 (nmol/l)	36.7	26.8** (−27%)	32.4	26.1* (−19%)
TSH (ng/ml)	4.44	4.41 (−1%)	4.47	4.89* (+9%)
Liver weight, absolute (g)	1.30	1.36 (+5%)	1.31	1.60** (+22%)
Liver weight, relative (% of body weight)	5.70	6.32 (+11%)	5.38	6.65** (+23%)
Hepatocellular hypertrophy	0/5	4/5	0/5	5/5
Increased number of mitoses	0/5	3/5	0/5	0/5
CYP (nmol/mg protein)	0.94	2.31** (+146%)	0.98	1.33* (+36%)
EROD (pmol/mg protein per minute)	48.08	190.65** (+297%)	35.34	167.88** (+375%)
PROD (pmol/mg protein per minute)	6.01	88.99** (+1381%)	4.98	71.97** (+1345%)
BROD (pmol/mg protein per minute)	17.33	871.66** (+4930%)	18.82	554.00** (+2844%)
UDPGT (nmol/mg protein per minute)	16.24	17.23	15.18	12.96

From Rouquie (2008b)
* $P < 0.05$; ** $P < 0.01$
[a] % change from control shown in parentheses.

C57BL/6J mice for 3 days at a dietary concentration of 2000 ppm (equivalent to about 286 mg/kg bw per day) or at a dose level of 80 mg/kg bw per day, respectively. A similarly constituted group of 10 males received control diet for 3 days and acted as a control group. Clinical signs were recorded daily, and body weight was measured on study days 1 and 4. At final sacrifice, liver was weighed and sampled for gene expression analyses by quantitative polymerase chain reaction (PCR).

There were no mortalities or body weight effects during the course of the study. Animals treated with phenobarbital showed reduced motor activity throughout the treatment period. At necropsy, mean absolute and relative liver weights were increased by 60–61% for fluopyram-treated animals and by 17–19% for phenobarbital-treated animals, compared with the control animals (Table 39).

Quantitative PCR analyses of transcripts of genes known to be implicated in the hepatic inactivation of thyroid hormones revealed in fluopyram-treated animals an upregulation of sulfotransferase transcripts (+92% to +463%) and UDPGT transcripts (+173% to +273%). Similarly, an upregulation of sulfotransferase transcripts *Sult1a1* and *Sultn* (+62% and +96%, respectively), but not *Sult2a2*, and of UDPGT transcripts (+82% to +119%) was observed in phenobarbital-treated animals. In contrast, an equivalent effect of both substances could not be shown for *Cyp1a1*, which was induced by fluopyram but repressed by phenobarbital. For most genes, effects were more pronounced with fluopyram than with phenobarbital (Table 39).

In conclusion, this study demonstrates that both fluopyram at 2000 ppm and phenobarbital at 80 mg/kg bw per day administered to the C57BL/6J mouse for 3 days induced an upregulation of the sulfotransferase and UDPGT gene transcripts in the liver. These transcripts are known to encode enzymes that inactivate T_3 and T_4 via glucuronide and sulfate derivatives. However, several differences between fluopyram and phenobarbital in terms of gene expression were detected (Rouquie, 2008c).

In a mechanistic study (non-GLP compliant) conducted to determine the potential effects of fluopyram on the clearance of T_4 in the blood, fluopyram (purity 94.7%) was administered via the

Table 39. Summary of selected findings from a mechanistic study on gene expression in mice

	Control group	Fluopyram: 2000 ppm	Phenobarbital: 80 mg/kg bw per day
Liver weight, absolute (g)	1.20	1.93** (+61%)	1.40** (+17%)
Liver weight, relative (% of body weight)	5.34	8.55** (+60%)	6.34** (+19%)
CYP (*Cyp1a1*)	1.29	4.81** (+272%)	1.20 (−7%)
CYP (*Cyp2b9*)	14.71	48.61* (+230%)	21. 11 (+43%)
CYP (*Cyp3a11*)	1.51	43.59** (+2783%)	7.76** (+413%)
Sulfotransferase (*Sult1a1*)	1.19	2.29** (+92%)	1.92* (+62%)
Sulfotransferase (*Sult2a2*)	0.51	2.90** (+463%)	0.63 (+22%)
Sulfotransferase (*Sultn*)	1.41	5.93** (+321%)	2.76** (+96%)
UDPGT (*Ugt1a1*)	1.08	4.03** (+273%)	2.36** (+119%)
UDPGT (*Ugt2b1*)	1.04	2.84** (+173%)	1.97** (+90%)
UDPGT (*Ugt2b5*)	1.30	4.30** (+231%)	2.36** (+82%)

From Rouquie (2008c)
* $P < 0.05$; ** $P < 0.01$
[a] % change from control shown in parentheses.

diet at a concentration of 2000 ppm (equivalent to about 286 mg/kg bw per day) to a group of five male C57BL/6J mice for 3 days; a similarly constituted group of five males received untreated diet and acted as a control group. A further group of five males received phenobarbital sodium salt (purity 99.6%; suspended in a 0.5% aqueous solution of methylcellulose) at a dose level of 80 mg/kg bw per day by oral gavage for 3 days. Phenobarbital acted as a reference compound known to induce an increase in T_4 clearance in the mouse through induction of T_4 glucuronidation. On study day 4, each animal received by intravenous injection via the tail 250 µl of diluted ^{125}I-labelled T_4 solution corresponding to a dose of 37 kBq of ^{125}I-labelled T_4 per animal. Approximately 3 hours post-administration with ^{125}I-labelled T_4, each animal received 0.1 mg of sodium iodide in 250 µl of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal 1.3, 2, 4, 6 and 24 hours after administration of ^{125}I-labelled T_4. The level of ^{125}I radioactivity in each sample was measured using a gamma scintillation counter. The decrease in the level of ^{125}I radioactivity was indicative of the rate of T_4 clearance from the blood. Animals were checked daily for mortality and clinical signs. Body weights were recorded on study days 1 and 4. Owing to technical difficulties encountered with the intravenous injection of ^{125}I-labelled T_4, T_4 clearance data for nine animals only (five control animals, one fluopyram-treated animal and three phenobarbital-treated animals) were obtained from this first group of animals (subgroup I). Consequently, five additional animals were incorporated into the study (subgroup 2). One animal acted as a control and received untreated diet, whereas the remaining four animals were treated with 2000 ppm fluopyram. The results obtained in the first and the supplementary experiments were combined.

There were no mortalities or clinical signs during the course of the study. There was no statistically significant effect on body weight. The results show that following an intravenous injection of ^{125}I-labelled T_4, the radioactivity level in the blood of fluopyram-treated animals was lower than that in the blood of the corresponding control animals. This decrease in the level of radioactivity in the blood of fluopyram-treated animals was observed at all time points examined and reflects a more rapid clearance of T_4 in these animals over a 24-hour period, compared with the controls. A partly similar response was seen in animals treated with the reference compound phenobarbital (Table 40).

In conclusion, the results suggest a more rapid T_4 clearance after treatment with fluopyram or phenobarbital than in untreated mice. Phenobarbital is known to induce an increase in T_4 clearance

Table 40. Whole blood radioactivity after a single administration of ^{125}I-labelled T_4 to mice in a mechanistic study

Time point	Whole blood radioactivity (cpm)		
	Control group	Fluopyram: 2000 ppm	Phenobarbital: 80 mg/kg bw per day
Number of animals	*6*	*5*	*3*
1 h 20 min	11 434 ± 1 624	4 767 ± 1 953	5 775 ± 2 615
2 h	11 025 ± 1 415	4 686 ± 1 999	5 905 ± 2 095
4 h	9 811 ± 1 756	4 984 ± 1 491	5 651 ± 995
6 h	8 692 ± 1 397	4 566 ± 1 342	6 021 ± 1 046
24 h	2 686 ± 454	1 955 ± 199	2 309 ± 446

From Rouquie (2008d)
cpm, counts per minute

in the mouse through induction of glucuronidation of this hormone. A similar mechanism may be assumed for fluopyram (Rouquie, 2008d).

In a second mechanistic study conducted to determine the potential effects of fluopyram on the clearance of T_4 in the blood, fluopyram (purity 94.7%) was administered via the diet at a concentration of 2000 ppm (equivalent to about 286 mg/kg bw per day) to a group of eight male C57BL/6J mice for 4 days; a similarly constituted group of eight males received untreated diet and acted as a control group. A further group of eight males received phenobarbital sodium salt (purity 99.6%; suspended in a 0.5% aqueous solution of methylcellulose) at a dose level of 80 mg/kg bw per day by oral gavage for 4 days. On study day 5, each animal received by intravenous injection via the tail 250 µl of diluted ^{125}I-labelled T_4 solution corresponding to a dose of 37 kBq of ^{125}I-labelled T_4 per animal. Approximately 3 hours post-administration with ^{125}I-labelled T_4, each animal received 0.1 mg of sodium iodide in 250 µl of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal 40 minutes and 1.5, 4 and 24 hours post-administration of ^{125}I-labelled T_4. The level of ^{125}I radioactivity in each sample was measured using a gamma scintillation counter. The decrease in the level of ^{125}I radioactivity was indicative of the rate of T_4 clearance from the blood. Animals were checked daily for mortality and clinical signs. Body weights were recorded on study days 1 and 5.

There were no clinical signs during the course of the study. One animal from the phenobarbital-treated group was sacrificed after intravenous injection of the radiolabelled T_4 due to a technical problem during the injection. There was no statistically significant effect on body weight.

The results show that following an intravenous injection of ^{125}I-labelled T_4, the radioactivity level in the blood of fluopyram-treated animals was lower than that in the blood of the corresponding control animals. This decrease in the level of radioactivity in the blood of fluopyram-treated animals was observed at all time points examined and reflects a more rapid clearance of T_4 in these animals over a 24-hour period, compared with the controls. A partly similar response was seen in animals treated with the reference compound, phenobarbital (Table 41).

In conclusion, the results indicate that the clearance of T_4 from the blood after treatment with fluopyram or phenobarbital was increased when compared with untreated mice over a 24-hour period (Rouquie, 2009).

In a mechanistic study conducted to determine the potential effects of fluopyram on thyroid hormone synthesis at the level of thyroid peroxidase (TPO), interactions with TPO-catalysed reactions

Table 41. Whole blood radioactivity after a single administration of ^{125}I-labelled T_4 to mice in a second mechanistic study

Time point	Whole blood radioactivity (cpm)		
	Control group	Fluopyram: 2000 ppm	Phenobarbital: 80 mg/kg bw per day
Number of animals	*8*	*8*	*7*
40 min	19 726 ± 1 468	6 163** ± 2 025	10 691** ± 1 197
1.5 h	16 930 ± 1 001	6 388** ± 1 982	10 592** ± 1 245
4 h	13 781 ± 1 099	6 111** ± 1 304	9 312** ± 1 330
24 h	3 889 ± 561	2 562** ± 482	2 653** ± 547

From Rouquie (2009)
** $P < 0.01$
cpm, counts per minute

were studied in vitro using solubilized hog thyroid microsomes as an enzyme source. Amitrole and ethylenethiourea served as positive control substances.

Amitrole, a potent inhibitor of TPO, strongly inhibited TPO-catalysed oxidation of guaiacol and formation of iodine. About 50% inhibition was observed in the presence of amitrole at a concentration of 1 µmol/l for guaiacol oxidation and 0.1 µmol/l for iodine formation. Ethylenethiourea, which is not a TPO inhibitor, but a trap of the iodinating intermediate generated by TPO from iodide, temporarily suppressed iodine formation (Table 42).

In contrast, fluopyram did not affect TPO-catalysed guaiacol oxidation up to 300 µmol/l, the highest concentration tested. Similarly, TPO-catalysed iodine formation was not affected by fluopyram at 300 µmol/l. These findings strongly suggest that fluopyram does not affect thyroid hormone synthesis at the level of TPO (Freyberger, 2008).

(c) Toxicology of metabolites

For two plant metabolites of fluopyram, studies of acute toxicity, genotoxicity and short-term toxicity were conducted: fluopyram-pyridyl-carboxylic acid (AE C657188) or 3-chloro-5-(trifluoromethyl)pyridine-2-carboxylic acid; and fluopyram-methyl-sulfoxide (AE 1344122) or 3-(methylsulfinyl)-5-(trifluoromethyl)pyridine-2-carboxylic acid. The metabolite fluopyram-pyridyl-carboxylic acid (AE C657188) is also found in the metabolism of rats.

(i) Fluopyram-pyridyl-carboxylic acid (AE C657188)

In an acute oral toxicity study, AE C657188 (purity 99.7%) in 1% aqueous methylcellulose was administered by gavage to groups of fasted male and female Hsd:Sprague-Dawley (CD) rats at volumes of 10–20 ml/kg bw. In a preliminary test, one male and one female rat were dosed at 4000 mg/kg bw, whereas in the main test, groups of three male and three female rats were dosed at 500 and 2000 mg/kg bw. The observation period lasted for at least 14 days. Both rats dosed at 4000 mg/kg bw were found dead on day 3. Clinical signs comprised piloerection, hunched posture, lethargy, abnormal gait, shallow respiration, reduced body temperature, dull, partially closed eyes, body tremors, pallor of the extremities and lacrimation. At 500 and 2000 mg/kg bw, no mortalities occurred, and clinical signs were confined to piloerection on day 1 in males at both dose levels and in females at 500 mg/kg bw. All animals had recovered by day 2. Under the conditions of this study, the LD_{50} was greater than 2000 mg/kg bw and less than 4000 mg/kg bw for both male and female rats (Coleman, 2000).

In a reverse gene mutation assay in bacteria conducted according to OECD test guideline 471, *S. typhimurium* (strains TA98, TA100, TA1535, TA1537) and *Escherichia coli* (strain WP2*uvr*A/

Table 42. Effect of fluopyram and amitrole on TPO-catalysed reactions

	Control	Fluopyram			Amitrole
	Concentration (µmol/l)				
	0	3	30	300	1 or 0.1
Guaiacol oxidation (ΔE/min)	0.121	0.122	0.123	0.124	0.054
(% of control)	(—)	(101)	(101)	(102)	(44.5)
Iodine formation (ΔE/min)	0.259	0.269	0.246	0.260	0.131
(% of control)	(—)	(104)	(95)	(100)	(50.5)

From Freyberger (2008)
E, extinction (absorption) of light

pKM101) were exposed to AE C657188 (purity 99.7%), using DMSO as solvent, in the presence and absence of S9 metabolic activation in two independent sets of experiments. For the initial plate incorporation test using doses up to and including 5000 µg/plate, three plates were used for each strain, condition and dose. Vehicle and positive controls were included in each experiment. The independent repeat was performed with preincubation for 30 minutes at 37 °C. Other conditions remained unchanged. Doses up to and including 5000 µg/plate did not cause any bacteriotoxic effects. No substance precipitation was observed. No evidence for mutagenic activity was seen at any dose level of AE C657188. The concurrent positive controls induced the appropriate responses in the corresponding strains. Therefore, AE C657188 was considered to be not mutagenic in the bacterial strains tested, in either the presence or absence of metabolic activation (Kitching, 2000).

In an in vitro mammalian cell gene mutation test conducted according to OECD test guideline 476, AE C657188 (purity 99.7–99.9%) dissolved in DMSO was tested for its ability to induce forward mutations at the HPRT locus in Chinese hamster V79 cells. Two independent sets of experiments were conducted at concentrations of 16–5000 µg/ml in the presence and absence of S9 metabolic activation. As precipitation was observed at 4000 µg/ml and above, no evaluation was possible at 5000 µg/ml. Ethyl methanesulfonate and dimethylbenzanthracene served as positive controls in the experiments without and with metabolic activation, respectively. The cells were treated for 5 hours in both experiments, without and with metabolic activation. After this, the incubation media were replaced by culture medium and the cells were incubated for about 1 week for expression of mutant cells. This was followed by incubation of cells for 1 week in selection medium containing 6-thioguanine. Neither in the initial nor in the confirmatory studies was any increase in the mutant frequency observed. In contrast to this, the positive control substances ethyl methanesulfonate and dimethylbenzanthracene resulted in a marked increase in mutant frequency. Based on the results of the study, AE C657188 was considered to be not mutagenic in the V79/HPRT forward mutation assay, in either the presence or absence of metabolic activation (Herbold, 2003a).

In an in vitro mammalian chromosomal aberration test conducted according to OECD test guideline 473, the clastogenic potential of AE C657188 (purity 99.1%) dissolved in DMSO was tested in duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. In experiment 1, treatment was for 3 hours and harvest at 20 hours, in the absence of S9 at 739–2256 µg/ml and in the presence of S9 at 379–2256 µg/ml. In experiment 2, treatment in the absence of S9 was continuous for 20 hours at 739–2256 µg/ml, whereas treatment in the presence of S9 was for 3 hours at 379–2256 µg/ml and harvest at 20 hours. Vehicle (DMSO) and positive controls (cyclophosphamide and 4-nitroquinoline 1-oxide for the tests with and without

metabolic activation, respectively) were included to demonstrate the sensitivity of the test system. About 2 hours prior to cell harvest, colchicine was added to arrest cells in metaphase. After slide preparation and staining of the cells, 200 metaphases per dose and treatment condition were analysed for chromosomal aberrations. None of the cultures treated with AE C657188 in the absence or in the presence of S9 mix showed any biologically relevant or statistically significant increase in the numbers of aberrant metaphases. The positive controls cyclophosphamide and 4-nitroquinoline 1-oxide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the S9 mix used. Based on the results of this test, AE C657188 was considered not to be clastogenic for mammalian cells in vitro, in either the presence or absence of metabolic activation (Lloyd, 2003a).

In a study of toxicity conducted according to OECD test guideline 407, groups of five male and five female Sprague-Dawley Crl: CD(SD)IGS Br rats were given diets containing AE C657188 (purity 99.1%) at a concentration of 0, 20, 200, 2000 or 20 000 ppm, equal to 0, 1.50, 15.0, 149 and 1574 mg/kg bw per day in males and 0, 1.63, 15.9, 162 and 1581 mg/kg bw per day in females, for 28 days. Animals were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded once weekly. During the acclimatization phase, all animals were subjected to an ophthalmic examination. All animals at 0 and 20 000 ppm were re-examined at the end of week 3. Haematology, plasma chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically. The only treatment-related findings occurred at the high dose of 20 000 ppm and consisted of a slight decrease in feed consumption in females of 9% on weeks 2 and 4 and 15% ($P \leq 0.05$) on week 3 and a slight decrease in inorganic phosphorus concentration of 12% ($P \leq 0.01$) in males, compared with the controls. No other treatment-related changes were observed.

The NOAEL was 20 000 ppm, equal to 1574 mg/kg per day, the highest dose tested (Kennel, 2001).

(ii) Fluopyram-methyl-sulfoxide (AE 1344122)

In an acute oral toxicity study conducted according to OECD test guideline 423 (acute toxic class method), AE 1344122 (purity 98.8%) in demineralized water and 2% Cremophor EL was administered by gavage to three female HsdCpb:WU rats at a single dose of 2000 mg/kg bw and a volume of 10 ml/kg bw. Because there was no mortality, a second group of three female rats was additionally dosed at 2000 mg/kg bw. The observation period lasted for at least 14 days. No mortality occurred, and no clinical signs were observed. The oral LD_{50} was greater than 2000 mg/kg bw for male and female rats. According to the Globally Harmonised System for the classification of chemicals, the LD_{50} cut-off of AE 1344122 was greater than or equal to 5000 mg/kg bw (Schuengel, 2003).

In a reverse gene mutation assay in bacteria conducted according to OECD test guideline 471, *S. typhimurium* (strains TA98, TA100, TA102, TA1535, TA1537) were exposed to AE 1344122 (purity 98.8%), using DMSO as solvent, in the presence and absence of S9 metabolic activation in two independent sets of experiments. For the initial plate incorporation test using doses up to and including 5000 µg/plate, three plates were used for each strain, condition and dose. Vehicle and positive controls were included in each experiment. The independent repeat was performed with preincubation for 20 minutes at 37 °C. Other conditions remained unchanged. Bacteriotoxic effects were observed at 500 µg/plate and above as well as at 500 µg/tube and above. Owing to the weakness of this effect in the plate incorporation test, up to 5000 µg/plate could be used for assessment, whereas 5000 µg/tube could not be used for assessment. No substance precipitation was observed. No evidence for mutagenic activity was seen at any dose level of AE 1344122. The concurrent positive controls induced the appropriate responses in the corresponding strains. Therefore, AE 1344122

was considered to be not mutagenic in the bacterial strains tested, in either the presence or absence of metabolic activation (Herbold, 2003b).

In an in vitro mammalian cell gene mutation test conducted according to OECD test guideline 476, AE 1344122 (purity 98.8%) dissolved in DMSO was tested for its ability to induce forward mutations at the HPRT locus in Chinese hamster V79 cells. Two independent sets of experiments were conducted at concentrations of 75–2400 µg/ml in the presence and absence of S9 metabolic activation. Testing was performed up to the limit of solubility of AE 1344122, with no cytotoxic effects observed. Ethyl methanesulfonate and dimethylbenzanthracene served as positive controls in the experiments without and with metabolic activation, respectively. The cells were treated for 5 hours in both experiments, without and with metabolic activation. After this, the incubation media were replaced by culture medium and the cells were incubated for about 1 week for expression of mutant cells. This was followed by incubation of cells for 1 week in selection medium containing 6-thioguanine. Neither in the initial nor in the confirmatory studies was any increase in the mutant frequency observed. In contrast to this, the positive control substances ethyl methanesulfonate and dimethylbenzanthracene resulted in a marked increase in mutant frequency. Based on the results of the study, AE 1344122 was considered to be not mutagenic in the V79/HPRT forward mutation assay, in either the presence or absence of metabolic activation (Herbold, 2003c).

In an in vitro mammalian chromosomal aberration test conducted according to OECD test guideline 473, the clastogenic potential of AE 1344122 (purity 98.8%) dissolved in DMSO was tested in duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. In experiment 1, treatment was for 3 hours and harvest at 20 hours, in the absence of S9 at 1296–2532 µg/ml and in the presence of S9 at 1620–2532 µg/ml. In experiment 2, treatment in the absence of S9 was continuous for 20 hours at 306–1123 µg/ml, whereas treatment in the presence of S9 was for 3 hours at 1829–2532 µg/ml and harvest at 20 hours. Vehicle (DMSO) and positive controls (cyclophosphamide and 4-nitroquinoline 1-oxide for the test with and without metabolic activation, respectively) were included to demonstrate the sensitivity of the test system. About 2 hours prior to cell harvest, colchicine was added to arrest cells in metaphase. After slide preparation and staining of the cells, 200 metaphases per dose and treatment condition were analysed for chromosomal aberrations. None of the cultures treated with AE 1344122 in the absence or in the presence of S9 mix showed any biologically relevant or statistically significant increase in the numbers of aberrant metaphases, with one exception. In experiment 2, following 20 hours of treatment in the absence of S9, a single culture at the highest concentration (1123 µg/ml) exhibited an aberrant cell frequency that marginally exceeded the historical negative control range. However, the aberrant cell frequency of the replicate culture, and all other treated cultures in both experiments, fell within the normal range; therefore, this observation was not considered biologically significant. The positive controls cyclophosphamide and 4-nitroquinoline 1-oxide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the S9 mix used. Based on the results of this test, AE 1344122 was considered not to be clastogenic for mammalian cells in vitro, in either the presence or absence of metabolic activation (Lloyd, 2003b).

In a study of toxicity conducted according to OECD test guideline 407, groups of 10 male and 10 female Wistar Rj: WI (IOPS HAN) rats were given diets containing AE 1344122 (purity 98.8%) at concentrations of 0, 20, 200, 2000 and 20 000 ppm, equal to 0, 1.5, 14.9, 152 and 1495 mg/kg bw per day in males and 0, 1.7, 16.8, 167 and 1616 mg/kg bw per day in females, for 28 days. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. In addition, grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes were examined once during the acclimatization phase and during week 4 of the study. Body weight and

feed consumption were recorded once weekly. During the acclimatization phase, all animals were subjected to an ophthalmic examination; all animals in the control and 20 000 ppm groups were re-examined during week 4. Haematology, clinical chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

Toxicological findings were confined to the highest dose level of 20 000 ppm and consisted of scabs around the nose/head region (three males, together with soiling around the eye in one male), chromodacryorrhoea (two other males) and anogenital soiling (one female). Body weight in males was reduced by 7%, with overall body weight gain reduced by 18% by day 28, compared with the controls. In females, body weight was reduced by 4% on day 28, whereas overall body weight gain was 18% lower than in controls. Feed consumption was reduced by 13% during week 1 in males and by 9% over the course of the study in females. Organic phosphorus concentration was reduced by 10% by day 28 in males. Urinalysis revealed coarsely granular casts in the urine of 1 of 10 males and 9 of 10 females, slightly lower pH values and ketone levels in males, and higher urinary volume in both sexes. Microscopically, minimal to moderate tubular degeneration/regeneration and single-cell necrosis of tubular epithelial cells of minimal to moderate severity were observed in the kidneys of 8 of 10 females. These kidney changes were correlated with the coarsely granular casts observed in the urine.

At 2000 ppm, treatment-related clinical findings were limited to scabs around the nose/head region in 2 of 10 males and nasal soiling noted in one male. In the absence of any other treatment-related effects in either sex at this dose level, these findings were considered to have no toxicological relevance.

The NOAEL was 2000 ppm, equal to 152 mg/kg bw per day, based on reduced body weight gain and feed consumption in both sexes and urinalysis findings and related histological changes in the kidney (tubular degeneration and single-cell necrosis) in females at 20 000 ppm (McElligott, 2003).

3. Observations in humans

There were no reports of adverse health effects in manufacturing plant personnel. Also, there were no reports of poisonings with fluopyram.

Comments

Biochemical aspects

In rats given [^{14}C]phenyl ring–labelled or [^{14}C]pyridyl ring–labelled fluopyram orally by gavage, absorption was rapid and accounted for ≥ 93% of the total recovered radioactivity after a single dose of 5 mg/kg bw (both labels) or 250 mg/kg bw (phenyl ring label). The maximum plasma concentrations of radiolabelled material were reached after 0.7–3 hours with the pyridyl ring label and after 8–48 hours with the phenyl ring label. Radiolabel was widely distributed throughout the body. Residues in tissues 168 hours after a single dose of 5 mg/kg bw accounted for less than 0.5% (pyridyl ring label) or 3–5% (phenyl ring label) of the administered dose, with liver and kidney containing the highest concentrations of residues. Elimination of the radiolabel was via the faeces (39–64%) and the urine (35–60%), with evidence of significant enterohepatic circulation. In bile duct–cannulated rats, extensive biliary excretion (79–87%) was demonstrated. The terminal elimination half-lives of radiolabelled material ranged from 24 to 53 hours for the phenyl ring–labelled fluopyram and from 56 to 73 hours for the pyridyl ring–labelled fluopyram.

Fluopyram was extensively metabolized, and more than 20 metabolites were identified. The metabolism was principally oxidative and took place mainly at the ethylene bridge of the molecule.

Hydrolytic cleavage of the molecule and subsequent oxidation were also observed, as was conjugation of several hydroxylated metabolites with glucuronic acid and, to a lesser extent, sulfate.

Toxicological data

Fluopyram was of low toxicity after oral and dermal exposure in rats (LD_{50} > 2000 mg/kg bw), and neither mortality nor systemic toxicity occurred at this limit dose. After inhalation exposure in rats, fluopyram was also of low toxicity (LC_{50} > 5.1 mg/l), and the clinical signs observed were nonspecific and reversible within 1–5 days. Fluopyram was not a skin irritant in rabbits, was only minimally irritating to the eye of rabbits and was not a skin sensitizer in the local lymph node assay in mice.

Following repeated administration of fluopyram to mice, rats and dogs, the liver was the major target organ in all species tested. The effects noted at lower doses (increased liver weights, hepatocellular hypertrophy) were consistent with the induction of hepatic CYP, whereas effects observed at higher doses included hepatocellular degeneration or necrosis and related clinical chemistry findings (e.g. increased serum levels of liver enzymes, cholesterol and triglycerides). In mice, the adrenals were an additional target. The thyroid effects seen in mice and rats were considered to be secondary to the enhanced hepatic clearance of thyroid hormones. The hyaline droplet nephropathy observed in male rats was considered not to be relevant to humans, as this effect is due to an accumulation of α_{2u}-globulin, a protein that is found only in trace amounts in humans, in the proximal tubules.

In a 28-day range-finding study in mice, the NOAEL was 150 ppm (equal to 24.7 mg/kg bw per day), based on effects in the liver (hepatocellular necrosis) and the adrenals (hypertrophy of the zona fasciculata) at 1000 ppm and above. In a 90-day study in mice, the NOAEL was 150 ppm (equal to 26.6 mg/kg bw per day), based on effects in the liver (hepatocellular necrosis) and the adrenals (cortical vacuolation) at 1000 ppm.

In a 28-day range-finding study in rats, the NOAEL was 400 ppm (equal to 31.0 mg/kg bw per day), based on effects in the liver (increased liver weight, hepatocellular hypertrophy, enzyme induction) and the thyroid (hypertrophy of follicular cells, colloid depletion) at 3200 ppm. In a 90-day study in rats, the NOAEL was 200 ppm (equal to 12.5 mg/kg bw per day), based on effects in the liver (hepatocellular hypertrophy and vacuolation) and the thyroid (hypertrophy of follicular cells) at 1000 ppm and above. Effects at higher doses (3200 ppm, equal to 204 mg/kg bw per day) included decreased body weight gain and feed consumption, decreased haemoglobin and haematocrit, clinical chemistry changes and increased levels of T_3, T_4 and TSH. It was noted that levels of these hormones more often change in opposite directions, with decreases in T_3 and/or T_4 being associated with increases in TSH. This pattern was subsequently observed in mechanistic studies described below.

In a 28-day range-finding study in dogs, the NOAEL was 150 mg/kg bw per day, based on treatment-related clinical signs (liquid faeces) and liver toxicity (increased liver weight, clinical chemistry changes, histopathological findings) at 750 mg/kg bw per day. In a 90-day study in dogs, the NOAEL was 800 ppm (equal to 28.5 mg/kg bw per day), based on liver toxicity (increased liver weight, histopathological findings, including necrosis, related clinical chemistry changes) at 5000 ppm and above. In a 1-year study in dogs, the NOAEL was 400 ppm (equal to 13.2 mg/kg bw per day), based on liver toxicity (hepatocellular hypertrophy, increased serum levels of alkaline phosphatase) at 2000 ppm. The overall NOAEL for the 90-day and 1-year dog studies was 28.5 mg/kg bw per day.

Long-term studies of toxicity and carcinogenicity were conducted in mice and rats. In an 18-month study of carcinogenicity in mice, the NOAEL for oncogenicity was 150 ppm (equal to 20.9 mg/kg bw per day), based on an increased incidence of follicular cell adenoma in the thyroid in males at 750 ppm. The NOAEL for non-neoplastic changes was 30 ppm (equal to 4.2 mg/kg bw per day), based on liver toxicity (hepatocellular single-cell degeneration/necrosis) and thyroid changes (follicular cell hyperplasia) in males at 150 ppm and above.

In a mechanistic study on thyroid tumorigenesis in male mice, increased liver weights, hepatocellular hypertrophy, increased hepatic CYP content and marked increases in hepatic activities of PROD (CYP2B) and BROD (CYP3A) were observed after 3 and 14 days of exposure to fluopyram at 2000 ppm (equal to 308–314 mg/kg bw per day), whereas EROD (CYP1A2) activity was only slightly increased and UDPGT activity (using 4-nitrophenol as a substrate) was unaffected. However, decreased T_4 and increased TSH levels were noted after 3 and 14 days. The pattern of changes in the liver and thyroid end-points was similar to the profile observed in male mice treated with phenobarbital at 80 mg/kg bw per day in a parallel study.

In a 3-day study of effects on gene expression in the liver of male mice, quantitative PCR analyses demonstrated that both fluopyram (2000 ppm, equivalent to approximately 286 mg/kg bw per day) and phenobarbital (80 mg/kg bw per day) induced an upregulation of the sulfotransferase and UDPGT gene transcripts. For most genes, at the doses used, effects were more pronounced with fluopyram than with phenobarbital. Further mechanistic studies in male mice demonstrated that both fluopyram (2000 ppm, equivalent to approximately 286 mg/kg bw per day) and phenobarbital (80 mg/kg bw per day) significantly increased the clearance of intravenously administered T_4. In vitro studies showed that fluopyram did not affect the TPO-catalysed oxidation of guaiacol.

The Meeting concluded that for the thyroid tumours in male mice, there was evidence that the MOA was likely to be secondary to enhanced hepatic clearance of T_4, leading to hormone imbalance. The marked quantitative species differences in the inherent susceptibility for neoplasia in response to thyroid hormone imbalance allowed for the conclusion that the fluopyram-induced thyroid tumours in mice are not relevant to humans.

In a 24-month study of toxicity and carcinogenicity in rats, the NOAEL for oncogenicity was 150 ppm (equal to 8.6 mg/kg bw per day), based on an increased incidence of liver cell tumours (adenoma and carcinoma) in females at 1500 ppm (equal to 89 mg/kg bw per day). The NOAEL for non-neoplastic changes was 30 ppm (equal to 1.2 mg/kg bw per day), based on increased incidences of findings in the liver (hepatocellular hypertrophy and eosinophilic foci in males) at 150 ppm and above. The changes in the thyroid (follicular cell hypertrophy, colloid alteration) at 150 ppm and above were attributable to the apparent susceptibility of rats to thyroid hormone imbalance and were therefore not considered relevant to humans.

In a mechanistic study on liver tumorigenesis in female rats, increased liver weights, hepatocellular hypertrophy, increased cell proliferation in the centrilobular and periportal zones of the hepatic lobules, increased hepatic CYP content and moderate to marked increases in hepatic activities of EROD, PROD, BROD and UDPGT were observed after 7 days of exposure to fluopyram (3000 ppm, equal to 193 mg/kg bw per day). The pattern of changes was similar to the profile observed in female rats treated with phenobarbital at 80 mg/kg bw per day in a parallel study.

The Meeting concluded that the relevance of the liver tumours in female rats to humans could not be discounted, as the results of the mechanistic studies were only partly sufficient to support the proposed phenobarbital-like MOA. In particular, activation of the constitutive androstane receptor (CAR) by fluopyram has not been clearly demonstrated, and there is a lack of dose–response concordance with key precursor events and tumour incidence (see Appendix 1). However, the Meeting noted that the MOA for the observed liver tumours is a high-dose phenomenon that would be anticipated to exhibit a threshold.

Fluopyram was tested for genotoxicity in vitro and in vivo in an adequate range of assays. It was not found to be genotoxic in mammalian and microbial test systems.

The Meeting concluded that fluopyram was unlikely to be genotoxic.

On the basis of the absence of genotoxicity, the human non-relevance of the thyroid tumours in mice and the fact that the dose–response relationship for the liver tumours in rats would be anticipated to exhibit a threshold, the Meeting concluded that fluopyram is unlikely to pose a carcinogenic risk to humans at dietary exposure levels.

In a two-generation reproductive toxicity study in rats, the NOAEL for effects on fertility was 1200 ppm (equal to 82.4 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 220 ppm (equal to 13.9 mg/kg bw per day), based on decreased body weight and/or body weight gain in females and liver toxicity in both sexes at 1200 ppm. The NOAEL for offspring toxicity was 220 ppm (equal to 13.9 mg/kg bw per day), based on decreased body weight gain and decreased spleen and thymus weights at 1200 ppm.

In a prenatal developmental toxicity study in rats, the NOAEL for maternal toxicity was 30 mg/kg bw per day, based on decreased body weight gain and feed consumption and increased liver weights and hepatocellular hypertrophy at 150 mg/kg bw per day and above. The NOAEL for prenatal developmental toxicity was 150 mg/kg bw per day, based on lower fetal body weights and an increased incidence of visceral and skeletal variations at 450 mg/kg bw per day.

In a prenatal developmental toxicity study in rabbits, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on reduced body weight gain and feed consumption at 75 mg/kg bw per day. The NOAEL for prenatal developmental toxicity was 25 mg/kg bw per day, based on reduced fetal body weights and an increased number of small fetuses ("runts") at 75 mg/kg bw per day.

The Meeting concluded that fluopyram caused developmental toxicity only at doses that were maternally toxic and that it was not teratogenic.

In an acute neurotoxicity study in rats, the NOAEL for neurotoxicity was 50 mg/kg bw, based on decreased motor and locomotor activities at 100 mg/kg bw and above. In a subchronic study of neurotoxicity in rats, the NOAEL for neurotoxicity was 2500 ppm (equal to 164.2 mg/kg bw per day), the highest dose tested. The NOAEL for general toxicity was 500 ppm (equal to 33.2 mg/kg bw per day), based on decreased body weight, body weight gain and feed consumption and increased liver weight at 2500 ppm.

Fluopyram-pyridyl-carboxylic acid (AE C657188), a plant metabolite of fluopyram, was of low acute oral toxicity in rats (LD_{50} > 2000 mg/kg bw) and showed no genotoxic potential in vitro in mammalian or microbial test systems. In a 28-day oral toxicity study in rats, there was no evidence of toxicity up to dietary concentrations of 20 000 ppm (equal to 1574 mg/kg bw per day), the highest dose tested.

Fluopyram-methyl-sulfoxide (AE 1344122), a plant metabolite of fluopyram, was of low acute oral toxicity in rats (LD_{50} > 2000 mg/kg bw) and showed no genotoxic potential in vitro in mammalian or microbial test systems. In a 28-day oral toxicity study in rats, the NOAEL was 2000 ppm (equal to 152 mg/kg bw per day), based on reduced body weight gain and feed consumption in both sexes and kidney toxicity (tubular degeneration and single-cell necrosis, urinalysis findings) in females at 20 000 ppm.

There were no reports of adverse health effects in manufacturing plant personnel. Also, there were no reports of poisonings with fluopyram.

The Meeting concluded that the existing database on fluopyram was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for fluopyram of 0–0.01 mg/kg bw, based on a NOAEL of 1.2 mg/kg bw per day for changes in the liver (hepatocellular hypertrophy, eosinophilic foci) at 6.0 mg/kg bw per day in a 2-year rat study. A safety factor of 100 was applied. The ADI provides a margin of at least 860-fold relative to the NOAEL for liver tumours in rats.

The Meeting established an acute reference dose (ARfD) for fluopyram of 0.5 mg/kg bw, based on the NOAEL of 50 mg/kg bw for decreases in measures of motor and locomotor activities at 100 mg/kg bw in an acute neurotoxicity study in rats. A 100-fold safety factor was applied.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Thirteen-week study of toxicity	Toxicity	150 ppm, equal to 26.6 mg/kg bw per day	1000 ppm, equal to 188 mg/kg bw per day
	Eighteen-month study of toxicity and carcinogenicity	Toxicity	30 ppm, equal to 4.2 mg/kg bw per day	150 ppm, equal to 20.9 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 20.9 mg/kg bw per day	750 ppm, equal to 105 mg/kg bw per day
Rat	Thirteen-week study of toxicity	Toxicity	200 ppm, equal to 12.5 mg/kg bw per day	1000 ppm, equal to 60.5 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity	Toxicity	30 ppm, equal to 1.2 mg/kg bw per day	150 ppm, equal to 6.0 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 8.6 mg/kg bw per day	1500 ppm, equal to 89 mg/kg bw per day
	Multigeneration study of reproductive toxicity	Fertility	1200 ppm, equal to 82.4 mg/kg bw per day[a]	—
		Parental toxicity	220 ppm, equal to 13.9 mg/kg bw per day	1200 ppm, equal to 82.4 mg/kg bw per day
		Offspring toxicity	220 ppm, equal to 13.9 mg/kg bw per day	1200 ppm, equal to 82.4 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	30 mg/kg bw per day	150 mg/kg bw per day
		Embryo and fetal toxicity	150 mg/kg bw per day	450 mg/kg bw per day
	Acute neurotoxicity study[b]	Neurotoxicity	50 mg/kg bw	100 mg/kg bw
	Subchronic neurotoxicity study	Neurotoxicity	2500 ppm, equal to 164.2 mg/kg bw per day[a]	—
Rabbit	Developmental toxicity study[b]	Maternal toxicity	25 mg/kg bw per day	75 mg/kg bw per day
		Embryo and fetal toxicity	25 mg/kg bw per day	75 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity[c]	Toxicity	800 ppm, equal to 28.5 mg/kg bw per day	2000 ppm, equal to 66.1 mg/kg bw per day

[a] Highest dose tested.
[b] Gavage administration.
[c] Two or more studies combined.

Estimate of acceptable daily intake for humans

0–0.01 mg/kg bw

Estimate of acute reference dose

0.5 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to fluopyram

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; approximately 93%
Distribution	Wide; highest concentrations in liver and kidney
Rate and extent of excretion	> 95% within 168 h (35–60% in urine; 39–64% in faeces; up to 79–87% in bile)
Potential for accumulation	None
Metabolism in animals	Extensive; hydroxylation, oxidation and hydrolytic cleavage of the molecule, followed by conjugation (glucuronic acid, sulfate)
Toxicologically significant compounds (animals, plants and the environment)	Fluopyram
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.1 mg/l (4 h, nose-only exposure)
Rabbit, dermal irritation	Not irritating
Rabbit, eye irritation	Minimally irritating
Mouse, dermal sensitization	Not sensitizing (local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Liver (enzyme induction, hypertrophy, single-cell necrosis) in mice, rats and dogs, adrenals (cortical hypertrophy and vacuolation) in mice
Lowest relevant oral NOAEL	12.5 mg/kg bw per day (90-day study in rats)
Lowest relevant dermal NOAEL	300 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data
Long-term toxicity and carcinogenicity	
Target/critical effect	Liver (hypertrophy, single-cell degeneration/necrosis) in mice and rats
Lowest relevant NOAEL	1.2 mg/kg bw per day (2-year study in rats)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans at levels of dietary exposure
Genotoxicity	
	No genotoxic potential
Reproductive toxicity	
Reproductive target/critical effect	No effects on fertility at highest dose tested; decreased body weight gain in pups at parentally toxic dose
Lowest relevant reproductive NOAEL	13.9 mg/kg bw per day for offspring toxicity (two-generation study in rats)
Developmental target/critical effect	Decreased fetal weight and increased number of small fetuses at maternally toxic dose
Lowest relevant developmental NOAEL	25 mg/kg bw per day (rabbit)

Neurotoxicity	
Acute neurotoxicity	Decrease in motor and locomotor activity; NOAEL: 50 mg/kg bw
Subchronic neurotoxicity	No evidence of neurotoxicity at highest dose tested
Other toxicological studies	
Mechanistic studies	Studies on liver enzyme induction (rats, mice) and thyroid hormone levels (mice) suggest a non-genotoxic threshold mechanism for carcinogenicity
Studies on plant metabolites	Fluopyram-pyridyl-carboxylic acid (AE C657188): lower toxicity than parent compound, not genotoxic in vitro
	Fluopyram-methyl-sulfoxide (AE 1344122): lower toxicity than parent compound, not genotoxic in vitro
Medical data	
	Limited data; no adverse health effects reported in manufacturing plant personnel

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw	Two-year study of toxicity in rat	100
ARfD	0.5 mg/kg bw	Acute neurotoxicity study in rat	100

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Appendix 1: Application of the IPCS conceptual framework for cancer risk assessment

(IPCS framework for analysing the relevance of a cancer mode of action for humans)

This framework, developed by an International Programme on Chemical Safety (IPCS) working group, provides a generic approach to the principles commonly used in evaluating a postulated MOA for tumour induction by a chemical (Sonich-Mullin et al., 2001; Boobis et al., 2006). Thus, the framework was used by the 2010 JMPR to provide a structured approach to the assessment of the overall weight of evidence for the postulated MOA for the increased incidences of thyroid follicular cell adenomas in male mice and of hepatocellular adenomas and carcinomas in female rats observed after long-term administration of fluopyram.

Thyroid follicular cell adenomas in mice

1. Introduction

In the 18-month study of carcinogenicity in mice, increased incidences of thyroid follicular cell adenomas were observed in male mice after administration of fluopyram at a dietary concentration of 750 ppm, equal to 105 mg/kg bw per day (see section 2.3).

2. Postulated mode of action (theory of the case)

The postulated MOA for fluopyram-induced thyroid follicular cell tumours involves the perturbation of homeostasis of the pituitary–thyroid axis by an extra-thyroidal mechanism. Specifically, fluopyram induces hepatic microsomal enzymes, including sulfotransferases and UDPGT, which increases the metabolism of T_4 by conjugation and increased excretion of the conjugated hormone. The pituitary gland responds to a decrease in circulating serum T_4 levels by enhancing the release of TSH. Prolonged elevation of circulating TSH levels stimulates the thyroid gland to increase thyroid hormone synthesis and release, thus leading to thyroid follicular cell hypertrophy and hyperplasia. With chronic exposure, thyroid follicular cell hyperplasia eventually progresses to neoplasia.

3. Key events

The key events in the fluopyram MOA for thyroid tumour formation in mice include:

- induction of hepatic microsomal enzyme activity,
- increase in hepatic metabolism and excretion of T_4,
- decrease in serum T_4 half-life and concentration,
- increase in serum TSH concentration,
- thyroid follicular cell hypertrophy and follicular cell hyperplasia.

The key events as described above include changes in liver metabolism, alterations in hormone levels, increase in thyroid growth and lesion progression in the thyroid. These effects have been investigated and observed in male mice in short-term mechanistic studies and at interim and terminal sacrifices in a long-term study. The dose–response relationships and the temporal analyses of the key events and tumour response are presented below.

4. Concordance of dose–response relationships

The lowest-observed-adverse-effect levels (LOAELs) for the key effects in the MOA of fluopyram in the thyroid are provided in Table A1.

In all studies evaluated, increased liver weight and hepatocellular hypertrophy appeared to be the most sensitive indicators of changes in liver metabolism, occurring at dose levels of 4.2 and 20.9 mg/kg bw per day and above, respectively, in the 18-month study. A significant induction of hepatic microsomal enzymes was demonstrated in 3-day and 14-day mechanistic studies at dose levels of 308 and 314 mg/kg bw per day, respectively, whereas an upregulation of sulfotransferase and UDPGT gene transcripts was observed in a 3-day mechanistic study at a dose level of 286 mg/kg bw per day. Consistent with the enhanced clearance of T_4 in 3-day and 4-day mechanistic studies at a dose level of 286 mg/kg bw per day, decreases in serum T_4 levels and subsequent increases in TSH were confirmed in 3-day and 14-day mechanistic studies at dose levels of 308 and 314 mg/kg bw per day, respectively. Prolonged TSH stimulation leads to follicular cell hyperplasia of the thyroid. In the 18-month mouse study, thyroid hyperplasia was seen at dose levels of 20.9 mg/kg bw per day and above, whereas an increased thyroid tumour incidence was found at 105 mg/kg bw per day, the highest dose level tested.

Generally, there was a good correlation between the doses causing induction of liver enzymes and subsequent T_4 and TSH effects with those causing an increased incidence of thyroid hyperplasia and thyroid tumours.

Table A1. LOAELs for key effects in the MOA of fluopyram in mice

Effect	LOAEL (mg/kg bw per day)
Liver	
Induction of microsomal enzymes (total CYP, EROD, PROD, BROD)	308/314 (3-day/14-day mechanistic study)
Upregulation of sulfotransferase and UDPGT gene transcripts	286 (3-day mechanistic study)
Increase in liver weight	308/314 (3-day/14-day mechanistic study)
	26.6 (90-day study)
	4.2 (18-month study)
Hepatocellular hypertrophy	308/314 (3-day/14-day mechanistic study)
	26.6 (90-day study)
	20.9 (18-month study)
Hormones	
Increase in serum T_4 clearance	286 (3-day/4-day mechanistic study)
Decrease in serum T_4 level	308/314 (3-day/14-day mechanistic study)
Increase in serum TSH level	308/314 (3-day/14-day mechanistic study)
Thyroid	
Increase in thyroid hyperplasia	20.9 (12-month interim and terminal sacrifice, 18-month study)
Increase in thyroid tumours	105 (18-month study)

5. Temporal association

The key events, such as induction of hepatic microsomal enzymes, upregulation of sulfotransferase and UDPGT gene transcripts, increased clearance of thyroid hormones (decreased T_4 levels) and increased TSH levels, were observed after only a 3- to 14-day exposure to fluopyram. In the 18-month mouse study, thyroid follicular cell hyperplasia was first observed at the 12-month interim sacrifice, whereas an increased incidence of thyroid follicular cell adenoma was seen at the terminal sacrifice after 18 months. Thus, there is a logical temporal response, with all key events preceding tumour formation.

6. Strength, consistency and specificity of association of tumour response with key events

Based on information from the studies in mice described in the main part of this monograph, there is sufficient weight of evidence that the key events (e.g. induction of hepatic microsomal enzymes, upregulation of sulfotransferase and UDPGT gene transcripts, decrease in T_4, increase in TSH) are linked to the precursor lesions in the thyroid (as follicular cell hyperplasia) and the ultimate tumour response. The key events were observed consistently in a number of studies with differing experimental designs.

Data from a 90-day study in rats also support the observation that the key events (e.g. increase in TSH level, thyroid follicular cell hypertrophy) were reversible after cessation of exposure to fluopyram. These data presume that a similar recovery of TSH and non-neoplastic pathological changes in the thyroid would also occur in mice.

7. Biological plausibility and coherence

The relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by TSH and the progression of thyroid follicular cells

to hypertrophy, hyperplasia and eventually neoplasia is considered to be biologically plausible and has been shown in several studies in rodents and particularly in rats. Increased secretion of TSH may result via different mechanisms including increased hepatic clearance of T_4, as is the case with fluopyram.

Data described by Dellarco et al. (2006) support the perspective that increased TSH concentrations alone may cause rodent thyroid follicular cells to begin proliferation, and continued stimulation by TSH leads to hypertrophy and hyperplasia. These data are consistent with the data from fluopyram-exposed mice that developed thyroid follicular cell tumours following induction of hepatic enzymes that metabolize thyroid hormone, decreased circulating T_4 and sustained increased TSH.

8. Other modes of action

Genotoxicity is always one possible MOA to consider, but no genotoxic potential was demonstrated for fluopyram in the following tests:

- mutation in five strains of *Salmonella typhimurium*,
- mutation at the HPRT locus of Chinese hamster V79 cells,
- chromosomal aberration in Chinese hamster V79 cells,
- micronucleus induction in bone marrow cells of mice treated in vivo.

Therefore, the available evidence indicates that genotoxicity is not an alternative MOA for fluopyram.

In addition, a possible direct effect of fluopyram on TPO was investigated. However, a special study confirmed that fluopyram had no direct effect on TPO in vitro.

9. Uncertainties, inconsistencies and data gaps

No major data gaps were identified in the database for fluopyram with regard to the postulated MOA for thyroid follicular cell tumours.

Although no increase in UDPGT activity could be demonstrated in the 3-day and 14-day mechanistic studies with both fluopyram and phenobarbital, there was clear evidence for an upregulation of sulfotransferase and UDPGT gene transcripts, which are known to encode for enzymes that inactivate thyroid hormones via sulfate and glucuronide derivatives.

Also, the 3-day and 14-day mechanistic studies were conducted at a dose level (about 300 mg/kg bw per day) that was higher than the dose level with a tumour response in the 18-month study (105 mg/kg bw per day). However, this is not considered a critical data gap, because sufficient biological indicators representing the key events in the MOA are available and the associations between the key events and the tumour development are strong and consistent. The exposure to a higher dose for a shorter time achieves a target systemic concentration more rapidly than the lower dose, which allows a measurable response to occur following a short exposure.

10. Assessment of postulated mode of action

The data presented are considered, with a high degree of confidence, to be adequate to explain that the development of thyroid follicular cell adenoma in male mice following chronic exposure to fluopyram is secondary to enhanced hepatic clearance of thyroid hormones leading to increased secretion of TSH and enhanced thyroid growth.

11. Conclusion

There is sufficient experimental evidence that fluopyram induces thyroid follicular cell adenoma in male mice by a process including increased hepatic clearance of thyroid hormones and disruption of the hypothalamic–pituitary–thyroid axis. Although the postulated MOA could theoretically operate in humans, the particular sensitivity of rodents for neoplasia due to alterations in thyroid homeostasis allows for the conclusion that fluopyram does not pose a carcinogenic risk to humans at exposure levels relevant to residues in food.

Liver cell tumours in rats

1. Introduction

In the 24-month study of chronic toxicity and carcinogenicity in rats, increased incidences of liver cell tumours (adenoma and carcinoma) were observed in female rats after administration of fluopyram at a dietary concentration of 1500 ppm, equal to 89 mg/kg bw per day (see section 2.3).

2. Postulated mode of action (theory of the case)

The development of liver tumours in rats after long-term administration of fluopyram is initiated by activation of nuclear receptors, in particular CAR in hepatocytes, which is followed by altered expression of CAR-regulated genes and subsequent induction of liver enzymes (including CYP isoenzymes), hepatocellular hypertrophy, increase of cell proliferation and suppression of apoptosis and perturbation of liver function. The resulting single-cell necrosis is associated with increased cell replication, and suppression of normal apoptotic processes is associated with clonal expansion of potentially mutated cells that leads to foci of altered hepatocytes. Such altered foci ultimately progress to neoplasia (Bannasch, Haertel & Su, 2003).

The postulated MOA for fluopyram is considered to be similar to the MOA for phenobarbital, which induces liver cell tumours in rodents by a non-genotoxic mechanism. A key effect of phenobarbital is the induction of liver CYP enzymes, which is mediated through activation of nuclear receptors, in particular CAR.

3. Key events

The key events in the fluopyram MOA for liver tumour formation in rats include the following:

- *Activation of nuclear receptors, in particular CAR*: Activation of nuclear receptors, in particular CAR, is most probably an early step, although activation of CAR by fluopyram has not been demonstrated conclusively. Nevertheless, it is likely that this does occur, given that induction of liver enzymes resulted from short-term administration of the compound to rats and mice.
- *Induction of hepatic CYP enzymes*: Induction of activities of hepatic CYP enzymes, particularly BROD and PROD, was observed after administration of fluopyram for 7 days (193 mg/kg bw per day) in a pattern that was similar to that produced by phenobarbital (80 mg/kg bw per day) and may be considered to be indicative of a CAR-mediated response.
- *Increase of liver cell proliferation*: Increased liver cell proliferation was demonstrated by an increased BrdU labelling index in the liver after administration of fluopyram for 7 days at 193 mg/kg bw per day and by an increased number of mitoses in liver cells after administration of fluopyram for 12 and 24 months at 89 mg/kg bw per day.
- *Hepatocellular hypertrophy and hyperplasia*: Hepatocellular hypertrophy was observed after administration of fluopyram for 7 days at 193 mg/kg bw per day, after 90 days of administration at 70 mg/kg bw per day and after 12 and 24 months of administration at 89 mg/kg bw per day.

- *Increase in liver weight*: Increased liver weight was observed after administration of fluopyram for 7 days at 193 mg/kg bw per day, after 90 days of administration at 70 mg/kg bw per day and after 12 and 24 months of administration at 89 mg/kg bw per day.
- *Development of altered hepatic foci*: Foci of altered hepatocytes were increased after administration of fluopyram for 12 and 24 months at 89 mg/kg bw per day.

The key events as described have been observed in rats in short-term toxicity and mechanistic studies as well as at the interim and terminal sacrifices in a long-term study. The dose–response relationships and the temporal analyses of the key events and tumour response are presented below.

4. Concordance of dose–response relationships

The NOAELs and LOAELs for the identified key events in the MOA of fluopyram in the liver are provided in Table A2.

A dose-related increase in liver enzyme induction was observed in the 28-day study in rats, with no enzyme induction at 4.6 mg/kg bw per day, a moderate enzyme induction at 36.1 mg/kg bw per day and a marked enzyme induction at 263 mg/kg bw per day. Similarly in this study, liver weight was not changed at 4.6 mg/kg bw per day, was slightly increased at 36.1 mg/kg bw per day and was significantly greater than control at 263 mg/kg bw per day.

An increase in liver cell proliferation, using BrdU as a marker of deoxyribonucleic acid (DNA) synthesis, was observed after a 7-day exposure to fluopyram at 193 mg/kg bw per day, whereas an increased number of mitoses was observed after 1 year and 2 years of exposure to fluopyram at 89 mg/kg bw per day. Exposure to fluopyram at the same dose level resulted in an increase in the incidence of altered hepatic foci and of liver cell tumours.

5. Temporal association

The identified key events, such as induction of CYP enzymes, increased liver cell proliferation (as identified by increased DNA synthesis), liver cell hypertrophy and increased liver weights, were observed after only a 7-day or 28-day exposure to fluopyram. Altered hepatic foci were observed at first after 12 months of exposure (interim sacrifice in the long-term study), whereas liver cell adenoma and carcinoma occurred only at the terminal sacrifice in the 24-month study. Thus, there is a logical temporal response, with the key events preceding tumour formation.

6. Strength, consistency and specificity of association of tumour response with key events

The identified key events that are fundamental to the proposed MOA for liver tumour induction by fluopyram were reported consistently at several time points before tumours were identified. Early key events in the proposed MOA include induction of CYP enzymes, increased hepatocellular proliferation, hepatocellular hypertrophy, increased liver weight and, somewhat later, the development of altered hepatic foci. The early key events (see above) were first reported after 7 days and 28 days of exposure, and similar effects were reported after 90 days of exposure to fluopyram.

The key events identified for fluopyram are similar to those of phenobarbital. The proposed MOA for the induction of liver tumours in rodents (rats and mice) by phenobarbital involves activation of nuclear receptors, particularly CAR, and subsequent induction of CYP enzymes (particularly the CYP2B family), whereas additional phenobarbital responses that are key in its tumorigenic effect include increased cell proliferation, inhibition of apoptosis, hypertrophy and development of altered hepatic foci (Whysner, Ross & Williams, 1996; Holsapple et al., 2006).

Table A2. NOAELs/LOAELs for key events in the MOA of fluopyram in liver of rats

Effect	NOAEL/LOAEL (mg/kg bw per day)
Induction of CYP enzymes (total P450, EROD, PROD, BROD)	—/193 (7-day mechanistic study)
	4.6–36/36–263 (28-day study)
Increase in liver cell proliferation	—/193 (7-day mechanistic study)
	8.6/89 (12-month interim and terminal sacrifices, 24-month study)
Hepatocellular hypertrophy	—/193 (7-day mechanistic study)
	14.6/70 (90-day study)
	8.6/89 (12-month interim and terminal sacrifices, 24-month study)
Increase in liver weight	—/193 (7-day mechanistic study)
	36/263 (28-day study)
	14.6/70 (90-day study)
	8.6/89 (12-month interim and terminal sacrifices, 24-month study)
Increase in hepatic foci	8.6/89 (12-month interim and terminal sacrifices, 24-month study)
Increase in liver cell tumours	8.6/89 (terminal sacrifice, 24-month study)

7. Biological plausibility and coherence

The proposed MOA for the induction of liver tumours in female rats by fluopyram is plausible and cohesive, as the data show a substantial similarity to the MOA that has been proposed (and which appears to be widely accepted) for phenobarbital.

8. Other modes of action

Genotoxicity is always one possible MOA to consider, but no genotoxic potential was demonstrated for fluopyram in the following tests:

- mutation in five strains of *Salmonella typhimurium*,
- mutation at the HPRT locus of Chinese hamster V79 cells,
- chromosomal aberration in Chinese hamster V79 cells,
- micronucleus induction in bone marrow cells of mice treated in vivo.

Therefore, the available evidence indicates that genotoxicity is not an alternative MOA for fluopyram.

There was no indication of hepatotoxic effects such as peroxisome proliferation or chronic degeneration in the general toxicity studies performed on fluopyram that might suggest cycles of degeneration and regenerative hyperplasia. Also, none of the studies of general toxicity or toxicity to reproduction have suggested that there might be perturbation of estrogenic hormone homeostasis that could result in a mitogenic stimulus to the liver.

9. Uncertainties, inconsistencies and data gaps

No data were presented to demonstrate that fluopyram activates CAR to induce CYP enzymes, although this data gap might be considered to be of low relevance given the close similarities in enzyme induction between fluopyram and phenobarbital.

In the short-term mechanistic studies, fluopyram induced some CYP enzymes (BROD, PROD) with a pattern of changes that was similar to the profile observed in rats treated with phenobarbital.

Especially the induction of PROD, which is a marker for CYP2B, is considered a diagnostic effect of a phenobarbital-like MOA. However, the magnitude of changes was different between fluopyram and phenobarbital when EROD activity was concerned.

Also, there was a lack of dose–response concordance with the key precursor events and the development of liver tumours, as the dose levels used in the short-term mechanistic studies were more than 2-fold higher than the dose that produced an increased tumour incidence.

10. Assessment of postulated mode of action

The data presented are considered to be adequate to explain the development of liver cell tumours in female rats following chronic exposure to fluopyram. The key events for the MOA of fluopyram have been identified and documented, and they illustrate a strong dose–response relationship and temporal relationship to tumour formation. Induction of CYP is a well-known MOA for liver tumour development in rodents, and the major end-points accepted for documenting this MOA have been presented for fluopyram.

11. Conclusion

There are a number of elements in the toxicology of fluopyram that are consistent with an MOA based on nuclear receptor–mediated gene activation, and it might be concluded that the weight of evidence supports a phenobarbital-like MOA for hepato-tumorigenesis.

The nuclear receptor involved in phenobarbital-induced liver enzyme induction is mainly CAR. Although CAR is expressed in human hepatocytes, CYP induction in human liver may act more through pregnane X receptor (PXR) than through CAR (Moore et al., 2003). In addition, there are convincing data showing that patients receiving phenobarbital for many years do not show evidence of tumorigenic effects (IARC, 2001). For these reasons, the MOA for phenobarbital-like CYP induction is not considered to be relevant to humans regarding the induction of liver tumours (Holsapple et al., 2006).

However, the relevance of the liver tumours in female rats to humans cannot be discounted, as the results of the mechanistic studies were only partly sufficient to support the proposed phenobarbital-like MOA for fluopyram. In particular, activation of CAR by fluopyram has not been clearly demonstrated, and there is a lack of dose–response concordance with key precursor events and tumour incidence.

MEPTYLDINOCAP

First draft prepared by
Ian Dewhurst[1] and Roland Solecki[2]

[1] Chemicals Regulation Directorate, York, England
[2] Federal Institute for Risk Assessment, Berlin, Germany

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Explanation

Meptyldinocap is the International Organization for Standardization (ISO)–approved name for 2-(1-methylheptyl)-4,6-dinitrophenyl crotonate (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) No. 131-72-6. Meptyldinocap is a new dinitrophenolic fungicidal compound, which acts by uncoupling mitochondrial oxidative phosphorylation.

Meptyldinocap was reviewed for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) at the request of the Codex Committee on Pesticide Residues (CCPR).

Meptyldinocap is one of the six structural analogues present in the existing active substance dinocap. Dinocap was evaluated previously by the JMPR in 1969, 1974, 1989, 1998 and 2000. In 1998, the acceptable daily intake (ADI) and the acute reference dose (ARfD) for dinocap were established at 0–0.008 mg/kg body weight (bw) and 0.008 mg/kg bw, respectively. In 2000, two ARfDs were established for dinocap, one for women of childbearing age, at 0.008 mg/kg bw, and another for the general population, at 0.03 mg/kg bw. Dinocap contains approximately 22% meptyldinocap.

The database supporting meptyldinocap consists of some new studies performed with meptyldinocap together with earlier studies performed with dinocap. A number of the kinetic and metabolism studies that used dinocap had the radiolabel in the methylheptyl isomer that was subsequently developed as meptyldinocap (see Table 1). Studies with meptyldinocap have been summarized in detail in this monograph, whereas previously evaluated studies with dinocap are summarized in Appendix 1, with only the conclusions presented in the main part of the monograph. Most of the pivotal studies met the basic requirements of the relevant Organisation for Economic Co-operation and Development (OECD) or national test guidelines, although the level of detail in some of the older reports of studies performed with dinocap did not always meet current requirements. A number of studies using dinocap did not contain certificates of compliance with good laboratory practice (GLP).

Meptyldinocap has been known under the development codes DE 126, Dinocap II and RH-23-163. Dinocap was known as Karathane.

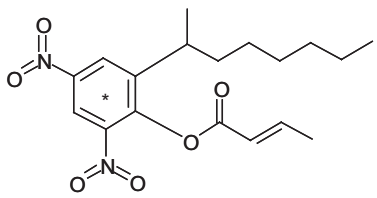
Table 1. Composition of dinocap

Isomer	Abbreviation	Nominal % in dinocap
2,4-dinitro-6-(1-methylheptyl)phenyl crotonate	2,4-DNHPC (meptyldinocap)	22
2,6-dinitro-4-(1-methylheptyl)phenyl crotonate	2,6-DNHPC	11
2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate	2,4-DNEHPC	22
2,6-dinitro-4-(1-ethylhexyl)phenyl crotonate	2,6-DNEHPC	11
2,4-dinitro-6-(1-propylpentyl)phenyl crotonate	2,4-DNPPC	22
2,6-dinitro-4-(1-propylpentyl)phenyl crotonate	2,6-DNPPC	11

Evaluation for acceptable daily intake

The structure of meptyldinocap is shown in Figure 1.

Figure 1. Structure of meptyldinocap with position of radiolabel (*)



1. Biochemical aspects

No new absorption, distribution, metabolism and excretion (ADME) studies on meptyldinocap in mammals have been conducted. However, in a number of the ADME studies with dinocap, the radiolabel was present on the methylheptyl analogue, which is the primary component of meptyldinocap.

Table 2. Recovery of ^{14}C label from male mice (n = 4) following a single oral dose of [^{14}C]dinocap (mean ± standard deviation)

^{14}C compound	^{14}C dose (mg/kg bw)	Recovery (% of administered dose)					
		Urine	Urine funnel wash	Faeces	Cage wash	Tissues	Total
2,4-DNHPC	0.5	32 ± 19	13 ± 10	40 ± 16	7.2 ± 3	3.9 ± 0.6	96 ± 20
2,4-DNHPC	3	20 ± 9	39 ± 6	37 ± 9	3.5 ± 1	0.9 ± 0.2	100 ± 9
2,4-DNHPC	25	23 ± 8	16 ± 14	32 ± 5	12 ± 11	0.2 ± 0.02	83 ± 0.9
2,6-DNHPC	25	23 ± 17	17 ± 9	43 ± 16	6 ± 3	0.16 ± 0.1	90 ± 13

From DiDonato et al. (1986)

1.1 Absorption, distribution and excretion

(a) Oral route

Mice

Groups of male CD-1 mice received gavage doses of [phenyl-^{14}C]dinocap (with the radiolabel present as either [^{14}C]2,4-dinitro-6-(1-methylheptyl)phenyl crotonate [2,4-DNHPC; meptyldinocap] or [^{14}C]2,6-dinitro-4-(1-methylheptyl)phenyl crotonate [2,6-DNHPC]). The vehicle was gum tragacanth, and the dose levels of dinocap were 0.5, 3 and 25 mg/kg bw. Blood samples were taken 6 times in the first 24 hours and then every 24 hours until 96 hours. Urine and faecal samples were taken at 6, 24, 48, 72 and 96 hours. At sacrifice (96 hours all doses; 6, 24, 48 and 72 hours at 25 mg/kg bw), tissue samples were taken and analysed for ^{14}C. Excretion was approximately equally divided between urine and faeces, but interpretation is confounded by the high residue in the urine funnel (Table 2), with no marked differences between analogues or among administered doses. The absorption half-life was calculated to be less than 1 hour. Elimination half-lives were longest for brain and eyes with 2,4-DNHPC and for liver and eyes with 2,6-DNHPC (DiDonato et al., 1986).

The rapid absorption and excretion of dinocap (containing [^{14}C]DNHPC) by male CD-1 mice were reported by Potter (1996), with 93% of the total urinary excretion occurring within 24 hours of gavage dosing at 25 mg/kg bw in corn oil. Urinary excretion (+ funnel wash) at 96 hours accounted for approximately 58% of the administered dose.

Rats

In an initial study, albino rats (one per sex) received 62 mg/kg bw per day of [^{14}C]dinocap (phenyl ring labelled) by oral gavage for 7 days. The [^{14}C]dinocap was a mixture of 2,4-dinitro-6-(2,3,4-octyl)phenyl crotonate (2,4-DNOPC) and 2,6-dinitro-4-(2,3,4-octyl)phenyl crotonate (2,6-DNOPC) (specific activity 20 MBq/g). Urine, faeces, exhaled carbon dioxide and cage wash were collected daily over the course of the study. After a 4-day withdrawal period, animals were sacrificed, and tissues were analysed for radioactivity. Quantification of the radioactive material was performed by liquid scintillation counting. In males, excretion was evenly split between urine and faeces; in females, faecal excretion predominated (about 65% versus 35% in urine). At sacrifice, tissue levels were low (< 0.5% of the administered dose), highest levels of ^{14}C being found in kidney, liver and skin (Graham, 1970).

Groups of male Sprague-Dawley rats received gavage doses of dinocap containing [^{14}C]2,4-DNHPC (meptyldinocap). The vehicle was gum tragacanth, and dose levels were 3, 25 or 100 mg/kg bw. Blood samples were taken 6 times in the first 24 hours and then every 24 hours until 96 hours.

Table 3. Recovery of ^{14}C label from male rats (n = 4) following a single oral dose of [^{14}C]dinocap (mean ± standard deviation)

^{14}C dose (mg/kg bw)	Recovery (% of administered dose)					
	Urine	Urine funnel wash	Faeces	Cage wash	Tissues	Total
3	38 ± 4.5	12 ± 9	61 ± 17	1.1 ± 1	0.3 ± 0.1	113 ± 12
25	29 ± 11	4.0 ± 2	57 ± 17	0.4 ± 0.2	0.13 ± 0.03	90 ± 29
100	35 ± 9	5.4 ± 2	64 ± 4	0.9 ± 0.3	0.15 ± 0.04	105 ± 12

From DiDonato et al. (1986)

Table 4. Findings in female rabbits (n = 4) treated orally with [^{14}C]2,4-DNHPC (means)

[^{14}C]2,4-DNHPC dose (mg/kg bw)	Number of animals	Specific activity (MBq/g)[a]	Peak plasma concentration at 6 h (ppm)	% of administered dose in urine + funnel wash	% of administered dose in faeces	% of dose absorbed[a]
0.5	4	205	0.29	45	72	69
3	4	146	1.3	31	67	60
25	4	18	15	44	57	64

From DiDonato & Longacre (1985)

ppm, parts per million

[a] % of administered dose in urine versus % of intravenous dose in urine.

Urine and faecal samples were taken at 6, 24, 48, 72 and 96 hours. At sacrifice (96 hours all doses; 6, 24, 48 and 72 hours at 25 mg/kg bw), tissue samples were taken and analysed for ^{14}C. Excretion was primarily via faeces (about 60% versus 30–40% in urine) (Table 3), with no marked differences among administered doses. The absorption half-life was calculated to be less than 1 hour. Elimination half-lives were longest for brain and eyes (DiDonato et al., 1986).

The rapid absorption and excretion of dinocap (containing [^{14}C]DNHPC) by male Sprague-Dawley rats were reported by Potter (1996), with 95% of the total urinary excretion occurring within 24 hours of gavage dosing at 100 mg/kg bw in corn oil. Urinary excretion (+ funnel wash) at 96 hours accounted for approximately 31% of the administered dose.

Rabbits

Female New Zealand White rabbits (four per group) received [^{14}C]2,4-DNHPC (meptyldinocap), uniformly radiolabelled in the phenyl ring, by gavage at 0.5, 3 or 25 mg/kg bw (Table 4). Peak plasma levels increased with dose, whereas the proportion of ^{14}C in faeces and urine remained unchanged. In the region of 60–70% of the oral dose was calculated to be absorbed when related to the urinary excretion of an intravenous dose of 3 mg/kg bw (52%) and corrected for recovery (DiDonato & Longacre, 1985).

(b) Dermal route

Rabbits

In a study to complement a dermal developmental study with dinocap, female New Zealand White rabbits (four per group) were exposed to [^{14}C]2,4-DNHPC (meptyldinocap) uniformly radiolabelled in the phenyl ring. A number of doses and vehicles were used (Table 5). The proportion of the applied dose that was absorbed varied between 3.7% and 13%. Peak plasma concentrations determined at 6 hours increased with applied dose, indicating little, if any, saturation, and were 50–100 times lower than those from an equivalent oral dose (Table 4) (DiDonato & Longacre, 1985).

Table 5. Findings in female rabbits treated dermally with [^{14}C]2,4-DNHPC

[^{14}C]2,4-DNHPC dose (mg/kg bw)	Application rate (mg/cm^2)	Vehicle	Number of animals	Specific activity (MBq/g)	Peak plasma concentration at 6 h (ppm)	% of dose absorbed[a]
25	2.2	Neat	4	33.5	0.15	3.7 ± 1.8
100	8.3	Neat	4	8.2	0.47	5.1 ± 2.5
220	18	Neat	4	3.8	1.5	4.8 ± 3.8
25 (7 days)	2.1	Neat	4	32.7	0.24	13.1 ± 6.1
25	2.4	Powder	4	32.7	0.19	9.2 ± 3.2
25	2.5	Liquid concentrate	4	32.7	0.24	4.0 ± 1.3
25	2.2	Acetone	4	32.7	0.28	7.3 ± 2.2
25 (× 13)	—	Neat	4	32.7	—	6.2 ± 0.9

From DiDonato & Longacre (1985)

ppm, parts per million

[a] % of administered dose in urine versus % of intravenous dose in urine.

Monkeys

Groups of four female Rhesus monkeys were exposed to [^{14}C]dinocap at dose levels of 0.2 mg/kg bw (6-hour exposures of abdominal skin). Plasma and urine samples were taken pretest and then repeatedly up to 96 hours. No ^{14}C was detected in plasma above the limit of quantification. Based on urinary excretion, corrected for approximately 49% urinary excretion of an intramuscular dose, the dermal absorption was estimated to be approximately 16% at an application rate of 40 µg/cm^2 and 5% at an application rate of 2500 µg/cm^2 (Wester & Maibach, 1985).

(c) Intravenous/intramuscular route

Rabbits

Female New Zealand White rabbits (four per group) received a single intravenous dose of [^{14}C]2,4-DNHPC (meptyldinocap) uniformly radiolabelled in the phenyl ring; the vehicle was dimethyl sulfoxide (DMSO). Total recovery was low (mean 76% ± 25%), with approximately 52% of the dose excreted in the urine, 11% in the urine funnel wash and 14% in the faeces (DiDonato & Longacre, 1985).

Monkeys

Four female Rhesus monkeys received a single dose of 670 µl (368 kBq) of ^{14}C-labelled dinocap by intramuscular injection. Urinary excretion from 0 to 4 hours accounted for 27% of the administered dose, with 52% excreted in urine by 24 hours (Maibach, 1985). Similar results were reported by Wester & Maibach (1985), with 49% of an intravenous dose of 200 µg/kg bw being excreted in urine in 96 hours.

1.2 Biotransformation

Mice

The hydrolysis of [^{14}C]2,4-DNHPC (meptyldinocap) to the free phenol (2,4-dinitro-6-(1-methylheptyl)phenol) [2,4-DNHP]) was investigated using CD-1 mouse plasma and liver microsomes. Incubations were performed at 37 °C. For plasma, a 2.5-minute reaction time and substrate concentration range of 10–100 µmol/l were used; for liver microsomes, the incubation period was 2.5 minutes, with a substrate concentration range of 5–100 µmol/l. The results, presented in Table 6, show

Table 6. Kinetic parameters for the hydrolysis of 2,4-DNHPC by rat and mouse plasma and liver microsomes

	Mouse plasma	Rat plasma	Mouse liver	Rat liver
V_{max} (nmol/min per milligram protein)	5.6	12.5	1.7	3.3
K_m (µmol/l)	14	20	5.9	33

From Anderson & Longacre (1986)

K_m, Michaelis-Menten constant; V_{max}, maximum rate

that while there are differences between rats (see next section) and mice, these do not appear to be significant in terms of the potential capacity to hydrolyse the crotonate bond (Anderson & Longacre, 1986).

An investigation of the metabolism of dinocap was performed by Udinsky, Tran & Frederick (1986). Groups of male CD-1 mice received a single dose of dinocap (containing [^{14}C]2,4-DNHPC [meptyldinocap]) at 0.5, 3 or 25 mg/kg bw by gavage in gum tragacanth. Excreta samples were taken over 24 hours and analysed for metabolites using liquid scintillation counting, high-performance liquid chromatography (HPLC) and ultraviolet (UV) absorption. There was a dose-related decrease in the proportion of the administered dose excreted in the urine (60% at 0.5 mg/kg bw, 24% at 25 mg/kg bw). Conjugation was found to be of limited significance following incubations with glucuronidases or arylsulfatases. In urine, there were three major metabolites accounting for over 60% of the urinary radioactivity. These were tentatively identified as being formed after hydrolytic cleavage of the crotonate moiety. In faeces, the main component was parent compound (approximately 30% of faecal radioactivity), with the hydrolysis product 2,4-DNHP representing about 20% (Udinsky, Tran & Frederick, 1986).

Fifteen male CD-1 mice received single doses of 25 mg/kg bw of an isotope mixture of [^{14}C]2,4-DNHPC (lot No. 742.0211; specific activity 500 MBq/g) and [^{13}C]2,4-DNHPC (lot No. 4133-0) dispersed in corn oil (total 2,4-DNHPC dose 2.5 mg/ml) by oral gavage. The isotope ratio of [^{14}C]2,4-DNHPC and [^{13}C]2,4-DNHPC administered to mice was 1:1 (no [^{12}C]2,4-DNHPC isotope was present). The isotopes were uniformly incorporated in the phenyl ring. Urine was collected over dry ice at 24-, 48-, 72- and 96-hour intervals after dosing. Aliquots (1 ml) of urine from 24-hour collection samples were pooled and used for identification of metabolites by HPLC and mass spectrometry and for quantification of metabolites by HPLC. Quantification of the radioactive material was performed by liquid scintillation counting. Identification was aided by comparison with synthetic standards.

Over 80% of the urinary radioactivity was eluted as 13 discrete HPLC peaks. The results are summarized in Table 7. None of the urinary metabolites contained the crotonate moiety, indicating that the initial reaction is probably hydrolysis of this component. Subsequent reactions include oxidation of the octyl chain, with β-oxidation the predominant metabolic pathway (approximately 47% of the urinary metabolites), whereas α-oxidation metabolites represent approximately 33%. Approximately 7% of the urinary metabolites were conjugated, but these were only partially characterized (Potter, 1996).

Rats

The hydrolysis of [^{14}C]2,4-DNHPC (meptyldinocap) to the free phenol (2,4-DNHP) was investigated using Sprague-Dawley rat plasma and liver microsomes. Incubations were performed at 37 °C. For plasma, a 2.5-minute reaction time and substrate concentration range of 10–100 µmol/l were used; for liver microsomes, the incubation time was 5 minutes, with concentrations of 10–100 µmol/l. The results are presented in Table 6 above and show that whereas there are differences between rats

Table 7. Main metabolites found in urine of mice and rats administered meptyldinocap

Chemical name	% urinary radioactivity in mice	% urinary radioactivity in rats
2-Acetamino-4-nitro-6-(1-methyl-α,β-pentenoate)phenol	< 1%	2
2,4-Diacetamino-6-(1-methylpentanoate)phenol	< 1%	2
2,4-Dinitro-6-(1-methyl-β-hydroxypentanoate)phenol	8	4
2,4-Dinitro-6-(1-methylpropanoate)phenol	6	2
2,4-Dinitro-6-(1-methylbutanoate)phenol	24	18
2,4-Dinitro-6-(1-methyl-α,β-pentenoate)phenol	9	3
2,4-Dinitro-6-(1-methylpentanoate)phenol	22	58
2,4-Dinitro-6-(1-methyl-α,β-buten-4-ol)phenol	3	< 1%

From Potter (1996)

and mice, these do not appear to be significant in terms of the potential capacity to hydrolyse the crotonate bond (Anderson & Longacre, 1986).

Male and female Wistar rats were treated with daily doses of 11.7 mg/kg bw per day of [^{14}C]2,4-DNOPC (isomeric mixture of 95% *trans*-[^{14}C]2,4-DNOPC and 5% *cis*-[^{14}C]2,4-DNOPC; specific activity 65 MBq/g; 95% radiochemical purity) by oral gavage (vehicle: methylcellulose–water solution) for 7 days. Samples of excreta were analysed for metabolites using liquid scintillation counting, gas chromatography, mass spectrometry and nuclear magnetic resonance. Excretion was predominantly in faeces (52–58%), with 15–20% in urine. Over 20 metabolites were detected, but only 3 of these were identified. There were clear differences between the metabolite patterns in males and females. The main metabolites in male rat excreta were identified as 2,4-dinitro-6-(1-methyl-5-hydroxy-heptyl)phenol (37% of urinary excretion; 4% of faecal excretion), 2,4-dinitro-6-(1-methyl-7-carboxy-heptyl)phenol (27% of urinary excretion) and 2,4-dinitro-6-(2-octyl)phenol (5% of faecal excretion) (Honeycutt & Garska, 1976a,b).

Groups of male Sprague-Dawley rats received a single dose of [^{14}C]2,4-DNHPC (meptyldinocap) at 3, 25 or 100 mg/kg bw by gavage in gum tragacanth. Excreta samples were taken over 24 hours and analysed for metabolites using liquid scintillation counting, HPLC and UV absorption. There was no evidence of any decrease in the proportion of the administered dose excreted in the urine with increasing dose level. Conjugation was found to be of limited significance following incubations with glucuronidases or arylsulfatases. In urine, there were two major metabolites accounting for over 80% of the urinary radioactivity. These were tentatively identified as having been formed after hydrolytic cleavage of the crotonate moiety. UV spectra indicated that the rat metabolites were not substituted on the phenolic oxygen, whereas mouse metabolites were. In faeces, the main component was parent compound (approximately 30–40% of faecal radioactivity), with the hydrolysis product 2,4-DNHP representing about 13–30% (Udinsky, Tran & Frederick, 1986).

Five male Sprague-Dawley rats received single doses of 25 mg/kg bw of an isotope mixture of [^{14}C]2,4-DNHPC (lot No. 742.0211; specific activity 500 MBq/g) and [^{13}C]2,4-DNHPC (lot No. 4133-0) dispersed in corn oil (total 2,4-DNHPC dose 2.5 mg/ml; meptyldinocap) by oral gavage. The isotope ratio of [^{14}C]2,4-DNHPC and [^{13}C]2,4-DNHPC administered was 1:1 (no [^{12}C]2,4-DNHPC isotope was present). The isotopes were uniformly labelled in the phenyl ring position. Urine was collected over dry ice at 24-, 48-, 72- and 96-hour intervals after dosing. Aliquots (1 ml) of urine from 24-hour collection samples were pooled and used for identification of metabolites by HPLC and mass spectrometry and for quantification of metabolites by HPLC. Quantification of the radioactive

Table 8. Acute toxicity studies with meptyldinocap

Test substance	Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Purity (%); batch	Vehicle	Reference
Meptyldinocap	Rat	F344	F	Oral	> 2000		92.9; SL1088R301	None	Merkel (2005a)
Meptyldinocap	Rat	F344	F	Dermal	> 5000		92.9; SL1088R301	None	Merkel (2005b)
Meptyldinocap	Rat	F344	M & F	Inhalation (nose only)[a]		1.2 (M) 1.6 (F)	91.5; SL1088R301	None	Krieger & Garlinghouse (2009)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male

[a] Mass median aerodynamic diameter 2.0–3.6 µm.

material was performed by liquid scintillation counting. Identification was aided by comparison with synthetic standards.

Over 90% of the urinary radioactivity was eluted as 12 discrete HPLC peaks. The results are summarized in Table 7 above. None of the urinary metabolites contained the crotonate moiety, indicating that the initial reaction is probably hydrolysis of this component. Subsequent reactions include oxidation of the octyl chain, with β-oxidation the predominant metabolic pathway (approximately 72% of the urinary metabolites), whereas α-oxidation metabolites represent approximately 18% (Potter, 1996).

The proposed metabolic pathway for rats, based primarily on the data of Potter (1996), is shown in Figure 2.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Meptyldinocap was of low acute toxicity via the oral and dermal routes and of moderate acute toxicity via the inhalation route (Table 8).

(b) Dermal and ocular irritation and dermal sensitization

Meptyldinocap was slightly irritating to rabbit skin (Merkel, 2005c), was a moderate eye irritant (Merkel, 2005d) and gave positive results in a local lymph node assay for skin sensitization (Woolhiser & Wiescinski, 2005).

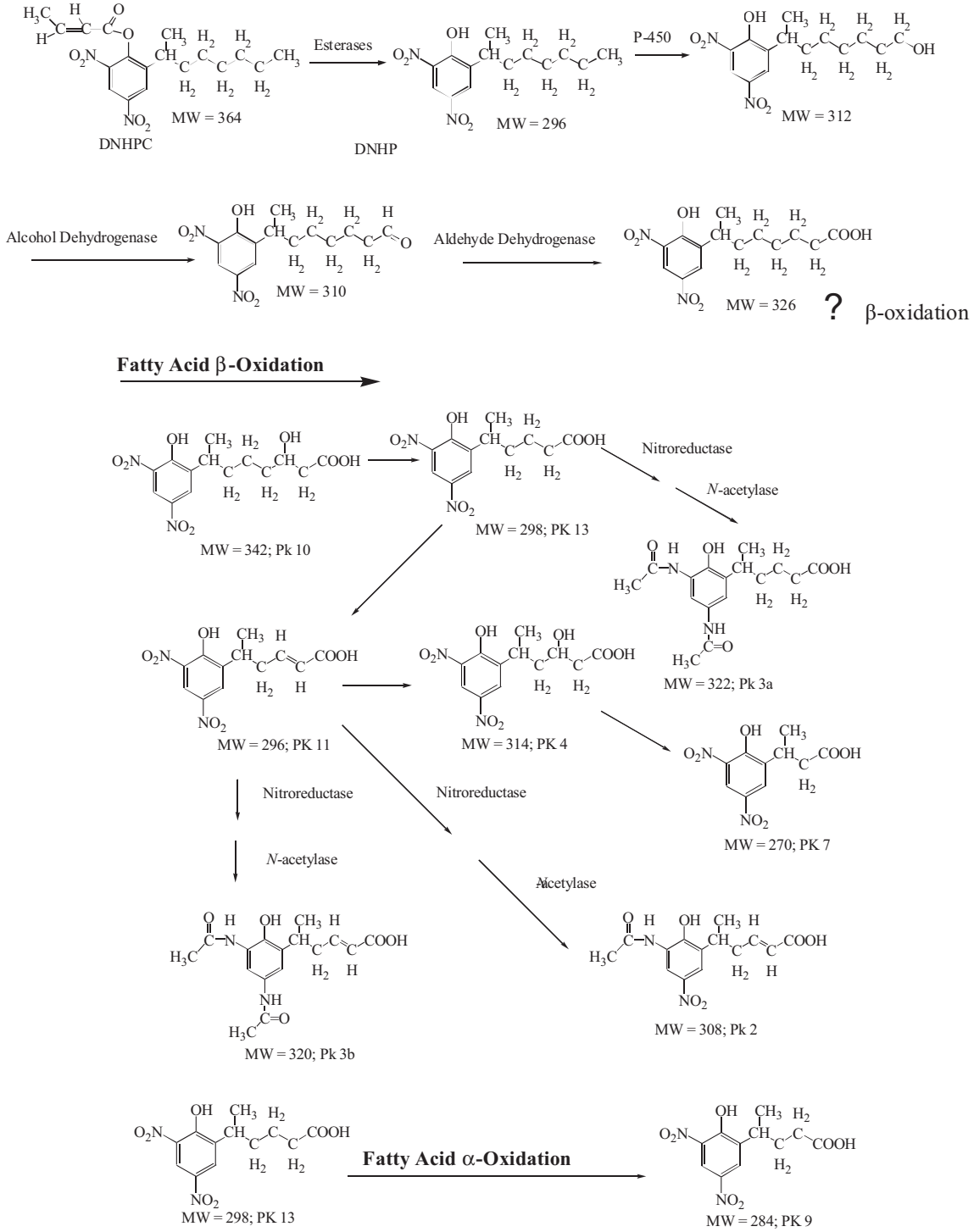
2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a GLP-compliant study, groups of CD-1 mice (five of each sex per dose) received meptyldinocap (purity 92.9%; lot No. SL1088R301) in the diet at 0, 100, 200 or 750 parts per million (ppm) for 28 days. Parameters evaluated included daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, fundic photographs, body weights, feed consumption, clinical chemistry, haematology (including prothrombin time), urinalysis and selected organ weights,

Figure 2. Proposed metabolic pathway of meptyldinocap in rats



with gross examinations of all animals and histopathological examinations (about 40 tissues) of control and top-dose animals plus any gross findings.

Achieved dietary concentrations and homogeneity were confirmed analytically; achieved mean intakes are given in Table 9. In the meptyldinocap groups, there were no deaths or clinical signs of note other than yellow urine staining in all treatment groups. Body weight gain was unaffected by treatment. Feed consumption was reduced in all treated male groups at the start of the study, but this is possibly related to unusually high consumption in control males; in females, feed consumption was

Table 9. Findings in mice (n = 5) receiving meptyldinocap in the diet for 28 days

	Males				Females			
	Dietary concentration (ppm)							
	0	100	200	750	0	100	200	750
Achieved intakes (mg/kg bw per day)	0	17	33	126	0	21	44	173
Body weight, week 4 (g)	39	37	38	38	29	27	28	27
Body weight gain, days 0–4 (g) (mean ± SD)	3.0 ± 2.1	1.2 ± 0.4	2.0 ± 0.5	2.0 ± 0.5	1.1 ± 0.8	0.9 ± 0.1	1.1 ± 0.6	1.2 ± 0.9
Feed consumption, days 0–4 (g/day) (mean ± SD)	7.0 ± 0.4	6.2 ± 0.1	5.9 ± 0.4	6.1 ± 0.7	5.3 ± 0.2	5.4 ± 0.7	5.4 ± 0.6	7.0 ± 1.1
Urine pH	7.5	7.0	7.0	6.5	6.0	6.5	7.0	6.0
Liver weight, absolute (g)	2.22	2.09	2.29	2.56	1.58	1.49	1.57	1.59
Liver weight, relative to body weight (%)	5.9	5.8	6.0	6.9*	5.5	5.5	5.6	6.0*
Aggregates of mononuclear cells, focal	0	0	0	1	0	0	0	0
Aggregates of mononuclear cells, multifocal	1	0	0	0	3	0	0	1
Necrosis, focal	0	0	0	0	0	0	1	0
Necrosis, with accompanying inflammation, focal	0	0	0	1	0	0	0	1

From Thomas, Yano & Dryzga (2005)

SD, standard deviation; * $P < 0.05$, both sexes combined due to small group size

increased in top-dose animals at the start of the study. There were no notable findings in ophthalmoscopy, clinical chemistry or haematology (although total white blood cell counts showed great variation across all groups). Urinary pH fluctuated between test groups; however, with no other indications of renal impairment, this finding is considered to be related to the production of acidic metabolites and not adverse. One top-dose male had a unilateral preputial abscess, and a top-dose female had a cloudy cornea, both of which are considered to be spontaneous; there were no other notable gross pathological findings. Liver weights were increased at 750 ppm, together with indications of very slight focal necrosis accompanied by inflammation (Table 9). No information is available on the mode of action behind the liver effects.

The no-observed-adverse-effect level (NOAEL) is 200 ppm (equal to 33 mg/kg bw per day), based on increased liver weights at 750 ppm (equal to 126 mg/kg bw per day) (Thomas, Yano & Dryzga, 2005).

Rats

In a GLP-compliant study, groups of Sprague-Dawley rats (10 of each sex per group) were given meptyldinocap (purity 92.9%; lot No. SL1088R301) in the diet at 0, 200, 650 or 2000 ppm for 90 days. Parameters evaluated were daily observations, detailed clinical observations, ophthalmological examinations, body weight, feed consumption, haematology (including prothrombin time), clinical chemistry, urinalysis and selected organ weights, with gross examinations of all animals plus histopathological examinations in controls and top-dose animals (about 40 tissues, including additional nervous system samples) plus all gross abnormalities. In addition to the above routine

Table 10. Findings in rats (n = 10) receiving meptyldinocap in the diet for 90 days

	Males				Females			
	Dietary concentration (ppm)							
	0	200	650	2000	0	200	650	2000
Achieved intakes (mg/kg bw per day)	0	12	40	122	0	14	44	137
Body weight, day 8 (g)	307	315	302	297	216	213	213	207
Body weight, day 92 (g)	527	563	503	485	298	298	293	279
Body weight gain, days 1–8 (g) (mean ± SD)	55 ± 4.6	58 ± 8.8	52 ± 6.4	45 ± 5.3	22 ± 8.3	22 ± 4.7	20 ± 9.0	15 ± 7.3
Feed consumption, days 1–8 (g/day) (mean ± SD)	24 ± 2.1	26 ± 2.2	25 ± 1.5	23 ± 1.1	17 ± 1.6	17 ± 0.9	17 ± 1.2	15* ± 0.9
Total protein (g/dl)	6.9	6.8	6.8	6.9	7.3	7.5	7.6	7.9*
Albumin (g/dl)	3.7	3.6	3.7	3.8	4.1	4.2	4.3	4.5*
Cholesterol (mg/dl)	52	54	46	64	61	72	71	82*
Liver weight, absolute (g)	12.9	14.7	12.9	13.6	7.0	7.2	7.2	7.8
Kidney weight, absolute (g)	3.5	3.6	3.3	3.4	1.9	1.9	1.9	2.0
Thyroid weight, absolute (g)	0.022	0.025	0.024	0.024	0.014	0.014	0.014	0.016
Hepatocyte hypertrophy, centrilobular/midzonal (very slight)	1/10	0/0	1/10	7/10*	0/10	0/0	0/10	3/10
Lacrimal glands, mononuclear cell infiltrate (very slight)	2/10	0/10	1/1	2/10	3/10	0/0	0/0	7/10*

From Johnson & Andrus (2005)
SD, standard deviation; * $P < 0.05$

parameters, potential neurotoxicity was assessed by an automated test of motor activity and a functional observational battery (FOB), including assessment of grip performance, landing foot splay and rectal temperature conducted pre-exposure and at the end of the study.

Achieved dietary concentrations and homogeneity were confirmed analytically; achieved mean intakes are given in Table 10. One animal from the 650 ppm group died the night before scheduled necropsy; the cause of death could not be ascertained. There were no clinical signs of note other than yellow urine staining. Body weight gain was consistently reduced at 2000 ppm; although the magnitude was relatively small, the study authors considered it to be treatment related. Feed consumption was reduced in top-dose females at most time points. There were no notable findings in the FOB, haematology, ophthalmoscopy or urinalysis investigations other than yellow coloration of the urine. Top-dose females had increased levels of total protein, albumin and cholesterol. Absolute liver, kidney and thyroid weights were increased (approximately 10%) in top-dose females, with marked increases in relative organ weights consequent to the low body weights. Histopathological findings were limited to very slight hepatocyte hypertrophy and mononuclear cell infiltrate of the lacrimal glands in top-dose animals (Table 10).

The NOAEL is 650 ppm (equal to 40 mg/kg bw per day), based on reduced feed consumption, altered clinical chemistry parameters and histopathological findings in the lacrimal glands at 2000 ppm (equal to 122 mg/kg bw per day) (Johnson & Andrus, 2005).

Dogs

Studies in dogs with dinocap had found marked ocular lesions at dose levels of 60 ppm and above (see Annex 1, references *58* and *85*, and Appendix 1).

Table 11. Acclimatization protocol for dietary incorporation of meptyldinocap

Day −7	Day −6	Day −5	Day −4	Day −3	Day −2	Day −1	Day +1
—	—	—	—	—	—	—	0 ppm
—	—	—	—	—	—	—	15 ppm
—	—	—	15 ppm	15 ppm	30 ppm	30 ppm	60 ppm[a]
15 ppm	15 ppm	30 ppm	30 ppm	60 ppm	60 ppm	60 ppm	120 ppm

From Stebbins, Brooks & Lacher (2005)
[a] For dinocap and meptyldinocap.

In a GLP-compliant study, groups of Beagle dogs (four of each sex per group or eight of each sex at 120 ppm) received meptyldinocap (purity 92.9%; lot No. SL1088R301) in the diet at 0, 15, 60 or 120 ppm for 90 days; another group received dinocap (purity 93.4%; lot No. SB03088R301) at 60 ppm. The dogs were introduced to test material gradually to try to overcome palatability problems (Table 11). Parameters evaluated included daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, fundic photographs, body weights, feed consumption, clinical chemistry, haematology, prothrombin time, urinalysis, selected organ weights and gross and histopathological examinations (about 40 tissues) of all animals.

Achieved dietary concentrations and homogeneity were confirmed analytically. The achieved mean intakes are given in Table 12. In the meptyldinocap groups, there were no deaths or clinical signs of note other than yellow urine staining at 60 and 120 ppm. Reduced body weight gain was seen in males but not in females after the first week of dosing with 120 ppm (equal to 3.9 mg/kg bw per day), following the gradual introduction to the treated diets. The company proposed that the reduced body weight gain was due to reduced feed consumption, but this was not supported by data from individual animals or overall patterns of feed consumption (Table 13). Terminal body weights were not affected in the meptyldinocap groups, but interpretation is confounded by the large difference in initial body weights in males (8.9 kg in controls, 10.6 kg at 120 ppm). The overall body weight gain in top-dose males was reduced by 41% over the duration of the study. There were no notable findings in ophthalmoscopy, clinical chemistry or haematology. Fluctuations were seen in a number of clinical chemistry parameters, but there were no consistent patterns across or within test groups, and concurrent control values were often outside typical ranges. Uterine weights were increased in 15 ppm females, but this was attributed to estrous cycle stages. There were no treatment-related gross pathological findings. Very slight kidney tubule degeneration was present in a small number of animals (Table 12) receiving meptyldinocap, but it is not considered to be toxicologically adverse, given the low severity and absence of effects on renal function. One top-dose female had very slight degeneration of individual tibial nerve fibres.

In the animals receiving diet containing dinocap at 60 ppm, a range of findings was seen, including body weight loss, markedly reduced feed consumption, eye lesions and tibial nerve fibre degeneration (Table 12).

The NOAEL for meptyldinocap is 60 ppm (equal to 1.6 mg/kg bw per day), based on reduced body weight gain at 120 ppm (equal to 3.9 mg/kg bw per day) in males (Stebbins, Brooks & Lacher, 2005).

In an extension of Stebbins, Brooks & Lacher (2005), groups of Beagle dogs (four of each sex) received meptyldinocap (purity 92.9%; lot No. SL1088R301) in the diet at 120 ppm for 1 year. The dogs were introduced to meptyldinocap gradually (see Table 11 above) to overcome palatability issues. Parameters evaluated, which were relatively limited, included daily cage-side observations, weekly detailed clinical observations, monthly ophthalmic examinations, fundic photography, body weights, feed consumption and complete gross and selected histopathological examinations (eyes, tibial nerve and heart).

Table 12. Findings in dogs exposed to meptyldinocap and dinocap for 90 days.

	Control	Meptyldinocap			Dinocap
		Dietary concentration (ppm)			
		15	60	120	60
Males					
Compound intake (mg/kg bw per day)	0	0.5	1.6	3.9	1.9
Terminal body weight (kg)	10.6	11.1	11.4	11.6	10.4
Body weight gain, days 0–90 (kg)	1.7	1.7	1.3	1.0	0.5
Body weight gain, days 0–8 (g)	395	373	332	156*	−6
Eye, hyper-reflective fundus	0/4	0/4	0/4	0/4	4/4
Eye, blood vessel attenuation	0/4	0/4	0/4	0/4	3/4
Eye, hyperpigmentation associated with retinopathy	0	0	0	0	0
Kidney, tubule degeneration, focal (very slight)	0/4	0/4	0/4	0/4	1/4
Kidney, tubule degeneration, multifocal (very slight)	0/4	0/4	0/4	1/4	0/4
Tibial nerve fibre degeneration, focal (very slight)	0/4	0/4	0/4	0/4	3/4
Females					
Compound intake (mg/kg bw per day)	0	0.5	2.3	4.2	2.1
Body weight gain, days 0–90 (kg)	0.87	0.87	1.1	1.3	0.3
Body weight gain, days 0–8 (g)	144	254	334	193	−124
Eye, hyper-reflective fundus	0/4	0/4	0/4	0/4	3/4
Eye, blood vessel attenuation	0/4	0/4	0/4	0/4	2/4
Eye, hyperpigmentation associated with retinopathy	0	0	0	0	2/4
Kidney, tubule degeneration, focal (very slight)	0/4	0/4	0/4	1/4	0/4
Kidney, tubule degeneration, multifocal (very slight)	0/4	0/4	1/4	0/4	0/4
Tibial nerve fibre degeneration, focal (very slight)	0/4	0/4	0/4	1/4	3/4

From Stebbins, Brooks & Lacher (2005)
* $P < 0.05$

Achieved dietary concentrations and homogeneity were confirmed analytically. The achieved mean intake over the 12-month study was 3.5 mg/kg bw per day. There were no deaths or clinical signs other than yellow urine staining. Body weight gains in males were reduced for the first 90 days, but after that, the dogs maintained body weights consistent with controls in other 1-year studies. The only gross pathological or histopathological findings of note were very slight, unilateral degeneration of the tibial nerve fibres in one male and one female and slight inflammation of the skin/subcutis in two females and one male. The authors noted that spontaneous degeneration of peripheral nerves in young dogs has been reported at an incidence of less than 3%, and affected dogs were without clinical evidence of neuromuscular disease. A number of incidental ophthalmoscopic changes were reported: clear periocular soiling, well-defined lenticular nucleus, retinal folds and unilateral prolapse of the nictitans gland. Prolapse of the nictitans gland was considered to be genetic in origin. The original supplier of the Beagle dogs used in this study has indicated that the Beagle colony has a 5% incidence rate of spontaneous prolapse of the nictitans gland. Hypopigmented areas of the fundus were also noted in some dogs. This finding, when not associated with other retinal changes such as hyperreflectivity, was considered by the examining ophthalmologist to be incidental pigment variation.

This study shows that the ocular effects seen in dogs administered 60 ppm dinocap for 90 days are not produced with higher doses of meptyldinocap (120 ppm for 1 year) (Stebbins, Brooks & Lacher, 2006a,b).

Table 13. Body weight and feed consumption data for individual dogs fed meptyldinocap for 90 days

Group	Animal number	Body weight (kg)		Body weight gain over days 1–8 (kg)	Feed consumption, days 1–8 (kg/day)	Pretest feed consumption, days −7 to −1 (kg/day)
		Test day 1	Test day 8			
0 ppm	264	7.542	7.841	0.299	0.307	—
	265	9.696	10.090	0.394	0.385	—
	266	9.359	9.879	0.520	0.361	—
	267	8.938	9.304	0.366	0.372	—
	Mean	**8.884**	**9.279**	**0.395**	**0.356**	**—**
	SD	0.947	1.014	0.093	0.034	—
120 ppm	280	9.524	9.766	0.242	0.354	0.319
	281	9.995	10.301	0.306	0.405	0.393
	282	10.899	11.161	0.262	0.351	0.320
	283	10.091	10.251	0.160	0.344	0.351
	284	10.727	10.683	−0.044	0.367	0.411
	285	10.297	10.099	−0.198	0.329	0.404
	286	11.840	12.045	0.205	0.427	0.465
	287	11.643	11.951	0.318	0.437	0.413
	Mean	**10.626**	**10.782**	**0.156**	**0.377**	**—**
	SD	0.809	0.856	0.183	0.041	—

From Stebbins, Brooks & Lacher (2005)
SD, standard deviation

(b) Dermal application

No data were submitted.

(c) Exposure by inhalation

No data were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

All studies were performed with dinocap. Dinocap contains approximately 22% of the analogue present in meptyldinocap. The weight of evidence from short-term and developmental studies is that meptyldinocap is of lower toxicity than dinocap. It is considered that the carcinogenic potential of meptyldinocap, in terms of exposures from pesticidal use, can be based on the existing studies performed with dinocap.

Mice

Groups of CD-1 rats (60 of each sex per group) received dinocap (purity 96%; lot No. 2893) incorporated into the diet at levels of 0, 15, 100 or 200 ppm for 78 weeks. The NOAEL for general toxicity is 15 ppm (equal to 2.8 mg/kg bw per day for females), based on body weight deficits at 100 ppm (equal to 18 mg/kg bw per day). The NOAEL for carcinogenicity is 150 ppm (equal to 23 mg/kg bw per day), the highest dose tested (Moore, 1991) (see Appendix 1 for further details).

Rats

Groups of Crl:CD®BR rats (20 of each sex per group) received dinocap (purity 97%; batch No. 632 6C21) in the diet at 0, 20, 200 or 2000 ppm for 52 weeks. The NOAEL is 200 ppm (equal to 11.4 mg/kg bw per day), based on reduced body weight gain and feed consumption, liver and thyroid changes and increased blood urea nitrogen at 2000 ppm (equal to 121 mg/kg bw per day) (Ferguson, Morrison & Bernacki, 1999) (see Appendix 1 for further details).

Groups of Wistar rats (80 of each sex per group) received dinocap technical (purity 77.5%; batch not specified) incorporated into the diet at a level of 0, 20, 200 or 2000 ppm for 30 months (130 weeks). Interim kills were performed after 13, 26 and 52 weeks. The NOAEL for general toxicity is 200 ppm (equal to 6.4 mg/kg bw per day for males and 8.05 mg/kg bw per day for females) based on body weight deficits at 2000 ppm (equal to 71 mg/kg bw per day). The NOAEL for carcinogenicity is 2000 ppm (equal to 71 mg/kg bw per day), the highest dose tested (Maita et al., 1981) (see Appendix 1 for further details).

Dogs

In a pre-GLP study, groups of Beagle dogs (four of each sex per dose) received dinocap technical; purity 78%; lot No. 2-0375) in the diet at 0, 15, 60 or 240 ppm for up to 2 years. The NOAEL is 15 ppm (equivalent to 0.4 mg/kg bw per day), based on retinal atrophy seen at 60 ppm (equivalent to 1.5 mg/kg bw per day) (Weatherholtz et al., 1979) (see Appendix 1 for further details). These ocular lesions were not evident in the 1-year dog study with meptyldinocap described above (Stebbins, Brooks & Lacher, 2006a,b).

2.4 Genotoxicity

Testing of the genotoxicity of meptyldinocap has been performed in a range of assays. The overall database is considered adequate to conclude that meptyldinocap is not genotoxic.

Data on the genotoxicity of meptyldinocap are summarized in Table 14.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of CD:Crl®BR rats (26 of each sex per group) received dinocap (purity 96%; batch No. 2893) in the diet at 0, 40, 200 or 1000/400 ppm for 70 days before mating and during the mating and gestation periods of two generations. Dinocap produced no adverse effects on reproduction at the highest dose tested (1000 ppm, equal to 65 mg/kg bw per day in the first mating; 400 ppm, equal to 27 mg/kg bw per day in the second mating). The NOAEL for pup development is 200 ppm (equal to 13 mg/kg bw per day), based on reduced body weight and survival in the F_1 generation at 1000 ppm). The NOAEL for parental toxicity is 200 ppm (equal to 13 mg/kg bw per day), based on reduced body weight gains (Morseth, 1990) (see Appendix 1 for further details).

(b) Developmental toxicity

Developmental toxicity was a particular concern with dinocap, with a variety of findings reported in mice, rats, hamsters and rabbits (see Annex 1, references *58* and *85*).

Table 14. Results of studies of genotoxicity with meptyldinocap

Test substance (vehicle)	End-point	Test object	Concentration	Purity (%); batch	Result	Reference
In vitro						
Meptyldinocap (DMSO)	Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* (WP2*uvrA*)	1, 3, 10, 33, 100,[a] 333, (1000,[b,c] 5000[b]) µg/plate, with S9 mix 3, 10, 33, 100, 333, 1000,[a,c] 5000 µg/plate, without S9 mix	92.9; SL1088R301	Negative	Mecchi (2005)
Meptyldinocap (DMSO)	Gene mutation (*HPRT*)	Chinese hamster ovary cells (K_1-BH_4)	0.5, 1, 2, 3, 4, 5, 6, 7, 8,[a] 9, 10, 15, 20 µg/ml without S9 mix 2, 4, 10, 20, 30, 40, 50, 52[a] µg/ml with S9 mix	92.9; SL1088R301	Negative	Seidel, Schisler & Kleinert (2005)
Meptyldinocap (DMSO)	Chromosomal aberration	Sprague-Dawley rat lymphocytes	1, 3, 10 µg/ml −S9 (4 h) 0.3, 1, 3 µg/ml −S9 (24 h) 20, 30, 40 µg/ml +S9 (4 h)	92.9; SL1088R301	Negative	Charles, Schisler & Kleinert (2005)
In vivo						
Meptyldinocap (0.5% aqueous methylcellulose)	Micronucleus assay	CD-1 mouse bone marrow (gavage)	0, 500, 1000, 2000 mg/kg bw (24 h) 0, 2000 mg/kg bw (48 h)	92.9; SL1088R301	Negative	Charles, Grundy & Schisler (2005)

DMSO, dimethyl sulfoxide; S9, 9000 × *g* supernatant from livers of rats

[a] Cytotoxicity became evident.

[b] *E. coli* only.

[c] Precipitation became evident.

Mice

In a GLP-compliant study, groups of CD-1 mice (15 mated females per group) received meptyldinocap (purity 98.5%; lot No. 2003-03220-64) by gavage in 0.5% methylcellulose on days 6–17 of gestation. Dose levels were 0, 100, 250 or 500 mg/kg bw per day. The study focused on investigations on the inner ear and cleft palate, which had been identified as targets for dinocap, and included a positive control group that received dinocap at 25 mg/kg bw per day. Dams were subjected to routine in-life observations. On gestation day (GD) 18, they were sacrificed and examined grossly, and the uterine contents were examined. All fetuses were examined for external alterations. Full visceral and skeletal examinations were not performed.

The pregnancy rate was low in the 100 and 500 mg/kg bw per day groups (Table 15), but this is not related to treatment, as pregnancy is determined before the dosing with meptyldinocap started. The only clinical sign of note in the meptyldinocap groups was the presence of bright yellow urine staining from the first day of dosing. One dam in the dinocap positive control group died on GD 18. One dam from the top-dose group delivered on GD 18. Body weights were reduced in the dinocap group but not in animals exposed to meptyldinocap. There were no significant findings in the meptyldinocap-treated groups; marked fetotoxicity was seen in the dinocap-treated group (Table 15). The single cleft palate seen in the low-dose meptyldinocap group is within the historical background range and, as it is not reproduced at higher dose levels, is not considered to be treatment related.

Table 15. Findings in mice exposed to meptyldinocap or dinocap during gestation

	Dose (mg/kg bw per day)				
	0	25 (dinocap)	100 (meptyldinocap)	250 (meptyldinocap)	500 (meptyldinocap)
No. bred	15	15	15	15	15
Pregnant (%)	12/15 (80.0)	13/15 (86.7)	9/15 (60.0)	12/15 (80.0)	8/15 (53.3)
No. of deaths	0	1	0	0	0
No. of litters with viable fetuses	12	10	9	12	7
No. of implantations/dam	13.5 ± 1.4	12.6 ± 1.4	13.2 ± 3.8	13.5 ± 1.8	13.4 ± 2.5
No. of resorptions/litter	0.5 ± 0.8	0.9 ± 1.5	2.4 ± 3.6	1.0 ± 1.1	0.7 ± 0.8
Resorptions/litters with resorptions	1.5 (6/4)	2.2 (11/5)	3.7 (22/6)	1.7 (12/7)	1.3 (5/4)
Mean % postimplantation loss	3.98 ± 6.4	24.20 ± 37.0*	18.3 ± 23.0	8.27 ± 9.20	5.23 ± 5.8
Viable fetuses/litter	13.0 ± 1.9	9.3 ± 4.5*	10.8 ± 4.4	12.4 ± 2.3	12.7 ± 2.4
Fetuses with cleft palate	0	109*	1	0	0
Mean otoconia score	11.8 ± 0.4	0.8* ± 1.0	11.7 ± 0.5	11.7 ± 0.6	11.8 ± 0.4

From Carney & Tornesi (2005a)

* $P < 0.05$

The data show that meptyldinocap does not have the same teratogenic potential in mice as dinocap. The NOAEL for maternal and fetotoxicity is 500 mg/kg bw per day, the highest meptyldinocap dose tested (Carney & Tornesi, 2005a).

In a non-GLP study, the developmental effects of dinocap and some of its component analogues were investigated in small groups of CD-1 mice. In the first phase, groups of 6–9 pregnant mice received dinocap (purity 84%; lot No. 3-83122) or the analogues 2,4-DNHPC (meptyldinocap) and 2,6-DNHPC (purity 95%) singly or in combination at 25 mg/kg bw per day. Dosing was by gavage in corn oil vehicle on days 7–16 of gestation. On GD 18, dams were killed and fetuses examined for cleft palate and external lesions. The only treatment that produced adverse effects was dinocap, which reduced fetal body weight by about 20% and produced cleft palate in 84% of the fetuses.

In the second phase, groups of 7–12 pregnant mice received dinocap (purity 84%; lot No. 3-83122) or the analogues 2,4-DNHPC (meptyldinocap) or 2,6-DNHPC (purity 95%) singly at 25 mg/kg bw per day. Dosing was by gavage in corn oil vehicle on days 7–16 of gestation; dams were allowed to litter normally. On day 45 postpartum, 20 pups per group were investigated for their swimming ability, head tilt (tortocollis) and otolith score. Results for corn oil and individual analogue groups were similar, with no adverse findings. The dinocap group showed reduced pup weights, reduced pup survival, increased incidences of tortocollis and inability to swim and a marked reduction in otolith score.

This study indicates that the developmental effects of dinocap in mice are not due to the two methylheptyl analogues tested and therefore not relevant to meptyldinocap, which is more than 90% (w/w) 2,6-DNHPC (Rogers et al., 1987).

In a further investigation of dinocap analogues, groups of 10 mated CD-1 mice received one of four different dinocap technical analogues on GD 6–17. The compounds were suspended in 0.5% methylcellulose and administered by gavage at dose levels of 0 or 10 mg/kg bw per day for the

Table 16. Findings (mean ± standard deviation) in mice exposed to dinocap analogues during gestation

	Control	4-EH	4-PP	6-EH	6-PP
	Dose (mg/kg bw per day)				
	0	5	5	10	10
Litters with viable fetuses	8	8	6	10	9
No. of implantations/dam	13.0 ± 3.9	13.6 ± 3.2	14.8 ± 1.8	13.7 ± 3.5	14.9 ± 1.7
Mean % postimplantation loss	7.05 ± 5.64	0.96* ± 2.72	38.77* ± 47.64	7.61 ± 17.09	7.23 ± 4.98
Viable fetuses/litter	12.0 ± 3.5	13.5 ± 3.3	9.0* ± 7.1	12.8 ± 4.3	13.8 ± 1.4
Mean fetal weight (g)	1.42 ± 0.11	1.37 ± 0.07	0.76* ± 0.22	1.38 ± 0.16	1.34 ± 0.08
Fetuses with cleft palate	0	0	83*	0	0
Fetuses with ablepharia	1	0	4	0	0
Fetuses with hindlimb club foot	0	0	5*	0	0
Mean otolith score	11.8 ± 0.2	11.6 ± 0.3	1.8* ± 2.3	11.8 ± 0.3	11.7 ± 0.3

From Carney & Tornesi (2005b)
* $P < 0.05$

2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate (6-EH) and 2,4-dinitro-6-(1-propylpentyl)phenyl crotonate (6-PP) analogues and 5 mg/kg bw per day for the 2,6-dinitro-4-(1-ethylhexyl)phenyl crotonate (4-EH) and 2,6-dinitro-4-(1-propylpentyl)phenyl crotonate (4-PP) analogues. Maternal investigations included clinical observations, body weight and body weight gain. On GD 18, all surviving mice were killed and examined for gross pathological alterations. Gravid uterine weights and numbers of uterine implantations, resorptions and live/dead fetuses were recorded. All fetuses were weighed and examined for external alterations, including cleft palate, and otoconia. Adverse effects were seen only with the 4-PP analogue; severe maternal toxicity, requiring euthanasia in four dams, was evident from GD 12, with a range of fetal effects typical of dinocap (Table 16).

This study indicates that the developmental effects produced in mice by dinocap are associated with the 4-PP analogue (Carney & Tornesi, 2005b).

Rats

In a GLP-compliant study, groups of CD rats (26 mated females per group) received meptyldinocap (purity 97.4%; lot No. 2004-03140-64) by gavage in 0.5% methylcellulose on GDs 6–20. Dose levels were 0, 50, 150 or 500 mg/kg bw per day. The study complied with OECD guideline 414. Dams were subjected to routine in-life observations. On GD 21, they were sacrificed and examined grossly, and their uterine contents were examined. All fetuses were examined for external alterations; approximately half the fetuses were examined for visceral and craniofacial alterations, with the remainder examined for skeletal and otoconial alterations.

Bright yellow urine staining was evident from the beginning of dosing. Marked maternal toxicity (e.g. mortality, poor condition and body weight loss) was evident at 500 mg/kg bw per day, and this group was terminated on or before GD 11. At the next highest dose level of 150 mg/kg bw per day, there were reductions from GD 6 to GD 9 in feed consumption (approximately 18 g per rat) and maternal body weight gain (approximately 14 g per rat) at the start of the dosing period, with a body weight deficit persisting until termination. There was no evidence of maternal toxicity at 50 mg/kg bw per day; minor effects (< 10%) on feed consumption on GDs 6–9 and liver weights were considered not to be adverse. There was no fetotoxicity at 150 mg/kg bw per day (Table 17).

The NOAEL for maternal toxicity is 50 mg/kg bw per day, based on increased (> 20%) absolute liver weights and decreased body weight gain at 150 mg/kg bw per day. The NOAEL for devel-

Table 17. Findings in rats exposed to meptyldinocap during gestation[a]

	Dose (mg/kg bw per day)		
	0	50	150
Body weight gain, GDs 6–9 (g)	20 ± 4.5	16 ± 5.1	5.7* ± 6.6
Body weight, GD 18 (g)	361 ± 21	357 ± 17	346* ± 21
Feed consumption, GDs 6–9 (g/day)	23 ± 1.8	21* ± 1.8	17* ± 2.4
Absolute liver weight (g)	14.4 ± 1.9	15.7* ± 1.4	17.7* ± 1.5
Number bred	26	26	26
Pregnant (%)	25/26 (96.2)	25/26 (96.2)	26/26 (100)
Number of litters totally resorbed	0	0	0
Number of litters with viable fetuses	25	25	26
Number of corpora lutea/dam	13.8 ± 1.9	13.8 ± 0.9	14.0 ± 2.3
Number of implantations/dam	13.1 ± 2.1	13.5 ± 1.0	13.2 ± 2.0
Number of resorptions/litter	0.1 ± 0.3	0.2 ± 0.5	0.2 ± 0.6
Viable fetuses/litter	13.0 ± 2.2	13.2 ± 1.1	13.0 ± 2.0
Fetal weight, sexes combined (g)	5.8 ± 0.30	5.9 ± 0.24	5.8 ± 0.25
Gravid uterine weight (g)	102 ± 14.8	108 ± 8.5	105 ± 13.7
Fetuses with external malformations	0/325	0/331	0/338
Fetuses with craniofacial malformations	0/168	0/172	0/176
Fetuses with visceral malformations	0/168	4/172	0/176
Fetuses with skeletal malformations	2/157	1/159	0/162
Mean otoconia scores	12.0 ± 0.1	11.8 ± 0.4	11.9 ± 0.32

From Carney & Tornesi (2005c)

* $P < 0.05$

[a] Findings are ± standard deviation.

opmental toxicity is 150 mg/kg bw per day, the highest dose tested for the full duration of the study (Carney & Tornesi, 2005c).

Rabbits

In a GLP-compliant study, groups of New Zealand White rabbits (26 mated females per group) received meptyldinocap (purity 97.4%; lot No. 2004-03140-64) by gavage in 0.5% methylcellulose on GDs 7–27. Dose levels were 0, 3, 12 or 48 mg/kg bw per day. The study complied with OECD guideline 414. Dams were subjected to routine in-life observations. On GD 28, they were sacrificed and examined grossly, and their uterine contents were examined. All fetuses were examined for external, visceral and skeletal alterations; approximately half the fetuses were examined for craniofacial alterations or for otoconia.

Yellow urine staining was evident in most top-dose animals, four mid-dose animals and two low-dose animals. Two top-dose animals died on day 20 after a period of inanition; one of the dams had a low feed consumption even before dosing commenced. Maternal body weight loss was seen in several dams before dosing commenced and in half the dams exposed at 48 mg/kg bw per day early in the dosing period. From GD 7 to GD 10, dams in the top-dose group had a mean deficit of 44 g in body weight gain relative to controls; although this was similar in magnitude to the decrease in feed consumption over the same period, there was no apparent consistency in individual body weight gains or feed consumption. Over the remainder of the study, the body weight gain in the top-dose group was similar to that in controls, and from GD 20 to GD 28 (during which dosing continued), the body

Table 18. Findings in rabbits exposed to meptyldinocap during gestation

	Dose (mg/kg bw per day)			
	0	3	12	48
Body weight gain, GDs 7–10 (g)	42 ± 32	16 ± 42	33 ± 31	−4* ± 56
Body weight gain, GDs 10–13 (g)	50 ± 36	63 ± 41	65 ± 44	43 ± 57
Body weight gain, GDs 20–28 (g)	117	132	114	151
Body weight, GD 28 (g)	3582	3493	3532	3549
Feed consumption, GDs 7–10 (g/day)	545	522	551	513
Pregnant (%)	25/26 (96.2)	25/26 (96.2)	23/26 (88.5)	24/26 (92.3)
Number of litters with viable fetuses	25	25	23	22
Number of corpora lutea/dam	9.4 ± 1.6	9.4 ± 2.7	9.6 ± 1.6	8.9 ± 1.6
Number of implantations/dam	8.7 ± 1.6	8.7 ± 2.5	8.6 ± 1.8	8.1 ± 1.4
Number of resorptions/litter	0.5 ± 0.7	0.4 ± 1.1	0.5 ± 0.8	0.1 ± 0.4
Viable fetuses/litter	8.2 ± 1.3	8.3 ± 2.0	8.1 ± 1.7	8.0 ± 1.4
Fetal weight, sexes combined (g)	37 ± 2.7	36 ± 4.0	37 ± 3.3	35 ± 4.0
Gravid uterine weight (g)	460 ± 70	444 ± 100	445 ± 77	418 ± 52
Fetuses with external malformations	0/206	3/207	0/187	0/175
Fetuses with craniofacial malformations	0/109	2/109	0/99	0/92
Fetuses with visceral malformations	7/206	2/207	1/187	4/175
Fetuses with skeletal malformations	2/192	0/207	1/186	0/175
Mean otoconia scores	11.9 ± 0.2	11.9 ± 0.2	11.7 ± 0.8	11.9 ± 0.2

From Carney & Tornesi (2005d)
* $P < 0.05$

weight gain was greater than that in controls. An increase in kidney weight of approximately 8% was seen in the top-dose group, but this is not considered to be adverse. There were no adverse effects on pregnancy outcome or on the pattern of fetal findings, and ossification was similar in control and treated groups (Table 18).

The NOAEL for maternal toxicity is 12 mg/kg bw per day, based on body weight loss at the beginning of dosing. The NOAEL for developmental effects is 48 mg/kg bw per day, the highest dose tested (Carney & Tornesi, 2005d).

2.6 Special studies

There are no special studies.

3. Observations in humans

Meptyldinocap is a relatively new molecule, and no workplace monitoring data are available.

Comments

Biochemical aspects

No new ADME studies on meptyldinocap in mammals have been conducted. However, in a number of the ADME studies with dinocap, the radiolabel was present on the methylheptyl analogue, which is the primary component of meptyldinocap. The Meeting considered that these ADME studies were applicable to meptyldinocap.

Meptyldinocap is relatively well absorbed, with approximately 60–70% of the radiolabel absorbed in rabbits. Absorption is rapid, with peak plasma radioactivity seen 1–6 hours after oral administration. Radiolabel was widely distributed, with tissue levels generally low and below those in blood. The compound did not tend to concentrate in any particular organ or tissue; highest levels were found in the liver, kidneys and skin. Metabolism was extensive, consisting of hydrolytic cleavage to release the crotonate moiety and subsequent oxidation of the methylheptyl chain. The basic metabolic pathways are similar in rats and mice. Excretion of radiolabel was extensive via urine (39–58% in mice; 31–50% in rats) and faeces and mainly occurred within 48 hours.

Toxicological data

Meptyldinocap is of low acute toxicity when administered orally or dermally (LD_{50} > 2000 mg/kg bw) but is of moderate toxicity by inhalation (LC_{50} 1.2 mg/l). Meptyldinocap is a slight irritant to skin and a moderate irritant to the eye; it has been found to produce skin sensitization in a local lymph node assay in mice.

Short-term studies of toxicity with meptyldinocap were performed in mice, rats and dogs. Yellow urine staining was a consistent finding, but this is considered not to be adverse, as it is associated with the excretion of coloured metabolites of meptyldinocap. In a 28-day dietary study of meptyldinocap in mice, there were increases in liver weight (approximately 10–15%) at 750 ppm (equal to 126 mg/kg bw per day), with a NOAEL of 200 ppm (equal to 33 mg/kg bw per day). In a 90-day dietary study of meptyldinocap in rats, altered clinical chemistry parameters and mononuclear cell infiltration of the lacrimal glands were seen at 2000 ppm (equal to 122 mg/kg bw per day), with a NOAEL of 650 ppm (equal to 40 mg/kg bw per day).

In a 90-day dietary study, groups of dogs were exposed to meptyldinocap at 0, 15, 60 or 120 ppm, with a positive control group receiving 60 ppm dinocap. Reduced body weight gain was seen in males after the first week of dosing with 120 ppm (equal to 3.9 mg/kg bw per day), following a gradual introduction to the treated diets. These initial body weight effects showed no consistency between animals or with feed consumption patterns. The body weight gain over 90 days was 41% lower in males receiving 120 ppm than in controls. Ocular changes seen in the dinocap-exposed group were not evident in the meptyldinocap-treated groups. The NOAEL for meptyldinocap was 60 ppm (equal to 1.6 mg/kg bw per day), based on the effects on body weight gain over the duration of the study. In an extension to this 90-day study, a satellite group was exposed to meptyldinocap for 1 year at 120 ppm (equal to 3.5 mg/kg bw per day). The examinations were limited to tibial nerves, eyes and heart. This segment of the study showed that there were no significant eye, heart or nerve lesions evident after exposure to meptyldinocap for 1 year. The reduced body weight gain seen in the 90-day phase was not evident over the extended dosing period. The 1-year study was not designed to permit the identification of a NOAEL.

No evidence of carcinogenicity was seen in long-term studies of toxicity and carcinogenicity with dinocap at the highest doses tested, 150 ppm (equal to 23 mg/kg bw per day) in mice and 2000 ppm (equal to 71 mg/kg bw per day) in rats. The NOAEL for general toxicity in the chronic study in mice with dinocap was 15 ppm (equivalent to 2.8 mg/kg bw per day), based on body weight deficits in females. In the 30-month study of dinocap in rats, there was a significant increase in survival in both sexes at the top dose of 2000 ppm (equal to 71 mg/kg bw per day), which had an impact on the incidences of a number of age-related changes. The NOAEL for general toxicity of dinocap was 200 ppm (equal to 6.4 mg/kg bw per day).

The potential genotoxicity of meptyldinocap has been investigated in an adequate range of tests in vitro and in vivo. No evidence of mutagenicity or clastogenicity was noted.

The Meeting concluded that meptyldinocap is unlikely to be genotoxic.

The Meeting concluded that dinocap is not carcinogenic and that this conclusion could be extrapolated to meptyldinocap.

No effects on fertility, reproductive parameters, sperm or reproductive tissues were seen in a two-generation dietary study with dinocap at doses up to 400 ppm (equal to 27 mg/kg bw per day), the highest dose tested in the second generation. In the first generation, at 1000 ppm (equal to 65 mg/kg bw per day), reduced pup survival led to a reduction in the dose level to 400 ppm for the second generation, which was without effect on pups. The NOAEL for pup development and parental toxicity was 200 ppm (equal to 13 mg/kg bw per day).

In a developmental toxicity study in mice investigating effects on the palate and inner ear, meptyldinocap did not produce any such effects on fetuses at the highest dose of 500 mg/kg bw per day, whereas a dinocap dose of 25 mg/kg bw per day produced cleft palate in nearly all fetuses and had marked effects on otoconia formation. Additional studies showed that the teratogenicity of dinocap in mice was associated with the 4-propylpentyl analogue and not the methylheptyl analogue present in meptyldinocap. In a developmental toxicity study in rats, marked maternal toxicity and marked reductions (approximately 50%) in feed consumption were seen with a meptyldinocap dose of 500 mg/kg bw per day, such that this dose level had to be terminated by GD 11. At the next highest dose level of 150 mg/kg bw per day, there were reductions from GD 6 to GD 9 in feed consumption (approximately 18 g per rat) and maternal body weight gain (approximately 14 g per rat) at the start of the dosing period. The body weight deficit and increased absolute liver weights (23%) were evident at the end of the study, but there were no indications of fetotoxicity. The NOAEL for maternal toxicity was 50 mg/kg bw per day, with the NOAEL for fetotoxicity being 150 mg/kg bw per day. In a rabbit developmental toxicity study with meptyldinocap, maternal body weight loss was seen in several dams before dosing commenced and in half the dams exposed at 48 mg/kg bw per day early in the dosing period. From GD 7 to GD 10, dams in the top-dose group had a mean deficit of 44 g in body weight gain relative to controls; although this was similar in magnitude to the decrease in feed consumption over the same period, there was no apparent consistency in individual body weight gains or feed consumption. Over the remainder of the study, the body weight gain in the top-dose group was similar to that in controls, and from GD 20 to GD 28 (during which dosing continued), the body weight gain was greater than that in controls. There were no effects on the fetuses at any dose level. The maternal NOAEL was 12 mg/kg bw per day, and the NOAEL for fetotoxicity was 48 mg/kg bw per day.

The Meeting concluded that meptyldinocap did not induce developmental toxicity and that it was not teratogenic.

There are no specific neurotoxicity studies on meptyldinocap, but there were no indications of neurotoxicity in routine studies, including a 90-day study in rats that included an FOB.

No information on medical surveillance or poisoning incidents was available.

The Meeting concluded that the existing database on dinocap and meptyldinocap was adequate to characterize the potential hazards of meptyldinocap to fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.02 mg/kg bw on the basis of the NOAEL of 1.6 mg/kg bw per day in the 90-day dietary study in dogs, based on reduced body weight gain in males at 3.9 mg/kg bw per day. A safety factor of 100 was applied.

The Meeting concluded that an ARfD was unnecessary, as there were no effects that could be attributed to a single exposure. Meptyldinocap did not produce neurotoxicity, fetotoxicity or reproductive effects and has an oral LD_{50} of greater than 2000 mg/kg bw. The Meeting reviewed in depth the reduced body weight gains and feed consumption seen in the early stages of the 90-day study

in dogs and the developmental toxicity studies in rats and rabbits. In the rat developmental toxicity study, the body weight deficits were considered secondary to reduced feed consumption, which was probably associated with palatability issues. The Meeting concluded that the body weight and feed consumption patterns seen in the early stages of the dog and rabbit studies were not consistent between individual animals. The findings in these three studies did not provide an appropriate basis for establishing an ARfD for meptyldinocap.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Twenty-eight-day study of toxicity with meptyldinocap[a]	Toxicity	200 ppm, equal to 33 mg/kg bw per day	750 ppm, equal to 126 mg/kg bw per day
	Seventy-eight-week study of toxicity and carcinogenicity with dinocap[a]	Toxicity	15 ppm, equal to 2.8 mg/kg bw per day	100 ppm, equal to 18 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 23 mg/kg bw per day[b]	—
Rat	Ninety-day study of toxicity with meptyldinocap[a]	Toxicity	650 ppm, equal to 40 mg/kg bw per day	2000 ppm, equal to 122 mg/kg bw per day
	Thirty-month study of toxicity and carcinogenicity with dinocap[a]	Toxicity	200 ppm, equal to 6.4 mg/kg bw per day	2000 ppm, equal to 71 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 71 mg/kg bw per day[b]	—
	Multigeneration study of reproductive toxicity with dinocap[a]	Reproductive toxicity	400 ppm, equal to 27 mg/kg bw per day[b]	—
		Parental toxicity	200 ppm, equal to 13 mg/kg bw per day	400 ppm, equal to 27 mg/kg bw per day
		Offspring toxicity	200 ppm, equal to 13 mg/kg bw per day	400 ppm, equal to 27 mg/kg bw per day
	Developmental toxicity study with meptyldinocap[c]	Maternal toxicity	50 mg/kg bw per day	150 mg/kg bw per day
		Embryo and fetal toxicity	150 mg/kg bw per day[b]	—
Rabbit	Developmental toxicity study with meptyldinocap[c]	Maternal toxicity	12 mg/kg bw per day	48 mg/kg bw per day
		Embryo and fetal toxicity	48 mg/kg bw per day[b]	—
Dog	Ninety-day study of toxicity with meptyldinocap[a]	Toxicity	60 ppm, equal to 1.6 mg/kg bw per day	120 ppm, equal to 3.9 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.

Estimate of acceptable daily intake for humans

0–0.02 mg/kg bw

Estimate of acute reference dose

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to meptyldinocap

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; moderately well absorbed (60–70%)
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Relatively rapid
Metabolism in animals	Extensively metabolized, initially hydrolysis to remove the crotonate side-chain and then via oxidation of the methylheptyl chain
Toxicologically significant compounds (animals, plants and the environment)	Meptyldinocap
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	1.2 mg/l (4 h, nose only)
Rabbit, dermal irritation	Slight
Rabbit, ocular irritation	Moderate
Mouse, dermal sensitization	Sensitizer (local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Body weight gain (males)
Lowest relevant oral NOAEL	Dog: 1.6 mg/kg bw per day (meptyldinocap)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Body weight
Lowest relevant NOAEL	Mouse: 2.8 mg/kg bw per day (dinocap)
Carcinogenicity	Not carcinogenic in rats or mice
Genotoxicity	
	Not genotoxic in vitro or in vivo
Reproductive toxicity	
Reproduction target/critical effect	Pup survival
Lowest relevant reproductive NOAEL	Rat: 13 mg/kg bw per day (dinocap)
Developmental target/critical effect	None
Lowest relevant developmental NOAEL	Rabbit: 48 mg/kg bw per day (meptyldinocap)
Neurotoxicity/delayed neurotoxicity	
	No indications in routine studies
Other toxicological studies	
	No data
Medical data	
	No data

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Ninety-day study of toxicity in dogs	100
ARfD	Unnecessary		

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Appendix 1: Summaries of studies performed with dinocap and relevant to the evaluation of meptyldinocap

Long-term studies of toxicity and carcinogenicity

Mice

In a non-GLP chronic toxicity/carcinogenicity study, groups of CD-1 rats (60 of each sex per group) received dinocap technical (purity 96%; lot No. 2893) incorporated into the diet at levels of 0, 15, 100 or 200/150 ppm for 78 weeks. Mortality, clinical signs, body weight, behaviour, and feed and water consumption were recorded routinely. Ophthalmoscopy examinations were performed on all animals at weeks 52 and 78. Haematology samples were obtained from all animals at weeks 52 and 78. Gross examinations were conducted for all animals sacrificed or dying during the course of the study, main organs were weighed and an extensive range of organs from all control and top-dose animals was examined histopathologically. Lung, liver, kidneys, gross lesions and suspected tumours from all groups were examined histopathologically.

Homogeneity and stability of the test diets were confirmed analytically; although there was a degree of variability in the level of exposure over the duration of the study, this is considered insignificant. Mean intakes were 0, 2.3, 15 and 23 mg/kg bw per day in males and 0, 2.8, 18 and 27 mg/kg bw per day in females. As a result of deaths in females at the 200 ppm dose level during week 1, the top dose level was reduced to 150 ppm; subsequently, survival was similar in all groups (> 55% in all groups at termination). There were no clinical signs associated with treatment other than urine stains in top-dose males. Body weights and feed consumption were reduced throughout the study in top-dose animals of both sexes, with mean body weight gain also reduced significantly in mid-dose females (Table 19). Slight deficits in feed consumption and body weight in 15 ppm males were not considered to be adverse due to the small magnitude (< 10%) and absence of an effect on body weight in 100 ppm males. There were no treatment-related ophthalmoscopy or haematological findings. Testicular atrophy (bilateral) was increased at 150 ppm; absolute seminal vesicle weights were also decreased at 150 ppm, but this appeared to be secondary to the body weight deficits. A slight reduction in absolute testes weight at 100 ppm is not considered to be adverse, as there are no related pathological changes and the value is within normal variation (Table 19). There were no increases in tumour incidences in treated animals.

The NOAEL for general toxicity is 15 ppm (equal to 2.8 mg/kg bw per day for females), based on body weight deficits at 100 ppm (equal to 18 mg/kg bw per day). The NOAEL for carcinogenicity is 150 ppm (equal to 23 mg/kg bw per day), the highest dose tested (Moore, 1991).

Rats

In a GLP-compliant 1-year toxicity study, groups of Crl:CD®BR rats (20 of each sex per group) received dinocap (purity 97%; batch No. 632 6C21) in the diet at 0, 20, 200 or 2000 ppm for 52 weeks. Rats were observed routinely for mortality, clinical signs, body weight, feed consumption and water consumption. After 13, 26 and 52 weeks of treatment, samples from 10 rats of each sex per group were collected for haematology, clinical chemistry and urinalysis examinations. Ophthalmological examinations were performed on all animals prior to initiation and prior to termination of treatment. The weights of seven organs were recorded after autopsy from 10 animals of each sex per group. An extensive range of organs from all animals was preserved in buffered 10% formalin for histopathological examination. Histopathological examinations were performed on tissues of all animals in the control and 2000 ppm groups and for all animals found dead or killed as moribund during the course of the study. Homogeneity and stability of the test diets were confirmed analytically. Achieved intakes were 0, 1.1, 11 and 121 mg/kg bw per day in males and 0, 1.4, 14 and 144 mg/kg bw per day in females.

Table 19. Findings in mice (n = 60) receiving dinocap in the diet for 78 weeks

	Males				Females			
	Dietary concentration (ppm)							
	0	15	100	150	0	15	100	150
Body weight, week 1 (g)	30.9	30.3	29.7*	28.8*	23.8	23.5	23.7	20.5*
Body weight, week 78 (g)	39.2	37.4*	37.9	37.0*	32	31.5	30.3	28.9*
Body weight gain, weeks 0–78 (g) (mean ± SD)	9.5 ± 2.9	8.5 ± 2.5	8.6 ± 2.5	7.7* ± 3.4	9.3 ± 3.9	9.2 ± 3.4	7.2* ± 2.4	6.2* ± 2.1
Mean feed consumption, week 1 (g/week)	38.3	37.2	35.0*	31.4*	36.0	35.1	31.3*	26.2*
Mean total feed consumption, weeks 0–78 (g)	1224	1165*	1158*	1121*	1143	1132	1111	1059*
Testicular atrophy (bilateral)	11	4	11	25*	—	—	—	—
Testes (small)	3	1	6	12*	—	—	—	—
Testes weight, absolute (g)	0.21 ± 0.05	0.23 ± 0.04	0.19* ± 0.04	0.16* ± 0.06	—	—	—	—
Seminal vesicle weight, absolute (g)	0.56 ± 0.19	0.55 ± 0.20	0.54 ± 0.29	0.45* ± 0.12	—	—	—	—
Testes weight, relative to body weight (%)	0.61 ± 0.14	0.68* ± 0.12	0.56 ± 0.13	0.48* ± 0.18	—	—	—	—
Seminal vesicle weight, relative to body weight (%)	0.40 ± 0.05	0.42 ± 0.06	0.39 ± 0.05	0.40 ± 0.06	—	—	—	—

From Moore (1991)
* $P < 0.05$

Five animals died or were sacrificed during the study, but, as none of these were in the top-dose groups, they are considered to be unrelated to treatment. The only clinical sign was bright yellow–stained urine in both sexes at 200 and 2000 ppm and yellow-stained anogenital area in both sexes at 2000 ppm. Body weight deficits consistent with reduced feed consumption were evident throughout the study at 2000 ppm. There were no notable changes in haematological values. Statistically significant reductions in levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol and triglycerides were seen at 2000 ppm at some sampling times, but values were within normal variation at week 52. Blood urea nitrogen was increased in top-dose females at all sampling times. Statistically significant increases in relative organ weights (liver, brain and kidney of both sexes; testis of males; adrenals, lungs, spleen and ovaries of females) were observed in animals in the 2000 ppm group; absolute weights were similar to control values. Histopathological findings were limited to hypertrophy of the liver and thyroid and altered colloid in the thyroid at 2000 ppm (Table 20).

Table 20. Findings in rats receiving dinocap in the diet for 1 year

	Males				Females			
	Dietary concentration (ppm)							
	0	20	200	2000	0	20	200	2000
Body weight, week 1 (g)	243	243	240	213*	178	175	177	160*
Body weight, week 49 (g)	672	665	666	540*	380	375	373	279*
Mean feed consumption, week 1 (g/week)	160	163	157	120*	199	193	193	182
Mean feed consumption, week 49 (g/week)	124	122	121	86*	146	149	144	127*
AST activity, week 52 (U/l) (mean ± SD)	168 ± 41	163 ± 52	201± 199	133 ± 42	209 ± 176	139 ± 51	136 ± 39	125 ± 23
ALT activity, week 52 (U/l) (mean ± SD)	70 ± 54	67 ± 47	98 ± 150	44 ± 41	110 ± 124	62 ± 45	52 ± 27	41* ± 14
Triglycerides, week 52 (mg/dl) (mean ± SD)	123 ± 61	104 ± 41	99 ± 71	70 ± 27	76 ± 35	109 ± 35	101 ± 45	51 ± 31
Cholesterol, week 52 (mg/dl) (mean ± SD)	109 ± 46	83 ± 20	76* ± 22	62* ± 14	95 ± 21	99 ±27	103 ± 27	96 ± 18
Blood urea nitrogen, week 52 (mg/dl) (mean ± SD)	13.8 ± 1.7	13.6 ± 1.9	14.5 ± 3.6	13.9 ± 3.2	14.2 ± 1.7	14.2 ± 1.9	13.9 ± 2.3	17.8* ± 3.8
Liver panlobular hypertrophy	0/20	0/20	1/20	14/20	0/20	0/20	0/20	0/20
Thyroid follicular cell hypertrophy	7/20	5/20	8/20	13/20	7/20	10/20	9/20	15/20
Thyroid altered colloid	7/20	6/20	7/20	13/20	2/20	3/20	3/20	7/20

From Ferguson, Morrison & Bernacki (1999)
SD, standard deviation; U, units; * $P < 0.05$

The NOAEL is 200 ppm (equal to 11 mg/kg bw per day), based on reduced body weight gain and feed consumption, liver and thyroid changes and increased blood urea nitrogen at 2000 ppm (equal to 121 mg/kg bw per day) (Ferguson, Morrison & Bernacki, 1999).

In a non-GLP lifetime toxicity/carcinogenicity study, groups of Wistar rats (80 of each sex per group) received dinocap (purity 77.5%; batch not specified) incorporated into the diet at a level of 0, 20, 200 or 2000 ppm for 30 months (130 weeks). Eight animals of each sex per dose level were sacrificed after 13, 26 and 52 weeks. Mortality, clinical signs, body weight, behaviour, feed consumption and water consumption were recorded routinely. Urinalysis, clinical chemistry and haematology were examined in samples obtained prior to the sacrifice of the satellite groups. Gross examinations were conducted for all animals sacrificed or dying during the course of the study, and weights of 11 major organs were recorded. In addition to the major organs, a range of other organs/tissues (approximately 15) and grossly observable lesions were examined histopathologically. Statistical analyses were not performed on a number of parameters.

Table 21. Findings in rats receiving dinocap in the diet for 30 months

	Males				Females			
	Dietary concentration (ppm)							
	0	20	200	2000	0	20	200	2000
Body weight, week 1 (g)	190	187	190	167*	138	140	136	120*
Body weight, week 110 (g)	697	712	692	566	557	554	533	354*
Mean feed consumption, week 1 (g/day)[a]	19.2	19.5	19.1	13.7	14.3	15.6	14.4	8.6
Mean feed consumption, week 110 (g/day)[a]	18.9	17.3	18.2	15.0	13.9	15.4	14.4	11.7
Mean feed efficiency (g weight gain/100 g feed)[a]	2.9	3.0	3.1	2.5	3.2	3.0	3.4	2.4
Liver fatty degeneration	22/80	20/80	13/80	3*/80	17/80	19/80	13/80	5/80
Liver necrosis	15/80	12/80	13/80	5*/80	12/80	6/80	8/80	3*/80
Liver hyperplasia	21/80	26/80	22/80	36*/80	22/80	15/80	22/80	25/80
Kidney tubular atrophy/cast	20/80	29/80	20/80	34*/80	17/80	27/80	21/80	16/80
Kidney chronic nephrosis	22/80	13/80	18/80	3*/80	5/80	3/80	4/80	1/80

From Maita et al. (1981)

* $P < 0.05$

[a] Standard deviation not calculated, statistics not performed.

Mean dinocap intakes were 0, 0.65, 6.4 and 71 mg/kg bw per day in males and 0, 0.8, 8.1 and 90 mg/kg bw per day in females. Significantly reduced mortality was observed in both sexes at the high dose, survival at 30 months in controls and 2000 ppm groups, respectively, being 4 of 56 versus 25 of 56 males and 6 of 56 versus 24 of 56 females. Group mean body weight gains and feed consumption were reduced in both sexes at the high dose level from initiation of the study to termination (Table 21). The only clinical sign of note was yellowing of the urine and fur at the high dose level. Both males and females at 2000 ppm showed decreased haematocrit, haemoglobin and red blood cell counts intermittently during the first year of the study. There were no notable or consistent effects on clinical chemistry results. Absolute kidney weights were increased significantly in top-dose males at week 52; relative weights of many organs were increased at 2000 ppm, consistent with the lower body weights. Decreased fat deposition was noted in the 2000 ppm dose groups. The pattern of neoplastic findings was similar in test and control groups, with the exception of mammary gland tumours, which were significantly less prevalent in top-dose females. The pattern of non-neoplastic lesions in some organs was altered in the top-dose groups, probably related to the markedly increased survival (Table 21); these changes are not considered to be adverse. An increase in total number of top-dose animals with calf muscle or sciatic nerve degeneration and cataracts was associated primarily with surviving animals and is considered secondary to the increased survival (Table 22).

The NOAEL for general toxicity is 200 ppm (equal to 6.4 mg/kg bw per day), based on body weight deficits at 2000 ppm (equal to 71 mg/kg bw per day). The NOAEL for carcinogenicity is 2000 ppm (equal to 71 mg/kg bw per day), the highest dose tested (Maita et al., 1981).

Dogs

In a pre-GLP study, groups of Beagle dogs (four of each sex per group) received dinocap (purity 78%; lot No. 2-0375) in the diet at 0, 15, 60 or 240 ppm for up to 2 years. Because of high toxicity seen at 240 ppm, the top dose was varied downwards during the study, and this group was terminated at week 62. Mortality, clinical signs, body weight, behaviour, feed consumption and water consumption were recorded routinely. Samples for urinalysis, clinical chemistry and haematology were taken pretest and on five occasions during the study. Ophthalmoscopy was performed pretest, at

Table 22. Incidence of cataracts and degeneration of muscle and nerves in rats receiving dinocap in the diet for 30 months

Parameter	Males								Females							
	Dietary concentration (ppm)															
	0		20		200		2000		0		20		200		2000	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
No. of animals examined	28 / 4	52	26 / 2	52	28 / 4	52	49 / 25	31	30 / 6	50	30 / 6	50	28 / 4	52	48 / 24	32
Calf muscle degeneration / atrophy	4	38	3	34	4	31	24	19	6	16	5	11	4	21	22	12
Sciatic nerve degeneration / atrophy	4	28	4	15	4	18	25	16	6	12	6	8	2	13	24	11
Incidence of cataract	—	5	2	5	1	3	6	4	—	3	1	2	—	3	3	—

From Maita et al. (1981)

a. Killed on schedule / surviving to 30 months.

b. Dead or killed in extremis.

week 52 and then every 13 weeks. Main organs from all animals were weighed, and a wide range of tissues was examined histopathologically. The only finding of note at the intermediate dose level was bilateral retinal atrophy with greyish discoloration of the tapetum lucidum, seen in seven animals at week 52 and subsequently; this was seen in three males during histopathological examination.

The NOAEL is 15 ppm (equivalent to 0.4 mg/kg bw per day), based on retinal atrophy seen at 60 ppm (equivalent to 1.5 mg/kg bw per day) (Weatherholtz et al., 1979).

Multigeneration studies

Rats

In a GLP-compliant study, groups of CD:Crl®BR rats (26 of each sex per group) received dinocap (purity 96%; batch No. 2893) in the diet at 0, 40, 200 and 1000/400 ppm for 70 days before mating, during mating and during gestation until terminal sacrifice. At weaning of the F_1 pups, the highest dose was reduced to 400 ppm because of a high rate of mortality. Pairing was one male to one female. On day 4 postpartum, litter size was reduced to eight pups, ideally four of each sex. Each litter was weaned on lactation day 21. Observations included clinical signs and mortality and recording of body weight, feed consumption and reproductive performance. In addition, gait, righting reflex and behaviour were evaluated during the growth phase of the F_0 and F_1 parental animals. All adult animals of the F_0 and F_1 generations were subjected to a gross necropsy, and their reproductive organs, liver and thyroid were weighed. Pups culled on day 4 postpartum and postweaning offspring not selected for continuation of the study were examined externally and internally for macroscopic abnormalities. Histological examinations were performed on all gross lesions and the reproductive organs, liver, thyroid (including parathyroid) and eyes from the control and high dose group parental animals.

In addition to the guideline requirements, a supplemental investigation of effects of dinocap on male reproductive capabilities was conducted on selected F_1 males at 0, 40 and 200 ppm (12 rats per dose group). Clinical observations, body weights and feed consumption were evaluated weekly until termination after 16 weeks. At necropsy, terminal body weight, left testicular, epididymal and caudal weights, sperm motility and sperm count per gram caudal tissue were recorded.

Table 23. Intakes of dinocap in the rat reproduction study

Dietary concentration (ppm)	Dose (mg/kg bw per day)					
	Males (premating)		Females (premating)		Females (gestation)	
	F_0	F_1	F_0	F_1	F_0	F_1
40	2.6	3.1	3.2	3.7	2.8	2.8
200	12.9	14.9	15.7	18.0	13.9	13.7
1000/400	65.1	32.4	77.3	38.7	69.5	27.4

Homogeneity and stability were confirmed analytically. Achieved intakes are outlined in Table 23. There were no deaths or overt clinical signs of systemic toxicity related to treatment. Yellow-stained fur and orange-discoloured urine were seen in most animals of the two highest dose groups (200 and 1000/400 ppm). This is not considered adverse, as it is most likely related to the presence of dinocap or its metabolites in the urine. Body weight gain and feed consumption were reduced at 1000 ppm from the start of treatment, by 10–20% and 10%, respectively. Body weight gain during gestation was not adversely affected by dinocap treatment. At 400 ppm, body weight gain was similar to that of controls. There were no treatment-related effects on mating performance, fertility, litter size, gestation length and index, parturition or viability index (number of pups surviving to day 4) at all doses of both generations. At the top dose (1000 ppm), the body weight gain of the F_1 offspring during the lactation phase was decreased compared with controls, and more pups appeared to be weak/thin and dehydrated. Pup survival during lactation was not affected by treatment; however, after weaning, the mortality rate of the high-dose pups increased. A total of 28 pups died within 7 days after weaning (mortality rate 26%). No adverse effects on parameters investigated (litter size, body weight, clinical observations, viability) were noted in F_2 pups at any dose level. In parental animals of the F_0 and F_1 generations receiving 1000/400 ppm, the relative liver and testes weights were increased (< 10%), with no evidence of a histological correlate. No other effects on organ weights were observed. In addition, necropsy (including histomorphological evaluation) of parental animals and gross pathology of F_1 and F_2 pups did not reveal any treatment-related effects at dose levels up to 400/1000 ppm dinocap. In F_1 pups that had been culled after weaning, the cause of mortality could not be determined, but the presence of orange or red discoloration of the contents of the gastrointestinal tract and bladder was noted, indicating the presence of dinocap or its metabolites. In the investigation of male reproductive tissues, no adverse effects were seen on reproductive organs or sperm counts/motility.

Dinocap produced no adverse effects on reproduction at the highest dose tested (1000 ppm, equal to 65 mg/kg bw per day in the first mating; 400 ppm, equal to 27 mg/kg bw per day in the second mating). The NOAEL for pup development is 200 ppm (13 mg/kg bw per day, based on reduced body weight and survival at 1000 ppm). The NOAEL for parental toxicity is 200 ppm (13 mg/kg bw per day), based on reduced body weight gains (Morseth, 1990).

TEBUCONAZOLE

First draft prepared by
P.V. Shah,[1] Midori Yoshida[2] and Maria Tasheva[3]

[1] Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, United States of America (USA)
[2] Division of Pathology, National Institute of Health Sciences, Tokyo, Japan
[3] National Service for Plant Protection, Sofia, Bulgaria

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Explanation

Tebuconazole is the International Organization for Standardization (ISO)–approved name for (*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (International Union of Pure and Applied Chemistry [IUPAC]), for which the Chemical Abstracts Service (CAS) No. is 107534-96-3. Tebuconazole is a triazole fungicide that acts by inhibiting sterol biosynthesis in fungi (demethylation inhibitor).

The toxicity of tebuconazole was first evaluated by the 1994 Joint FAO/WHO Meeting on Pesticide Residues (JMPR). That Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) on the basis of a no-observed-adverse-effect level (NOAEL) of 2.9 mg/kg bw per day for histopathological alterations in the adrenal glands seen at 4.4 mg/kg bw per day and above in two 52-week toxicity studies in dogs and using a safety factor of 100.

Tebuconazole was re-evaluated by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). Three new studies (an acute neurotoxicity study, a subacute neurotoxicity study and a developmental neurotoxicity study) since the last review by the JMPR were made available. All pivotal studies with tebuconazole were certified as complying with good laboratory practice (GLP) unless otherwise stated.

Evaluation for acceptable daily intake

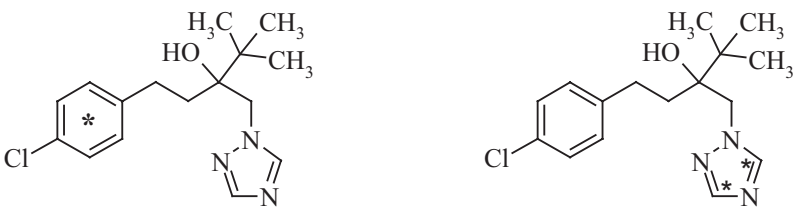
Unless otherwise stated, studies evaluated in this monograph were performed by GLP-certified laboratories and complied with the relevant Organisation for Economic Co-operation and Development (OECD) and/or United States Environmental Protection Agency (USEPA) test guideline(s).

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution and elimination of tebuconazole were studied in rats after oral dosing of tebuconazole radiolabelled with ^{14}C, as shown in Figure 1.

Figure 1. Position of the radiolabel on tebuconazole used in pharmacokinetic studies in rats



* position of radiolabel

Table 1. Summary of tests performed to investigate biokinetics in rats

Test No.	Administered dose (mg/kg bw)	Sex and number of animals	Kind of test
1	20	5 males	Excretion in expired air, urine, faeces
2	2	5 males	Excretion in bile, urine, faeces
3	2	5 males	Excretion in urine, faeces; plasma levels
4	2	5 females	Excretion in urine, faeces; plasma levels
5	2	5 males	Excretion in urine, faeces; plasma levels, pretreatment[a]
6	2	5 females	Excretion in urine, faeces; plasma levels, pretreatment[a]
7	20	5 males	Excretion in urine, faeces; plasma levels
8	20	5 females	Excretion in urine, faeces; plasma levels
9	20	5 males	Excretion in urine, faeces; plasma levels, repetition of test No. 7

From Weber (1987)

[a] Pretreatment with 2 mg/kg bw non-radiolabelled dose once daily for 14 consecutive days followed by a 2 mg/kg bw radiolabelled dose on day 15.

The absorption, distribution and metabolism (see section 1.2) of tebuconazole uniformly labelled with ^{14}C in the benzene ring (Figure 1) were studied in BOR: WISW (SPF Cpb) Wistar rats. Tebuconazole, both ^{14}C labelled (radiochemical purity 99.5%) and non-radioactive, suspended in 0.5% aqueous tragacanth gel was used in the study. The test substance was administered to male and female rats at an oral dose of 2 or 20 mg/kg bw. In addition, rats of both sexes were first subjected to 14 days of treatment with a daily oral dose of 2 mg/kg bw of unlabelled test substance, followed by a single radioactive dose of 2 mg/kg bw 24 hours after the last of these doses. Furthermore, excretion of the radioactivity in the exhaled air (dose 20 mg/kg bw) and in the bile (dose 2 mg/kg bw) was studied in male rats. The study design and sampling times are summarized in Table 1.

(a) Absorption and distribution

The amounts of radioactivity excreted in the bile (90.7% of the administered dose) and urine (7.4%) by the bile duct–cannulated animals plus the residues in the total body at the time of sacrifice (0.23%) showed that the radioactivity was completely absorbed after oral administration (Table 2). The analysis of the plasma curves (Tables 3 and 4) showed that the test compound was rapidly absorbed from the gastrointestinal tract of male and female rats in all test groups, as indicated by the times at which the maximum plasma radioactivity concentrations were reached (T_{max} values between 0.33 and 1.7 hours). The maximum dose-normalized equivalent concentrations (P_{max}) were achieved between 0.11 and 0.20 hour, indicating good tissue accessibility of the radioactivity administered with the test compound. The values calculated for the terminal half-lives (Table 4) ranged from 31.9 to 52.5 hours and were therefore short in relation to the observation period of 72 hours. The dose-corrected areas under the plasma concentration–time curves (AUC) yielded a relatively wide range of AUC_{total} values (1.7–5.2 hours); correspondingly high total plasma clearances, ranging from 0.6 to 1.9 ml/min, were calculated. The mean residence time of the radioactivity in the plasma ranged from 26.9 to 48.6 hours and was therefore short in relation to the observation period of 72 hours. The relatively high values determined for the distribution volume at steady state of 8.7–17.9 ml/g indicate that the radioactivity continued to be distributed unevenly in the organism at later times after administration.

A statistical analysis of the above-mentioned parameters revealed sex-dependent differences (Table 4) between the test groups. The males in all groups were found to have significantly larger (by a factor of 1.7–3) AUCs, which led to correspondingly lower total clearance values. The males treated with the high dose also exhibited a significantly longer mean residence time; the pretreated males were found to have a significantly shorter terminal elimination half-life. Analysis of the dependence

Table 2. Cumulative excretion of total radioactivity in urine, faeces, bile and exhaled air and radioactive residues in the male rat 48 or 72 hours after oral administration of [phenyl-UL-^{14}C]-tebuconazole

Sample material	Time (h)	% of administered dose	
		Dose (mg/kg bw)	
		20	2
		Test No. 1 (n = 5)	Test No. 2 (n = 4)
Bile	1	—	15.0
	2	—	39.2
	3	—	52.8
	4	—	61.7
	6	—	72.7
	8	—	85.8
	12	—	88.0
	18	—	88.7
	24	—	89.6
	30	—	90.1
	36	—	90.5
	42	—	90.6
	48	—	90.7
Exhaled air	0–72	0.03	—
Urine	3	—	1.0
	4	1.0	2.4
	6	—	4.3
	8	3.2	5.5
	12	—	6.7
	18	—	7.1
	24	13.4	7.3
	32/30	14.5	7.3
	48	15.8	7.4
	56	16.0	—
	72	16.2	—
Faeces	24	62.7	1.5
	48	74.7	1.5
	56	74.9	—
	72	75.8	—
Total excreta	—	92.0	99.0
Total body	—	0.8	0.23
Total recovery	—	92.8	99.2

From Weber (1987)

Table 3. Time course of the dose-normalized equivalent concentrations (P) in the plasma of rats after administration of a single oral dose of [phenyl-UL-^{14}C]tebuconazole

Time post-application (h)	Dose-normalized equivalent concentration (P) in plasma (% of administered dose)						
	Male	Female	Male	Female	Male	Female	Male
	Dose (mg/kg bw)						
	2	2	2[a]	2[a]	20	20	20
	Test No. 3	Test No. 4	Test No. 5	Test No. 6	Test No. 7	Test No. 8	Test No. 9[b]
	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)
0.17	0.0986	0.1659	0.0501	0.0781	0.0209	0.0522	0.0450
0.33	0.1493	0.1974	0.0953	0.1081	0.0779	0.0794	0.1025
0.67	0.1481	0.1545	0.1266	0.0973	0.0961	0.0941	0.1622
1	0.1455	0.1392	0.1409	0.1048	0.1627	0.0902	0.1852
1.5	0.1504	0.1128	0.1284	0.1120	0.1471	0.0875	0.1782
2	0.1419	—	0.1285	0.1141	0.1312	0.0781	0.1562
3	0.1295	0.0956	0.1228	0.1170	0.1013	0.0680	0.1265
4	0.1308	0.0908	0.1114	0.0886	0.0933	0.0646	0.1131
6	0.1190	0.0720	0.1040	0.0821	0.1070	0.0560	0.1140
8	0.1118	0.0648	0.0991	0.0306	0.0842	0.0507	0.1111
24	0.0457	0.0225	0.0416	0.0267	0.0439	0.0182	0.0571
32	0.0385	0.0169	0.0737	0.0192	0.0387	0.0113	0.0595
48	0.0231	0.0096	0.0245	0.0118	0.0274	0.0061	0.0317
56	0.0218	0.0087	0.0222	0.0109	0.0240	0.0051	0.0319
72	0.0165	0.0072	0.0155	0.0082	0.0168	0.0044	0.0193

From Weber (1987)

[a] Pretreated with 2 mg/kg bw daily for 14 days.

[b] Repetition of test No. 7.

of the biokinetic characteristics on the size of the dose (Table 4) revealed, for both sexes, significant differences only in those parameters describing the course of the plasma radioactivity concentration. In males, the rise in the plasma radioactivity concentration took place about 2 times more slowly after administration of the high dose. In addition, the terminal elimination of the radioactivity from the plasma was found to proceed significantly more rapidly after the high dose. The females exhibited two significant effects additional to the above. After administration of the high dose, the T_{max} was observed to increase by a factor of 3. In addition, the dose-corrected AUC was significantly smaller in the females after administration of the high dose. Pretreatment with the unlabelled test substance led, in both sexes, to some differences compared with the situation after a single dose. These differences were related mainly to the characteristics derived from the time course of the plasma radioactivity concentrations. Those differences, found to be statistically significant, were a slowdown in the rise of the plasma concentration in females and a shorter terminal half-life in males after pretreatment with unlabelled test substance (Weber, 1987).

(b) Excretion and terminal radioactive residues in tissues

The radioactivity was excreted in exhaled air only to a very small extent (0.03% of the administered dose) (Table 2). The cumulative excretion, radioactivity in the body and total recoveries are shown in Table 5. After 72 hours, between 86.5% and 98.4% of the administered dose (approximately 99% of the recovered dose) was excreted in the urine and faeces. The major route of excretion was faecal (61.5–82.1%). The males of all experimental groups excreted about half as much radioactivity

Table 4. Comparison of biokinetic parameters of plasma radioactivity after oral administration of [phenyl-UL-^{14}C]tebuconazole

Parameter	Male	Female	Male	Female	Male	Female
	Dose (mg/kg bw)					
	2	2	2[a]	2[a]	20	20
	Test No. 3 (n = 5)	Test No. 4 (n = 5)	Test No. 5 (n = 5)	Test No. 6 (n = 5)	Test No. 9[b] (n = 5)	Test No. 8 (n = 5)
Terminal half-life (h)	48.46	52.46[c]	31.93	43.68	34.45[c]	34.81[d]
AUC_{exp} (h)	3.57	2.00	3.61[c]	1.96	4.24	1.52[c]
AUC_{total} (h)	4.75	2.51	4.35[c]	2.51	5.24	1.74[d]
P_{max} (h)	0.17	0.20	0.14	0.13	0.18[c]	0.11[c]
T_{max} (h)	0.87	0.33	1.70	1.67	1.67[c]	1.06
Clearance total (ml/min)	0.71	1.35	0.71	1.35	0.64[c]	1.85[c]
Clearance renal (ml/min)	0.15	0.55	0.13[c]	0.54	0.13[c]	0.57[d]
Mean residence time (h)	48.63[c]	41.89	41.55	44.27	42.73[c]	26.87[d]
Volume steady state (ml/g)	10.90	16.74	8.71	17.93	8.18[c]	14.87[d]

From Weber (1987)
AUC, area under the curve; exp, experimental
[a] Pretreated with 2 mg/kg bw daily for 14 days.
[b] Repetition of test No. 7.
[c] Values based on four animals.
[d] Values based on three animals.

in the urine as did the females; the proportion of radioactivity excreted in the faeces was correspondingly higher in the males. Bile duct–cannulated rats eliminated, within 48 hours, about 91% of the recovered radioactivity in the bile, about 7% in the urine and only 1.5% in the faeces (Table 2). The large quantities of radioactivity determined in the faeces of the other dose groups must have been, therefore, radioactivity excreted into the intestinal lumen with the bile. The animals with biliary fistulae excreted in the urine, within 48 hours after administration, about half of the quantity of radioactivity, compared with the corresponding males of the other test groups, indicating an enterohepatic recirculation of the radioactivity.

The radioactivity remaining in the body excluding the gastrointestinal tract was low in all test groups. After 72 hours, less than 1% of the applied radioactivity could be detected in the organs, tissues and the remaining carcass. Highest residues were found in the liver and kidney, those organs responsible for metabolism and excretion of the test compound and its metabolites. Sex-dependent differences between the corresponding groups could be observed; the radiolabelled residues determined in most tissues and organs at the end of the study were approximately 1.5–2.5 times higher in males of all groups than in the corresponding females (Weber, 1987).

In a whole-body autoradiography study, [phenyl-UL-^{14}C]tebuconazole (radiochemical purity 99%) was administered in a 0.5% aqueous tragacanth gel solution at a dose level of 20 mg/kg bw to male Bor: WISC SPF Cpb Wistar rats. Seven male rats were used in this study. The animals were sacrificed at 1, 4, 8, 24, 48 and 72 hours after administration of the test substance. After deep-freezing, sagittal sections of the animals (50 µm thick) were cut with a microtome and placed onto an X-ray film. The autoradiographs were visually inspected to estimate the relative concentrations of radioactivity in the various tissues and organs of the rats.

Table 5. Cumulative excretion of total radioactivity in urine and faeces and radioactive residues in the rat 72 hours after oral administration of [phenyl-UL-^{14}C]tebuconazole

Sample material	Time (h)	% of administered dose						
		Male	Female	Male	Female	Male	Female	Male
		Dose (mg/kg bw)						
		2	2	2[a]	2[a]	20	20	20[b]
		Test No. 3 (n = 5)	Test No. 4 (n = 5)	Test No. 5 (n = 5)	Test No. 6 (n = 5)	Test No. 7 (n = 5)	Test No. 8 (n = 5)	Test No. 9 (n = 5)
Urine	4	3.2	8.6	1.8	5.1	1.2	2.1	1.9
	8	6.6	14.2	5.0	9.9	3.4	8.0	5.0
	24	13.9	28.1	12.8	25.8	11.7	23.3	14.4
	32	14.9	29.9	13.9	28.5	12.6	25.2	15.3
	48	15.9	32.0	14.5	31.1	13.9	27.8	16.6
	56	16.1	32.5	14.8	31.7	14.1	28.3	16.7
	72	16.3	32.9	15.0	32.3	14.4	28.8	17.0
Faeces	24	71.1	52.2	64.3	47.1	61.3	50.8	63.8
	48	79.9	60.9	75.0	58.7	70.8	60.8	77.3
	56	81.3	61.0	76.9	61.2	71.1	61.3	77.7
	48–72	82.1	62.5	78.8	61.5	72.1	62.7	78.7
Total excreta	—	98.4	95.4	93.8	93.8	86.5	91.5	95.7
Total body	—	0.81	0.69	1.01	1.38	0.81	0.52	1.03
Total recovery	—	99.2	96.1	94.8	95.2	87.3	92.0	96.7

From Weber (1987)

[a] Pretreated with 2 mg/kg bw daily for 14 days.

[b] Repetition of test No. 9.

One hour after administration, tebuconazole-derived radioactivity was detectable in almost all tissues and organs, with the exception of compact bone substance. The radioactivity of the parent compound was unevenly distributed in the animal body. Very high concentrations of radioactivity were discernible in the contents of the stomach and some portions of the small intestine, in the preputial gland as well as in areas of the mucosa of nose and tongue and the epithelium of the oesophagus. High concentrations were found in the liver, the cortex of the adrenal gland, the infraorbital gland and the hair follicles of the dorsal skin. Low-level radioactivity was detected in the fatty tissues, renal papilla, musculature, thymus, bone marrow and skin. During the entire investigation, the ratio of the radioactivity concentrations among the tissues and organs showed only slight alterations. With increasing time after administration, the concentrations declined more rapidly in the mucosa of the nasopharyngeal tract, the fat tissues, the brain and spinal marrow as well as the infraorbital gland and the preputial gland, compared with the mean body concentration. At the end of the investigation, radioactivity concentrations were relatively low in most organs and tissues; only the cortex of the adrenal gland showed a high level of radioactivity. Additionally, the evaluation of the autoradiographs showed high biliary excretion combined with a long-lasting enterohepatic circulation as well as a relatively low renal elimination rate (Weber, 1988).

1.2 Biotransformation

The metabolism of the test substance after administration of either [phenyl-UL-^{14}C]tebuconazole (purity > 99%) or [triazol-3,5-^{14}C]tebuconazole (purity 98.4%) to several groups of BOR:

Table 6. Summary of tests performed to investigate the metabolism of tebuconazole in rats

Test No.	Administered dose (mg/kg bw)	Sex and number of animals	Kind of test
1	2	5 males	Single oral low dose, phenyl label
2	2	5 females	Single oral low dose, phenyl label
3	2	5 males	Oral low dose, pretreatment,[a] phenyl label
4	2	5 females	Oral low dose, pretreatment,[a] phenyl label
5	20	5 males	Single oral high dose, phenyl label
6	20	5 females	Single oral high dose, phenyl label
7	20	5 males	Single oral high dose, triazole label (excretion experiment)
8	20	5 males	Single oral high dose, triazole label (metabolism)
9	20	5 females	Single oral high dose, triazole label (metabolism)

From Ecker et al. (1987)

[a] Pretreatment with 2 mg/kg bw of non-radiolabelled dose once daily for 14 consecutive days followed by a 2 mg/kg bw radiolabelled dose on day 15.

WISW (SPF Cpb) rats under varying experimental conditions was assayed. The dose groups were a single oral low dose of 2 mg/kg bw; 14 daily single oral non-radioactive doses of 2 mg/kg bw, followed by a radioactive dose of 2 mg/kg bw on the 15th day; and a single oral high dose of 20 mg/kg bw (Table 6).

In the main study, [phenyl-UL-^{14}C]tebuconazole was used. Each group consisted of five male and five female animals. In addition to these trials, the high dose of the triazole-labelled test substance was orally administered to both sexes. Because more than 90% of the recovered radioactivity was excreted via faeces and urine within 48 hours, pooled samples from the sampling intervals 0–48 hours were prepared in order to include all major metabolites. The metabolites were extracted and purified from urine and faeces with suitable solvents. The identification was conducted by comparative high-performancee liquid chromatography and gas chromatography using authentic reference compounds, as well as by employing gas chromatography with mass spectrometry and nuclear magnetic resonance spectroscopic techniques. The radioactivity of urine and extracts of faeces and urine was determined by liquid scintillation counting; solid samples were combusted, and the radioactivity of the trapped carbon dioxide was measured by liquid scintillation counting. The quantitative distribution of the identified metabolites in urine and faeces is given in Table 7.

The excretion balances showed nearly identical patterns compared with those in the biokinetic study (Weber, 1987). Approximately 14–33% of the dose was eliminated in the urine, and 61–82% of the dose was eliminated in the faeces, with no apparent differences between the two dose groups. After dosing with the [^{14}C]phenyl-labelled compound, males excreted significantly less radioactivity in the urine (14–17% of the dose) than did females (29–33% of the dose); in the case of the [^{14}C]-triazole-labelled compound, there were no sex-dependent differences in the urine to faeces ratio of radioactivity excretion. The parent compound was not detected in the urine; only between 0.5% and 2.2% could be found in the faeces of all dose groups. Regarding the excreta as a whole, tebuconazole-1-hydroxy (M 03) and tebuconazole-carboxylic acid (M 06) were the main metabolites in all test groups and accounted for 15.7–28.2% (M 03) and 14.1–36.2% (M 06) of the administered radioactivity, with a slight tendency towards higher amounts in females. Sex-related differences between the test groups were found in the quantitative distribution of some of the minor metabolites in the excreta. One compound, tebuconazole-1,5-di-OH-glucuronide (M 12), was detected in the excreta of male animals only and accounted for 0.7–1.3% of the administered radioactivity. Two further

Table 7. Quantitative distribution of metabolites in urine and faeces after oral administration of [phenyl-UL-^{14}C]tebuconazole or [triazole-3,5-^{14}C]tebuconazole to the rat

Metabolites[a]	% of administered dose							
	Male	Female	Male[b]	Female[b]	Male	Female	Male	Female
	Dose (mg/kg bw)							
	2	2	2	2	20	20	20	20
	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Test No. 5	Test No. 6	Test No. 2	Test No. 3
	Urine/ faeces (0–48 h)	Urine/ faeces (0–48 h)	Urine/ faeces (0–48 h)	Urine/ faeces (0–48 h)	Urine/ faeces (0–48 h)	Urine/ faeces (0–48 h)	Urine (0–48 h)	Urine (0–48 h)
	Phenyl	Phenyl	Phenyl	Phenyl	Phenyl	Phenyl	Triazole	Triazole
Tebuconazole	0.46	0.57	0.69	0.49	2.2	0.47	—	—
M 02	2.4	3.1	3.4	3.1	4.7	5.1	—	—
M 03	15.7	18.9	16.8	21.6	19.7	28.2	2.2	0.30
M 04	1.3	0.47	2.2	0.78	5.6	0.37	—	—
M 06	32.6	36.2	27.1	35.3	14.1	29.8	1.6	9.7
M 07	3.4	1.1	5.6	0.78	2.3	1.0	3.4	0.7
M 10	—	2.0	0.10	2.2	0.09	2.3	0.20	2.7
M 11	0.46	4.8	0.29	3.0	0.19	3.7	0.30	2.9
M 12	1.3	—	0.70	—	1.0	—	0.50	0.20
M 14	0.65	0.66	0.69	0.88	1.1	0.28	—	—
M 26	—	—	—	—	—	—	5.4	1.5
Unknown 1	1.4	1.1	1.2	0.98	1.0	0.65	—	—
Unknown 2	2.0	0.57	1.5	0.59	3.5	0.19	—	—
Unknown 3	1.8	0.09	0.98	0.20	2.3	0.19	—	—
Unknown 4	0.93	0.66	1.3	0.59	2.2	0.84	—	—
Unknown 5	0.56	0.28	0.88	0.29	1.6	0.28	—	—
Total identified[c]	**58.3**	**67.9**	**57.6**	**68.1**	**51.0**	**71.2**	**13.6**	**18.0**
Sum unknown 1–5	6.7	2.7	5.9	2.7	10.6	2.2	—	—
Not assigned[d]	19.8	19.9	26.0	21.4	20.4	13.7	10.3	6.4
Total unidentified[c]	26.5	22.6	32.0	24.1	31.0	15.9	10.3	6.4
Unextracted solids	6.9	3.7	7.7	4.3	9.5	4.4	—	—
Faeces	—	—	—	—	—	—	70.7	72.7
Body[e]	0.83	0.57	1.2	1.1	1.6	1.8	5.9	3.0
Total recovery[c]	**92.5**	**94.8**	**98.3**	**97.5**	**93.2**	**93.4**	**100.6**	**100.2**

From Ecker et al. (1987)

[a] For chemical names and codes, see Figure 2 below.

[b] Pretreated with 2 mg/kg bw daily for 14 days.

[c] Any lack of correspondence between the sum of the individual values and the "total" values is due to rounding.

[d] Radioactivity not in discrete fractions.

[e] Values included for balance reasons.

compounds, tebuconazole-1,5-dihydroxy (M 04) and tebuconazole-ketocarboxylic acid (M 07), were detected in higher amounts in the excreta of the males compared with those of the females. The corresponding values for the males were 1.3–5.6% (M 04) and 2.3–5.6% (M 07) compared with 0.4–0.8% (M 04) and 0.8–1.1% (M 07) of the administered radioactivity in the females. Two compounds were found in greater amounts in the excreta of the females. Tebuconazole-1-hydroxysulfate (M 10) accounted for 2.0–2.3% and tebuconazole-1-OH-glucuronide (M 11) 3.0–4.8% of the administered radioactivity in females. Both compounds were detected in the excreta of the males in amounts of less than one tenth of these values. Two additional compounds, tebuconazole-*o*-hydroxy (M 02) and tebuconazole-desmethyl (M 14), were detected in minor amounts and showed no significant dose- or sex-dependent differences. Neither the dose level nor the pretreatment showed a significant influence on the metabolic pattern in any of the dose groups. Five unidentified compounds were detected in all dose groups, none of them exceeding 3.5% of the administered radioactivity. Sex-related differences between the test groups were detected in the distribution of these compounds. In general, females excreted less than half as much of these compounds as did males. In total, 15.9–32.0% of the administered dose remained unidentified after extraction. This unidentified activity consisted of the above-mentioned unidentified compounds and of background activity not assigned to specific metabolites or fractions. Between 3.7% and 9.5% of the administered radioactivity remained unextractable in the faeces. The rate of identification was high; after administration of [phenyl-UL-^{14}C]tebuconazole at doses of 2 or 20 mg/kg bw, between 51.0% and 71.2% of the administered radioactivity could be identified. The identification balance did not take into account the amount of 1,2,4-triazole ("free triazole", M 26) found in the study with [triazole-3,5-^{14}C]tebuconazole. In a total material balance, the amount of identified radioactivity should include the figures for 1,2,4-triazole as well. The triazole-labelled metabolites from faecal extracts were not quantified in this study because the comparison of the metabolic profiles in faecal extracts shows identity regardless of the label. Comparison of the metabolic profiles in urine of the animals treated with differently labelled tebuconazoles raised one significant difference. One additional metabolite, identified as 1,2,4-triazole (M 26), accounted for 5.4% of the administered radioactivity in males and 1.5% in females. The metabolic profiles revealed sex-related differences similar to those already observed in the animals treated with the phenyl-labelled test substance (Ecker et al., 1987).

The proposed metabolic pathway is given in Figure 2. The first step in the biotransformation of tebuconazole was the hydroxylation of the *t*-butyl group, resulting in tebuconazole-1-hydroxy. This compound was either excreted or further transformed via oxidation to the carboxylic acid, sulfonylation or conjugation with glucuronic acid at the *t*-butyl group. Further steps of minor importance involve hydroxylation of tebuconazole-1-hydroxy in the benzylic position followed by conjugation with glucuronic acid at the *t*-butyl group, hydroxylation of the parent compound's phenyl ring, oxidation in the benzylic position, decarboxylation at the *t*-butyl group and cleavage of the triazole moiety (Neumann & Hartmann, 2009).

2. Toxicological studies

2.1 Acute toxicity

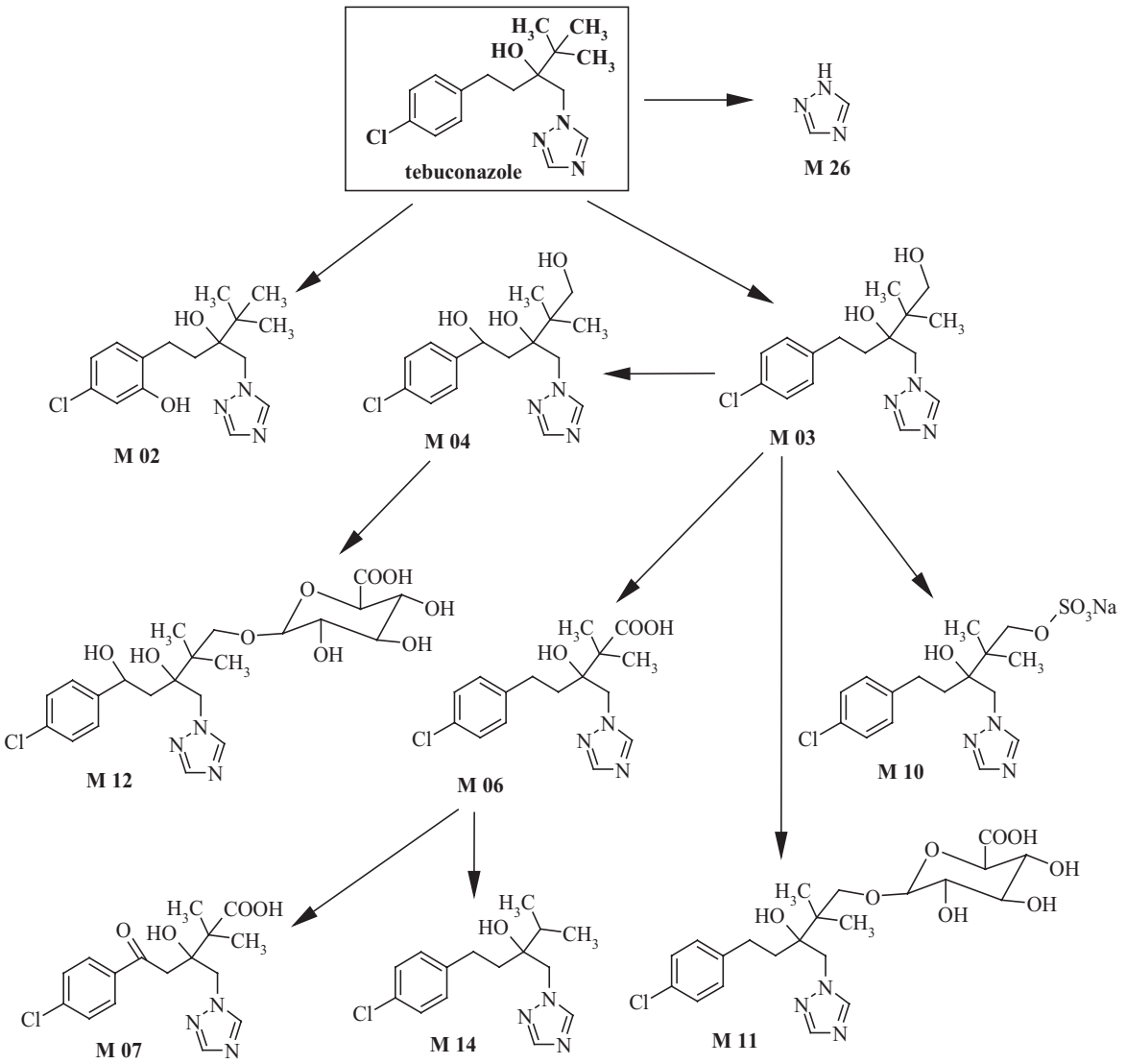
The acute toxicity of tebuconazole is summarized in Table 8.

(a) Oral administration

Mice

Groups of male and female young adult Crj:CD ICR mice (five of each sex per dose) were given tebuconazole (purity 98.0%) as a single dose of 0 mg/kg bw or in the range 1600–5000 mg/kg

Figure 2. Proposed metabolic pathway of tebuconazole in rats



M 02: tebuconazole-*o*-hydroxy
M 03: tebuconazole-1-hydroxy
M 04: tebuconazole-1,5-dihydroxy
M 06: tebuconazole-carboxylic acid
M 07: tebuconazole-ketocarboxylic acid
M 10: tebuconazole-1-hydroxysulfate
M 11: tebuconazole-1-OH-glucuronide
M 12: tebuconazole-1,5-di-OH-glucuronide
M 14: tebuconazole-desmethyl
M 26: 1,2,4-triazole

bw by gavage. The test material was formulated in polyethylene glycol 400. The dose volume was 10 ml/kg bw. Animals were fasted overnight prior to dosing. Animals were observed frequently for clinical signs on day 1 and once or twice a day thereafter. Body weights were recorded on days 1, 7 and 14. Necropsy was performed on all animals.

Sedation and abnormal gait were observed between 1 minute and 1 hour after administration. The main clinical signs observed were sedation, abnormal gait, paralytic gait, hypnosis, half-closed eyes, rough coat, abnormal breathing and chick-like vocalization. The clinical signs disappeared within 3 days post-dosing in surviving animals except for rough coat in females of the 5000 mg/kg bw dose group, which disappeared 5 days after dosing. Changes in the digestive system (mucosal redness, dark reddish brown focus in the stomach, dilated lumen, yellowish contents and mucosal redness in the small intestine), lungs (dark reddish brown) and testis (atrophy) were observed in

Table 8. Acute toxicity of tebuconazole

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/m^3)	Reference
Mouse	Crj:CD ICR	M, F	Oral	Fasted M: 2800 F: 5200	—	Ohta (1991b)
	NMRI	M, F	Oral	Fasted M: 1615 F: 3023	—	Heimann & Pauluhn (1983)[a]
Rat	Crj:CD Sprague-Dawley	M, F	Oral	Fasted M: 4000 F: 1700	—	Ohta (1991a)
	Bor:WISW (SPF Cpb) Wistar	M, F	Oral	Fasted M: > 5000 F: 3933 Non-fasted M: 4264 F: 3352	—	Heimann & Pauluhn (1983)[a]
	Bor:WISW (SPF Cpb) Wistar	M	Oral	> 5000	—	Flucke (1987)
Rabbit	HC:NZW	M, F	Oral	Fasted M and F > 1000	—	Heimann & Pauluhn (1983)[a]
Rat	Bor:WISW (SPF Cpb) Wistar	M, F	Intraperitoneal	Fasted M: 751 F: 395	—	Heimann & Pauluhn (1983)[a]
Rat	Bor:WISW (SPF Cpb) Wistar	M, F	Dermal	M and F > 5000	—	Heimann & Pauluhn (1983)[a]
	Crj:CD SPF Sprague-Dawley	M, F	Dermal	> 2000	—	Ohta (1991c)
Rat	Bor:WISW (SPF Cpb) Wistar	M, F	Inhalation	—	> 818	Heimann & Pauluhn (1983)[a]
	Bor:WISW (SPF Cpb) Wistar	M, F	Inhalation	—	> 5093 (dust) > 371 (aerosol)	Pauluhn (1988)
	Hsd:Cpb:WU Wistar	M, F	Inhalation	—	> 2118	Pauluhn (2007)
Rabbit	HC:NZW	M	Dermal	Non-irritating	—	Heimann & Pauluhn (1983)[a]
	New Zealand White	M, F	Dermal	Non-irritating	—	Sheets (1988)
Rabbit	HC:NZW	M	Ocular irritation	Non-irritating	—	Heimann & Pauluhn (1983)[a]
	New Zealand White	M, F	Ocular irritation	Mildly irritating	—	Eigenberg & Sheets (1988)

Table 8 (continued)

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/m^3)	Reference
Guinea-pig	Pirbright White	F	Dermal sensitization (mazimization test)	Non-sensitizing	—	Heimann (1983)
	Hsd Poc:DH (SPF-bred)	F	Dermal sensitization (mazimization test)	Non-sensitizing	—	Stropp (1996)
	DHPW (SPF-bred)	M	Dermal sensitization (Buehler test)	Non-sensitizing	—	Heimann (1987)
	Dunkin-Hartley	M	Dermal sensitization (Buehler test)	Non-sensitizing	—	Sheets (1990)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male
[a] Non-GLP study.

animals that died during the observation period. Under the study conditions utilized, the oral median lethal dose (LD_{50}) of tebuconazole in mice was calculated to be 2800 and 5200 mg/kg bw for males and females, respectively (Ohta, 1991b).

Groups of male and female young adult NMRI mice (five of each sex per dose) were given tebuconazole (purity 97.1%) as a single dose of 0 mg/kg bw or in the range 100–3350 mg/kg bw for males and 500–5000 mg/kg bw for females by gavage formulated in Cremophor EL/water. The dose volume was 10 ml/kg bw. Animals were fasted overnight prior to dosing. The treated animals were observed for 14 days after dosing. Animals were observed for clinical signs and mortality frequently on day 1 and once per day thereafter. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

The primary clinical signs noted in the study included behavioural and motility disturbances, dyspnoea, staggering, spastic gait, stiff posture, rolling, reduced reflexes, prostration on side and stomach, occasional twitching and weight loss (males only). Animals that died during the post-treatment observation period exhibited spotted and distended lungs; patchy, pale and enlarged liver and liver lobulation; and reddened glandular stomach. No treatment-related findings were observed in animals sacrificed at termination. Under the study conditions utilized, the oral LD_{50} of tebuconazole in fasted mice was calculated to be 1615 and 3023 mg/kg bw for males and females, respectively (Heimann & Pauluhn, 1983).

Rats

Groups of male and female young adult Crj:CD Sprague-Dawley rats (five of each sex per dose) were given tebuconazole (purity 98.0%) as a single dose of 0 mg/kg bw or in the range from 1600 to 5000 mg/kg bw for males and from 730 to 5000 mg/kg bw for females by gavage formulated in polyethylene glycol 400 suspension. Animals were fasted overnight prior to dosing. Animals were observed for mortality and clinical signs at 1-hour intervals for the first day and twice daily thereafter for 13 days. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

No treatment-related effects on body weight were observed. Clinical signs of toxicity were manifested as sedation, abnormal gait, paralytic gait and emaciation. The symptoms were observed from 20 minutes to 4 days after administration in both sexes. Generally, the abnormal gait, paralytic gait and marked emaciation were observed 2 days post-dosing. At termination, abnormal findings in the liver (yellow-white patchy areas) and the testis (atrophy) for males were observed. Changes in the urinary bladder (reddish content), the adrenals (redness and hypertrophy) and the trachea (retention of foamy fluid) were observed in animals that died during the observation period. Under the study

conditions utilized, the oral LD_{50} of tebuconazole in rats was calculated to be 4000 and 1700 mg/kg bw for males and females, respectively (Ohta, 1991a).

In a second study, fasted young adult Bor:WISW (SPF Cpb) Wistar rats (five of each sex per dose) were given tebuconazole (purity 97.1%) as a single dose by gavage in Cremophor EL/water at a dose of 0 mg/kg bw or in the range from 1000 to 5000 mg/kg bw for fasted male and female rats and from 500 to 5000 mg/kg bw for non-fasted male and female rats. Animals were observed for 14 days after dosing. Animals were observed for clinical signs and mortality frequently on day 1 and once per day thereafter. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

Body weight losses were noted in the first week of the post-dosing period but were normalized at the end of the study. The primary clinical signs noted in the study included behavioural, breathing and motility disturbances, staggering, spastic gait, sternal or lateral recumbency, cramped posture, increased urine excretion and poor reflexes. Under the study conditions utilized, the oral LD_{50} of tebuconazole in fasted rats was calculated to be greater than 5000 and 3933 mg/kg bw for males and females, respectively. The oral LD_{50} of tebuconazole in non-fasted rats was calculated to be 4264 and 3352 mg/kg bw for males and females, respectively (Heimann & Pauluhn, 1983).

Rabbits

Groups of male and female young adult HC:NZW albino rabbits (three of each sex per dose) were given tebuconazole (purity 97.1%) as a single dose of 500 or 1000 mg/kg bw by gavage formulated in Cremophor EL/water. The dose volume was 5 ml/kg bw. Animals were fasted overnight prior to dosing. The treated animals were observed for 14 days after dosing. Animals were observed for clinical signs and mortality frequently on day 1 and once per day thereafter. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

No mortality occurred. A general loss of appetite was observed; however, body weights were not affected by the treatment. In animals sacrificed at termination, slightly distended and spotted lungs and slightly patchy kidneys were observed. Under the study conditions utilized, the oral LD_{50} of tebuconazole in fasted rabbits was greater than 1000 mg/kg bw for males and females, respectively (Heimann & Pauluhn, 1983).

(b) Intraperitoneal administration

Rats

Groups of young adult Bor:WISW (SPF Cpb) Wistar rats (five of each sex per dose) were given tebuconazole (purity 97.1%) intraperitoneally in Cremophor EL/water as a single dose in the range from 50 to 1000 mg/kg bw for fasted males and from 50 to 560 mg/kg bw for fasted females. The dose volume was 10 ml/kg bw. Animals were observed for 14 days. Animals were observed for clinical signs and mortality frequently on day 1 and once a day thereafter. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

Behavioural, breathing and motility disturbances, staggering, spastic gait, uncoordinated movements, poor reflexes, narcosis, convulsions, and lateral or sternal recumbency were observed. Animals dying during observation exhibited patchy to dark red, distended lungs; patchy, sometimes pale spleen and kidneys; patchy liver, slightly swollen from 500 mg/kg bw onwards, with the individual lobes adhering to each other and to pancreas, diaphragm, stomach and fatty tissue; reddened glandular stomach; thin, unpatterned walls of stomach; clear fluid in abdomen; and whitish deposits on all abdominal organs. Animals sacrificed at the end of the observation period exhibited swollen liver, with the liver lobes adhering to each other, and spleen covered with a white coat. Under the

study conditions utilized, the intraperitoneal LD_{50} of tebuconazole in fasted rats was calculated to be 751 and 395 mg/kg bw for males and females, respectively (Heimann & Pauluhn, 1983).

(c) Dermal application

Rats

Five male and five female young adult Bor:WISW (SPF Cpb) Wistar rats were exposed dermally to tebuconazole (purity 97.1%) at 5000 mg/kg bw applied to a shaved dorsal area of the body surface. The test substance was mixed with physiological saline solution. The exposure period was 24 hours. The treated area was covered by means of occlusive dressings and aluminium foil. The treated site was rinsed with water and soap 24 hours after the treatment. Animals were observed for mortality and clinical signs several times a day on day 1 and once a day for the remainder of the 14-day observation period. Body weights were recorded on days 1, 7 and 14. A gross necropsy was performed on all animals.

No treatment-related mortality, clinical signs, body weight changes, skin irritation or pathological findings were observed. The dermal LD_{50} of tebuconazole in rats was greater than 5000 mg/kg bw for males and females (Heimann & Pauluhn, 1983).

Five male and five female young adult Crj:CD SPF Sprague-Dawley rats were exposed dermally to tebuconazole (purity 98.0%) at 2000 mg/kg bw applied to a shaved dorsal area of the body surface. The test substance was mixed with polyethylene glycol 400 (5 ml/kg bw). The exposure period was 24 hours. The treated area was covered with gauze and sponge held in place by a non-irritating bandage. The treated site was rinsed with warm water 24 hours after the treatment. Animals were observed for mortality and clinical signs several times a day on day 1 and once a day for the remainder of the 14-day observation period. Body weights were recorded on days 1, 7 and 14. A gross necropsy was performed on all animals.

No treatment-related mortality, clinical signs, body weight changes, skin irritation or pathological findings were observed. The dermal LD_{50} of tebuconazole in rats was greater than 2000 mg/kg bw for males and females (Ohta, 1991c).

(d) Exposure by inhalation

Rats

Groups of 5–10 male and female young adult Bor:WISW (SPF Cpb) Wistar rats were exposed by nose only to tebuconazole (purity 97.1%) for 4 hours at nominal concentrations of 100, 250, 2500 or 5000 mg/m^3 (equivalent to analytical concentrations of 16, 49, 387 and 818 mg/m^3) for 4 hours. Another group was exposed to tebuconazole for 6 hours per day for 5 days at nominal concentrations of 100, 300 or 1000 mg/m^3 (equivalent to analytical concentrations of 24, 60 and 240 mg/m^3). The vehicle used in this study was ethanol:polyethylene glycol 400 (1:1). Rats were observed for 14 days. The animals were observed for clinical signs of toxicity several times during the first day and once daily thereafter. Individual body weights were recorded at day 1 (pretest), day 8 and day 15 of the test. Necropsy was performed on all animals.

The particle size was determined in the repeated-exposure study. The mass median aerodynamic diameter (MMAD) was 7.1, 5.0 and 4.6 μm for the 100, 300 and 1000 mg/m^3 dose groups, respectively.

There were no treatment-related effects on mortality or body weights in either study group. In the single-exposure study, reduced motility (lassitude) was observed in the 250, 2500 and 5000 mg/m^3 dose groups. In the repeated-dose study, nonspecific disturbed behaviour (lassitude) was observed in all groups. At necropsy, there were no indications of concentration-related grossly apparent lung

or organ damage. Under the study conditions utilized, it can be concluded that the inhalation median lethal concentration (LC_{50}) of tebuconazole following a single 4-hour exposure and five daily 6-hour exposures in rats was greater than 818 mg/m^3 and greater than 240 mg/m^3, respectively (Heimann & Pauluhn, 1983).

In a second study, groups of five male and five female young adult Bor:WISW (SPF Cpb) Wistar rats were exposed by nose only to tebuconazole (purity 96.2%) for 4 hours at a concentration of 0 or 4000 mg/m^3 (dust and aerosol) for 4 hours. The analytical concentrations were 371 mg/m^3 and 5093 mg/m^3 for aerosol and dust, respectively. The vehicle used in this study was ethanol:polyethylene glycol 400 (1:1). Control and vehicle control groups were also included in the study. Rats were observed for 14 days. The animals were observed for clinical signs of toxicity several times during the first day and daily thereafter. Individual body weights were recorded on the day of treatment and 3, 7 and 14 days following exposure. Necropsy was performed on all animals.

The particle size for the aerosol exposure was less than or equal to 5 µm. For the exposure via dust, approximately 8% of the particles were less than or equal to 5 µm.

No clinical signs of toxicity were observed in this study. No mortality occurred in the study. No treatment-related effects were observed in the group exposed to dust. In aerosol-treated groups, slight body weight loss was observed on day 3. The rats sacrificed at the end of the observation period did not provide any indications of grossly apparent lung or other organ damage. Under the study conditions utilized, it can be concluded that the inhalation LC_{50} of tebuconazole following a single 4-hour exposure was greater than 5093 mg/m^3 for dust exposure and greater than 371 mg/m^3 for aerosol exposure (Pauluhn, 1988).

In a third study, groups of five male and five female young adult Hsd Cpb:WU (SPF) Wistar rats were exposed by nose only to tebuconazole (purity 97.1%) for 4 hours at an aerosol concentration of 0 or 5000 mg/m^3 (target concentration). The gravimetrically measured concentration was 2118 mg/m^3. All animals were observed for mortality, signs of gross toxicity and behavioural changes at least once daily for 14 days after dosing. Body weights were recorded prior to administration and again on days 1, 3, 7 and 14. At the end of the scheduled period, the animals were killed and subjected to a gross examination.

Atmospheres generated had mean aerodynamic particle sizes of 2.76 µm, with a geometric standard deviation of 1.84 µm.

No mortality was observed in the study. All rats tolerated the exposure without specific signs but displayed an ungroomed hair-coat on post-exposure days 1–2. From post-exposure day 3 onwards, all rats appeared to be indistinguishable from the control. Transient body weight loss was observed in females. Statistically significant decreased body temperature was observed in treated groups compared with the controls; however, the extent of the change was too small to be of any toxicological significance. Necropsy findings were unremarkable between the control and the treated groups. Under the study conditions utilized, it can be concluded that the inhalation LC_{50} of tebuconazole in rats was greater than 2118 mg/m^3 (Pauluhn, 2007).

(e) Dermal irritation

In a study of primary dermal irritation, three young adult male HC:NZW rabbits were dermally exposed to 0.5 g of tebuconazole (purity 97.1%) mixed to a paste in water applied to 6 cm^2 of skin and fastened with an elastic adhesive tape. The test material was in contact with the skin for 4 hours. The other side of the back region was treated with water in a similar manner to serve as the control. The treated area was washed with water following a 4-hour exposure. Dermal irritation was scored according to the method of Draize after 30 minutes and then daily for 3 days.

No irritation was observed on any rabbits following application of tebuconazole. Under the conditions of this study, it is concluded that tebuconazole is non-irritating to the skin of rabbits (Heimann & Pauluhn, 1983).

In a second study of primary dermal irritation, three young adult male and female New Zealand White rabbits were dermally exposed to 0.5 g of tebuconazole (purity 96.6%) mixed to a paste in water applied to 6 cm^2 of skin and covered with gauze that was secured with hypoallergenic tape, then covered with a square of plastic and secured with adhesive bandage. Plastic covers were also placed on rabbits to prevent access to the exposure site. The application site was wiped with a paper towel following a 4-hour exposure period. Dermal irritation was scored at 0.5 hour, 1 hour and 1, 2 and 3 days using the method of Draize.

No irritation was observed in this study. No mortality or any adverse clinical signs were observed in the study. Under the conditions of this study, it is concluded that tebuconazole is non-irritating to the skin of rabbits (Sheets, 1988).

(f) Ocular irritation

In a study of primary eye irritation, 100 µl (approximately 50 mg) of tebucobazole (purity 97.1%) was instilled into the conjunctival sac of one eye of each of three male HC:NZW rabbits. The eyes were washed with saline 24 hours after the instillation. Irritation was scored by the method of Draize at 1 hour and 1, 2, 3, 7, 14 and 21 days after exposure.

There were no signs of corneal opacities or lesions involving the iris in any animal during the study. Reddening of conjunctiva was observed in one animal (average score 0.3; reversible at 48 hours). No chemosis was observed. Under the conditions of this study, it is concluded that tebuconazole is non-irritating to the eyes of rabbits (Heimann & Pauluhn, 1983).

In a second study of primary eye irritation, 100 mg of tebucobazole (purity 96.3%) was instilled into the conjunctival sac of one eye of each of three male and female New Zealand White rabbits. The treated eyes were not washed. Irritation was scored by the method of Draize at 1, 24, 48 and 72 hours after exposure.

There were no signs of corneal opacities or lesions involving the iris in any animal during the study. All six rabbits developed redness (grade 1), chemosis (grades 1 and 2) and discharge (grades 2 and 3) of the conjunctiva 1–24 hours after dosing. Chemosis and discharge had resolved in five animals by 72 hours after dosing and in all animals by day 7. Redness had resolved in four rabbits by 72 hours after dosing and in the one remaining animal by day 8. Under the conditions of this study, it is concluded that tebuconazole is mildly irritating to the eyes of rabbits (Eigenberg & Sheets, 1988).

(g) Dermal sensitization

In a study of dermal sensitization with tebuconazole (purity 97.1%), young male Pirbright White guinea-pigs were tested using the maximization method of Magnusson & Kligman. Twenty guinea-pigs were assigned to the test group, and another 20 served as the controls. In this study, the test concentrations chosen were 1% for intradermal induction and 25% for epidermal induction and challenge. The test material was formulated in 1% Cremophor EL in distilled water for intradermal induction and challenge. The topical induction was performed 1 week after the intradermal induction with 25% test substance (test group) (not irritating to the skin) and without test substance (control group) in a 48-hour exposure period. The challenge was performed 3 weeks after the intradermal induction with 25% test substance to the test and control group in a 24-hour exposure period.

Observations on skin effects, clinical signs and body weights were performed. The skin reactions were assessed after 24 and 48 hours.

Five animals in the control group and two animals in the treatment group died during the study. No treatment-related changes in body weights or any other clinical signs were observed. Evaluation revealed the same number of positive skin reactions on flanks in the test compound group and the control group. Under the study conditions utilized, it is concluded that tebuconazole is not a skin sensitizer in male guinea-pigs as determined by the method of Magnusson & Kligman (Heimann, 1983).

In a second study of dermal sensitization with tebuconazole (purity 96.9%), young female Hsd Poc:DH (SPF-bred) guinea-pigs were tested using the maximization method of Magnusson & Kligman. Twenty guinea-pigs were assigned to the test group, and another 10 served as the controls. In this study, the test concentrations chosen were 5% for intradermal induction, 50% for epidermal induction and 40% for challenge. These doses were selected based on the results of the range-finding study conducted on five animals. The test material was formulated in 2% Cremophor EL in saline for intradermal induction, topical induction and challenge. The topical induction was performed 1 week after the intradermal induction with 50% test substance (test group, in vehicle) and without test substance (control group, vehicle only) in a 48-hour exposure period. The challenge was performed 3 weeks after the intradermal induction with 40% test substance to the test and control groups in a 24-hour exposure period. Observations on skin effects, clinical signs and body weight were performed. The skin reactions were assessed after 24 and 48 hours. The sensitivity of the test was assessed using 2-mercaptobenzothiazole.

Five animals in the control group and two animals in the treatment group died during the study. The stability and homogeneity of the test material were confirmed analytically. No treatment-related mortality, changes in body weight or any other clinical signs were observed. No skin effects were observed in the treatment or control groups. Under the study conditions utilized, it is concluded that tebuconazole is not a skin sensitizer in female guinea-pigs as determined by the method of Magnusson & Kligman (Stropp, 1996).

In a third study of dermal sensitization with tebuconazole (purity 97.4%), young adult male DHPW (SPF-bred) guinea-pigs were tested using the method of Buehler (closed-patch test). Three groups of guinea-pigs were randomly established (test group and two control groups consisting of 12 animals each). For the main study, the concentration of the test substance was 25% by weight in 2% Cremophor in sterile water for three weekly induction and challenge exposures. The test concentrations were selected based on the results of the pilot study. For the dermal induction, animals were dermally treated with patches containing 25% test substance formulation (hypoallergenic dressing loaded with the test substance formulation) 3 times at intervals of 7 days. This was the highest usable concentration. After 6 hours of exposure, the patches were removed and the skin was visually assessed. The animals from the control group were exposed to hypoallergenic patches moistened with physiological saline solution. The first challenge was performed 5 weeks after the dermal induction, and patches containing 25% test substance formulation were applied to animals in the control and test groups. Control patches were also applied to the test group. After 6 hours of exposure, the patches were removed. The skin reactions were assessed at 48 and 72 hours after patch removal. The animals were observed for clinical signs at least once daily throughout the entire study period. The body weights of the animals were recorded before initiating the study and weekly thereafter, as well as at the end of the study.

No treatment-related mortality, changes in body weight or any other clinical signs were observed. No skin effects were observed in the treatment or control groups. The second challenge

was not performed because of lack of a response following the first challenge. Dinitrochlorobenzene (DNCB) was used as a positive control, which produced skin reactions as expected. Under the study conditions utilized, it is concluded that tebuconazole is not a skin sensitizer in male guinea-pigs as determined by the method of Buehler (Heimann, 1987).

In a fourth study of dermal sensitization with tebuconazole (purity 94.6%), young adult male Dunkin-Hartley guinea-pigs were tested using the method of Buehler (closed-patch test). Four groups of guinea-pigs were randomly established: a test group (15 animals), a control group (5 animals), a DNCB positive control group (5 animals) and a DNCB control group (5 animals). A dose range–finding study was performed to estimate doses for the induction (highest dose that causes mild irritation) and first challenge (highest non-irritating dose). Animals were dermally treated with patches containing 0.4 g test substance formulation (moistened with deionized water) 3 times at intervals of 7 days. After 6 hours of exposure, the patches were removed and the skin was visually assessed. The animals from the control group were exposed to patches moistened with deionized water. The challenge was performed 4 weeks after the dermal induction, and patches containing 0.4 g test substance formulation were applied to animals in the control and test groups. Control patches were also applied to the test group. DNCB test and control groups were included as positive and non-induced controls. After 6 hours of exposure, the patches were removed. The skin reactions were assessed at 48 and 72 hours after the patch removal. The animals were observed for clinical signs at least once daily throughout the entire study period. The body weights of the animals were recorded before initiating the study and at the end of the study.

No treatment-related mortality, changes in body weights or any other clinical signs were observed in the test or DNCB-treated groups. No skin effects were observed in the treatment or control groups. The DNCB test group showed an average dermal score of 1.0/0.9 after the third induction and 1.0/1.3 after the challenge. Under the study conditions utilized, it is concluded that tebuconazole is not a skin sensitizer in male guinea-pigs as determined by the method of Buehler (Sheets, 1990).

2.2 Short-term studies of toxicity

(a) Oral administration

Rats

In a non-GLP, 28-day dose range–finding study of toxicity, groups of 20 male and 20 female Bor:WISW (SPF Cpb) Wistar rats were given tebuconazole (purity 97.0%) at a dose of 0, 30, 100 or 300 mg/kg bw per day by gavage in Cremophor EL and deionized water (0.2 ml Cremophor diluted with water to 10 ml) for 4 weeks. Ten rats of each sex per dose served as a recovery group for 4 weeks. Rats were observed for clinical signs of toxicity daily. All animals were weighed at the beginning of each week of the study and prior to necropsy. Blood, liver tissue and urine samples were evaluated at the end of the 28-day treatment period and at the end of the recovery period (five rats of each sex per dose). All animals were necropsied, and selected organs were weighed. A complete macroscopic and microscopic examination was performed.

Mild lethargy was observed in a few animals of the 300 mg/kg bw per day dose group. One control female and one high-dose female were found dead on days 19 and 21 of the treatment. The cause of death was not established for either rat. Statistically significant decreases in body weights or body weight gains were observed for males and females of the high-dose group (weeks 1–4) during the treatment period. Doses of 100 and 300 mg/kg bw per day were associated with decreased haemoglobin concentration and haematocrit values. In females at 300 mg/kg bw per day, the leukocyte count was increased. All haematological parameters had normalized after the recovery period.

Clinical chemistry revealed slight (not statistically significant) increases in the activities of glutamate–oxalate transaminase (aspartate aminotransferase [AST]) and glutamate–pyruvate transaminase (alanine aminotransferase [ALT]) in males at 300 mg/kg bw per day and marked increases in liver enzyme activities (glutamate–oxalate and glutamate–pyruvate transaminases and alkaline phosphatase) in females at the same dose. Treatment at 100 and 300 mg/kg bw per day induced the microsomal enzyme system, and the activities of *N*- and *O*-demethylases and cytochrome P450 and the triglyceride concentration in liver were increased. All these changes were reversible. Urinalyses revealed no abnormal findings. At 100 and 300 mg/kg bw per day, absolute and relative weights of the liver and spleen were increased in animals of both sexes, and the absolute weight of the kidney was increased in females. Histopathological findings at 300 mg/kg bw per day consisted of fatty changes in the liver and bile duct proliferation in females; enlargement of the centrilobular hepatocytes was found in male rats. Histopathological changes were also found in the adrenal cortex, consisting of proliferated endothelial cells and an increased incidence of fat vacuoles. Sclerosis of the red pulp of the spleen, associated with sideropenia, was observed in males at 300 mg/kg bw per day; sideropenia was also found in females at 100 mg/kg bw per day.

The NOAEL in the 28-day gavage study in rats was 30 mg/kg bw per day on the basis of changes in haematological and clinical chemical parameters and organ weights seen at the lowest-observed-adverse-effect level (LOAEL) of 100 mg/kg bw per day and above (Heimann & Kaliner, 1984).

In an oral study of toxicity in rats, tebuconazole (purity 93.4%; 4.8% symmetrical isomer) was administered for 13 weeks to Bor:WISW Wistar rats (10 of each sex per dose) in the diet at a dose level of 0, 100, 400 or 1600 parts per million (ppm) (equal to 0, 8.6, 34.8 and 171.7 mg/kg bw per day for males and 0, 10.8, 46.5 and 235.2 mg/kg bw per day for females, respectively). Diets were analysed for homogeneity, stability and concentrations. The analytical data indicated that the mixing procedure was adequate (pilot study) and that the variation between nominal and actual dosage to the animals was acceptable (within 10% of the nominal). Animals were inspected at least twice daily (once daily on weekends) for signs of morbidity and mortality; detailed physical examinations were performed weekly. Body weights, feed consumption and water consumption were recorded at weekly intervals. Ophthalmological examinations were performed on 10 males and 10 females in the control and 1600 ppm dose groups at week 4 and prior to termination. Blood was collected at 1 and 3 months for haematology and clinical analyses from five rats of each sex per group (1 month) and all survivors at 3 months. Urine was analysed at week 4 and at termination. At study termination, all animals were subjected to a detailed gross pathological examination. Organs were collected, weighed and prepared for histopathology.

The treatment had no effect on appearance, behaviour or the findings of haematological examinations or urinalyses. Feed intake was increased in animals of each sex at 1600 ppm. One male in the control group died at week 4, and a second control group male died at week 12; deaths were reported to be associated with blood sampling. In the high-dose group, one male died during the first week of dosing, and one female died during week 4; the authors concluded that both deaths were probably due to haemorrhagic diathesis and considered them to be compound related. Retardation of body weight gain was observed at 400 ppm in females during the first 6 weeks and at 1600 ppm in animals of both sexes. There were no eye abnormalities at the 4-week examination. The authors stated that there was no indication of substance-induced damage to the eyes in the 1600 ppm dose group; however, one high-dose male exhibited corneal erosion, and one high-dose female had a right corneal lens cataract. Few of the changes in clinical chemistry showed a dose-related or time-consistent pattern, and all changes were regarded as toxicologically insignificant. An increase in urea concentration at 1600 ppm and a decrease in triglyceride concentration in animals at 400 ppm and above were seen only after the first 4 weeks and not at the end of the study. Pronounced increases in *N*-demethylase

activity and cytochrome P450 content were found in male animals at 1600 ppm, and increased liver weights were seen in females at this dose. Histopathological examination revealed an increased incidence of intraplasmatic vacuoles in the cells of the zona fasciculata of the adrenals (probably lipid accumulation) in some females at 400 ppm and in all females at 1600 ppm. This effect was less pronounced in males because of a higher background incidence of adrenal vacuole formation in control animals.

The NOAEL in this 90-day dietary toxicity study in rats was 100 ppm, equal to 10.8 mg/kg bw per day, on the basis of retardation of body weight gain and histopathological changes in the adrenals at higher doses seen in females at the LOAEL of 400 ppm, equal to 46.5 mg/kg bw per day (Bomhard & Schilde, 1986).

Dogs

In a 90-day study of toxicity, groups of four male and four female Beagle dogs were given diets containing tebuconazole (purity 93.4%; 4.8% symmetric isomer) at a dose of 0, 200, 1000 or 5000 ppm (equal to 0, 8.5, 41.0 and 212 mg/kg bw per day for males and females, combined) for 13 weeks. The dietary levels were calculated by the formula (compound intake per animal per day)/[(body weight week −1 + body weight week 13)/2]. Test diets were prepared weekly. Homogeneity and stability of the diets were assessed at regular intervals. The test diets were homogeneous (97–104%) and were stable for 14 days. Animals were inspected for signs of morbidity and mortality several times daily. Body temperatures were measured, and reflex tests (pupil reaction, corneal reflex, patellar tendon reflex, stretch, bending and righting reflex) were conducted prior to study initiation and during study weeks 3, 7 and 13. Body weights were recorded weekly. Feed consumption was calculated on a weekly basis. Ophthalmological examinations were performed prior to study initiation and during study weeks 3, 7, 10 and 12; mid- and high-dose dogs were also examined during study week 14. Haematology, clinical chemistry and urinalysis were conducted prior to initiation of the study and at weeks 3, 7 and 13. At the end of the study, a complete gross postmortem was done. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

One dog at 5000 ppm was found dead after the first dose, with no previous clinical signs. All remaining dogs survived until the scheduled terminal sacrifice. Body temperature, pulse rates and reflexes were similar in dosed and concurrent control dogs. At 1000 ppm and above, the mean body weight gain was retarded from week 7 to study termination. Mean body weight gains calculated over 13 weeks were decreased in mid- and high-dose males (25% and 46%, respectively) and females (19% and 48%, respectively) compared with controls. Body weights and body weight gains of low-dose animals were similar to those of concurrent controls. Most of the animals' feed consumption at 5000 ppm was repeatedly incomplete, as the dogs did not eat all the feed served to them. A few dogs at 1000 ppm and most of those at 5000 ppm had a deteriorated nutritional status. Ophthalmic examination revealed lens opacities in all animals at 5000 ppm, which first appeared after 7 weeks of treatment. These results were confirmed by histopathology, where morphological degeneration was observed. Anisocytosis and a change in erythrocyte morphology were found at study termination and were accompanied by a histological increase in siderosis of the liver and spleen in high-dose animals. Increased platelet counts and increased spleen weights were also exhibited in these animals. Effects on the liver were indicated by increased alkaline phosphatase, decreased albumin, increased globulin and increased cytochrome P450 activity at the high dose, as well as a dose-related increase in *N*-demethylase activity. The *N*-demethylase activity in the liver had increased slightly by the end of the study in animals at 1000 ppm. No treatment-related effects on triglyceride, ALT or AST levels were observed. Urinalyses revealed no treatment-related effects. Changes in organ weights did not follow a consistent pattern, except that an increase in spleen weights was seen in animals of both sexes at 5000 ppm. The increased level of iron pigment accumulation in conjunction with mechanisms for

adapting to the increased metabolic rate are considered to be the reasons for the higher mean absolute and relative spleen weights in the highest-dose group. Histopathological examination confirmed lens degeneration, indicating the induction of cataracts in animals at 5000 ppm. Other histopathological alterations observed in this group included slightly increased accumulation of ferriferous pigments in Kupffer cells of the liver and of siderocytes in spleen.

The NOAEL was 200 ppm, equal to 8.5 mg/kg bw per day, on the basis of reduced body weight gain and feed consumption and liver enzyme induction at 1000 ppm, equal to 41 mg/kg bw per day, and higher. The NOAEL for cataract induction was 1000 ppm, equal to 41 mg/kg bw per day (von Keutz & Schilde, 1987a).

In a 1-year study of oral toxicity, groups of four male and four female Beagle dogs were given diets containing tebuconazole (purity 96.9%) at a dietary concentration of 0, 40, 200 or 1000 ppm (weeks 1–39) and 2000 ppm (weeks 40–52), equal to 0, 1.5, 7.5 and 47 mg/kg bw per day in males and females combined, for 53 weeks. Diets were prepared at weekly intervals. Homogeneity and stability of the diets and dietary concentrations were confirmed analytically. The homogeneity and stability of diets were within the acceptable range. The dogs were inspected several times a day for mortality, moribundity and clinical signs. Body weights were recorded weekly. Feed consumption was measured daily for each animal. Ophthalmological examination was performed on all animals 2 weeks prior to study initiation and at weeks 13, 26, 32, 46 (controls and high dose) and 52. Blood was collected from all animals 2 weeks before treatment and at weeks 6, 13, 26, 39, 46 (controls and high dose) and 52 for haematology and clinical chemistry analysis. Urinalysis was conducted on all animals 2 weeks prior to the initiation of the study and at weeks 6, 13, 26, 39, 46 (controls and high dose) and 52. At the end of the study, a complete gross postmortem examination was done. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically. Liver enzyme activities and protein electrophoresis were evaluated.

No treatment-related effects were observed on survival rate, appearance, behaviour, organ weights, haematological examination, urinalyses, body temperature, pulse rates, reflexes, feed and water consumption or body weight gain. Ophthalmic examination revealed lens opacities in two dogs at 200 ppm and one at 1000 ppm; the opacities appeared between weeks 26 and 32 and were of the same intensity at all subsequent examinations. In the single dog at 1000 ppm that showed this ocular change, corneal opacity was also found, which persisted until the end of the study, whereas the lens opacities disappeared after week 32 of treatment. The lack of a dose–response relationship with regard to lens opacities does not preclude an association with treatment but may be due to the small number of animals in the group and differences in individual sensitivities. None of the other animals in this group had lens stars (physiological structures found occasionally in juvenile dogs); in single animals, faint lens stars were already present before treatment started but did not become more pronounced with the treatment. Incipient lens stars observed in animals at 40 and 200 ppm also remained stable, and most disappeared before the end of the treatment period. Clinical observation did not reveal impairment in any animal's vision. The findings of the haematological examination did not reveal any damage to the red blood cells. Nevertheless, the histopathological examination at the end of the study detected a slightly increased siderin level in the spleen in five of eight animals at 1000/2000 ppm. The incidence of this finding may point to an increased rate of breakdown of the red blood cells, which was so marginal, however, that it was not apparent from the haematological data. Clinical chemistry analyses revealed slight, dose-related changes in the activity of alkaline phosphatase: whereas the age-dependent reduction in activity was similar in control animals and in those at 40 and 200 ppm, the mean activity in animals at 1000/2000 ppm indicated slight induction, resulting in a retardation in the physiological fall in alkaline phosphatase activity. The activity of *N*-demethylase and the triglyceride content of the liver were slightly increased in animals at 1000/2000 ppm. Most of the gross pathological findings, such as a dose-related increase in the incidence of livers with

marked lobulation in animals treated with 200 ppm and above, were not correlated with histopathological alterations. Histopathological findings included intracytoplasmic vacuoles in cells of the zona fasciculata of the adrenals in animals at 200 and 1000/2000 ppm and slight siderosis in the spleen in most animals at 1000/2000 ppm.

The NOAEL was 40 ppm, equal to 1.5 mg/kg bw per day, on the basis of histopathological changes in the adrenals seen at the LOAEL of 200 ppm, equal to 7.5 mg/kg bw per day (von Keutz & Schilde, 1987b).

In a second 1-year study of oral toxicity, groups of four male and four female Beagle dogs were given diets containing tebuconazole (purity 96.0%) at a dietary concentration of 0, 100 or 150 ppm (equal to 0, 2.96 and 4.39 mg/kg bw per day for males and 0, 2.94 and 4.45 mg/kg bw per day for females, respectively) for 53 weeks. Diets were prepared every 2–4 weeks. Homogeneity and stability of the diets and dietary concentrations were confirmed analytically. The homogeneity and stability of the diets were within the acceptable range. The dogs were inspected daily for mortality, moribundity and clinical signs. Body weights were recorded weekly. Feed consumption was measured daily for each animal. Ophthalmological examination was performed on each dog at 3 and 6 months and prior to terminal sacrifice. Blood and urine were collected at 3 and 6 months and prior to terminal sacrifice. At the end of the study, a complete gross postmortem examination was done. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

The treatment did not affect mortality, body weight gain, feed consumption, biochemical, haematological or urinary parameters, ophthalmoscopic findings (including cataracts), gross pathological appearance or organ weights. One control female stopped eating during the 8th week of the study and showed a body temperature of 40.6 °C and elevated white blood cell count. The animal was isolated in a separate room and sacrificed during the 10th week of the study. Another female from the same shipment of animals replaced this female on day 70. The only histopathological alteration was slight hypertrophy of adrenal zona fasciculata cells in all animals at 150 ppm; only one control animal had similar changes. The enlargement was accompanied by an increased incidence of large fatty vacuoles.

The NOAEL was 100 ppm, equal to 2.94 mg/kg bw per day, on the basis of histopathological alterations in the adrenals seen at the LOAEL of 150 ppm, equal to 4.39 mg/kg bw per day. The NOAEL for cataract induction was 150 ppm, equal to 4.39 mg/kg bw per day (Porter et al., 1989, 1993).

(b) Dermal application

Rabbits

In a non-GLP repeated-dose dermal toxicity study, groups of six New Zealand White rabbits of each sex per dose (abraded and intact skin) received a dermal application of tebuconazole (purity 97.1%) formulated in Cremophor EL in Lewatit water at a dose of 0, 50 or 250 mg/kg bw per day, 6 hours per day, 5 days per week, for 3 weeks. The test material was applied to previously shaved skin (abraded and intact skin). The appropriate volume (0.5 ml/kg bw) of tebuconazole suspension was applied to the skin. The treated area was left uncovered. After 6 hours, the treated sites were rinsed with soap and water. Animals were examined for mortality and signs of toxicity daily except on weekends and holidays. Body weights were recorded weekly. The skin irritation was scored according to the method of Draize. Blood and urine samples were collected from all rabbits before the start of the study and at study termination. At termination, all animals were examined externally and internally for macroscopic changes. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

There were no treatment-related deaths or clinical signs and no biologically significant treatment-related effects on body weight, body weight gain or feed consumption. Abraded skin showed slight redness for the first 3 days; however, this was also present before the treatment. No treatment-related effects were observed on the urinalysis, haematological or clinical chemistry parameters evaluated. No induction of microsomal enzymes was observed. Slightly distended lungs, swollen spleens, livers with slight lobulation and patchy kidneys were observed upon macroscopic examination; however, these observations were not corroborated by histopathological findings. Slightly higher absolute and relative spleen and kidney weights were observed in males at 250 mg/kg bw per day. This was a result of two animals having high organ weights and an infestation. Histopathological examination did not reveal treatment-related abnormalities.

The NOAEL in this 21-day dermal toxicity study in rabbits was 250 mg/kg bw per day. A LOAEL was not observed (Heimann & Schilde, 1984).

In a second repeated-dose dermal toxicity study, groups of five New Zealand White rabbits of each sex per dose received a dermal application of tebuconazole (purity 97.4%) formulated in Cremophor EL (2% by volume) in demineralized water at a dose of 0 or 1000 mg/kg bw per day, 6 hours per day, 5 days per week, for 3 weeks. The test material was applied to previously shaved skin (11 cm × 12 cm). The treated area was covered with a non-occlusive dressing for 6 hours. After 6 hours, the treated sites were rinsed with soap and water. Animals were examined for mortality and signs of toxicity daily except on weekends and holidays. Body weights were recorded weekly. The skin irritation was scored according to the Draize method. Blood and urine samples were collected from all rabbits before the start of the study and at study termination. At termination, all animals were examined externally and internally for macroscopic changes. Selected organs were weighed, and a comprehensive range of tissues was preserved and examined microscopically.

No treatment-related effects were observed on clinical signs of toxicity, mortality, body weight, body weight gain, feed consumption, urinary, haematological and clinical chemistry parameters and gross macroscopy. No treatment-related inductions of liver enzymes (microsomal enzymes) were observed in this study. Slightly increased liver weights (absolute and relative) were observed in the high-dose females; however, they were not considered to be an adverse effect, as no corroborative clinical or histological findings were noted. The histopathological examination of the treated skin revealed minimal thickening of the epidermis, in comparison with the untreated skin, in two males and four females. In the case of four females, minimal hyperkeratosis was also noted. The alterations are presumably attributable to mechanical irritation of the skin, as the test compound formulation was a suspension of viscous consistency or slurry, and the pressure of the occlusive dressing presumably resulted in skin friction.

The NOAEL in this 21-day dermal toxicity study in rabbits was 1000 mg/kg bw per day. A LOAEL was not established (Heimann & Schilde, 1988).

(c) Exposure by inhalation

Rat

In a 21-day study of inhalation toxicity after repeated doses, groups of 10 male and 10 female Wistar rats (Bor:WISW) were exposed to nominal aerosol concentrations of tebuconazole (purity 96.2%) at 0, 5, 50 or 500 mg/m^3 in polyethylene glycol by head and nose exposure for 6 hours per day, 5 days per week, for 3 weeks (15 days). The analytical concentrations were 0, 1.2, 11 and 156 mg/m^3 (0, 0.0012, 0.011 and 0.156 mg/l). About 90% of the particle mass had an aerodynamic diameter of less than 5 μm. During the 3 weeks of exposure, body weights, clinical signs and mortality were recorded. At the end of the study, clinical chemistry, haematology, urinalysis, gross pathological and histopathological examinations were performed.

The treatment had no effect on mortality rate, body weight gain, haematological or clinical chemical parameters or organ weights; urinalysis showed no abnormal findings. Rats treated with 156 mg/m^3 had piloerection after each exposure. Mixed-function oxidases in the liver were induced. At the end of the study, *N*-demethylase activity in the liver was increased in animals of both sexes at the highest dose. Males in this group also had increased *O*-demethylase activity. No treatment-related gross pathological or histopathological alterations were observed. The changes in enzyme levels were considered to be an adaptive response and not adverse.

The no-observed-adverse-effect concentration (NOAEC) was greater than or equal to 156 mg/m^3 ($\geq$ 0.156 mg/l). The study author established a no-observed-effect concentration (NOEC) of 0.11 mg/m^3 on the basis of liver enzyme induction (Pauluhn, 1985, 1987).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Two combined chronic toxicity and carcinogenicity studies were performed using mice (Bomhard & Ramm, 1988; Bomhard, 1991; Sander, 1992).

In the first study (Bomhard & Ramm, 1988), groups of 50 male and 50 female NMRI mice were given tebuconazole at a concentration of 0, 20, 60 or 180 ppm (equal to 0. 5.9, 18 and 53 mg/kg bw per day for males and 0, 9.0, 26 and 81 mg/kg bw per day for females) for 21 months in the diet. Groups of 10 similarly treated male and female animals (satellite groups) were sacrificed after a period of 12 months. The tebuconazole doses were based on the results of two previous feeding studies lasting 4 and 8 weeks, respectively, in NMRI mice of the same strain (Ramm & Karbe, 1986; Ramm & Schilde, 1986). The animals were inspected for any clinical signs of toxicity. Individual body weights were recorded weekly for the first 13 weeks and once every 2 weeks thereafter. Feed and water intakes were determined groupwise once a week from the start of the study up to and including week 13 and every 2 weeks from week 15. Haematology, clinical chemistry, urinalysis, measurement of organ weights, and macroscopic and microscopic examinations were conducted after 12 and 21 months. Animals that died spontaneously during the study or were moribund and sacrificed were dissected and their organs/tissues subjected to detailed gross pathological examination.

Clinical signs, feed and water intakes, growth and mortality were unaffected in both sexes of all treated groups, including 180 ppm. In the haematological examination, erythrocytes, haemoglobin and haematocrit concentration were decreased in females of the satellite group at 180 ppm in 12 months, but not at 21 months. At the same dose, the male mice showed significantly lower erythrocyte counts at 12 and 21 months (Table 9). In clinical chemistry, the total bilirubin concentration was significantly increased in females at 180 ppm at 12 months and at 20 ppm and higher at 21 months. Any changes indicating hepatotoxicity were not detected in the liver from animals of both sexes at 20 or 60 ppm at 12 months. Slight increases in total bilirubin at 20 and 60 ppm at 21 months in females are considered to be incidental findings, because no changes were observed in these animals (20 and 60 ppm dose groups) at 12 months, and the increases may have been statistically significant as a result of the unusually low value in the control group at 21 months. The plasma cholesterol levels were significantly lower in males and females in the 180 ppm satellite group and showed a lower tendency in males at 21 months (Table 9).

In both sexes at 180 ppm, absolute and relative liver weights were increased compared with the controls at 12 and 21 months. However, the variations were statistically significant only for the relative liver weights in males at 21 months. The means in this dose group for females were greatly affected by three extreme figures at the end of the study, whereas values for the other animals were largely in the range for control females.

Table 9. Clinical chemistry and haematological examinations in the carcinogenicity study of tebuconazole in mice

Parameter	Sacrifice time	Sex	Dietary concentration (ppm)			
			0	20	60	180
Bilirubin (µmol/l)	12 months	Male	3.9	3.8	3.8	3.9
		Female	2.7	3.2	3.3	3.7**
	21 months	Male	3.3	3.2	3.3	3.4
		Female	2.2	2.6*	3.4**	3.6**
Cholesterol (mmol/l)	12 months	Male	4.66	4.38	4.36	3.61*
		Female	3.86	4.32	3.43	2.44**
	21 months	Male	4.31	3.93	3.93	3.27
		Female	3.57	3.53	2.97*	3.46
Erythrocyte count (10^{12}/l)	12 months	Male	8.30	8.57	8.41	8.73*
		Female	8.30	8.07	8.42	7.88*
	21 months	Male	8.20	8.55	8.15	7.65**
		Female	7.87	7.26*	7.13	7.56
Haemoglobin (g/l)	12 months	Male	137	140	136	140
		Female	142	140	141	133**
	21 months	Male	144	154	145	139
		Female	143	135	131	141
Haematocrit (l/l)	12 months	Male	0.46	0.48	0.47	0.49
		Female	0.47	0.46	0.47	0.45*
	21 months	Male	0.428	0.432	0.426	0.410
		Female	0.409	0.390	0.393	0.392

From Bomhard & Ramm (1988)
* $P \leq 0.05$; ** $P \leq 0.01$

Incidences of periportal vacuolization of the liver were marginally increased in females. The incidences of periportal vacuolization in females were 1/50 for all treated dose groups, whereas an incidence of 1/50 was observed only at the high dose (180 ppm) in males (Table 10). In addition, incidences of centrilobular fine vacuolization were higher in males of the 60 and 180 ppm dose groups than in the control group. Further examination showed that the vacuoles in the liver were identified as lipids. In this study, there were no treatment-related effects in animals of the satellite and the main groups.

The LOAEL was 60 ppm (equal to 18 mg/kg bw per day) based on the increased incidence of centrilobular fine vacuolization in the liver of males. The NOAEL was 20 ppm (equal to 5.9 mg/kg bw per day). There was no evidence for carcinogenic potential, but the effects on the liver at the LOAEL and above were not very marked in intensity, posing a question as to whether a maximum tolerated dose (MTD) had been reached in this study.

The objective of the second study (Bomhard, 1991) was to examine the carcinogenic potential of tebuconazole at doses higher than those used in the first study. Groups of 50 male and 50 female NMRI mice were administered tebuconazole at a concentration of 0, 500 or 1500 ppm (equal to 0, 85 and 279 mg/kg bw per day in males and 0, 103 and 357 mg/kg bw per day in females, respectively) in their diet for 21 months. Groups of 10 male and 10 female animals (satellite groups) were analogously treated and sacrificed after a study duration of 12 months for haematological and clinical examinations.

Table 10. Incidences of periportal and centrilobular fine vacuolization of hepatocytes in the carcinogenicity study of tebuconazole in mice

Parameter	Incidence of vacuolization of hepatocytes[a]							
	Dietary concentration (ppm)							
	0		20		60		180	
	M	F	M	F	M	F	M	F
Focal periportal vacuolization, minimal	0/50	0/50	0/50	0/50	1/50	1/50	8/50	2/50
Periportal vacuolization	0/50	0/50	0/50	1/50	0/50	1/50	1/50	1/50
Focal centrilobular fine vacuolization, minimal	0/50	1/50	2/50	0/50	5/50	1/50	2/50	0/50
Centrilobular fine vacuolization, minimal	0/50	2/50	1/50	1/50	4/50	1/50	8/50	8/50
Centrilobular fine vacuolization, moderate	0/50	0/50	0/50	0/50	1/50	0/50	4/50	0/50

From Bomhard & Ramm (1988)
F, females; M, males
[a] Number of animals affected/total number of animals.

Table 11. Results of the combined chronic toxicity/carcinogenicity study of tebuconazole in mice (main groups)

Parameter	Dietary concentration (ppm)						Dose–response	
	0		500		1500			
	M	F	M	F	M	F	M	F
Number of animals examined	*50*	*50*	*50*	*50*	*50*	*50*	*n/a*	*n/a*
Mortality[a]	20/50	30/50	18/50	32/50	23/50	32/50	−	−
Body weight, week 91 (g)	46.5	41.3	44.5	39.3	46.6	44.1*	−	−
Feed consumption (g/kg bw per day)	146.6	188.8	169.8	206.1	186.0	237.7	+	+
Water consumption (g/kg bw per day)	208.3	314.0	213.2	297.7	204.1	279.4	−	−

From Bomhard (1991)
F, females; M, males; n/a, not applicable; * $P \leq 0.05$
[a] Number of animals that died/total number of animals.

Clinical signs, water intakes and mortality were unaffected in both treatment groups. The incidence of animals exhibiting increases in abdominal girth was elevated at the 1500 ppm level. Growth retardation was observed in males at 500 and 1500 ppm in a dose-related manner. At 1500 ppm, the differences in body weights were slightly greater than 10% at times, whereas they were around 6–7% at most at 500 ppm. Body weight was reduced in females at 1500 ppm, and deviations from the control group were less than 10%. Body weights at 21 months were comparable to those of the controls (Table 11).

The haematological examination did not provide any indication of damage to the blood in females at 500 ppm. Marginal effects on the haematocrit value and the mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were observed in the males at 500 and 1500 ppm, but these changes were not considered toxicologically significant because they were minimal and no clear dose–response relationship was observed. The erythrocyte count, haemoglobin content, haematocrit value and thromboplastin time were generally reduced at 1500 ppm, whereas the thrombocyte and leukocyte counts were elevated, in some cases to a marked extent (Table 12).

Table 12. Major changes in clinical chemistry and haematological examinations in the carcinogenicity study of tebuconazole in mice

Clinical chemistry and haematology	Sacrifice time	Sex	Dietary concentration (ppm)		
			0	500	1500
ALAT (U/l)	12 months	Male	38.0	53.2*	236.3**
		Female	31.7	51.7*	272.5**
	21 months	Male	74.9	123.1**	480.8**
		Female	39.2	64.9*	419.4**
ASAT (U/l)	12 months	Male	31.9	37.5	121.3**
		Female	38.3	47.2*	144.0**
	21 months	Male	46.1	60.7	251.8**
		Female	36.9	59.0**	303.8**
Alkaline phosphatase (U/l)	12 months	Male	74	117**	181**
		Female	174	212	292
	21 months	Male	126	156	531**
		Female	182	328	517**
Cholesterol (mmol/l)	12 months	Male	3.71	1.99**	1.66**
		Female	2.84	1.48**	1.92*
	21 months	Male	3.88	1.57**	4.55
		Female	3.76	2.25**	3.59
Bilirubin (µmol/l)	12 months	Male	1.8	1.3**	1.3**
		Female	2.2	2.1	1.9
	21 months	Male	2.1	1.6**	5.0
		Female	2.7	2.1*	4.9
Inorganic phosphate (mmol/l)	12 months	Male	1.90	1.73*	2.14*
		Female	1.63	1.69	1.94**
	21 months	Male	1.60	1.75**	2.06**
		Female	1.62	1.64	1.93**
Leukocyte count (10^9/l)	12 months	Male	5.2	5.6	10.2**
		Female	3.9	3.7	10.7**
	21 months	Male	6.6	5.0*	9.8*
		Female	7.6	4.3	9.5
Erythrocyte count (10^{12}/l)	12 months	Male	8.90	9.11	8.51
		Female	8.40	8.99	7.49
	21 months	Male	9.13	8.25	7.95*
		Female	8.36	8.63	7.51
Haemoglobin (g/l)	12 months	Male	142	144	129**
		Female	139	148**	132
	21 months	Male	143	150	117**
		Female	132	131	125
Haematocrit (l/l)	12 months	Male	0.435	0.414	0.373**
		Female	0.422	0.432	0.381*
	21 months	Male	0.427	0.375**	0.375**
		Female	0.407	0.401	0.380

Table 12 (continued)

Clinical chemistry and haematology	Sacrifice time	Sex	Dietary concentration (ppm)		
			0	500	1500
MCHC (g/l erythrocytes)	12 months	Male	327	348**	347**
		Female	331	344	347*
	21 months	Male	334	402**	313**
		Female	324	328	328
MCH (pg)	12 months	Male	16.0	15.8	15.3
		Female	16.6	16.6	17.7
	21 months	Male	15.7	18.3**	14.6*
		Female	15.8	15.3	16.7
Thrombocyte count (10^9/l)	12 months	Male	1311	1323	1650**
		Female	1024	1274*	1284

From Bomhard (1991)
U, units; * $P \leq 0.05$; ** $P \leq 0.01$

The clinical laboratory tests, gross pathology, organ weights and histopathology afforded evidence for marked and dose-related hepatotoxicity in both treatment groups, with dose dependency (Tables 12 and 13). The main findings included a marked increase in the activities of ALT and AST, in some cases major enlargement of the liver, single-cell and focal necrosis, inflammation, bile duct hyperplasia and steatosis. The incidences of hepatocellular tumours (adenomas and carcinomas in males and carcinomas in females) were increased at 1500 ppm and were markedly above the range of spontaneous incidences observed in this mouse strain (Table 13). In addition, hepatocellular alteration and focal hyperplasia of hepatocytes, which are considered to be precancerous lesions, were also increased in males and females at 1500 ppm. The incidence of hepatocellular tumours was unaffected in both sexes at 500 ppm.

In addition, the incidences of histiocytic sarcoma at 0, 500 and 1500 ppm were 1/48, 2/49 and 3/48 in males and 1/47, 3/45 and 5/46 in females of the main groups, respectively. This tumour is not rare in aged mice. The review of historical data of nine studies (starting at 1982–1985) on NMRI mice from Bayer indicates that the incidence of this tumour in the 500 and 1500 ppm groups was incidental and not related to treatment. The histopathology of the interim necropsy of females at 1500 ppm showed an increase in the incidence of hyperkeratosis and acanthosis of the forestomach mucosa (Table 13). The incidence of these findings did not result in a treatment-related increase in the animals of the main groups. No evidence for carcinogenic effects of tebuconazole on other organs may be inferred from the incidence, type, location or distribution among the study groups of the neoplasms observed.

The rates of liver tumours in males and females were elevated to a highly significant extent at 1500 ppm and were markedly above the range of spontaneous incidences observed in this mouse strain. Especially in mice, liver tumours caused by a variety of chemical substances at hepatotoxic doses occur frequently, and it is thought that under these circumstances, elevated incidences of spontaneous, relatively frequent tumours in rodents have no relevance for humans if the exposure to humans lies in a non-toxic range. The relatively major effects, particularly those on the liver, represent unequivocal evidence that the MTD has been exceeded at both concentrations and underscore the correctness of dose selection in the first study (Bomhard & Ramm, 1988).

The LOAEL was 500 ppm (equal to 85 mg/kg bw per day), based on liver toxicity. The NOAEL for systemic toxicity was not established. The NOAEL for carcinogenicity effects was 500 ppm (equal to 85 mg/kg bw per day) based on the increased incidence of tumours seen at the LOAEL of 1500 ppm (equal to 279 mg/kg bw per day).

Table 13. Results of the combined chronic toxicity/carcinogenicity study in mice

Parameter	Incidence[a]						Dose–response	
	Dietary concentration (ppm)							
	0		500		1500			
	M	F	M	F	M	F	M	F
Liver								
Relative weight (mg/100 g)	5214	6060	6345**	6642	18 313**	21 141**	+	+
Absolute weight (mg)	2409	2524	2822**	2623	8522**	9405**	+	+
Gross pathology								
Enlarged	1/50	0/50	2/50	5/50	35/50***	32/50***	+	+
Irregular surface	1/50	3/50	0/50	3/50	30/50***	26/50***	+	+
Masses	6/50	1/50	3/50	1/50	13/50**	8/50*	+	+
Non-neoplastic changes								
Necrosis of single hepatocytes	3/47	0/47	11/48*	2/45	2/48	1/46	−	−
Focal hyperplasia of hepatocytes	6/47	1/47	2/48	0/45	23/48***	12/46***	+	+
Panacinar fine fatty vacuolation	0/47	1/47	14/48***	4/45	25/48***	19/46***	+	+
Centriacinar fatty vacuolation	1/47	3/47	1/48	13/45**	0/48	4/46	−	−
Periacinar hepatocytic hypertrophy	0/47	0/47	0/48	0/45	2/48	13/46***	−	+
Oval cell proliferation	0/47	0/47	0/48	0/45	23/48***	17/46***	+	+
Pigment-laden Kupffer cells	1/47	1/47	0/48	3/45	6/48	7/46*	+	+
Extramedullary haematopoiesis	0/47	5/47	2/48	1/45	7/48*	12/46	+	+
Hepatocellular alteration	0/47	0/47	2/48	0/45	3/48	7/46**	+	+
Focal hyperplasia of hepatocytes	6/47	1/47	2/48	0/45	23/48***	12/46***	+	+
Neoplastic changes								
Hepatocellular adenoma	3/47	0/47	2/48	0/45	17/48***	2/46	+[b]	−
Hepatocellular carcinoma	0/47	1/47	0/48	0/45	10/48***	12/46***	+[b]	+[b]
Stomach (main group)								
Hyperkeratosis and acanthosis	6/47	12/46	8/48	16/45	8/48	13/46	−	−
Stomach (interim sacrifice)								
Hyperkeratosis and acanthosis	1/10	2/10	2/10	6/10	6/10	8/10*	−	+
Adrenals								
Relative weight (mg/100 g)	24	28	21	33	23	34*	−	+
Absolute weight (mg)	11	12	10	13	11	15**	–	+

From Bomhard (1991)

F, female; M, male; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

[a] Number of animals affected/total number of animals.

[b] Not relevant for humans.

Rats

Groups of 50 male and 50 female Wistar rats were given tebuconazole at a concentration of 0, 100, 300 or 1000 ppm (equal to 0, 5.3, 15.9 and 55.0 mg/kg bw per day for males and 0, 7.4, 22.8 and 86.3 mg/kg bw per day for females, respectively) for 2 years in their diet (Bomhard & Ramm, 1988; Sander & Schilde, 1993 [review of the historical control data]). Ten similarly treated male and female animals were sacrificed after a study period of 12 months as satellite groups. The animals were inspected at least twice daily, and any clinical signs and special features were noted. Detailed

Table 14. Treatment-related effects on non-neoplastic lesions in the carcinogenicity study of tebuconazole in rats

Parameter	Incidence[a]							
	Dietary concentration (ppm)							
	0		100		300		1000	
	M	F	M	F	M	F	M	F
Liver								
Kupffer cell pigmentation	0/49	2/49	1/49	2/50	0/50	1/50	1/50	7/50
Spleen								
Increased haemosiderin	0/49	2/50	0/49	3/50	1/50	3/50	0/50	19/50
Adrenals								
Haemorrhagic degeneration in cortex	3/49	23/50	4/49	15/50	4/50	13/50	1/50	4/50

From Bomhard & Ramm (1988)
F, female; M, male
[a] Number of animals affected/total number of animals.

individual inspections took place once a week. Ophthalmological examinations were made at the start of the study, after 12 months and before the end of the study, covering groups of 10 males and 10 females in the control group and the 1000 ppm dose group. Individual body weights were recorded weekly for the first 13 weeks and once every 2 weeks thereafter. Feed and water intakes were determined groupwise once a week from the start of the study up to and including week 13 and every 2 weeks from week 15. Haematology, clinical chemistry and urinalysis were performed after 6, 12, 18 and 24 months from 10 animals per group. Animals that died spontaneously during the study or were moribund and sacrificed were dissected and their organs/tissues subjected to detailed gross pathological examination. After 12 and 24 months, all the survivors of the satellite groups and main groups, respectively, were sacrificed and autopsied, and their organs/tissues were subjected to detailed gross pathological and histopathological examinations.

The appearance, general behaviour and mortality were unaffected in the treated groups. In the 1000 ppm group, decreased water intake was noted in females, and growth of the males and females in this dose group was retarded. In the females at 1000 ppm, feed consumption was increased. The haematology, urinalysis and ophthalmological examination did not provide any indication of damage. In clinical chemistry, the females at 1000 ppm showed induction of microsomal enzyme systems. Absolute and relative weights of the liver, lungs and spleen were significantly increased in the females at 1000 ppm at interim sacrifice, but these increases were not observed at termination. Relative weights of the liver in the females at 300 ppm were also increased at the interim sacrifice. The absolute and relative weights of the adrenals were decreased in females in the 30 ppm and higher groups at termination. Gross pathological examination did not show any treatment-related changes. Histopathologically, treatment-related non-neoplastic lesions were detected in the liver, spleen and adrenals in females at 1000 ppm (Table 14). They were haemosiderin accumulation in the spleen (not confirmed by Berlin blue staining) and pigment deposits in the Kupffer star cells in the liver of females at 1000 ppm. The clearly reduced number of females with haemorrhagic degeneration of the adrenal cortex at 1000 ppm, which corresponded to the reduction of adrenal weights, was considered to be most likely a treatment-related functional effect, but not toxicologically significant.

With respect to neoplastic lesions, the incidence of C-cell adenomas in the thyroid showed very slight, but not statistically significant, increased tendency in all treated male rats compared with controls (Table 15). The incidence of C-cell hyperplasia, a precancerous lesion of C-cell adenoma,

Table 15. Thyroid C-cell tumours and C-cell hyperplasias in the carcinogenicity study of tebuconazole in rats

Parameter	Incidence[a]							
	Dietary concentration (ppm)							
	0		100		300		1000	
	M	F	M	F	M	F	M	F
C-cell hyperplasia	1/50	1/49	3/50	2/50	7/50	3/50	6/50	0/50
C-cell adenoma (b)	0/50	1/49	1/49	0/50	3/50	1/50	2/50	1/50
C-cell carcinoma (m)	0/50	0/49	1/49	0/50	0/50	0/50	1/50	0/50

From Bomhard & Ramm (1988)
(b), benign neoplasms; F, female; (m), malignant neoplasms; M, male
[a] Number of animals affected/total number of animals.

was also increased in the males in the two higher dose groups, but not significantly. There was, however, no clear dose–response relationship, and the incidences were within the range of spontaneously occurring thyroid C-cell tumours in old male Wistar rats (Bomhard, Karbe & Loeser, 1986). Furthermore, the histopathology data revealed no evidence of progression from adenoma to carcinoma. A review of historical data (Sander & Schilde, 1993) and data from the study provides strong evidence that the incidence and dose dependency of thyroid tumours observed in the study were not treatment related. In female rats, a higher frequency of endometrial adenocarcinoma was found in comparison with controls. These incidences were small and not dose related.

The LOAEL was 1000 ppm (equal to 55.0 mg/kg bw per day), based on body weight depression in both sexes and an increased incidence of pigment deposits in the Kupffer cells in the liver of females. The NOAEL was 300 ppm (equal to 15.9 mg/kg bw per day). Tebuconazole was not carcinogenic (Bomhard & Ramm, 1988).

2.4 Genotoxicity

Various in vitro and in vivo studies have been conducted on the genotoxicity of tebuconazole. The results are summarized in Table 16. No genotoxic activity was found in any study.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a two-generation study of reproductive toxicity, groups of 25 male and 25 female Wistar (Bor:WISW (SPF Cpb)) rats were given diets containing tebuconazole (purity 95.2%) at a concentration of 0, 100, 300 or 1000 ppm (equal to 0, 7.1, 21.6 and 72.3 mg/kg bw per day for males and 0, 9.1, 27.8 and 94.8 mg/kg bw per day for females, respectively). The rats were treated with active ingredient throughout the study, including mating period, gestation and pup lactation. Prepared diets were analysed for stability, homogeneity and concentrations before the start of the treatment and every 3 months thereafter; diets were stable and homogeneously distributed. All animals were examined twice daily for mortality and clinical signs and once daily during holidays and weekends. Body weights and feed consumption were monitored at weekly intervals. Parturition was observed and described. Litter size, sex distribution and malformations of pups were recorded. Sacrificed parental

Table 16. Results of studies of genotoxicity with tebuconazole

End-point	Test system	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Reverse mutation (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537	20.0–12 500 µg/plate ± metabolic activation in DMSO[a]	97.0	Negative	Herbold (1983a)
	S. typhimurium strains TA98, TA100, TA1535, TA1537 and TA1538	37.5–2400 µg/plate 39.5–450 µg/plate ± metabolic activation in DMSO[b]	96.6	Negative	Herbold (1983b)
	S. typhimurium strains TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2uvrA	15.6–500 µg/plate 31.3–1000 µg/plate without metabolic activation 156–5000 µg/plate with metabolic activation in DMSO	98.0	Negative	Ohta (1991d)
Forward mutation (CHO/HGPRT)	Chinese hamster ovary cell line HGPRT locus	80.0–100 µg/ml[c] without metabolic activation in DMSO 12.5–200 µg/ml with metabolic activation in DMSO	96.6	Negative	Lehn (1988)
Recombination repair capacity (rec-assay)	*Bacillus subtilis* H17 (rec^+) and M45 (rec^-)	0.3–20 µg/plate ± metabolic activation in DMSO	98.0	Negative	Ohta (1992)
Cytogenicity	Human lymphocytes	3.0–30.0 µg/ml without metabolic activation in DMSO 30.0–300 µg/ml with metabolic activation in DMSO	96.6	Negative	Herbold (1988a)
DNA polymerase repair capacity	*E. coli* (K12) p 3478 (pol^-) W 3110 (pol^+)	3.0–300.0 µg/ml without metabolic activation in DMSO 625–10 000 µg/ml with metabolic activation in DMSO	97.1	Negative	Herbold (1988b)
Unscheduled DNA synthesis	Rat hepatocytes	0.5–25.2 µg/ml	96.5	Negative	Cifone (1987)
Sister chromatid exchange	Chinese hamster ovary cells	4.0–30.0 µg/ml without metabolic activation in DMSO 15.0–120 µg/ml with metabolic activation in DMSO	96.5	Negative	Putman (1987)
In vivo					
Micronucleus formation	NMRI mouse bone marrow (5 males and 5 females per group)	200, 500 or 2000 mg/kg bw (single oral dose in 1% Cremophor)[d]	95.3	Negative	Herbold (1985)
Dominant lethal assay	NMRI male mice (50 males per group)	2000 mg/kg bw (single oral dose in 1% Cremophor)	93.5	Negative	Herbold (1986)

DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid

[a] Toxic at doses ≥ 500 µg/plate.

[b] Toxic at doses ≥ 60 µg/plate.

[c] Toxic at doses ≥ 75 µg/plate with and without metabolic activation.

[d] Doses reduced in second and third trials owing to inhibition of erythropoiesis by the test compound.

animals were necropsied. Organs or tissues with major macroscopic abnormalities were removed and processed for histopathology. Selected organs were weighed. Selected organs were also examined histopathologically.

At doses up to 1000 ppm, appearance, behaviour, general condition, mortality, fertility, insemination rate, gestation, duration of gestation, rate of stillbirths and male/female ratio were unaffected. No malformations were observed up to 1000 ppm. Mean body weights were consistently depressed in both male and female adult rats exposed to tebuconazole at 1000 ppm prior to mating, after mating, during lactation and following the lactation period in both the F_0 and F_{1B} parental generations. No statistically significant effects on feed consumption were observed in either parental generation prior to mating. However, there was a small, generally consistent depression in feed consumption observed in the 1000 ppm males or females of both the F_0 and F_{1B} parents over the entire measurement period (F_0 males: 10%; F_{1B}: males 8%, females 11%) compared with the respective controls. The fertility index ranged from 75% to 96% and fluctuated considerably, but changes in the index did not appear to be a dose-related effect in either the F_0 or F_{1B} generation. The insemination index, gestation index and mean gestation period were not different among the dose groups in either generation. There were statistically significant depressions in the viability index of the F_0 generation and the F_{1A} parents at 100 and 1000 ppm (90.3% and 88.1%, respectively, versus 98.5% in controls) and in the lactation index of F_{1A} or F_{1B} generation animals at 1000 ppm. However, in the F_{1B} generation, neither the F_{2A} nor F_{2B} litters were affected in a compound-related manner. At 1000 ppm, neonatal weights from birth through week 3 or 4 of lactation were consistently and statistically significantly depressed in both the F_0 and F_{1B} generations. Reductions in the mean litter size at birth, in the viability index (survival until day 5 after birth) and in the lactation index were seen at 1000 ppm. No treatment-related effects on any organs were noted on gross and microscopic examination. No treatment-related histopathological findings were observed. Absolute and relative liver weights were decreased in F_{1B} males but not females at the middle and high dose levels, respectively. Absolute and relative kidney weights were also somewhat lower at the highest dose tested compared with the controls.

The NOAEL for parental systemic toxicity and offspring toxicity is 300 ppm (equal to 21.6 mg/kg bw per day), based on reduced feed consumption, decreased body weights and decreased liver and kidney weights seen at the LOAEL of 1000 ppm (equal to 72.3 mg/kg bw per day). The NOAEL for reproductive toxicity is 1000 ppm, equal to 72.3 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity is 300 ppm (equal to 21.6 mg/kg bw per day), based on decreased pup body weights and decreased litter size seen at the LOAEL of 1000 ppm (equal to 72.3 mg/kg bw per day) (Eiben, 1987).

(b) Developmental toxicity

(i) Oral administration

Mice

In a developmental toxicity study, tebuconazole (purity 93.6%) was administered to groups of 25 presumed pregnant NMRI/ORIG Kisslegg mice by gavage at a dose level of 0, 10, 30 or 100 mg/kg bw per day from gestation day (GD) 6 to GD 15. The vehicle was 0.5% aqueous Cremophor EL emulsion (dose volume 5 ml/kg bw). The dams were inspected daily with respect to mortality, appearance and behaviour and were weighed daily. The dams were sacrificed on GD 18, followed by gross examination of all internal organs. The uteri and contents were examined, the numbers of implantations and live and dead fetuses were recorded, and the sex of all live fetuses was determined. The placenta was weighed; each fetus was weighed individually. Fetuses were examined for external and visceral abnormalities using a modified Wilson's technique and skeletal abnormalities after clearing and staining. In a supplementary study to examine maternal toxicity, groups of 10 mated and presumed pregnant mice were administered tebuconazole (purity 97.4%) by gavage at a dose of 0,

10, 20, 30 or 100 mg/kg bw per day from GD 6 to GD 15 and sacrificed on GD 16. The animals were observed for mortality and clinical signs, and one half of the mice were subjected to gross examination. The liver, kidneys, spleen and adrenal glands were weighed, and the livers were processed for microscopic examination. Blood was drawn from five anaesthetized mice per group for haematology and clinical chemistry evaluation.

No maternal deaths or treatment-related clinical signs or body weight gains were observed in the main or supplemental study. Feed consumption was not measured. In the supplemental study, haematocrit was significantly decreased at 30 and 100 mg/kg bw per day, and mean corpuscular volume (MCV) was significantly decreased at 20, 30 and 100 mg/kg bw per day; no clear dose-related trend was observed for either parameter. Serum enzyme activities (AST, ALT and alkaline phosphatase) were increased at most doses, but no clear dose-related trend was observed for these parameters. Liver triglycerides were elevated at 100 mg/kg bw per day. No clear trend was observed for the increase in absolute liver weight, but relative (to body weight) liver weight was increased at all doses, particularly at 100 mg/kg bw per day. Gross examination showed pale lobular livers in 100 mg/kg bw per day mice compared with none of five in controls. Microscopically, mild to severe liver cell vacuolation and moderate to severe lipidosis (ORO stain) were observed in five of five mice at 100 mg/kg bw per day (2/5 in control mice). It should be noted, though, that the livers (and the blood) were obtained from pregnant and non-pregnant animals, so the importance of inseminating the dams is not obvious. The livers used for the homogenates in the high-dose group were taken from pregnant mice only, whereas three of the livers examined histopathologically were from non-pregnant mice.

No treatment-related effect was observed on the number of live fetuses per dam, mean fetal weight, number of resorptions per dam, per cent postimplantation loss or sex ratio. The total number of resorptions was marginally increased at 100 mg/kg bw per day. There was a dose-dependent and statistically significant increase in the number of runts (fetuses weighing less than 1.3 g) per litter at 30 mg/kg bw per day (20/234; 8.6%) and 100 mg/kg bw per day (26/202; 13%) compared with the controls (5/236; 2.1%), but without a correlation to the mean fetal weight or stage of ossification at 30 mg/kg bw per day. Thus, the retarding effect at 30 mg/kg bw per day is considered only marginal. An increased incidence of common malformations was found at 100 mg/kg bw per day (13/202; 6.5%), which consisted most frequently of cleft palates and individual cases of micrognathia, rib fusion and spinal dysplasia; the incidence of malformations in the control group was 1/236 (0.4%). The incidence of cleft palate at this dose, 6/202 (3%), was also markedly higher than the mean incidence in historical controls (0.7%).

When the results of the main study and supplementary study are combined, the maternal toxicity LOAEL is 100 mg/kg bw per day, based on increased hepatic triglycerides, pale lobular liver and increased severity of hepatic vacuoles and lipidosis. The NOAEL for maternal toxicity is 30 mg/kg bw per day. The LOAEL for developmental toxicity is 30 mg/kg bw per day, based on a marginal retarding effect (an increased number of runts). The NOAEL for developmental toxicity is 10 mg/kg bw per day (Renhof, 1988b; Renhof & Karbe, 1988). The study authors concluded that the doses from 10 mg/kg bw per day must be regarded as exerting slight maternal toxicity, and doses from 30 mg/kg bw per day display clear maternal toxicity (Renhof & Karbe, 1988). The JMPR in 1994 (Annex 1, reference *71*) established the NOAEL for maternal toxicity as less than 10 mg/kg bw per day from the Renhof & Karbe (1988) study. The difference in maternal NOAELs (10 versus 30 mg/kg bw per day) between the previous JMPR and the present JMPR is probably due to the fact that increased activities of AST, ALT and alkaline phosphatase and increased liver weights at all doses were considered as adverse effects by the JMPR in 1994 (Annex 1, reference *71*).

Below is the summary of a combined report on developmental toxicity in mice (two main studies) and two satellite studies to evaluate maternal toxicity parameters.

Table 17. Liver toxicity parameters in tebuconazole-treated pregnant female mice

Clinical chemistry and liver homogenate parameters	Dose (mg/kg bw per day)			
	0	10	30	100
	(*n* = 10 dams)	(*n* = 10 dams)	(*n* = 10 dams)	(*n* = 10 dams)
Cytochrome P450 (nmol/g)	29.4	42.3	73.7**	116.4**
N-Demethylase activity (nmol/g per minute)	260.4	413.9	752.9**	975.4**
O-Demethylase activity (nmol/g per minute)	2.52	2.95	3.67	8.16**
Liver triglyceride (µmol/g)	8.8	7.0	11.9	14.8
AST (U/l)	2.74	2.60	2.12	3.17
ALT (U/l)	1.07	1.38	1.05	1.78
Alkaline phosphatase (U/l)	2.21	2.22	3.77*	3.37

From Becker & Biedermann (1995a)
U, units; * $P \leq 0.05$; ** $P \leq 0.01$

In a developmental toxicity study, 35 presumed pregnant NMRI KFM-HAN mice were administered tebuconazole (purity 95.8–96.8%) in 0.5% Cremophor EL by gavage at a dose of 0, 10, 30 or 100 mg/kg bw per day on GDs 6–15, inclusive. On GD 18, dams were sacrificed and subjected to gross necropsy, and all fetuses were examined externally. One half of the fetuses were examined viscerally, and the remaining fetuses were examined for skeletal malformations/variations. An additional 10 mated dams per group were included in a satellite study to assess the effects on haematology and clinical biochemistry parameters and pathological changes of target organs. In the satellite study, blood was collected on GD 16, and the dams were then euthanized and subjected to gross necropsy. Liver tissue samples were collected to determine cytochrome P450 content and *N*- and *O*-demethylase activities or triglyceride content. The spleens, kidneys, adrenals and livers from all females (both main and satellite groups) were weighed and examined histologically. Because of marginal developmental effects observed at all dose levels, a second study was conducted in which 30 mated dams per group received nominal doses of tebuconazole at 0, 1 or 3 mg/kg bw per day orally by gavage on GDs 6–15, inclusive. The evaluations of the groups were the same as those in the main study except that only liver and adrenals were weighed and examined histologically. A satellite study was also again conducted: an additional seven mated dams per group were added to assess the effects of tebuconazole on target organs. Satellite group animals were sacrificed on GD 16, and the liver and adrenals were weighed and examined histologically.

No treatment-related mortalities or clinical signs of toxicity were observed in the study. Feed consumption was marginally decreased in the 30 and 100 mg/kg bw per day dose groups during GDs 6–11. A marginal decrease in body weight gain was noted in the 100 mg/kg bw per day group during GDs 6–16. Effects on the liver were seen at 10 mg/kg bw per day and above. The dose of 10 mg/kg bw per day resulted in enzyme induction (cytochrome P450, *N*-demethylase) and increased vacuolization of the liver (Table 17); however, the liver enzyme induction at 10 mg/kg bw per day was not statistically significant. Higher doses of 30 and 100 mg/kg bw per day resulted in further signs of liver toxicity, such as increases in liver weight and lipid storage in livers. Liver toxicity was also evident from clinical chemistry and histopathology observations. *O*-Demethylase activity in the liver was increased at 30 mg/kg bw per day and above, as was the alkaline phosphatase level in the blood. Transaminases (AST and ALT) in the blood were increased at 100 mg/kg bw per day. In addition, the 100 mg/kg bw per day dose resulted in slight effects on the blood turnover, evident by an increase in reticulocytes combined with a shift from low fluorescent to high fluorescent reticulocytes and an increase in spleen weight.

At 30 mg/kg bw per day, a marginal but test article–related increase in postimplantation loss was evident compared with the control group. Treatment with 100 mg/kg bw per day resulted in statistically significant increases ($P < 0.05$ or 0.01) in postimplantation loss (35.3% versus 8.4% in controls) and the number of resorptions per dam (4.3 versus 1.0 in controls), including early resorptions (3.5 versus 0.8 in controls) and late resorptions (0.8 versus 0.2 in controls), and statistically significant decreases in mean fetal weights (1.1 g versus 1.2 g in controls) and the number of live fetuses per litter (8.1 versus 10.9 in controls). The combined incidence rate of litters containing fetuses with external, visceral and skeletal malformations was 1/21, 6/20 and 3/18 for the 0, 1 and 3 mg/kg bw per day groups, respectively, and 3/29, 7/28, 6/24 and 15/26 ($P < 0.01$) for the 0, 10, 30 and 100 mg/kg bw per day groups, respectively (historical control data 8/25 or 6/23). The incidences of exencephaly (partial acrania), cleft palate, open eyes, fused/bifurcated ribs, vertebral defects (includes dysplastic vertebral bodies and missing cervical and lumbar vertebral bodies, sacral vertebrae and coccygeal vertebrae) and retarded ossification of individual phalanges of the forelimbs and hindlimbs were statistically significantly increased ($P < 0.05$ or 0.01) in litters in the 100 mg/kg bw per day group. Increases in the incidence of exencephaly, acrania and skull malformations were also seen in the 10 and 30 mg/kg bw per day groups (but each finding was within the range of historical control data). There was also an apparently dose-related increase in the incidence of small growth on forepaw/toe, seen in 1 fetus (1 litter) in controls, 2 fetuses (2 litters) at 10 mg/kg bw per day, 5 fetuses (4 litters) at 30 mg/kg bw per day and 7 fetuses (4 litters) at 100 mg/kg bw per day, but no litters in the second study at 0, 1 or 3 mg/kg bw per day. This finding was observed at external examination only and was not confirmed at skeletal examination. Other malformations or variations were either not statistically significant or not treatment related at the lower doses; no increase in the incidence of malformations was seen in the second study at 1 or 3 mg/kg bw per day. Cleft palates occurred at similar incidences without a dose–response relationship and were within the range of current or historical control data at 1, 3, 10 and 30 mg/kg bw per day.

The maternal toxicity NOAEL is 30 mg/kg bw per day, based on increased hepatic enzyme induction (increased cytochrome P450 content and *N*-demethylase activity) and severity of vacuolization in liver cells at the LOAEL of 100 mg/kg bw per day. The developmental toxicity NOAEL is 10 mg/kg bw per day, based on a marginal increase in postimplantation loss and statistically significant increase of common malformations or variations at the LOAEL of 30 mg/kg bw per day (Becker & Biedermann, 1995a).

Suitability of mice for developmental toxicity study

The expert opinion of Christian (2005) contains a discussion of the evolution of the current teratology guidelines and a history of the use of mice in regulatory toxicology studies. Christian (2005) suggested that NMRI mice are not a suitable model for the evaluation of teratogenic effects because of lack of an adequate historical control database, high incidence of spontaneous malformations (cleft palate), high rate of spontaneous genetic defects and high reactivity to environmental stress. Christian (2005) concluded that "observations of malformations in the fetuses of NMRI mice should not be considered an appropriate criterion for calculation of a NOEL [no-observed-effect level]".

Similarly, the suitability of the mouse as a test species for developmental toxicity studies is addressed by Neubert (2000). He stated that "It should be noted that the mouse as a species has a strong tendency to display unspecific reactions in response to 'stress' (e.g. hunger, restraint, etc.). It is therefore not used routinely in prenatal toxicology, but only for special investigations."

Rats

In a range-finding study, groups of 25 female Wistar rats were treated by gavage with tebuconazole (purity 99.5%) at a dose of 0 or 100 mg/kg bw per day on GDs 6–15. The treatment had no effect on mortality rates, but body weight gain was retarded. The fertilization rate was not reduced by

treatment, but most litter parameters (e.g. number of implantations, litter size and losses) indicated an adverse effect. More runts and fetuses with malformations (micrognathia, hydronephrosis and hydroureter) were found in treated animals.

The NOAEL for maternal toxicity, embryotoxicity and teratogenicity was less than 100 mg/kg bw per day (Renhof, 1984).

In a second developmental toxicity study, tebuconazole (purity 93.4%) was administered to groups of 25 pregnant Wistar (Bor:WISW (SPF Cpb)) rats by gavage at a dose level of 0, 10, 30 or 100 mg/kg bw per day from GD 6 to GD 15. The vehicle was 0.5% aqueous suspension of Cremophor emulsion (dose volume 10 ml/kg bw). Stability and homogeneity were evaluated, but the results were not presented in the study report. On GD 20, caesarean sections were performed, followed by gross examination of all internal organs with a focus on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The uteri plus contents were weighed; uteri in non-pregnant females were stained to identify implantation sites. Fetuses were sexed, individually weighed and examined for gross abnormalities. One half of the fetuses in each litter were processed for examination of visceral and brain abnormalities using modified Wilson's technique, and the remaining half were cleared and stained for skeletal examination.

No treatment-related deaths occurred. No treatment-related clinical signs of toxicity were observed. Faecal alterations (e.g. loose stools) occurred at 100 mg/kg bw per day in 11 dams. At 30 and 100 mg/kg bw per day, dose-related reductions in weight gain were observed throughout the treatment period; at 100 mg/kg bw per day, retarded body weight gain was observed throughout gestation. Effects on litter parameters included an increased number of losses and a decrease in mean fetal weight at 100 mg/kg bw per day. At this dose, there were also more runts (below 3 g) and fetuses with malformations (mostly microphthalmia): the runt incidence was 36%, compared with 7% in controls, and the incidence of fetuses with malformations was 7%, compared with 2% in controls.

The NOAEL for maternal toxicity is 30 mg/kg bw per day, based on decreased body weight gain throughout the dosing period seen at the LOAEL of 100 mg/kg bw per day and above. The NOAEL for developmental toxicity is 30 mg/kg bw per day, based on decreased fetal weight, increased incidence of malformations, increased incidence of postimplantation losses and increased number of runts seen at the LOAEL of 100 mg/kg bw per day (Renhof, 1985a).

In a third developmental toxicity study, tebuconazole (purity 98.3%) was administered to groups of 25 pregnant Wistar/NAN (Kfm:WIST, Outbred, SPF) rats by gavage at a dose level of 0, 30, 60 or 120 mg/kg bw per day from GD 6 to GD 15. The vehicle was 0.5% aqueous suspension of Cremophor emulsion (dose volume 10 ml/kg bw). The dosing solutions were prepared daily. Determination of concentration, homogeneity and stability of the dosing solution was performed on one occasion during the treatment period. The dams were sacrificed on GD 21 (caesarean section) followed by gross examination of all internal organs with a focus on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The uteri plus contents were weighed; uteri in non-pregnant females were stained with ammonium sulfide solution to identify implantation sites. Fetuses were sexed, individually weighed and examined for gross abnormalities. One half of the fetuses in each litter were processed for examination of visceral and brain abnormalities using Wilson's technique, and the remaining half were cleared and stained for skeletal examination.

The dose of 30 mg/kg bw per day affected neither maternal nor fetal parameters. No compound-related mortality or clinical signs of toxicity were reported at any dose level. Although body weight gains for GDs 6–21 were slightly decreased (85% of controls) at the high dose, corrected or uncorrected body weight gains were not significantly different from those of controls at any dose level. Mean daily feed consumption during days 6–16 was significantly decreased with respect to controls at the middle dose (−7%) and at the high dose (−15%). There was a slight increase in daily

feed consumption (about 5%) during the post-dosing period. A significant decrease in corrected body weight (minus uterine weights) was reported for the high dose, and a dose-dependent and statistically significant increase in absolute and relative liver weights was reported at the high and middle doses. The statistically significant increase in liver weights at the middle dose (60 mg/kg bw per day) was minimal and was not considered biologically relevant by the Meeting. Reproductive parameters (numbers of corpora lutea and implantations) were not adversely affected. An increase in the numbers of early and late resorptions and a decrease in mean fetal weights (−10.6%) and in total live fetuses (19.4% below controls) were observed at the high dose. External examination of fetuses revealed a missing tail in one high-dose fetus and agnathia (lower jaw), microstomia and anophthalmia in another fetus of a different litter at the highest dose tested. No malformed or anomalous fetuses were observed in the control, 30 mg/kg bw per day or 60 mg/kg bw per day dose groups. Visceral examination of fetuses revealed findings of excess fluid in the thoracic cavity at the high dose (three pups in one litter, one pup in another litter) and at the low dose (one pup). Skeletal examination revealed increased incidences of supernumerary ribs, non-ossified cervical vertebrae and incompletely ossified sternebrae, indicating retardation of fetal development.

The maternal toxicity NOAEL is 60 mg/kg bw per day, based on increased absolute and relative liver weights and decreased body weight gains at the LOAEL of 120 mg/kg bw per day. The NOAEL for developmental toxicity is 60 mg/kg bw per day, based on higher incidence of resorptions, decreased litter size, reduced fetal body weight and marginally increased incidence of skeletal variations at 120 mg/kg bw per day (Becker, Vogel & Terrier, 1988a).

Rabbits

In a developmental toxicity study, tebuconazole (purity 93.4%) was administered to groups of 15 presumed pregnant Himalayan (CHBB:HM) rabbits by gavage at a dose level of 0, 3, 10 or 30 mg/kg bw per day in 0.5% aqueous Cremophor emulsion from GD 6 to GD 18. The dams were sacrificed on GD 29, followed by gross examination of all internal organs with a focus on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The uteri and contents were weighed; uteri in non-pregnant females were stained with ammonium sulfide solution to identify implantation sites. The livers were weighed. Fetuses were sexed, individually weighed and examined for gross abnormalities. The internal examination included the thorax, abdomen and pelvis and sex determination. The crania were examined for ossification, and the heads were fixed, serially sectioned and examined. The carcasses were cleared and stained for skeletal examination.

No treatment-related clinical signs of toxicity were observed. One dam of the control group died prior to sacrifice, but the death was not related to dosing. There was reduced body weight gain (74% of control, but not statistically significant) in dams of the highest dose group during the dosing period. At 30 mg/kg bw per day, a significant increase in resorptions per dam (0.8) was observed, which has to be assessed with regard to the unusually low number of resorptions per dam (0.2) in the control group. The historical control data reveal a range from 0.2 to 2.6 resorptions per dam. Also, the slightly increased postimplantation losses (11.3%) are clearly within the range of historical control data (2.6–38.8%). Therefore, these findings are not considered to be treatment-related effects.

The maternal toxicity NOAEL in the developmental toxicity study in rabbits is 10 mg/kg bw per day, based on reduced body weight gain during treatment at the LOAEL of 30 mg/kg bw per day. The developmental toxicity NOAEL is 30 mg/kg bw per day, the highest dose tested. A developmental toxicity LOAEL was not observed in this study (Renhof, 1985b).

In a second developmental toxicity study, tebuconazole (purity 98.2%) was administered to groups of 16 presumed pregnant Chinchilla (Kfm:CHIN, hybrids, SPF Quality) rabbits by gavage at a dose level of 0, 10, 30 or 100 mg/kg bw per day in 0.5% Cremophor EL (4 ml/kg bw) from GD 6 to GD 18. The dams were sacrificed on GD 28, followed by gross examination of all internal organs

with a focus on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The uteri and contents were weighed; uteri in non-pregnant females were stained with ammonium sulfide solution to identify implantation sites. The livers were weighed. Fetuses were sexed, individually weighed and examined for gross abnormalities. The internal examination included the thorax, abdomen and pelvis and sex determination. The crania were examined for ossification, and the heads were fixed, serially sectioned and examined. The carcasses were cleared and stained for skeletal examination.

In the high-dose group, maternal weight gain was decreased by 38% during the dosing period, 29% during the post-dosing period and 27% from GD 6 to GD 28. After correcting for gravid uterus weight, the high-dose group had a small weight loss (−0.3 g versus 1.4 g weight gain for controls), whereas the controls had a small weight gain. Feed consumption was decreased by 12% in high-dose does during dosing, but was similar to or slightly greater than that of controls during the post-dosing period. Absolute and relative liver weights were not affected by treatment, and no treatment-related gross lesions were observed in maternal animals. A statistically significant increase in postimplantation loss was observed at the high dose, as evidenced by increased fetal resorptions and decreased numbers of live fetuses per dam. Slightly decreased fetal body weight, which was correlated with a slightly decreased retarded ossification, was observed at the high dose; however, it lacked a clear dose–response relationship, and values were within historical and concurrent control ranges (35.1, 33.5, 35.0 and 33 g at 0, 10, 30 and 100 mg/kg bw per day). External examination revealed frank malformations (peromelia, palatoschisis, malrotation of hindlimb, agenesis of claws of the hind paw) in the high-dose group. Examination of the fetal heads by Wilson's technique revealed one fetus with hydrocephalus internus at the high dose. Skeletal examination of fetuses revealed abnormalities and delayed ossification in the high-dose group only. Of 90 fetuses born to dams treated at 100 mg/kg bw per day, eight (9%) were malformed, and five of these (6%) had peromelia. Peromelia was seen in none of a total of 346 fetuses in the other three groups combined. This finding was not observed in the other developmental studies in rabbits and probably should be assessed as retardation. The study suffers from some technical deficiencies, as there was no double staining, there was an insufficiently long observation time of fetal development and many indications of incomplete development also exist in controls. At 100 mg/kg bw per day, the percentage of fetuses with absent or incomplete phalangeal ossification was also increased.

The maternal toxicity NOAEL is 30 mg/kg bw per day, based on decreased body weight gains and decreased feed consumption during dosing at the LOAEL of 100 mg/kg bw per day. The developmental toxicity NOAEL is 30 mg/kg bw per day, based on increased postimplantation loss, frank malformations, hydrocephalus and increased external and skeletal abnormalities at the LOAEL of 100 mg/kg bw per day (Becker, Vogel & Terrier, 1988b).

In a third developmental toxicity study (main study), tebuconazole (purity 96.3–96.8%) was administered to groups of 16 presumed pregnant Chinchilla (Kfm:CHIN, hybrids, SPF Quality) rabbits by gavage at a dose level of 0, 10, 30 or 100 mg/kg bw per day in 0.5% Cremophor EL (dose volume 4 ml/kg bw) from GD 6 to GD 18. The dams were sacrificed on GD 28, followed by gross examination of all internal organs with a focus on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea. The uteri and contents were weighed; uteri in non-pregnant females were stained with ammonium sulfide solution to identify implantation sites. The livers were weighed. Fetuses were sexed, individually weighed and examined for gross abnormalities. The internal examination included the thorax, abdomen and pelvis and sex determination. The crania were examined for ossification, and the heads were fixed, serially sectioned and examined. The carcasses were cleared and stained for skeletal examination.

In a supplementary study, five female rabbits per dose group (0, 10, 30 or 100 mg/kg bw per day) were exposed to tebuconazole using the identical dosing regimen as for the third main study.

The purpose of this study was to assess the effects of tebuconazole on specific parameters of maternal toxicity in pregnant Chinchilla rabbits. Blood specimens were taken from the marginal ear vein on GDs 6, 12 and 19 postcoitum and subjected to full haematological and clinical chemistry analysis. The dams were sacrificed just after the last blood specimens had been taken, and the gravid uteri were removed by caesarean operation and weighed. All reproduction parameters were recorded (as above). The adrenals, kidneys, liver and spleen from all gravid dams were weighed separately and prepared for histological examinations. Two portions of 10 g each were taken from each liver before the preparation for determination of cytochrome P450, *N*-demethylase and *O*-demethylase activities and the triglyceride content.

No treatment-related clinical signs of toxicity or mortality were observed. At 100 mg/kg bw per day, feed intakes were decreased, and body weight loss occurred from GD 6 to GD 11, which was not recovered during the remaining gestation period. Overall body weight gain during the treatment period was also decreased by 17% compared with the control group. No treatment-related findings were noted during postmortem examination. The reproduction parameters affected in the high-dose group were a slightly increased mean postimplantation loss and a decrease in mean fetus weight (statistically significant), which was correlated with slightly retarded ossification and increased incidence of common malformations. The decrease in mean fetal body weight was 31.5, 32.0, 31.6 and 30.0 g at 0, 10, 30 and 100 mg/kg bw per day, respectively, with a historical control range of 29.2–35.9 g (Becker, 2006). At 30 mg/kg bw per day, three fetuses with external malformations were observed, which are related to the high spontaneous variability but not to a treatment-related effect. Abnormal findings at 100 mg/kg bw per day were noted in four fetuses during external and visceral examinations. Additional findings were evident in two of the four fetuses during examination of heads by Wilson's technique and in two of the four fetuses during skeletal examinations for abnormal findings. Slightly increased incidences of non-ossification and incomplete ossification, which were correlated with statistically significant reductions in mean fetal body weight (individual basis), were also noted in this group.

There were no treatment-related effects on the haematological or clinical chemistry parameters evaluated on GDs 6, 12 and 19. In addition, there was no observable effect at GD 19 on liver cytochrome 450, *N*-demethylase, *O*-demethylase or triglycerides. No treatment-related gross abnormality was noted upon macroscopic examination (GD 19). The absolute and relative organ weights of adrenals, kidneys, liver and spleen were unaffected by the treatment. Based on the first amendment to the study report by Becker & Biedermann (1995b), histopathological evaluation of the liver showed single-cell necrosis with increased incidences in test article–treated animals, whereas liver necrosis was evident in one female of the 30 mg/kg bw per day group. The number of animals investigated was too low to permit definitive interpretation of these findings. However, the liver is known to be the main target organ in the other species examined. Therefore, a treatment-related effect for these findings (from 10 mg/kg bw per day upwards) has to be assumed (Becker & Biedermann, 1995b).

The NOAEL for maternal toxicity is 30 mg/kg bw per day, based on decreased feed consumption and decreased body weight gains observed at the LOAEL of 100 mg/kg bw per day. The developmental toxicity NOAEL is 30 mg/kg bw per day, based on increased postimplantation loss, increased incidence of common malformations and increased external and skeletal abnormalities at the LOAEL of 100 mg/kg bw per day (Becker & Biedermann, 1995c).

In a fourth developmental toxicity study, tebuconazole (purity 98.5%) was administered to groups of 14 or 15 presumed pregnant Chinchilla (CHB-W) rabbits by gavage at a dose level of 0 mg/kg bw per day (14 rabbits) or 100 mg/kg bw per day (15 rabbits) in 0.5% Cremophor EL (dose volume 4 ml/kg bw) from GD 6 to GD 19. All rabbits were inspected twice daily. Body weights were recorded on day 0 and daily from GD 6 to GD 19. A caesarean section was performed on GD 19, 2 hours post-dosing. Blood was drawn from an extremity vein just prior to caesarean section.

All reproductive parameters were recorded. The liver, ovaries, adrenals and placentas of all pregnant females (surviving) were weighed. The right liver lobes and two placentas with fetuses from each of the pregnant females were fixed in buffered 4% formaldehyde. The adrenals and ovaries of three females from each group were also fixed in buffered 4% formaldehyde. Enzyme activities of the livers (7-ethoxycoumarin deethylase, 7-ethoxyresorufin deethylase, aldrin epoxidase, epoxide hydrolase, gluthathione-*S*-transferase, uridine diphosphate-glucuronosyltransferase, testosterone metabolism assay) were determined. Steroid concentrations (cortisol, corticosterone, 11-deoxycorticosterone and progesterone) were determined in adrenal tissue. Additionally, the 11β-hydroxylation of 11-deoxycorticosterone in adrenal mitochondria was measured. The blood samples were analysed for content of tebuconazole, as were fetal tissues.

Two tebuconazole-treated animals died during the study because of intubation errors. Dosing did not affect appearance or behaviour of the animals. Feed consumption was decreased (GDs 6–12). Body weight gain was decreased (GDs 6–10). Urination and amount of faeces were decreased. The necropsy findings did not reveal treatment-related effects. Absolute and relative weights of the liver, ovaries and adrenals and the absolute placental weight did not differ statistically between treated groups and the control group. Histopathological findings showed a distinct hypertrophy of adrenocortical cells from the zona fasciculata. In addition, centrilobular cytoplasmic change of the liver was observed in two females. The activity of 7-ethoxycoumarin deethylase was statistically significantly increased (55.1%). A statistically significant decrease (27.6%) in the activity of glutathione-*S*-transferase was evident at the 100 mg/kg bw per day level, which could be indicative of impaired liver function. A slightly increased 11β-hydroxylation of 11-deoxycorticosterone in the adrenal mitochondria and slightly increased concentrations of 11-deoxycorticosterone and corticosterone in the adrenal tissue occurred. No reproductive parameters were affected by the treatment, except for a slight, statistically significant decrease in fetal body weights. No external finding occurred in any of the fetuses of both study groups (control and treated groups). There was no indication of an accumulation of tebuconazole in the plasma of fetuses, and there was a good correlation between maternal plasma and fetal tissue levels for the individual females. No examination of the fetuses was performed.

Tebuconazole shows maternal toxicity. This study discusses the possibility that formerly (in two studies) recorded increased numbers of external, skeletal and visceral anomalies and variations could have been due to elevated levels of corticosteroids produced by the dams themselves as a response to stress. This study and its results cannot support this theory, as the increase in plasma levels is marginal in this test, with distinct hypertrophy of steroid-forming areas of the adrenals in two out of three histologically examined adrenals; however, the investigators did not examine the fetuses for malformations, so a definite conclusion cannot be made. Also, there were no statistically significant changes in the concentrations of selected steroids in maternal adrenal tissues and mitochondria. However, maternal toxicity has not been shown to be responsible for the teratogenic activity of tebuconazole via stress induction of elevated levels of corticosteroidal compounds.

The LOAEL is 100 mg/kg bw per day, based on marginally increased plasma levels and histopathological changes in adrenals. The NOAEL for maternal and developmental toxicity is less than or equal to 100 mg/kg bw per day. The study authors suggested a threshold value of 100 mg/kg bw per day for fetal malformations (Holzum, Schmidt & Hartmann, 1999).

(ii) Dermal application

Mice

In a dermal developmental toxicity study, 25 pregnant NMRI KFM-HAN (outbred, SPF Quality) mice received repeated dermal applications of tebuconazole (purity 98.1%) in aqueous 4% carboxymethylcellulose (dose volume 2.5 ml/kg bw) at a dose level of 0, 100, 300 or 1000 mg/kg bw per day from GD 6 through GD 15 inclusive. The application was covered with an occlusive bandage

and left for 6 hours and was then rinsed off with lukewarm water. The dams were inspected at least twice daily for mortality, clinical signs and changes in appearance and behaviour. Weights of the animals and feed consumption were recorded at regular intervals. At day 18 postcoitum, caesarean sections were performed, gross macroscopic examination of all internal and all external organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed, and the data were recorded. The fetuses were removed from the uterus, sexed, weighed individually and examined for gross external abnormalities. Half of the live fetuses were allocated to Wilson's slicing techniques for examination of the viscera and brain. The other half were placed in potassium hydroxide solution, stained with alizarin red S and examined for skeletal abnormalities, and all abnormalities were recorded. The uteri and contents of all uteri with live fetuses were weighed at necropsy on day 18 postcoitum to enable calculation of the corrected body weight gain. If no implantation sites were evident, the uterus was placed in an ammonium sulfide solution.

As no overt maternal toxicity was observed in this main study, an additional supplementary study, employing the same treatment regimen and the same strain of mice, was conducted to further assess the maternal toxicity of the compound. In the supplementary study, groups of 2 × 10 mated females (parts A and B) were used for each dose level. The tebuconazole used was 96.0% pure. All dams were sacrificed on day 16 postcoitum and necropsied, the liver and adrenals of group A animals were weighed, the pregnancy status of the animals was recorded, and sections of liver and adrenals were examined histopathologically (group A). Before sacrifice of the study group B dams, blood samples were collected from non-fasted animals. Following this, the females were sacrificed and necropsied. The pregnancy status was recorded, and the entire liver was taken for analysis of the cytochrome P450 content and the *N*-demethylase and *O*-demethylase activities. The blood samples were analysed for AST, ALT, glutamate dehydrogenase and alkaline phosphatase activities.

Treatment had no effect on clinical signs, mortality, local skin reactions, body weight, feed consumption or reproductive parameters (numbers of corpora lutea, implantations, resorptions and live fetuses). In the supplementary study, clinical biochemical examinations revealed increased ALT activity at 1000 mg/kg bw per day; there was also increased cytochrome P450 content and *N*-demethylase activity in liver tissue above 300 mg/kg bw per day and a marginal increase in *O*-demethylase activity. None of these parameters showed a dose–response relationship. There was a dose-related reduction in relative adrenal weights in all dose groups, which was statistically significant at 1000 mg/kg bw per day; absolute adrenal weights were also reduced in all treatment groups, but not in relation to dose. No effect was seen on liver weights, but histological examination revealed fatty changes in periportal areas in most mice at 300 mg/kg bw per day and in all mice at 1000 mg/kg bw per day. In general, no dose-related effects were observed in the fetuses, but slightly increased numbers (not statistically significant) of palatoschises and supernumerary ribs were found in the fetuses of the 1000 mg/kg bw dose group.

When results of the main study and supplementary study are combined, the NOAEL for maternal toxicity is 100 mg/kg bw per day based on increased activities of cytochrome P450 and *N*-demethylase in liver tissue and fatty changes in the periportal liver area seen at the LOAEL of 300 mg/kg bw per day. The developmental toxicity NOAEL is 1000 mg/kg per day, the highest dose tested (Becker et al., 1990). The JMPR in 1994 established a developmental toxicity NOAEL of 300 mg/kg bw per day, based on slightly increased external anomalies and skeletal anomalies seen at 1000 mg/kg bw per day (Annex 1, reference *71*). The present Meeting did not consider the slightly increased anomalies as adverse, and no clear dose–response relationship was observed.

Rats

In a dermal developmental toxicity study, tebuconazole (purity 97.4%) in aqueous 1% Cremophor EL was administered dermally to pregnant Wistar (Bor:WISW (SPF Cpb)) rats on GDs 6–15 at a nominal level of 0, 100, 300 or 1000 mg/kg bw per day. The test material was applied to a 25 cm^2

area of shaved skin (nominal doses of 0, 0.87, 2.6 and 8.7 mg/cm^2 per day) for 6 hours, then removed, followed by washing of the application site with lukewarm water. Dams were sacrificed on GD 20, and gross macroscopic observation of all organs was performed. At caesarean section, the uteri and their contents were weighed to obtain corrected maternal body weight gains and were examined to determine the number of implantations. The fetuses were sexed, weighed and examined for external malformations. Approximately 50% of the fetuses were examined for visceral malformations by Wilson's technique, and the rest were cleared in potassium hydroxide and stained with alizarin S for evaluation of skeletal malformations by Dawson's method.

No evidence of maternal toxicity (changes in body weights, corrected body weights, feed consumption, clinical signs, pathology, deaths, abortions, premature deliveries) were noted at any dose level. No developmental toxicity was noted at any dose level based upon indices of mean number of corpora lutea per dam, number of implantations per dam, number of live or dead fetuses per dam, number of resorptions per dam (early and late), mean fetal weights, sex ratios (per cent male), mean crown–rump length, mean number of runts per dam, and incidence of variations or malformations.

For maternal and developmental toxicity by the dermal route, the NOAEL is 1000 mg/kg bw per day, the highest dose tested. A LOAEL could not be established (Renhof, 1988a).

In a second dermal developmental toxicity study, tebuconazole (purity 97.4%) in aqueous 1% Cremophor EL was administered dermally to pregnant Wistar (Hanlbm: WIST (SPF)) rats on GDs 6–15 at a nominal level of 0 or 1000 mg/kg bw per day. The test material was applied to a 25 cm^2 area of shaved skin and covered with an occlusive dressing. Six hours after the application, the bandage was removed, and the skin was rinsed with lukewarm tap water. The animals were inspected at least twice daily for mortality, clinical signs, changed appearance and behaviour. The animals were weighed daily, and feed consumption was recorded at regular intervals. Dermal irritation was evaluated daily according to the Draize method. Dams were sacrificed on GD 21, and gross macroscopic observation of all organs was performed. At caesarean section, the uteri and their contents were weighed to obtain corrected maternal body weight gains and were examined to determine the number of implantations. The fetuses were sexed, weighed and examined for external malformations. Approximately 50% of the fetuses were examined for visceral malformations by Wilson's technique, and the rest were cleared in potassium hydroxide and stained with alizarin S for evaluation of skeletal malformations by Dawson's method.

Only the local skin irritation on the treated area was more common and more severe in females treated with the active substance than in control animals treated with vehicle only (statistically significant). No other effects on any of the parameters examined were seen in this study.

For maternal and developmental toxicity by the dermal route, the NOAEL is 1000 mg/kg bw per day, the highest dose tested. A LOAEL could not be established (Becker & Biedermann, 1995d).

2.6 Special studies

(a) Acute neurotoxicity

Rats

In an acute oral neurotoxicity study, tebuconazole (purity 96.2–97.3%) was administered by gavage to 12 male Fischer 344 rats at a dose of 0, 100, 500 or 1000 mg/kg bw and to 12 female Fischer 344 rats at a dose of 0, 100, 250 or 500 mg/kg bw. Because a NOAEL did not appear to be attained in this study, a second (supplemental) acute oral neurotoxicity study using the same batch of tebuconazole and doses of 0, 20 and 50 mg/kg bw administered to 12 Fischer 344 rats of each sex per group was performed. The test substance was suspended in 0.5% methylcellulose–0.4% Tween

80 in deionized water and administered at a dose volume of 10 ml/kg bw. Based on analytical results, actual doses were 0, 0, 21, 50, 103, 497 and 950 mg/kg bw for male rats and 0, 0, 21, 50, 103, 239 and 497 mg/kg bw for female rats. In the main study, functional observational battery (FOB) and motor activity tests were performed pretreatment, on the day of test material administration (day 0) and on days 7 and 14 post-treatment; in the supplemental study, an abbreviated FOB and motor activity tests were performed on day 0 only. At the completion of the main study (day 14), six rats of each sex in the control and high-dose groups were subjected to perfusion, and brain and nervous tissues were examined microscopically. No histopathological evaluations were performed in the supplemental study.

The high dose of 1000 mg/kg bw resulted in mortality of 6 of 12 male rats within 2 days of treatment. One male rat in the 500 mg/kg bw dose group also died. There was no treatment-related mortality in females of any dose group. Clinical signs of incoordination, decreased activity, and nasal and perianal stains were observed on day 0, primarily in the two highest dose groups (males, 500 and 1000 mg/kg bw; females, 250 and 500 mg/kg bw). In general, the incidence of clinical signs increased with dose. Compound-related signs were apparent in both sexes on the day of treatment (day 0) and resolved by day 3 following treatment. Effects on body weight were minimal; weights were 96–101% of control weights on day 14. There was no effect of treatment on brain weight, and there were no histopathological findings in the brain or tissues of the nervous system that could be attributed to treatment.

FOB tests on the day of treatment showed treatment-related effects on gait (incoordination, ataxia); activity, arousal and response to stimuli (all decreased); hindlimb grip strength (decreased); footsplay (decreased in females only); and body temperature (decreased) in one or both sexes in the two highest dose groups (males, 500 and 1000 mg/kg bw; and females, 250 and 500 mg/kg bw). Most of these parameters were statistically significantly different from control values ($P < 0.05$). Increased arousal in the open field and decreased footsplay were also noted in females at 100 mg/kg bw. All effects except decreased footsplay in 250 and 500 mg/kg bw females were resolved by post-test day 7. No changes in FOB parameters were observed in males in the 100 mg/kg bw dose group. No treatment-related FOB effects were observed in either sex in the 20 and 50 mg/kg bw dose groups. Relative to concurrent controls, motor and locomotor activities were increased on day 0 in males and females in the 100 mg/kg bw dose group and decreased in the higher dose groups (males, 500 and 1000 mg/kg bw; and females, 500 mg/kg bw; all $P < 0.05$). There was no statistically significant change in 250 mg/kg bw females. No adverse effect of treatment on motor activity was observed in either sex in the 20 and 50 mg/kg bw dose groups, although there was a slight (non-statistically significant) increase in 50 mg/kg bw males that was considered compound related. No effect of treatment was observed on motor or locomotor activity on days 7 and 14. There were no compound-related gross lesions in males or females that survived to terminal sacrifice. Compound-related microscopic lesions were not evident in 500 mg/kg bw females or in surviving 1000 mg/kg bw males.

The NOAEL is 50 mg/kg bw, based on increased motor activity in male and female rats and decreased footsplay in female rats at the LOAEL of 100 mg/kg bw (Sheets, Gilmore & Hamilton, 1997).

(b) Short-term study of neurotoxicity

Rats

In a 90-day dietary neurotoxicity study, tebuconazole (purity 96.7–98.2%) was administered to 12 Fischer 344 rats of each sex per dose at a dietary level of 0, 100, 400 or 1600 ppm. Based on analytical measurements, doses were 0.0, 7.6, 29.2 and 107 mg/kg bw per day for males and 0.0, 8.8, 34.0 and 122 mg/kg bw per day for females. The test diets were prepared in corn oil at 1% by weight of the diet, and a small amount of acetone was used in the preparation of the diet. FOB and motor activity tests were performed pretreatment and during weeks 4, 8 and 13. At the completion of the

study, six rats of each sex per dose group were subjected to perfusion; brain and nervous tissues were examined microscopically in high-dose and control groups only.

No deaths occurred, and there were no clinical signs attributable to treatment. Relative to controls, body weights of male and female rats in the group receiving 1600 ppm in the diet were statistically significantly reduced after 1 week, by 7% in males and 5% in females (both $P < 0.05$). Lower body weights continued throughout the remainder of the study, with reductions of 8% for males and 7% for females at study termination (both $P < 0.05$). Feed consumption was reduced throughout the study in this group, with average daily feed consumption for males and females of 94% and 92% relative to respective controls. Feed efficiency was reduced by 11% and 13% in high-dose males and high-dose females, respectively. Relative to controls, there were no treatment-related effects on body weight or feed consumption or feed efficiency in the other dietary groups. No treatment-related effects were observed in FOB tests or motor activity tests. There were no gross pathological or histopathological findings that could be attributed to treatment with tebuconazole.

The NOAEL in this subchronic neurotoxicity study in rats is 1600 ppm (equal to 107 mg/kg bw per day), the highest dose tested. A LOAEL was not achieved in this study. The study authors concluded that the present study established that treatment with tebuconazole through the diet produces no evidence of neurotoxicity at the highest dose tested. These results support the highest dietary concentration of 1600 ppm as an MTD, based on decreased feed consumption and weight gain, and the middle dose of 400 ppm as a NOEL in both sexes (Sheets, Gilmore & Hamilton, 1998).

(c) Developmental neurotoxicity

Rats

In a developmental neurotoxicity study, tebuconazole (purity 96.0–96.9%) in corn oil was administered via the diet to pregnant Crl:CD®BR VAF/Plus® (Sprague-Dawley) rats (25 per dose) from GD 6 to lactation day (LD) 11 at a dose of 0, 100, 300 or 1000 ppm (equal to [GD/LD] 0/0, 8.8/16.3, 22.0/41.3 and 65.0/125.4 mg/kg bw per day). P dams were allowed to deliver naturally. On day 5 postpartum, litters were standardized to a maximum of 10 pups per litter. Pups were assigned to one of five subsets (20 pups of each sex per dose in each subset). Physical development landmarks were evaluated for all subsets (including surface righting, eye opening, pinna unfolding, acoustic startle response and pupil constriction); sexual maturation was evaluated in subsets 2–4. Subset 1 pups were sacrificed on postnatal day (PND) 12; brains were weighed for all subset 1 pups, and histopathological evaluations were performed on six pups of each sex in control and high-dose groups (morphometric analysis was performed on six pups of each sex in control, mid-dose and high-dose groups). Subset 2 pups were evaluated for learning and memory on days 23–25 (passive avoidance) and on days 59–63 (Water M-maze). Subset 3 pups were evaluated for motor activity (days 14, 18, 22 and 62) and for auditory startle habituation (days 23 and 63). Subset 4 pups received detailed weekly clinical evaluations. In addition, six animals of each sex per group in subset 4 were selected for neuropathological evaluations; brains were weighed, and the high-dose and control animals were evaluated histopathologically on day 83 (morphometric analysis was performed on six animals of each sex in control, mid-dose and high-dose groups). Subset 5 pups were sacrificed and necropsied on day 22.

At 1000 ppm, two P females died as a result of prolonged gestation. Body weights were slightly decreased ($P \leq 0.01$) in the P females during gestation (4–8%) and early lactation (6–12%). Body weight gains were decreased ($P \leq 0.01$ or 0.05) during GDs 6–9 (400%) and 6–21 (22%) and during LDs 1–12 (55–164%). When compared with concurrent controls, absolute (g/day per animal) feed consumption was reduced ($P \leq 0.05$ or 0.01) in the dams throughout gestation (9–23%) except during the GD 0–6 interval and during the LD intervals 4–7 (20%) and 7–12 (18%). Relative (g/kg bw per day) feed consumption was reduced ($P \leq 0.05$ or 0.01) starting on GD 6 (6–20%) and during

early lactation up to day 12 (8–12%). There was also an increase in alopecia in high-dose dams. The number of live fetuses per dam was decreased relative to concurrent controls (6%, $P \leq 0.01$), whereas the number of dead fetuses per dam was increased relative to concurrent controls (200%, $P \leq 0.01$). No treatment-related findings were observed in dams at 300 or 100 ppm.

At 1000 ppm, the stillborn index was increased (200%, $P \leq 0.01$), and the number of pup deaths (calculated by reviewers) was increased during days 1–5 (229%). Body weights were decreased ($P \leq 0.01$) in the males from PND 5 to PND 86 (7–23%) and in the females from PND 5 to PND 72 (5–24%). Pinna unfolding was delayed (19%, $P \leq 0.01$) relative to concurrent controls. There were decreases in several morphometric measurements of the brain, including decreased ($P \leq 0.01$) thickness of the cerebellum in the males and females on day 12 (10–14%) and on day 83 (7–9%) and an increased thickness of the germinal layer of the cerebellar cortex in the day 12 males (23%, $P \leq 0.01$). Absolute brain weights were decreased in the day 12 and day 83 animals (10–16%, $P \leq 0.01$ or 0.05). Relative (to body) brain weights were increased ($P \leq 0.01$ or 0.05) in the day 12 males and females (10–15%). There were also statistically significant changes in motor activity on days 14 (43% decrease in males [$P < 0.01$], 24% decrease in females [not significant]) and 22 (39% increase in males [$P < 0.05$], 19% increase in females [not significant]) and changes in auditory startle amplitude at both time points (decreased in both sexes on day 23 [14–33%], decreased in females [20%] and increased in males [71%] on day 63). At 300 ppm, there were also decreases in body weight (3–7%) and body weight gain (4–16%) (PNDs 5–23 and 72–86 in males, PNDs 5–51 in females). Pinna unfolding was delayed (16%). There were changes in auditory startle amplitude in both sexes: a dose-related decrease in females only on day 23 (26%), and a dose-related increase in males only on day 63 (18%). In addition, there was a decrease in absolute brain weight in both sexes (3–4%) on day 12 (statistically significant for females only) and in brain measurements (anterior/posterior cerebrum). At 100 ppm, there were decreases in body weight (3–7%) and body weight gain (5–13%) (PNDs 5–37 in males, PNDs 5–51 in females). There were decreases in motor activity (on days 14 and 18 in males [28–35%]) and changes in auditory startle amplitude (increased by 9% in day 14 females, increased by 16% in day 63 males, not significant). There was also a decrease in absolute brain weight in both sexes on day 12 (4%, statistically significant for both sexes) and in brain measurements (anterior/posterior cerebrum).

The LOAEL for maternal toxicity is 1000 ppm (equal to 65.0 mg/kg bw per day), based on decreased body weights, body weight gains and feed consumption, prolonged gestation with mortality and an increased number of dead fetuses. The NOAEL is 300 ppm (equal to 22.0 mg/kg bw per day). The LOAEL for offspring toxicity is 1000 ppm (equal to 65.0 mg/kg bw per day), based on decreased viability, decreases in body weights and absolute brain weights, brain measurements and evidence of developmental delay. The NOAEL is 300 ppm (equal to 22.0 mg/kg bw per day) (Parker, 2000).

A study was conducted by Moser et al. (2001) to evaluate adult neurological, immunological and reproductive parameters in rats. In this study, pregnant Tac:N(SD)fBR Sprague-Dawley rats were administered tebuconazole (purity 97.4%) in 0.7% methylcellulose by gavage at a dose of 0, 6, 20 or 60 mg/kg bw per day from GD 14 to PND 7; the pups were then dosed daily at the same levels from PND 7 to PND 42. Separate groups of rats were used for testing of immunological parameters, neurobehavioural testing using a screening battery of functional tests and cognitive evaluations. Other groups of rats were evaluated for reproductive development and function, whereas still others were sacrificed at the end of the dosing period for histological analyses of major organ systems, including neuropathological assessments.

Body weights of maternal animals were decreased at the highest dose tested during gestation. Pup viability and body weights were decreased in the highest dose group. There were no differences in the fertility indices in the exposed rats mated as adults. Developmental landmarks were mostly

unchanged by tebuconazole exposure. In the sheep red blood cell–immunized high-dose rats, spleen weights and cellularity were increased, and the ratio of cell types was altered compared with controls. There were, however, no biologically significant changes in the immune function of these rats. At necropsy on PND 46 or 152, kidney, liver and spleen weights were altered by tebuconazole treatment, but a dose–response relationship was not clear for most organs; only decreased kidney and increased liver weights were consistent in both sexes. Histological analyses were generally unremarkable outside of the brain. One month after the end of dosing, acquisition of learning the platform location in a water tank (i.e. Morris water maze) was impaired in the high-dose group; there were no differences in neuromuscular ability, motor activity or swim speed to account for this finding. Furthermore, there was no effect on recall of the position during a free-swim trial. Qualitative regional analysis of the brain indicated that the morphology was relatively normal across all regions with the exception of the neocortex and hippocampus. Neuropathological evaluations revealed pyknotic cells across hippocampal cell fields in animals of all tebuconazole treatment groups, with the highest incidence in the 20 and 60 mg/kg bw per day dose groups, coincident with cell loss within pyramidal cell layer of CA3-4 cell fields of the hippocampus and layer of the neocortex. Thus, perinatal exposure to tebuconazole produced neurobehavioural deficits and neuropathology in rats, but did not alter immunological or reproductive function.

The potential for developmental neurotoxicity produced by tebuconazole was investigated in two studies in rats: one by Bayer (Parker, 2000) and one by Moser et al. (2001). There were no neuropathological findings in the Bayer study (Parker, 2000), but Moser et al. (2001) reported that the neuropathological evaluations revealed pyknotic cells across hippocampal cell fields in animals of all tebuconazole treatment groups. An abbreviated neuropathology peer review was performed on slides of rat brain archived from the Moser et al. (2001) study. All available slides from rats in the high-dose and control groups were examined with knowledge as to treatment group. Although the slides were examined for the presence of any neuropathological alteration, particular emphasis was placed on scoring the numbers of dark neurons (referred to by Moser et al. [2001] as "pyknotic") in the hippocampus. Review of cresyl violet–stained sections from rats in the control and high-dose groups failed to confirm the presence of any treatment-related differences in the numbers of dark neurons within the hippocampus. Furthermore, the dark neurons present in the brain sections from this study are considered to be typical of those seen in association with handling artefact. Garman (2001) reported that "It is the opinion of this reviewing pathologist that it is inappropriate to refer to these dark neurons as 'pyknotic' and, furthermore, that these dark neurons are not indicative of any neuropathic process. This pathologist also found no evidence of any treatment-related neuron loss within the hippocampus".

These results from two studies in rats and neuropathology review were further analysed by Sheets (2002) to evaluate the potential of tebuconazole to produce neuropathology. Sheets (2002) concluded that, based on all of the available information, including that provided in the Moser et al. (2001) publication, Garman's (2001) review of the slides from that study and the absence of neuropathology in Bayer's developmental neurotoxicity study, there is no evidence that exposure to tebuconazole during development produces neuropathology at any dose level. The conclusion of the abbreviated neuropathology peer review regarding the Moser et al. (2001) study was that the dark neurons present in the brain sections were considered to be typical of those seen in association with handling artefact (Sheets, 2002). Subsequent to these findings by the neuropathology work group, Barone & Moser (2004) wrote a retraction "Letter to the Editor" about the findings in their original publication in 2001. Based on the above discussion, it is concluded that exposure to tebuconazole during development did not produce neuropathology at any dose level.

(d) Delayed neurotoxicity

No delayed toxicity studies were submitted.

(e) Combined toxicity

A study of acute oral toxicity was conducted to evaluate the potential potentiation effect of tebuconazole. An acute oral toxicity study in rats was conducted with tebuconazole plus triadimenol (combination) and tebuconazole plus dichlofluanid to evaluate the potential potentiation effect. Tebuconazole is a fungicide in the triazole class of fungicides, whereas triadimenol and dichlofluanid are fungicides belonging to the conazole class and phenylsulfamide class, respectively.

Groups of fasted adult male Bor:WISW (SPF Cpb) Wistar rats (five of each dose per group) were administered the test substance as a single dose by gavage formulated in Cremophor EL/demineralized water (2%). The dose volume was 10 ml/kg bw. The dosing regimen was as follows:

- Group 1 received tebuconazole (purity 94.7%) at a single dose of 5000 mg/kg bw.
- Group 2 received triadimenol (purity 97.1%) at a single dose of 710, 1000 or 1600 mg/kg bw.
- Group 3 received dichlofluanid (purity 99.4%) at a single dose of 5000 mg/kg bw.
- Group 4 received equitoxic doses (percentages based on the ratio of LD50 values) of a combination of tebuconazole (82.12%) and triadimenol (17.88%) at a single dose of 2000, 2240 or 3000 mg/kg bw.
- Group 5 received equitoxic doses of a combination of tebuconazole (50%) and dichlofluanid (50%) at a single dose of 5000 mg/kg bw.

The post-treatment observation period lasted 14 days. Clinical signs were recorded several times after dosing on day 1 and once a day thereafter. Body weight was recorded on the day of administration and on a weekly basis thereafter. Necropsy was performed at the end of the post-treatment period, and all animals were subjected to a gross pathological examination. Animals that died during the observation period were also subjected to a gross pathological examination.

The results of this study are summarized in Table 18.

Clinical signs such as bristled fur, pallor, apathy, reduced and later increased motility, spastic gait, staggering, cramps, slight convulsions, lateral recumbency, salivation, lacrimation, dyspnoea, diarrhoea and increased urine excretion were observed in these studies. Slight body weight loss was observed in these studies, but it was reversible until the end of the post-treatment observation period. Necropsy indicated changes in the lung (distended), liver (slight lobulation), spleen (pale), renal pelvis (dark red) and glandular stomach mucosa (reddened). No treatment-related macroscopic changes were observed in animals sacrificed at termination.

The results of these studies showed that equitoxic doses of tebuconazole and triadimenol administered orally to fasted rats resulted in a slightly potentiating effect. However, no potentiation was observed when equitoxic doses of tebuconazole and dichlofluanid were administered orally to fasted rats (Flucke, 1987).

(f) Cataract formation

Cats

Groups of four male and four female Forest of Dean cats received whole-body exposure to tebuconazole (purity 95.8%) in polyethylene glycol E 400 and ethanol at a mean analytical concentration of 61 or 309 mg/m^3 (99% of particles with an aerodynamic diameter of < 5 µm) for 6 hours per day for 4 weeks, followed by an observation period of 15 weeks. Control animals were exposed to aerosols of either the vehicle or about 20 mg/m^3 Scalex (KNJ 0953; positive controls for cataract).

Table 18. Acute toxicity and combined acute toxicity of tebuconazole

Dose (mg/kg bw)	Toxicological results[a]	Duration of clinical signs	Time of death	Mortality (%)
Group 1 tebuconazole				
5000	1/5/5	2 h – 12 days	6 days	20
LD_{50} > 5000 mg/kg bw				
Group 2 triadimenol				
710	1/5/5	30 min – 5 days	3 h	20
1000	2/5/5	20 min – 7 days	1–3 days	40
1600	4/5/5	40 min – 6 days	5 h – 2 days	80
LD_{50} 1089 mg/kg bw				
Group 3 dichlofluanid				
5000	0/5/5	7 h – 1 day	—	0
LD_{50} > 5000 mg/kg bw				
Group 4 tebuconazole (82.12%) + triadimenol (17.88%)				
2000	1/5/5	50 min – 8 days	4 days	20
2240	2/5/5	1 h – 8 days	1–6 days	40
3000	4/5/5	40 min –14 days	1–5 days	80
LD_{50} 3046 mg/kg bw (calculated); 2424 mg/kg bw (experimental) – slight potentiation				
Group 5 tebuconazole (50%) + dichlofluanid (50%)				
5000	2/5/5	3 h – 8 days	6 days	40
LD_{50} > 5000 mg/kg bw (calculated); approximately 5000 mg/kg bw (experimental) – no potentiation				

From Flucke (1987)

[a] Number of dead animals / number of animals with toxic signs / number of animals used.

During the weeks of exposure and observation period, body weights, clinical signs, mortality and ocular findings were recorded. At termination, gross pathological and histopathological examinations were performed.

Two low-dose males died during the study (death not attributed to test substance). At necropsy, it was found that the two male animals from the low-dose group, which died intercurrently, had thickening of the urinary bladder wall, mucous membrane inflammation, haemorrhage and haemorrhagic urine in the bladder. The treatment did not induce clinical symptoms and did not affect mortality rates or body weight gains. Cataracts due to lens fibre degeneration were found in all animals in the positive control group during the observation period of 4 months post-exposure. Exposure to tebuconazole did not result in cataract induction, but three females at 309 mg/m^3 and one positive control animal had yellow-tinged spots along the lens fissure. Examination of 42 untreated female cats aged 7–12 months showed that these ocular changes were not common spontaneous alterations; no such finding was seen in vehicle controls. The etiology of this finding and its toxicological relevance remain unclear. The 1994 JMPR established a NOAEL of 61 mg/m^3, equivalent to about 5 mg/kg bw per day, on the basis of ocular effects other than cataracts of unknown etiology at the highest concentration (Annex 1, reference *71*). The present Meeting affirmed the NOAEL of 61 mg/m^3 established by the JMPR in 1994 (Annex 1, reference *71*). The study authors established a NOAEL of 309 mg/m^3, the highest dose tested, for cataract development in cats (Märtins, Pauluhn & Kroetlinger, 1990).

Dogs

Groups of four female Beagle dogs were treated by head–nose exposure to tebuconazole (purity 97.1%) in polyethylene glycol and ethanol at a target aerosol concentration of 0, 150 or 800 mg/m^3 for 4 hours per day for 6 weeks and observed for 8 weeks. The analytical concentrations were 163 and

914 mg/m^3; about 90% of the particles had an aerodynamic diameter of less than 3 μm. No vehicle control group was included in the study. During the weeks of exposure and observation period, body weights, clinical signs, mortality, ocular findings, reflexes and feed intake were recorded, and lung function tests and blood examinations were performed. Measurements of the blood gases and the acid–base status were performed once near the end of exposure. At termination, organ weights, gross pathological and histopathological examinations were performed.

The treatment did not affect mortality rates. Most animals at 914 mg/m^3 began salivating immediately after exposure, and single animals also made tussive noises; these effects were reversed within 2 hours. In the reflex test, there were no treatment-related effects. A slight (statistically significant) drop in body temperature was recorded in both groups of exposed animals and was explained by the laboratory as being due to ethanol inhalation (central nervous system depression). Retardation of body weight gain was observed in both treated groups during the second half of the study, but with no dose–effect relationship. A lung function test revealed a marginal decrease in the mean minute volume in both treated groups and a slight reduction in the mean partial pressure of oxygen. The ophthalmic examinations showed no effects that could be related to treatment. Increased spleen weight observed at 914 mg/m^3 was the only change in organ weights. There were no deviations in other organ weights or other gross pathological changes at necropsy. No histopathological changes were recorded that could be related to inhalational treatment with tebuconazole.

The NOAEL was 163 mg/m^3, equivalent to 23 mg/kg bw per day, on the basis of clinical effects and questionable body weight effects seen at the LOAEL of 914 mg/m^3. The NOAEL for cataract induction was 914 mg/m^3, equivalent to 125 mg/kg bw per day (Märtins, 1991).

(g) Studies on metabolites

No toxicity studies were submitted; however, the registrant indicated that "Triazole fungicide metabolites were evaluated by the JMPR in 2008".

3. Observations in humans

In response to an inquiry, the Bayer company physician stated that "there are no known ill-effects on the health of the employees in the production of tebuconazole and production employees are subject to continuous medical supervision and are examined at least once annually" (Kollert, 1987). Bayer has monitored the health of personnel working in the production of tebuconazole since 1968. Routine occupational and medical examinations were conducted once a year, selected blood parameters were evaluated every 4 weeks, liver function tests were conducted every 2–3 years and thorax X-rays were performed every 3–6 years. Considering the occupational hygiene and use of personal protective equipment, the results of the monitoring programme indicate that no signs of changes in laboratory parameters, health impairments or permanent effects among employees have been recorded by the medical department (Faul & Krauthausen, 1995). Similarly, no ill-health effects have been reported in production employees during annual medical monitoring (including clinical chemistry, urinalysis, pulmonary functions, electrocardiogram) at the Kansas City, USA, plant since 1980 (Metz, Tice & Wey, 1996). In response to a separate inquiry, the company physician (J.D. Forbes) reported that "to date, with regard to our employees, we have had no reported overexposure situations nor have short-term or long-term health problems been identified which can be attributed to tebuconazole" during technical and formulation production (Wey & Forbes, 1997). No ill-health effects were reported in employees working in the production and formulation of tebuconazole in the Kansas City plant in the USA during annual routine checkups from 1991 to 2008. The medical examination included an annual checkup, clinical chemistry, blood counts, urinalysis, lung function testing, audiometry and vision testing. During the annual production period of 4–6 months per year, no worker accidents with tebuconazole occurred, and no consultations of the medical department due

to work or contact with tebuconazole were required (Steffens, 2009). Neumann & Hartmann (2009) reported that no relevant poisoning incidents were known to the company.

Comments

Biochemical aspects

In a toxicokinetic study, groups of male and female rats were given tebuconazole uniformly labelled with ^{14}C in either the phenyl ring or the 3,5-triazole ring as a single dose at 2 or 20 mg/kg bw or as 14 repeated doses of 2 mg/kg bw per day, followed by a single oral dose of radioactive tebuconazole at 2 mg/kg bw. Tebuconazole was rapidly absorbed from the gastrointestinal tract of rats and rapidly excreted from the body. Between 86% and 98% of the dose was excreted in the urine and faeces in 72 hours; most excretion occurred in the first 48 hours. Faecal excretion within 72 hours after administration was about 80% of the applied dose in males and about 65% in females; urinary excretion amounted to about 16% of the applied dose in males and about 33% in females. No significant differences in the absorption, distribution and excretion occurred following administration of a single oral low dose or high dose or repeated doses. Male rats with biliary fistulae excreted 90.7% of the dose with the bile, 7.4% in the urine and 1.5% in faeces within 48 hours, suggesting complete absorption of tebuconazole in intact rats. Only 0.3% of the radioactivity was detected in exhaled air within 72 hours following oral administration of tebuconazole. After 72 hours, less than 1% of the administered dose could be detected in the organs, tissues and the remaining carcass, indicating no potential for bioaccumulation. Highest residues were found in the liver and kidney. Tebuconazole was rapidly distributed (within 1 hour) in the body, as determined by whole-body autoradiography. The peak concentration of radioactivity in plasma was found at 0.33–1.7 hours. The terminal half-life of radiolabel was 31.9–52.5 hours.

Tebuconazole was extensively metabolized in the body following oral administration. Less than 0.7% of parent tebuconazole was detected in the excreta at 72 hours after administration. The metabolic pathway in rats also demonstrated sex-related differences. The main metabolites of tebuconazole in male rats were the oxidation products of one of the methyl groups of the tertiary butyl moiety (i.e. the alcohol and the carboxylic acid). Metabolism in female animals resulted preferentially in simple oxidation products (e.g. hydroxy and carboxy metabolites) and then conjugation to the glucuronide and sulfate, with only minor cleavage of the triazole moiety. In male animals, the primary oxidation products were further oxidized to triol and keto acid derivatives; in addition, cleavage of the triazole ring occurred, as indicated in trials with triazole-labelled compound. The free triazole accounted for about 5% of the administered dose in the urine of the males and 1.5% in that of females.

Toxicological data

Tebuconazole has low to moderate acute toxicity in mice and rats via the oral route. The oral LD_{50} of tebuconazole was 1700 and 4000 mg/kg bw in fasted female and male rats, respectively. The oral LD_{50} of tebuconazole in mice was 3023 and 1615 mg/kg bw in fasted female and male mice, respectively. The LD_{50} in rats treated dermally was greater than 2000 mg/kg bw. The LC_{50} in rats treated by inhalation (nose only) was greater than 0.82 mg/l. Tebuconazole was non-irritating to the eyes and skin of rabbits. Tebuconazole was not a skin sensitizer in guinea-pigs, as determined by the Magnusson & Kligman (maximization) test and the Buehler test.

In a non-GLP 28-day gavage study of toxicity in rats, decreases in haemoglobin concentration and haematocrit values were observed at 100 and 300 mg/kg bw per day. At 100 and 300 mg/kg bw per day, the absolute and relative weights of the liver and spleen were increased in both sexes, and the absolute weight of the kidney was increased in females. A reduced iron content was observed in

the spleen of females at 100 mg/kg bw per day. The NOAEL in the 28-day gavage study in rats was 30 mg/kg bw per day, on the basis of changes in haematological and clinical chemistry parameters and organ weights at 100 mg/kg bw per day. In a 90-day dietary toxicity study in rats, reduced body weight gain was observed at 400 ppm in females during the first 6 weeks. Histopathological examination revealed an increased incidence of intraplasmatic vacuoles in the cells of the zona fasciculata of the adrenals (probably lipid accumulation) in some females at 400 ppm and in all females at 1600 ppm. The NOAEL was 100 ppm (equal to 10.8 mg/kg bw per day), based on a reduction in body weights in females at 400 ppm (equal to 46.5 mg/kg bw per day).

The NOAEL in a 90-day dietary study of toxicity in dogs was 200 ppm (equal to 8.5 mg/kg bw per day), based on decreased body weight gain and feed consumption at 1000 ppm (equal to 41 mg/kg bw per day). Two 1-year dietary studies of toxicity were conducted in dogs with tebuconazole. The overall NOAEL was 100 ppm (equal to 2.9 mg/kg bw per day), based on intracytoplasmic vacuoles in cells of the zona fasciculata of the adrenals and slight hypertrophy accompanied by an increased incidence of large fatty vacuoles seen at 150 ppm (equal to 4.4 mg/kg bw per day) and above.

The carcinogenic potential of tebuconazole was studied in mice and rats. Two carcinogenicity studies were conducted in mice. In the first study, the NOAEL was 20 ppm (equal to 5.9 mg/kg bw per day), based on the increased incidence of centrilobular fine vacuolization in the liver of males at 60 ppm (equal to 18 mg/kg bw per day). There was no evidence of any carcinogenic potential, but the effects on the liver at the LOAEL and above were not very marked in intensity, posing a question as to whether an MTD had been reached in this study. Therefore, a second carcinogenicity study was conducted at higher doses. In the second study, no NOAEL was identified. The LOAEL was 500 ppm (equal to 85 mg/kg bw per day), based on liver toxicity. The incidence of liver tumours in male and female mice was significantly elevated at 1500 ppm (equal to 279 mg/kg bw per day) and was markedly above the range of spontaneous incidences observed in this mouse strain.

In the carcinogenicity study in rats, the NOAEL was 300 ppm (equal to 15.9 mg/kg bw per day), based on body weight depression in both sexes and an increased incidence of pigment deposits in the Kupffer cells in the liver of females at 1000 ppm (equal to 55 mg/kg bw per day). No treatment-related tumours were observed.

Tebuconazole was not genotoxic in an adequate range of in vitro and in vivo genotoxicity tests.

The Meeting concluded that tebuconazole is unlikely to be genotoxic.

In view of the absence of genotoxic potential, the absence of carcinogenicity in rats and no carcinogenicity in mice relevant to human dietary exposure levels, the Meeting concluded that tebuconazole is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproductive toxicity in rats, the reproductive parameters were not affected at doses up to 1000 ppm (equal to 72.3 mg/kg bw per day), the highest dose tested. The NOAEL for parental systemic toxicity and offspring toxicity was 300 ppm (equal to 21.6 mg/kg bw per day), based on reduced feed consumption and decreased body weights in parental animals and pups seen at 1000 ppm (equal to 72.3 mg/kg bw per day).

Several developmental toxicity studies in mice, rats and rabbits using gavage administration were submitted. The overall NOAEL for maternal toxicity in the oral gavage studies in mice, rats and rabbits was 30 mg/kg bw per day, mainly based on decreases in body weights and body weight gains (during the early treatment period) at 100 mg/kg bw per day. Marginal effects in studies in mice (haematological effects) and rats (reduced body weight gains) were not considered as adverse. Selected liver parameters (enzymes, weights and clinical chemistry) were evaluated in developmental toxicity studies in mice and rats. Changes in the liver parameters in these studies were considered an adaptive response and not considered as adverse. In one study in mice, there was an increase in the number of small fetuses (runts) at doses of 30 mg/kg bw per day and above. These small fetuses,

defined on the basis of low body weights, were considered unlikely to be due to a single exposure or a small number of exposures. The NOAEL for developmental toxicity in mice was 10 mg/kg bw per day. In other studies in mice, rats and rabbits, developmental effects included increased resorptions, a decreased number of live fetuses, decreased fetal weights, incomplete ossification and visceral and skeletal anomalies. In addition, postimplantation loss was observed in mice. These developmental effects were observed consistently at doses above 30 mg/kg bw per day and in the presence of maternal toxicity in all studies. The overall NOAEL for developmental toxicity was 30 mg/kg bw per day in rats and rabbits.

The Meeting concluded that tebuconazole caused developmental toxicity and teratogenic effects at doses that were maternally toxic in rats and rabbits.

In a study of acute neurotoxicity in rats with tebuconazole, the NOAEL was 50 mg/kg bw based on increased motor activity in male and female rats and decreased footsplay in female rats at 100 mg/kg bw. In a 90-day study of neurotoxicity in rats, no systemic or neurotoxic effects were seen at doses up to 1600 ppm (equal to 107 mg/kg bw per day), the highest dose tested. In a developmental neurotoxicity study in rats with dietary administration, the maternal NOAEL was 300 ppm (equal to 22 mg/kg bw per day), based on decreased body weights, body weight gains and feed consumption, prolonged gestation with mortality and an increased number of dead fetuses at 1000 ppm (equal to 65 mg/kg bw per day). The offspring toxicity NOAEL was 300 ppm (equal to 22 mg/kg bw per day), based on decreased pup viability, decreases in body weights and absolute brain weights, brain measurements and evidence of developmental delays seen at 1000 ppm (equal to 65 mg/kg bw per day), the highest dose tested. Tebuconazole did not produce neurobehavioural or neuropathological changes.

Workers did not report any adverse effects while handling tebuconazole in a production facility. The workers were monitored by routine physical examination and clinical chemistry measurements.

The Meeting concluded that the existing database on tebuconazole was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.03 mg/kg bw on the basis of an overall NOAEL of 2.9 mg/kg bw per day in two 1-year dietary toxicity studies in dogs, based on histopathological alterations in the adrenals seen at the LOAEL of 4.4 mg/kg bw per day, and using a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw on the basis of a maternal and developmental toxicity NOAEL of 30 mg/kg bw per day in studies of developmental toxicity in rats and rabbits based on maternal toxicity manifested as decreases in body weight gains in the early treatment period and visceral and skeletal anomalies seen at higher doses. The increased incidence of small fetuses, defined on the basis of low body weights, was considered unlikely to be due to a single exposure or a small number of exposures. The ARfD is supported by the NOAEL of 30 mg/kg bw per day observed in a 28-day oral (gavage) toxicity study in rats based on changes in haematological parameters seen at the LOAEL of 100 mg/kg bw per day, which might be produced by a single dose.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Twenty-one-month studies of toxicity and carcinogenicity[a,b]	Toxicity	20 ppm, equal to 5.9 mg/kg bw per day	60 ppm, equal to 18 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 85 mg/kg bw per day	1500 ppm, equal to 279 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
	Developmental toxicity[c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
Rat	Twenty-eight-day study of toxicity[c]	Toxicity	30 mg/kg bw per day	100 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[b]	Toxicity	300 ppm, equal to 15.9 mg/kg bw per day	1000 ppm, equal to 55 mg/kg bw per day
		Carcinogenicity	1000 ppm, equal to 55 mg/kg bw per day[d]	—
	Two-generation study of reproductive toxicity[a]	Parental toxicity	300 ppm, equal to 21.6 mg/kg bw per day	1000 ppm, equal to 72.3 mg/kg bw per day[d]
		Offspring toxicity	300 ppm, equal to 21.6 mg/kg bw per day	1000 ppm, equal to 72.3 mg/kg bw per day[d]
		Reproductive toxicity	1000 ppm, equal to 72.3 mg/kg bw per day[d]	—
	Developmental toxicity[c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
Rabbit	Developmental toxicity[c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
Dog	Two 1-year studies of toxicity[a,b]	Toxicity	100 ppm, equal to 2.9 mg/kg bw per day	150 ppm, equal to 4.4 mg/kg bw per day

[a] Dietary administration.
[b] Two or more studies combined.
[c] Gavage administration.
[d] Highest dose tested.

Estimate of acceptable daily intake for humans

0–0.03 mg/kg bw

Estimate of acute reference dose

0.3 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to tebuconazole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Complete and rapid
Dermal absorption	Not available
Distribution	Extensive
Potential for accumulation	None
Rate and extent of excretion	Rapid and extensive
Metabolism in animals	Extensive; metabolic pathways include hydrolysis, oxidation and conjugation

Toxicologically significant compounds in animals, plants and the environment	Tebuconazole and 1,2,4-triazole
Acute toxicity	
Rat, LD_{50}, oral	1700 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 0.82 mg/l, dust (4 h exposure, nose only)
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Guinea-pig, dermal sensitization	Not a sensitizer (Magnusson & Kligman and Buehler tests)
Short-term studies of toxicity	
Target/critical effect	Adrenals/hypertrophy of zona fasciculata cells (dogs)
	Liver, blood system and adrenals (rats)
Lowest relevant oral NOAEL	2.9 mg/kg bw per day (overall from two 1-year toxicity studies in dogs)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rabbits)
Lowest relevant inhalation NOAEC	0.0156 mg/l
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver toxicity (mice and rats)
Lowest relevant NOAEL	5.9 mg/kg bw per day (carcinogenicity study in mice)
Carcinogenicity	Not carcinogenic in rats, but hepatocarcinogenic in mice; unlikely to pose a carcinogenic risk at human dietary exposure levels
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	No reproductive effects
Lowest relevant reproductive NOAEL	1000 ppm, equal to 72.3 mg/kg bw per day, highest dose tested (rats)
Developmental target/critical effect	Developmental toxicity, including teratogenicity, only at maternally toxic doses in rats and rabbits
Lowest relevant developmental NOAEL	30 mg/kg bw per day (rats, rabbits); 10 mg/kg bw per day (mice; runts)
Neurotoxicity/delayed neurotoxicity	
Acute neurotoxicity	Increased motor activity in rats
Subchronic neurotoxicity	No neurotoxicity in rats
Developmental neurotoxicity	No neurodevelopmental toxicity in rats
Other toxicological studies	
	None
Medical data	
	No adverse effects reported

Summary

	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Two 1-year toxicity studies in dogs	100
ARfD	0.3 mg/kg bw	Developmental toxicity studies in rats and rabbits, supported by a 28-day study of toxicity in rats (gavage)	100

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THIAMETHOXAM

First draft prepared by
D.B. McGregor[1] and Roland Solecki[2]

[1] Toxicity Evaluation Consultants, Aberdour, Scotland
[2] Federal Institute for Risk Assessment, Berlin, Germany

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Explanation

Thiamethoxam is the International Organization for Standardization (ISO)–approved name for (*EZ*)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) No. 153719-23-4. It is a neonicotinoid insecticide active against a broad range of commercially important sucking and chewing pests. The biological effects of this chemical class in target species are mediated primarily by an interaction with nicotinic acetylcholine receptor sites.

Thiamethoxam is being reviewed for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies complied with good laboratory practice (GLP). Non-GLP studies were identified as such.

Evaluation for acceptable daily intake

1. Biochemical aspects

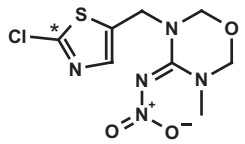
Kinetic studies on the absorption, distribution, metabolism and excretion of thiamethoxam were conducted in both mice and rats. This unusual extension of such studies to mice was considered necessary because neoplastic responses were observed in this species and it was believed that the additional information may go some way towards explaining the differences in response between mice and rats.

Mice

The studies in mice with thiamethoxam (identified in the original studies as CGA 293343) were mainly confined to males using unlabelled material (batch No. AMS 780/101; purity 99.3%) and a single, site-specifically radiolabelled thiamethoxam preparation: [thiazol-2-^{14}C]thiamethoxam (batch No. ILS-208.1; radiochemical purity > 99.4%) (Mewes, 1998; Briswalter, 1999) or [oxadiazin-4-^{14}C]thiamethoxam (batch No. ILS-235.1A; radiochemical purity 98.9%) (Mewes, 2000; Briswalter 2002a,c,d). Label positions were as shown in Figure 1.

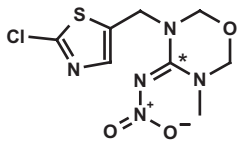
Figure 1. Structures of the radiolabelled molecules

[Thiazol-2-^{14}C]



* = ^{14}C position

[Oxadiazin-4-^{14}C]



* = ^{14}C position

The oral gavage administrations to primarily male mice included the following daily dosing regimens:

- [thiazol-2-^{14}C]thiamethoxam at 118 mg/kg bw for 14 consecutive days for the study of excretion and metabolism (Mewes, 1998) or for the identification of additional metabolites (Briswalter, 1999);
- dietary exposure to unlabelled thiamethoxam at a concentration of 0, 100, 500 or 2500 parts per million (ppm) for 29 days followed by one oral gavage dose of [oxadiazin-4-^{14}C]thiamethoxam at 10 mg/kg bw and a second oral gavage dose of the labelled compound 72 hours later for the study of absorption, metabolism and excretion (Mewes, 2000);

- a single oral gavage dose of [oxadiazin-4-^{14}C]thiamethoxam at 105.7 mg/kg bw for the study of blood kinetics and the identification of blood metabolites (Briswalter, 2002c);
- single oral doses of [oxadiazin-4-^{14}C]thiamethoxam at either 0.5 or 100 mg/kg bw on a single occasion for the study of metabolism (Briswalter, 2002a) and for the study of absorption, distribution and excretion (Briswalter 2002d).

Rats

The studies in rats with thiamethoxam were performed in young male and female Tif:RAIf(SPF) rats using unlabelled material (batch No. KI-4654-18, purity > 98%; and batch No. AMS 780/101, purity > 99%) and two different, site-specifically radiolabelled thiamethoxam preparations: [thiazol-2-^{14}C]thiamethoxam (radiochemical purity > 97%) and [oxadiazin-4-^{14}C]thiamethoxam (radiochemical purity > 96% or > 97%) (Müller & Stampf, 1996) (Figure 1).

The administrations to rats included single oral doses to males and females of either 0.5 or 100 mg/kg bw of either radiolabelled material or 14 daily oral doses of 0.5 mg/kg bw per day of the unlabelled compound followed by a single oral dose of 0.5 mg/kg bw of radiolabelled material.

Studies in rats included a single oral gavage dose of [oxadiazin-4-^{14}C]thiamethoxam at 106.5 mg/kg bw for the study of blood kinetics (Briswalter, 2002b).

The experimental programme also included investigation of bile duct–cannulated male rats and intravenously dosed male and female rats. Metabolism studies of specific metabolites are described below (see section 1.2). Urine, faeces, bile and expired air were individually and separately collected. For the kinetic studies in blood, samples were taken from three rats of each group and each sex by amputating the tip of the tail. Radioactivity in urine, plasma and other liquid samples was measured directly by liquid scintillation counting. Radioactivity in other collected materials was determined either after combustion or after digestion and liquid scintillation counting. Expired carbon dioxide was absorbed into a mixture of ethanolamine/ethylene glycol monomethyl ether 1:2 (volume per volume).

1.1 Absorption, distribution and excretion

Mice

Male mice received daily oral doses of [thiazol-2-^{14}C]thiamethoxam at a dose level of 118 mg/kg bw for 14 consecutive days (Mewes, 1998) (note that this dosing regimen is different from that used on mice in the [oxadiazin-4-^{14}C]thiamethoxam study and rats in both the [thiazol-2-^{14}C]thiamethoxam and the [oxadiazin-4-^{14}C]thiamethoxam studies, in which there were 14 days of dietary dosing with unlabelled thiamethoxam before gavage dosing with the radiolabelled compounds). Urine and faeces were collected daily until 72 hours after the last dose and were pooled at selected time intervals for analysis. Expired carbon dioxide was trapped during the first 3 days of the treatment.

Oral doses of thiamethoxam were apparently rapidly absorbed in mice, as administration was followed by its ready and rapid excretion, approximately 73% of the first dose appearing in urine and faeces within 24 hours. Only minor amounts of radioactivity were found in the excreta on days 2 and 3 after the last administration (time range of 360–384 hours). The administered radioactivity was predominantly excreted via the kidneys. A total of 71.8% of the administered dose was recovered in the urine, and 18.8% in the faeces. Low amounts of radioactivity were found in expired air (< 0.3% of the daily administered dose). A steady state of excretion was reached within 3 days after the first administration. The rates of excretion as percentages of the daily and cumulative doses are summarized in Table 1.

The quantitative analysis of excreted radioactivity in the urine and faeces of mice dosed with [thiazol-2-^{14}C]thiamethoxam (Mewes, 1998) indicated that 30–60% of the daily administered

Table 1. The rate of excretion of radioactivity as percentages of the daily and cumulative doses of [thiazol-2-^{14}C]thiamethoxam administered to male mice

Time point (h)	Percentage of daily dose		Percentage of cumulative dose	
	Mean	SD	Mean	SD
Urine				
0–24	66.03	14.69	5.29	1.18
24–48	58.83	9.75	4.65	0.27
48–72	69.01	3.13	5.49	0.63
72–96	80.32	3.51	5.92	0.87
96–120	56.85	3.46	4.18	0.64
120–144	56.51	9.28	4.18	1.14
144–168	66.91	8.57	4.95	1.16
168–192	71.75	5.69	4.71	0.15
192–216	64.14	12.20	4.25	0.57
216–240	84.13	3.96	5.59	0.36
240–264	81.17	13.12	5.37	0.65
264–288	88.02	8.38	5.91	0.56
288–312	77.77	8.59	5.19	0.19
312–336	78.71	19.18	5.19	1.12
336–360[a]	9.60	2.08	0.63	0.10
360–384[a]	3.76	0.40	0.25	0.04
Subtotal	—	—	71.75	3.50
Faeces				
0–24	6.99	1.35	0.56	0.10
24–48	34.98	11.36	2.86	1.17
48–72	14.41	3.65	1.13	0.23
72–96	9.93	1.42	0.73	0.14
96–120	18.73	3.83	1.37	0.29
120–144	21.26	11.10	1.57	0.83
144–168	16.54	7.86	1.21	0.56
168–192	14.89	2.63	0.99	0.22
192–216	19.32	4.27	1.30	0.38
216–240	27.44	8.65	1.84	0.64
240–264	18.55	4.08	1.25	0.37
264–288	14.09	3.46	0.96	0.31
288–312	17.60	7.57	1.20	0.56
312–336	17.29	10.21	1.17	0.77
336–360[a]	6.80	6.03	0.47	0.44
360–384[a]	3.07	3.25	0.21	0.24
Subtotal	—	—	18.84	3.46
Expired air				
0–24	0.17	0.01	0.01	< 0.01
24–48	0.25	0.06	0.02	< 0.01
48–72	0.26	0.03	0.02	< 0.01
Subtotal	—	—	0.06	< 0.01
Cage wash	—	—	3.81	0.31
Total recovery	—	—	94.46	1.27

From Mewes (1998)

[a] % of daily dose calculations are based on administered radioactivity at 312 h.

Table 2. Excretion of [oxadiazin-4-^{14}C]thiamethoxam administered to male mice after 29 days of dietary exposure to unlabelled thiamethoxam

Time point (h)	% of cumulative radioactive dose			
	Dietary concentration (ppm)			
	0	100	500	2500
Urine				
0–24	33.57	32.99	27.67	35.14
24–48	0.99	1.14	0.76	1.16
48–72	0.83	0.45	0.34	1.24
72–78	12.15	16.63	7.83	13.47
Subtotal	47.54	51.22	36.60	51.01
Faeces				
0–24	10.34	9.02	14.78	10.67
24–48	1.26	1.91	1.94	0.80
48–72	2.52	0.87	1.08	1.42
72–78	5.39	4.42	8.27	6.63
Subtotal	19.52	16.22	26.08	19.52
Cage wash				
78	10.74	7.23	8.14	8.02
Total excretion	77.79	74.67	70.82	78.56
Liver	0.43	0.73	0.72	0.53
Blood	0.08	0.11	0.11	0.09
Carcass	9.19	13.05	14.44	14.81
Subtotal	9.70	13.89	15.28	15.43
Total recovery	87.49	88.55	86.10	93.99

From Mewes (2000)

thiamethoxam was excreted as metabolites. As will be seen below (see section 1.2), this metabolism is much more extensive than in rats.

The effect of dietary administration of unlabelled thiamethoxam on the absorption, metabolism and excretion of [oxadiazin-4-^{14}C]thiamethoxam was studied in male and female mice (Mewes, 2000). The mice received the unlabelled compound at a dietary concentration of 0, 100, 500 or 2500 ppm for 29 days before receiving two oral doses of [oxadiazin-4-^{14}C]thiamethoxam, 10 mg/kg bw, 72 hours apart. Mice were killed 6 hours after the second radioactive dose. Excretion of radioactivity was very rapid at all dietary concentrations and was predominantly via the urine, and the route and rate of excretion were independent of the dietary dose level. Excretion was virtually complete by 72 hours after oral administration of a single oral dose of [oxadiazin-4-^{14}C]thiamethoxam, with approximately 70% of the dose being recovered in urine and a further 25% of the dose being recovered in faeces (Table 2). This result was similar to that found with the [thiazol-2-^{14}C]thiamethoxam label.

Considerable amounts of radioactivity were recovered in the liver, blood and carcass at termination (approximately 0.6%, 0.1% and 13% of the cumulative radioactive dose, respectively), as the mice were killed only 6 hours after administration of the second oral dose of [oxadiazin-4-^{14}C]-thiamethoxam. Very little radioactivity was recovered in the bile. Residues in tissues were similar at all dietary dose levels, with residues in blood and plasma amounting to about 1 µg-equivalents/g, and residues in liver amounting to 1.7–3.3 µg-equivalents/g.

Table 3. Absorption of radioactivity by rats after a single oral administration of [thiazol-2-^{14}C]-thiamethoxam or [oxadiazin-4-^{14}C]thiamethoxam

	% of dose											
	[Thiazol-2-^{14}C]thiamethoxam							[Oxadiazin-4-^{14}C]thiamethoxam				
	Low dose × 1[a]		Low dose × 14[a]		High dose × 1[a]		Low dose × 1 bile[b]	Low dose × 1[a]		High dose × 1[a]		Low dose × 1 bile[b]
	Male	Female	Male	Female	Male	Female	Male	Male	Female	Male	Female	Male
	Dose (mg/kg bw)											
	0.54	0.56	0.42	0.44	91.2	98.9	0.49	0.44	0.46	100.9	104.2	0.48
Urine	91.3	93.0	96.2	94.7	95.5	96.5	81.4	92.9	95.7	96.9	99.2	86.8
Bile	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.9	n.a.	n.a.	n.a.	n.a.	4.5
Tissues	0.4	0.3	0.4	0.5	0.3	0.3	1.5	0.3	0.2	0.3	0.7	1.6
Sum	91.7	93.3	96.6	95.2	95.8	96.8	86.8	93.2	95.9	97.2	99.9	92.9

From Müller & Stampf (1996)
n.a., not applicable (not collected)
[a] 168 h time interval.
[b] Bile duct–cannulated male rats, 48 h time interval.

Rats

Orally administered thiamethoxam was rapidly absorbed, and, as almost 100% of the oral doses were eliminated with the urine in all treatment groups, it can be assumed that thiamethoxam was virtually completely absorbed from the gastrointestinal tract irrespective of the dose level, pretreatment with non-radiolabelled thiamethoxam, the site of the label or the sex of the rats. The experiment with bile duct cannulation of male rats demonstrated that only a very small portion of the absorbed dose (approximately 4%) was excreted with the bile fluid into the duodenum (Table 3).

The routes of elimination were independent of the route of administration (oral and intravenous), the dose level, pretreatment with non-radiolabelled thiamethoxam, the site of the label and the sex of the rats. The absorbed amount was very rapidly excreted almost exclusively via the kidneys: within 24 hours, 84–95% and 3–6% of the dose were excreted in the urine and faeces, respectively. The bile duct–cannulated male rats excreted about 4%, 84% and 4% in bile, urine and faeces, respectively, within 48 hours, independent of the site of the radiolabel. Hence, these results obtained with bile duct–cannulated rats and with intravenously administered rats clearly show that the small amount eliminated with the faeces is derived from bile, providing strong evidence for the complete absorption of thiamethoxam from the gastrointestinal tract (Table 4).

After intravenous dosing, maximal blood concentrations averaged around 0.6 ppm thiamethoxam equivalents at the low dose. After oral dosing, maximal radioactivity in the blood was reached between 1 and 4 hours after administration in male and female rats independent of the dose level and the label; maximal concentrations averaged 0.18 and about 37 ppm thiamethoxam equivalents at the low and high doses, respectively. The radioactivity in the blood decreased rapidly, reaching half the maximal values 2–3 hours after intravenous administration and about 7 hours after oral administration in both sexes.

After oral administration, the areas under the concentration in blood–time curve ($AUC_{0-24\ h}$) were in the same range for both labels and both sexes, accounting for an average of 1.4 and 318 µg·h/g at the low and high dose levels, respectively. Comparisons of AUCs at the low and high doses indicate a linear relationship of the bioavailability with dose in the range 0.5–100 mg/kg bw. Independent of the dose and the labels used, the bioavailability determined by the $AUC_{0-24\ h}$ ratio after

Table 4. Excretion of radioactivity following a single intravenous or oral administration of thiamethoxam

Time point (h)	% of dose													
	[Thiazol-2-^{14}C]thiamethoxam									[Oxadiazin-4-^{14}C]thiamethoxam				
	Low dose × 1 iv		Low dose × 1 po		Low dose × 14 po		High dose × 1 po		Low dose × 1 po bile	Low dose × 1 po		High dose × 1 po		Low dose × 1 po bile
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Male	Female	Male	Female	Male
	Dose (mg/kg bw)													
	0.51	0.55	0.54	0.56	0.42	0.44	91.24	98.87	0.49	0.44	0.46	100.89	104.16	0.48
Urine														
0–24	83.8	89.1	87.8	88.2	94.0	92.3	92.9	93.0	78.6	89.6	92.5	92.0	95.2	77.4
24–48	1.7	2.1	1.8	2.2	1.3	1.3	1.5	2.1	2.9	1.8	1.4	2.8	2.2	9.4
48–168	1.3	1.6	1.7	2.6	0.8	1.0	1.1	1.4	n.a.	1.6	1.8	2.1	1.7	n.a.
Subtotal	86.8	92.7	91.3	93.0	96.2	94.7	95.5	96.5	81.4	92.9	95.7	96.9	99.2	86.8
Bile														
0–24	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.8	n.a.	n.a.	n.a.	n.a.	4.1
24–48	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.1	n.a.	n.a.	n.a.	n.a.	0.3
Subtotal	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.9	n.a.	n.a.	n.a.	n.a.	4.5
Faeces														
0–24	4.4	2.5	4.4	2.6	6.2	3.9	3.4	3.4	3.8	4.1	2.7	4.3	2.6	2.4
24–48	0.8	0.5	0.6	0.5	0.5	0.4	1.4	0.7	1.0	0.5	0.7	1.0	0.8	1.1
48–168	0.3	0.3	0.2	0.3	0.2	0.1	0.3	0.3	n.a.	0.4	0.7	0.4	0.6	n.a.
Subtotal	5.5	3.2	5.2	3.4	6.8	4.4	5.1	4.4	4.8	5.1	4.0	5.7	4.0	3.5
Expired air														
0–48	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.2	0.1	n.a.	n.a.	n.a.	0.1	0.1	n.a.
Cage wash	0.8	1.1	4.8	3.9	0.3	0.5	0.2	0.5	4.4	0.8	1.7	0.3	0.3	2.3
Total excretion	93.1	97.0	101.3	100.2	103.3	99.6	101.0	101.5	94.5	98.8	101.4	102.9	103.5	97.2

From Müller & Stampf (1996)

iv, intravenous; n.a., not applicable; po, by mouth (per os)

Table 5. Blood kinetics following single intravenous or oral administrations to rats of [thiazol-2-^{14}C]thiamethoxam or [oxadiazin-4-^{14}C]thiamethoxam

	[Thiazol-2-^{14}C]thiamethoxam						[Oxadiazin-4-^{14}C]thiamethoxam			
	Low dose × 1 iv		Low dose × 1 po		High dose × 1 po		Low dose × 1 po		High dose × 1 po	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
	Dose (mg/kg bw)									
	0.51	0.54	0.55	0.55	91.8	100.7	0.44	0.46	101.8	104.0
C_{max} (ppm)	—	—	0.174	0.168	43.22	34.45	0.201	0.186	35.74	32.95
T_{max} (h)	0.25	0.25	4	2	2	1	2	1	4	1
T_{max}/2 (h)	3	2	8	8	7	7	6	5	9	8
$AUC_{0-24\,h}$ (µg·h/g)	2.5	1.7	1.6	1.6	344.9	263.8	1.3	1.1	367.1	296.6
Bioavailability (%)	100	100	60	90	80	80	60	70	80	90

From Müller & Stampf (1996)

C_{max}, maximum concentration in plasma; iv, intravenous; po, by mouth (per os); T_{max}, time to reach the maximum concentration in plasma

oral and intravenous administration was about 0.7 and 0.8 for male and female rats, respectively. The major results are summarized in the Table 5.

Depletion from the broad range of tissues sampled is assumed to follow first-order kinetics. The half-lives in all tissues were in the range of 2–6 hours, independent of the dose level, the site of the label and the sex of the rats. Tissue residues were determined in male and female rats at four different time points after oral administration of [thiazol-2-^{14}C]thiamethoxam at both dose levels and [oxadiazin-4-^{14}C]thiamethoxam at the low dose level. As a consequence of the rapid depletion, the tissue residues were very low 7 days after oral administration of the low dose (0.5 mg/kg bw) when the tissue residues did not exceed 0.0033 ppm thiamethoxam equivalents (found in liver). The other tissue residues were close to or even below the limit of quantification. In some tissues (e.g. bone), the residues were below the limit of detection. At the low dose level, the limits of quantification were 0.0004–0.008 ppm for blood, brain, fat, heart, kidneys, liver, muscle, plasma, spleen and testes, 0.006–0.0011 ppm for bone, lungs and uterus and 0.0024–0.0031 ppm for ovaries. Treatment for 14 consecutive days with oral administrations of non-radiolabelled thiamethoxam (0.5 mg/kg bw) followed by a single oral dose of [thiazol-2-^{14}C]thiamethoxam did not influence the pattern of tissue residues.

After oral administration of the high dose (100 mg/kg bw), the concentrations in the tissues were proportionally (i.e. about 200 times) higher. The highest concentrations in tissues were again found in the liver. The liver residues in male rats were slightly higher after administration of the [oxadiazin-4-^{14}C] label (i.e. 0.56 ppm thiamethoxam equivalents) than after dosing of the [thiazol-2-^{14}C] label (0.37 ppm thiamethoxam equivalents), whereas female rats showed similar concentrations after receiving either label; however, as the residue levels in all the other tissues at the high dose and in all tissues at the low dose were essentially the same for both labels, a significant label difference cannot be reliably identified. Following liver, the tissues with the next highest concentration tended to be either kidney or blood.

1.2 Metabolism

Mice

No qualitative or quantitative differences were seen in the urinary metabolite profiles among the different dietary dose levels (Mewes, 2000). Unchanged thiamethoxam and the metabolites

CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] represented the majority of the radioactivity in urine, accounting for 36–44%, 10–15% and 8–11% of the dose, respectively. The minor metabolite CGA 353968 [1-(2-chlorothiazol-5-ylmethyl)-3-methylurea] accounted for 0.7–1.3% of the dose. Four other unidentified fractions (R1–R4) each accounted for 0.5–2% of the dose.

Minor metabolites that were detected in mouse urine are NOA 402988 [2-methylsulfanylthiazole-5-carboxylic acid], NOA 407475 [3-(2-chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylideneamine], NOA 404617 [1-(2-chlorothiazol-5-ylmethyl)-3-nitrourea], 4U [(2-chlorothiazol-5-ylmethyl)-urea], 6U [2-acetylamino-3-(2-chlorothiazol-5-ylmethylsulfanyl)-propionic acid], NOA 421275 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylguanidine] and NOA 421276 [*N*-(2-chlorothiazol-5-ylmethyl)-guanidine]. In addition to these metabolites, two unknown non-polar metabolites (R14 and R15) were detected in mouse urine.

Approximately 90% of the radioactivity in the faeces was extractable, and the metabolites in the faecal extracts were the same as in the urine. No qualitative or quantitative differences were seen in the metabolite profiles of the faecal extracts among the different dietary dose levels. Unchanged thiamethoxam and the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] represented the majority of the radioactivity in urine, accounting for 8–14%, 4–6% and 3–7% of the dose, respectively. The minor metabolite CGA 353968 [1-(2-chlorothiazol-5-ylmethyl)-3-methylurea] accounted for 0.3–0.4% of the dose. Four other unidentified fractions (R1–R4) each accounted for 0.5–2.5% of the dose.

Two-dimensional thin-layer chromatographic analysis of the faecal extracts revealed the presence of unchanged thiamethoxam and the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-N″-nitroguanidine], CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine], NOA 407475 [3-(2-chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylideneamine], NOA 402988 [2-methylsulfanylthiazole-5-carboxylic acid], NOA 421275 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylguanidine] and NOA 421276 [*N*-(2-chlorothiazol-5-ylmethyl)-guanidine]. Metabolite R6, accounting for 1.9–2.6% of the daily dose in mouse faeces, was isolated, with a structure proposed as 2-acetylamino-3-[5-(5-methyl-4-nitroimino-[1,3,5]oxadiazinan-3-ylmethyl)-thiazol-2-ylsulfanyl]-propionic acid, based on results from mass spectrometry. Quantitative aspects of these results are presented in section 1.3, in comparison with the findings from rats.

Bile metabolite profiles showed no qualitative or quantitative differences among the different dietary dose levels. The majority of the radioactivity in bile consisted of very polar metabolites, the most important of which was metabolite M04, representing 14–22% of the dose. Unchanged CGA 293343 and the metabolites CGA 322704 and CGA 265307 each accounted for 3–6% of the dose.

Plasma metabolite profiles also showed no qualitative or quantitative differences among the different dietary dose levels. Unchanged thiamethoxam and the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] represented the majority of the radioactivity in plasma, accounting for 17–26%, 19–26% and 43–50% of the dose, respectively.

Radioactive residues in the liver accounted for 0.4–0.7% of the total dose, and approximately 90% of this radioactivity was extractable. No qualitative or quantitative differences were seen in the metabolite profiles of the liver extracts among the different dietary dose levels. Unchanged thiamethoxam and the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] each accounted for 0.3–2.2% of the dose. Although most of the radioactivity in liver consisted of very polar metabolites, even the more prevalent of these amounted to less than 0.25%, individually, of the total radioactive dose.

The studies of Mewes (1998, 2000) and Briswalter (1999) demonstrated that the major reaction involved in the biotransformation of thiamethoxam in mice is the cleavage of the oxadiazine ring to result in the corresponding nitroguanidine compound, which is followed by its demethylation. In this way, the two major metabolites, CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine], are formed. Another important pathway is the substitution of the chlorine of the thiazole ring in thiamethoxam by glutathione, followed by its subsequent and complex degradation. Minor pathways (< 1% of the daily dose for each metabolite) include the cleavage of the nitroguanidine group from the oxadiazine ring of thiamethoxam to result in the corresponding guanidine. The guanidine metabolite can then be demethylated and hydrolysed, the latter to form a urea derivative. As these compounds retain the chlorine, this can react with glutathione to add more metabolites and increase complexity.

Substitution of the chlorine of the thiazole ring in thiamethoxam by glutathione and subsequent reactions on the glutathione moiety resulted in metabolite R6 found in mouse faeces. Minor pathways (< 1% of the daily dose for each metabolite) include the cleavage of the nitroguanidine group of thiamethoxam to result in the corresponding guanidine, yielding metabolite NOA 407475 [3-(2-chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylideneamine]. Cleavage of the nitroguanidine group and the oxadiazine ring yielded metabolite NOA 421275 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylguanidine]. Demethylation of NOA 421275 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methylguanidine] yielded metabolite NOA 421276 [*N*-(2-chlorothiazol-5-ylmethyl)-guanidine]. Hydrolysis of the guanidine group of CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] yielded the respective urea derivative, NOA 404617 [1-(2-chlorothiazol-5-ylmethyl)-3-nitrourea]. Substitution of the chlorine of the thiazole ring by glutathione and subsequent degradation resulted in the formation of metabolite NOA 402988 [2-methylsulfanylthiazole-5-carboxylic acid]. Metabolite 4U [(2-chlorothiazol-5-ylmethyl)-urea] is formed by hydrolysis of NOA 421276 [*N*-(2-chlorothiazol-5-ylmethyl)-guanidine]. More reactions of NOA 421276 with glutathione and subsequent metabolic transformations led to the formation of metabolite 6U [2-acetylamino-3-(2-chlorothiazol-5-ylmethylsulfanyl)-propionic acid], an *N*-acetylcysteinyl derivative of the thiazole moiety of thiamethoxam.

Minor pathways include the cleavage of the nitroguanidine group to result in the corresponding guanidine, yielding metabolite NOA 407475. Demethylation of NOA 407475 yielded metabolite N1. Cleavage of the nitroguanidine group and the oxadiazine ring of metabolite NOA 407475 yielded metabolite NOA 421275. Demethylation of NOA 421275 yielded metabolite NOA 421276. Hydrolysis of the guanidine group of CGA 265307 yielded the respective urea derivative, NOA 404617. Metabolite 4U is formed by hydrolysis of NOA 421276. Cleavage between the thiazole and oxadiazine moieties occurs to a very small extent, as shown by the presence of minor metabolites 6U, MO1, MO2, CGA 359683 and NOA 402988.

Based on the similarities in the metabolites identified, it appears that the metabolism of thiamethoxam in the mouse proceeds by the same major pathways as in the rat (see below). The new minor metabolites found were R14 [3-(2-chlorothiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylideneamine], R15: CGA 359683 [2-chlorothiazole-5-carboxylic acid], MO1 and MO2, which are glucuronic acid and acetylcysteine conjugates, respectively, of CGA 349208 and MO3, the *N*-acetyl cysteine conjugate of CGA 265307.

A proposed metabolic pathway for thiamethoxam in mice and other vertebrate species is shown in Figure 2 below.

Rats

The metabolic pathways of thiamethoxam were studied in male and female rats (Rumbëli, 1998; Thanei & Rümbeli, 1998) using the samples collected in the kinetic investigations described

above (Müller & Stampf, 1996) and stored at –18 °C. The urinary pattern of metabolites determined quantitatively by two-dimensional thin-layer chromatography was complex and essentially independent of sex, dose, preliminary treatment with non-radiolabelled thiamethoxam and route of administration. Very minor label-specific fractions were observed. The faecal pattern of metabolites was similar to, although less complex than, that found for urine, with some quantitative variations, but generally was independent of sex, dose, preliminary treatment with non-radiolabelled thiamethoxam and route of administration. The faecal pattern was only slightly dependent on the position of the label. The bile pattern was even less complex and independent of the position of the label, with some quantitative variations. All the patterns of metabolites in excreta were dominated by two major fractions that together accounted for approximately 60–80% of the dose.

Ultimately, 12 metabolites were isolated from the urine of the high-dose male and female rats and identified by spectroscopy. In addition, 13 metabolites were identified by co-chromatography with authentic reference substances.

The major fraction in urine, accounting for about 70–80% of the dose, was unchanged thiamethoxam. Based on the structures of the identified metabolites, the metabolic pathways of thiamethoxam in rats were derived and proposed to consist of the following:

- cleavage of the oxadiazine ring to the corresponding nitroguanidine compound (major pathway);
- reduction of the nitroguanidine group to a hydrazine followed by either acetylation or pyruvate conjugation (minor pathway);
- cleavage of the nitroguanidine group, yielding a guanidine derivative (minor pathway);
- hydrolysis of the guanidine group to the corresponding urea (minor pathway);
- demethylation of the guanidine group (minor pathway);
- substitution of the chlorine of the thiazole ring by glutathione (minor pathway);
- cleavage between the thiazole and oxadiazine rings (minor pathway).

The proposed metabolic pathway of thiamethoxam in all of the mammalian and avian species studied (rat, mouse and—as reported in the 2010 JMPR meeting report (Annex 1, reference *119*)—goat and fowl) is presented in Figure 2. The majority of metabolites were the result of more than one of the above-mentioned transformations. Identified metabolites are listed in Table 6.

Cleavage between the thiazole and oxadiazine rings occurs to a very small extent. It is initiated either by attack of glutathione on the bridging methylene group as long as the nitroguanidine moiety is intact, rendering a good leaving group, or by oxidative dealkylation. The former reaction gives rise to a thiazole-5-ylmethyl-glutathione derivative and a nitroguanidine compound, whereas the latter reaction ultimately produces the respective carboxylic acid derivative of thiazole and the corresponding cleavage counterpart.

Three hydrazine derivative metabolites of thiamethoxam have been found in rats. Two of these derivatives were detectable in rat excreta in small amounts. All of the hydrazine derivatives detected were acylated (pyruvate or acetate) and incorporated in an enamine structure (formed by reaction of a secondary amine with a carbonyl), which suggests a reduced reactivity of the hydrazine moiety.

The glutathione derivatives are prone to further degradation of the glutathione moiety, resulting in various sulfur-containing metabolites (e.g. mercapturates, sulfides and sulfoxides). These various sulfur-containing metabolites contribute significantly to the complex pattern of metabolites found in urine.

Figure 2. Proposed integrated metabolic pathways of thiamethoxam in mice, rats, goats and fowl

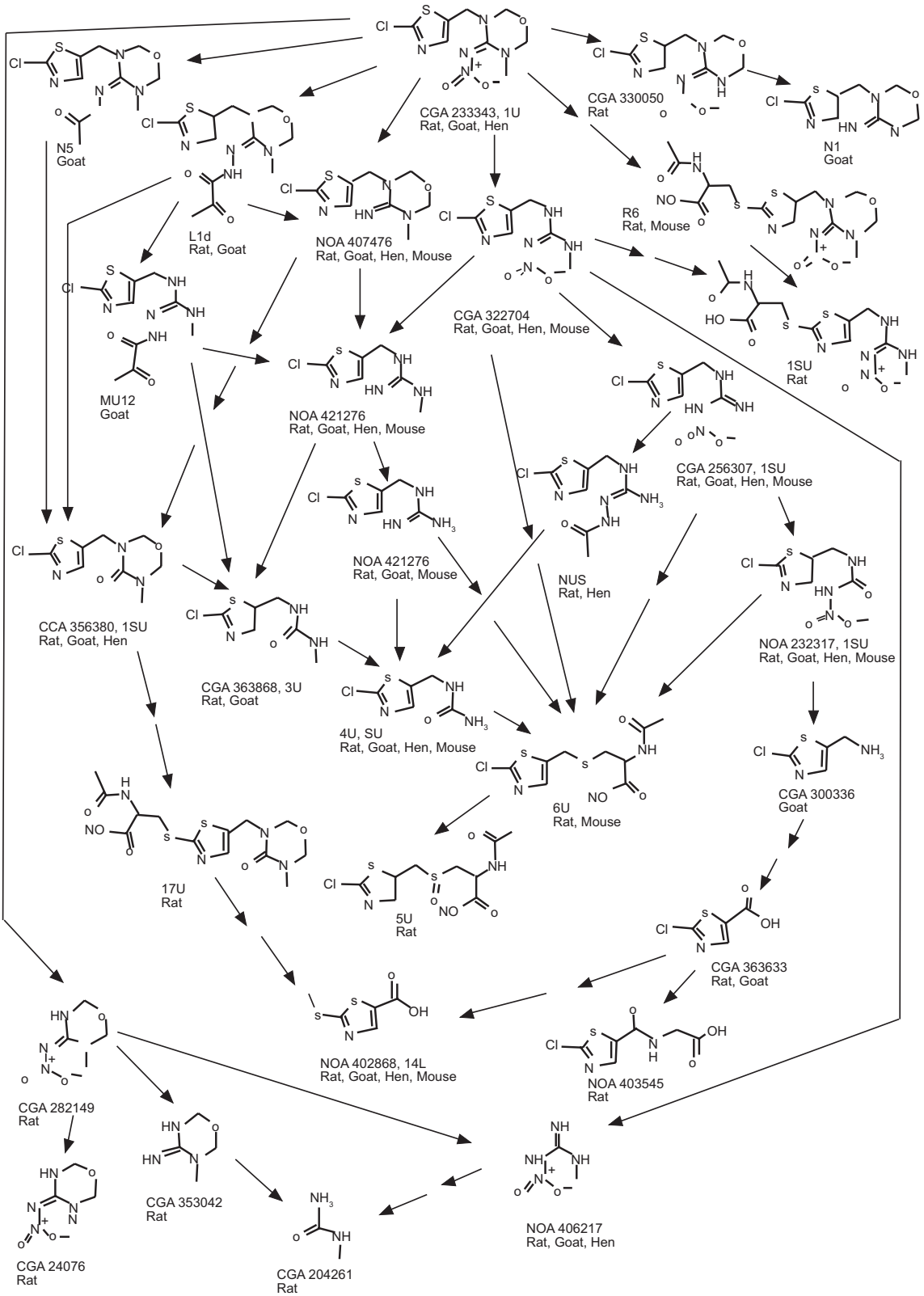


Table 6. Thiamethoxam and its metabolites as found in rats, mice, goats and fowl (hen)

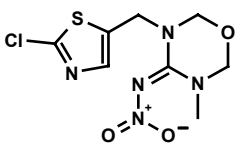
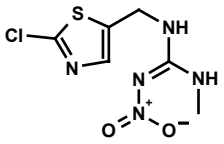
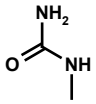
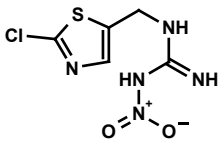
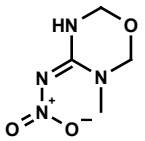
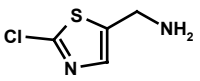
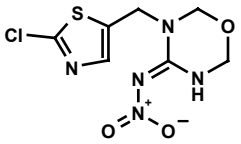
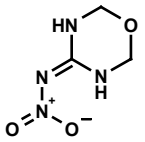
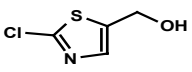
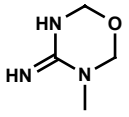
Code number	IUPAC name	Found in	Structure
CGA 293343 1U	3-(2-Chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-*N*-nitroamine	Rat, goat, hen	
CGA 322704 2U	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine	Rat, goat, hen	
CGA 204261	*N*-Methylurea	Rat	
CGA 265307 18U	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine	Rat, goat, hen	
MO3	*N*-Acetylcysteine conjugate of CGA 265307	Mouse	
CGA 282149	3,6-Dihydro-3-methyl-*N*-nitro-2H-1,3,5-oxadiazin-4-amine	Rat	
CGA 309335	(2-Chlorothiazol-5-yl)-methylamine	Goat	
CGA 330050	3-(2-Chlorothiazol-5-ylmethyl)-[1,3,5]-oxadiazinan-4-ylidene-*N*-nitroamine	Mouse, rat	
CGA 340575	3,6-Dihydro-*N*-nitro-2H-1,3,5-oxadiazin-4-amine	Rat	
CGA 349208	2-Chloro-5-thiazolemethanol	Mouse	
MO1	Glucuronic acid conjugate of CGA 349208	Mouse	
MO2	*N*-Acetylcysteine conjugate of CGA 349208	Mouse	
CGA 353042	3,6-Dihydro-3-methyl-2H-1,3,5-oxadiazin-4-amine	Rat	

Table 6 (continued)

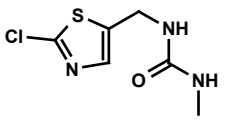
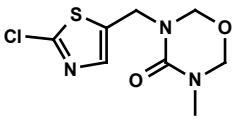
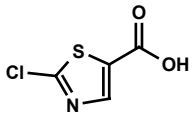
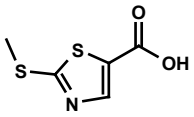
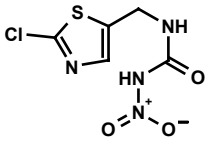
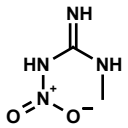
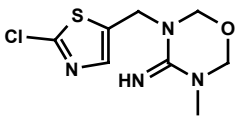
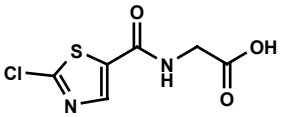
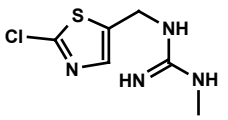
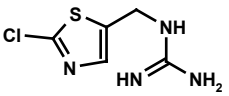
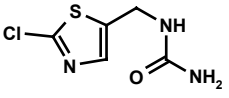
Code number	IUPAC name	Found in	Structure
CGA 353968 3U	1-(2-Chlorothiazol-5-ylmethyl)-3-methylurea	Rat, goat	
CGA 355190 19U	3-(2-Chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-one	Rat, goat, hen	
CGA 359683	2-Chlorothiazole-5-carboxylic acid	Mouse, rat, goat	
NOA 402988 14U	2-Methylsulfanylthiazole-5-carboxylic acid	Rat, goat, hen	
NOA 404617 15U	1-(2-Chlorothiazol-5-ylmethyl)-3-nitrourea	Rat, goat, hen	
NOA 405217	*N*-Nitro-*N*′-methylguanidine	Rat, goat, hen	
NOA 407475	3-(2-Chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylideneamine	Rat, goat, hen	
NOA 408445	[(2-Chlorothiazole-5-carbonyl)-amino]-acetic acid	Rat	
NOA 421275	*N*-(2-Chlorothiazol-5-ylmethyl)-*N*′-methylguanidine	Rat, goat, hen	
NOA 421276	*N*-(2-Chlorothiazol-5-ylmethyl)-guanidine	Rat, goat	
4U 8U 13U	(2-Chlorothiazol-5-ylmethyl)-urea	Rat, goat, hen	

Table 6 (continued)

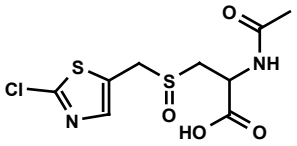
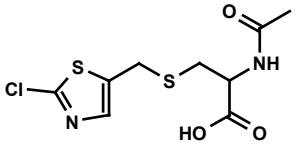
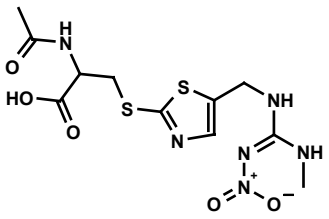
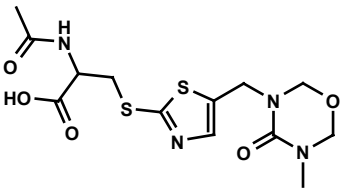
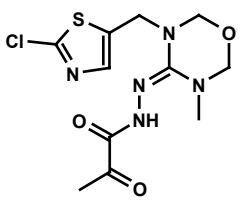
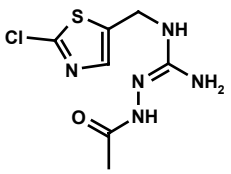
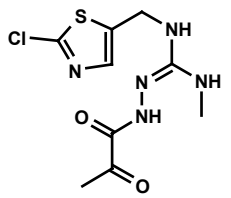
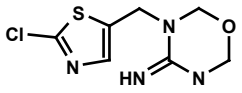
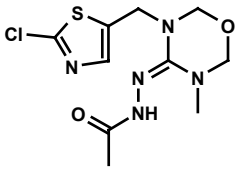
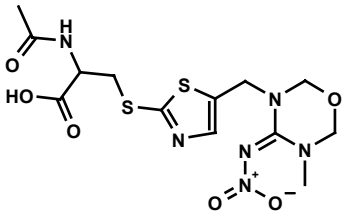
Code number	IUPAC name	Found in	Structure
5U	2-Acetylamino-3-(2-chlorothiazol-5-ylmethanesulfinyl)-propionic acid	Rat	
6U	2-Acetylamino-3-(2-chlorothiazol-5-ylmethylsulfanyl)-propionic acid	Rat	
16U	2-Acetylamino-3-[5-(*N*′-methyl-*N*″-nitroguanidinomethyl)-thiazol-2-ylsulfanyl]-propionic acid	Rat	
17U	2-Acetylamino-3-[5-(5-methyl-4-oxo-[1,3,5]-oxadiazinan-3-ylmethyl)-thiazol-2-ylsulfanyl]-propionic acid	Rat	
L14	2-Oxopropionic acid [3-(2-chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene]-hydrazide	Rat, goat	
MU3	Amino-([(2-chlorothiazol-5-ylmethyl)-amino]-methylene)-hydrazide	Rat, hen	
MU12	2-Oxopropionic acid ([(2-chlorothiazol-5-ylmethyl)-amino]-methylamino-methylene)-hydrazide	Goat	
N1, mouse R14	3-(2-Chlorothiazol-5-ylmethyl)-[1,3,5]-oxadiazinan-4-ylideneamine	Mouse, goat	

Table 6 (continued)

Code number	IUPAC name	Found in	Structure
N5	Acetic acid [3-(2-chlorothiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene]-hydrazide	Goat	
R6	2-Acetylamino-3-[5-(5-methyl-4-nitroimino-[1,3,5]oxadiazinan-3-ylmethyl)-thiazol-2-ylsulfanyl]-propionic acid	Mouse, rat	

From Briswalter (1999, 2002a,b,c); Mewes (2000)

Both the thiazole and oxadiazine moieties are susceptible to oxidative attack. These minor pathways proceed rapidly to small molecules, which may, at least partially, enter the general metabolism. As described previously (Müller & Stampf, 1996), a small but measurable amount of radioactivity was exhaled after administration of either label, most probably as carbon dioxide. The small metabolites resulting from thiazole and oxadiazine ring degradation also contribute to the metabolite complexity found in urine.

The degradation resulted in metabolites that, together with unchanged thiamethoxam, were eliminated very rapidly. The administered dose was almost completely absorbed and was degraded partially and eliminated almost completely via urine. Excretion via bile and ultimately via faeces was of very minor importance. Enterohepatic circulation was negligible.

1.3 Comparison of excretion and metabolism in mice and rats

In toxicity studies, described below, in which thiamethoxam was administered to rats and mice, a clear species difference was observed with respect to hepatotoxicity and the incidence of liver tumours. The latter was observed only in mice. Consequently, in an effort to understand this species difference, several metabolism studies were conducted in mice in order to compare the metabolic rate and fate of thiamethoxam in mice with those in rats.

There was about a 4-fold increased faecal excretion of radioactivity in mice (Mewes, 1998) compared with rats in the kinetic study (Müller & Stampf, 1996), revealing a species difference in the routes of excretion. About 19% of the total administered radioactivity was found in the mouse faeces, whereas only 5.1% of the dose was recovered in the rat faeces. The data are summarized in Table 7.

As the relative amounts excreted in mouse urine and faeces did not change markedly over the treatment period, it is likely that the different excretion patterns can be attributed to differences in the species rather than in the dose regimens. Of the daily administered thiamethoxam dose, 30–60% was metabolized and excreted in mice, whereas only 20–30% of the dosed thiamethoxam was metabolized and excreted in rats.

Identified metabolites were quantified in urine and faecal samples collected over 0–24 hours from rats receiving 91 mg/kg bw [thiazol-2-^{14}C]thiamethoxam on a single occasion and compared with samples collected over 0–24, 72–96, 144–168, 216–240, 288–312 and 360–384 hours from mice receiving 118 mg/kg bw [thiazol-2-^{14}C]thiamethoxam per day for 14 days. The 360- to 384-hour collection represented the collection from the last dose, which was administered at 312 hours.

Table 7. Species differences in metabolism of thiamethoxam in rats and mice

	Excretion (% of total dose)	
	Rat[a]	Mouse[b]
	Single oral, 91.2 mg/kg bw	Multiple oral, 14 × 118.2 mg/kg bw
Urine	95.49	71.75
Faeces	5.14	18.84
Expired air	0.18	0.06[c]
Cage wash	0.18	3.81
Tissue residues	0.29	Not determined
Total recovery	101.28	94.46

From Müller & Stampf (1996); Mewes (1998)

[a] Data are taken from Müller & Stampf (1996).

[b] Data are from Mewes (1998).

[c] Collection period 0–72 h after the first administration.

In urine, unchanged thiamethoxam represented 73% of the dose to rats, whereas it represented 33–41% of the dose to mice at the five collection times covering 0–312 hours. The metabolite CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] represented 12% of the dose to rats and about the same, 8–12%, of the dose to mice at the five successive collection times. Only for the metabolite CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] was there a marked difference in urinary representation, this being 1.9% of the dose to rats and 9.4–17.9% in mice. All other identified metabolites found in urine represented less than 0.9% of the dose in both rats and mice. There was, however, unresolved polar material that represented 0.3% of the dose to rats and between 4.7% and 8.1% of the dose to mice.

In faeces, unchanged thiamethoxam represented 1.2% of the dose to rats, whereas in mice, at the successive collection times, it represented 0.9%, 1%, 3.5%, 7.8% and 4.5% of the dose to mice. As there was but a single collection time from rats, it is not known whether the higher proportion of the administered dose found in mice at the three later collection times represents a true difference from rats or not. Similarly, the metabolite CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine] represented just 0.2% of the dose to rats and 0.3% of the dose to mice in faeces collected over 0–24 hours, whereas it tended to increase as a proportion of the administered dose at the successively later times to 0.5%, 1.7%, 3.2% and 1.8%. However, there did appear to be a species difference in the proportion of CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine], which represented just 0.04% of the dose in rats, but 0.59% in mice, over the 0- to 24-hour sampling time. At the successively later sampling times for mice, it represented 1.9%, 3.9%, 6.3% and 3.3% of the dose. The only other metabolite for which there was a substantial species difference, even when comparing the 0- to 24-hour sampling times, was R6 [2-acetylamino-3-[5-(5-methyl-4-nitroimino-[1,3,5]oxadiazinan-3-ylmethyl)-thiazol-2-ylsulfanyl]-propionic acid], which was not detected in rats but represented 2.15% of the dose to mice. This proportion in mice was maintained over the later sampling times, when it varied between 1.9% and 2.6%. However, eight other metabolites were also not detected in rat faeces but occurred in measurable amounts in mouse faeces, accounting for up to 0.8% of the administered dose (although more usually at much lower levels).

Comparisons of rats and mice are also possible from the results of three later studies (Briswalter, 2002a,b,c). First, aspects of these studies will be summarized.

Briswalter (2002a) studied the metabolism of [oxadiazin-4-^{14}C]thiamethoxam in mice after oral administration at a single oral dose of 0.5 or 100 mg/kg bw with a special emphasis on metabolite identification. The excretion and tissue distribution of thiamethoxam were investigated in a

Table 8. Summary of kinetic data following administration of a single oral dose of [oxadiazin-4-^{14}C]thiamethoxam at 106.5 mg/kg bw to male rats

Parameter	Total ^{14}C residues	Thiamethoxam	CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine]	CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine]
C_{max} (ppm)	50	41	8	0.6
T_{max} (h)	6	6	6	6
$t_{½}$ (h)	3	2	4	8
$AUC_{0–24\,h}$ (µg·h/g)	581	467	92	8

From Briswalter (2002b)

C_{max}, maximum concentration in plasma; $t_{½}$, half-life of elimination from plasma; T_{max}, time to reach the maximum concentration in plasma

previous, oral, multiple-dose study (Briswalter, 2002d) from which samples were used during this study. Approximately 72–73% of the administered dose was excreted in urine and a further 14–20% was excreted in faeces at the low dose level, and 82–90% and 11–15% were excreted in the urine and faeces, respectively, at the high dose level.

In urine, unchanged thiamethoxam and the metabolites CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine] and CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine] were identified and represented the majority of the radioactivity in urine, accounting for 25–41%, 10–16% and 13–19% of the dose, respectively. Two minor metabolites—M010 (*N*-formyl-*N′*-hydroxyurea) and M011 (*N*-formyl-*N′*-(hydroxymethyl)-urea)—accounted for 5–8% and 1–3% of the dose, respectively. Metabolites M012 [*N′*-formylureidoacetic acid] and CGA 330050 [3-(2-chlorothiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylidene-*N*-nitroamine] were detected in trace amounts.

The faecal extracts contained the same metabolites as urine. Unchanged thiamethoxam accounted for 2–4% of the dose, and metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine], CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine], M010 (*N*-formyl-*N′*-hydroxyurea) and M011 [*N*-formyl-*N′*-(hydroxymethyl)-urea] accounted for 1–3%, 2–6%, 1–2% and 0.1–0.6% of the dose, respectively.

Briswalter (2002b) studied the blood kinetics of thiamethoxam and its metabolites in male rats after a single oral administration of [oxadiazin-4-^{14}C]thiamethoxam at a dose of 107 mg/kg bw. Total residue levels in blood reached a maximum 6 hours after dosing of 50 µg of thiamethoxam equivalents per gram and declined rapidly thereafter. The half-life of elimination of total radioactivity in blood was 3 hours.

The radioactivity in the blood of rats was almost quantitatively extractable with acetonitrile. Thiamethoxam was the major component detected in blood, followed by CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine]. Only trace amounts of CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine] were found. As for the total radioactive residues, the maximum levels of thiamethoxam and metabolites in blood were reached 6 hours after dosing and declined rapidly thereafter. The maximum concentration of thiamethoxam in blood was 41 µg/g, with a half-life of elimination of 2 hours. The maximum concentrations of the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine] and CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine] in blood were 8 µg/g and 0.6 µg/g, respectively, with half-lives of 4 hours and 8 hours, respectively (Table 8).

Following administration of a single oral dose of [oxadiazin-4-^{14}C]thiamethoxam at a mean dose of 105.7 mg/kg bw to male mice (Briswalter, 2002c), radioactivity was rapidly absorbed into the systemic circulation. Total residue levels in blood reached a maximum 0.5 hour after dosing of 41 µg

Table 9. Summary of toxicokinetic data following administration of a single oral dose of [oxadiazin-4-^{14}C]thiamethoxam at 105.7 mg/kg bw to male mice

Parameter	Total ^{14}C residues	Thiamethoxam	CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine]	CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine]	CGA 330050 [3-(2-chlorothiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylidene-*N*-nitroamine]
C_{max} (ppm)	41	32	6	6	4
T_{max} (h)	0.5	0.5	2	2	2
$t_{½}$ (h)	4	3	3	3	3
$AUC_{0–24\,h}$ (µg·h/g)	277	122	39	66	28

From Briswalter (2002c)

C_{max}, maximum concentration in plasma; $t_{½}$, half-life of elimination from plasma; T_{max}, time to reach the maximum concentration in plasma

thiamethoxam equivalents per gram and declined rapidly thereafter. The half-life of elimination of total radioactivity in blood was 4 hours (Table 9).

The radioactivity in the blood of mice was almost quantitatively extractable, with the exception of a subgroup sampled 24 hours after dose administration. Thiamethoxam was the major component detected in blood. Lower amounts of the metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine], CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] and CGA 330050 [3-(2-chlorothiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylidene-*N*-nitroamine] were also detected in blood.

Of the radioactive residues identified, the maximum level of thiamethoxam in blood was reached 0.5 hour after dosing and declined rapidly thereafter. The maximum concentration of thiamethoxam in blood was 32 µg/g, with a half-life of elimination of 3 hours. The metabolites CGA 322704 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine], CGA 265307 [*N*-(2-chlorothiazol-5-ylmethyl)-*N*′-nitroguanidine] and CGA 330050 [3-(2-chlorothiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylidene-*N*-nitroamine] reached maximum concentrations in blood of 4–6 µg/g after 2 hours and had half-lives of elimination of 3 hours.

This study was followed by one in which [oxadiazin-4-^{14}C]thiamethoxam was administered to male and female mice by gavage at a nominal dose of 0.5 or 100 mg/kg bw. Urine and faeces were collected over a number of periods spanning 0–72 hours after dosing, and tissues were collected 72 hours after dosing.

Absorption of [oxadiazin-4-^{14}C]thiamethoxam from the gastrointestinal tract into the systemic circulation amounted to approximately 76% at the low dose level and about 88% at the high dose level, based on the recovery of radioactivity in urine, cage wash, tissues and carcass. The metabolite profiles were similar in urine and faeces (Briswalter, 2002a), and it was concluded that the portion of the dose excreted in faeces represents absorbed thiamethoxam excreted in the bile.

The routes and rates of elimination were independent of dose level and sex. Following oral administration of [oxadiazin-4-^{14}C]thiamethoxam at both the high and low dose levels, excretion was rapid and almost complete within 24 hours, with most of the radioactivity excreted via urine. At the low dose level, means of 72% (males) and 73% (females) of the dose were excreted in urine, and 20% (males) and 14% (females) of the dose were excreted via faeces. The routes and rates of excretion were similar at the high dose level, with urinary excretion accounting for 82% (males) and 90% (females) of the dose and faecal excretion accounting for 11% (males) and 15% (females) of the dose.

At both the low and high dose levels, radioactive residues remaining in the tissues and carcass 72 hours after dosing were low, accounting for approximately 1% of the dose in total. The distribution

of residues in the tissues and organs was essentially independent of dose level and sex, although tissue residues were slightly higher in female mice than in male mice.

At the low dose level, the highest tissue residue concentrations were found in the liver, amounting to 0.0139 ppm in males and 0.0271 ppm in females. Residues in all other tissues and organs were below 0.0032 ppm. Residues were correspondingly higher at the high dose level, with residues in liver amounting to 2.675 ppm in males and 5.114 ppm in females. Residues did not exceed 0.53 ppm in any other tissues.

Based on the metabolites identified in urine and faeces, the metabolism of thiamethoxam in mice proceeds by the same major pathway as in rats. There are, however, quantitative interspecies differences that deserve mention. Taking into consideration all of the absorption, distribution, metabolism and elimination studies summarized in this document, it can be concluded that about 20–30% of the dose was eliminated as metabolites of thiamethoxam in rats, whereas metabolism was more extensive in mice, with 30–60% of the dose appearing as metabolites of thiamethoxam. The predominant metabolites in rats were CGA 322704 and CGA 265307, which accounted for 10% and 1% of the dose, respectively, whereas the remaining 20 metabolites were below 1% of the dose. These same two metabolites, CGA 322704 and CGA 265307, were dominant in mice, accounting for about 20% and 15% of the dose, respectively. The $AUC_{0-24\ h}$ for CGA 265307 was significantly greater in mice than in rats (66 versus 8 µg·h/g blood). These species differences will be of particular interest in considering rat–mouse differences in neoplastic developments.

2. Toxicological studies

2.1 Acute toxicity

(a) Oral administration

In an acute oral toxicity study conducted according to Organisation for Economic Co-operation and Development (OECD) guideline 401 (1987), groups of five male and five female fasted Sprague-Dawley (Crj:CD SD) rats were each administered thiamethoxam (batch No. P.506006; purity 98.6% weight per weight) in 0.5% weight per volume (w/v) aqueous methylcellulose at a dose volume of 20 ml/kg bw by gavage as a single oral dose of 0, 900, 1500, 2300, 3800 or 6000 mg/kg bw. Mortality, clinical signs and body weight changes were recorded. Autopsies were performed on rats at termination on day 14.

Deaths occurred in both sexes at dose levels of 1500 mg/kg bw and above between 2 and 6 hours after dosing. In most instances, tonic convulsions preceded death. All rats died in the two highest dose groups, four of five of each sex at 2300 mg/kg bw and three of five of each sex at 1500 mg/kg bw. Ptosis occurred in all treated groups 1 hour after dosing, and survivors of both sexes treated at 1500 mg/kg bw and above showed reduced locomotor activity on the day of treatment. Clinical observations on the surviving rats returned to normal on the following day. Body weight loss or retarded weight gain was recorded for 2 days after dosing, after which body weight gain was normal. Autopsy and postmortem examination did not reveal any treatment-related abnormalities in either the rats that died or the survivors.

The acute oral median lethal dose (LD_{50}) of thiamethoxam in male and female rats was 1563 mg/kg bw (Oda, 1996a).

In an acute oral toxicity study conducted according to OECD guideline 401 (1987), groups of five male and five female fasted SPF (Crj:CD1(ICR) strain) mice were each administered thiamethoxam (batch No. P.506006; purity 98.6%) in 0.5% w/v aqueous methylcellulose at a dose volume of 20 ml/kg bw by gavage as a single oral dose of 0, 500, 700, 1000, 1400 or 2000 mg/kg bw. Mortality,

clinical signs and body weight changes were recorded. Autopsies were performed on mice at termination on day 14.

Deaths occurred in both sexes at dose levels of 700 mg/kg bw and above between 15 minutes and 24 hours after dosing. All mice died in the highest dose group, all males and four of five females at 1400 mg/kg bw, four of five males and three of five females at 1000 mg/kg bw and two of five males and one of five females at 700 mg/kg bw. Reduced locomotor activity or prostration occurred in all thiamethoxam-treated mice within 5–15 minutes of treatment, and clonic convulsions occurred 15 minutes to 4 hours after treatment. Clinical observations on the surviving mice returned to normal on the following day. The body weight development of all male treated groups was unaffected throughout the observation period, but the body weight gain of all female treated groups was slightly reduced on the day following dosing. Thereafter, body weight development of female groups returned to normal. Autopsy and postmortem examination did not reveal any treatment-related abnormalities in either the mice that died or the survivors.

The acute oral LD_{50} of thiamethoxam in mice was 783 mg/kg bw (95% confidence interval 619–1000 mg/kg bw) in males, 964 mg/kg bw (729–1271 mg/kg bw) in females and 871 mg/kg bw (735–1028 mg/kg bw) in both sexes combined (Oda, 1996b).

(b) Dermal application

In an acute dermal toxicity study conducted according to OECD guideline 402 (1987), five male and five female Sprague-Dawley (Crj:CD strain) rats were administered thiamethoxam (batch No. P.506006; purity 98.6%) applied to the closely clipped dorsum at a dose level of 0 or 2000 mg/kg bw. The applied thiamethoxam was covered by an occlusive dressing for 24 hours. Local or systemic signs of reaction to treatment were recorded during a subsequent 14-day period of observation. All rats were killed on day 15 and subjected to gross postmortem examination.

There were no deaths or clinical signs of reaction to treatment in any rat throughout the study. There was no evidence of a dermal response to treatment throughout the study. Body weight development was unaffected by treatment; a slight reduction in body weight was observed on the day following application in both control and treated groups. Autopsies performed on day 14 did not reveal any gross abnormalities.

The acute lethal dermal dose (LD_{50}) of thiamethoxam in rats was greater than 2000 mg/kg bw (Oda, 1996c).

(c) Exposure by inhalation

In an acute inhalation study conducted according to OECD guideline 403 (1981), groups of five male and five female Sprague-Dawley (Crl:CD strain) rats were exposed (nose only) for 4 hours to a dust aerosol of thiamethoxam (batch No. P.506006; purity 98.6%) at a nominal concentration of 10.9 or 56.6 g/m^3. The rats were observed for 14 days after exposure, then killed and autopsied. Parameters monitored included mortality, clinical signs and weekly body weights.

The characteristics of the achieved atmosphere were, respectively, mean achieved analytical atmospheric concentrations of 1.02 ± 0.05 and 3.72 ± 0.73 g/m^3; and mean mass aerodynamic diameters (MMAD) of 5.1 ± 0.3 and 5.6 ± 0.1 μm. The percentages of particles less than 7.07 μm were 67.5% and 66.9%, respectively. Higher atmospheric concentrations were not technically achievable.

There was no mortality and there were no treatment-related clinical signs of toxicity during the exposure and observation periods. Soiled fur in the nasorostral region was observed to a slight degree in all rats of both groups on the day of exposure, but this was considered to be related to the procedure rather than to thiamethoxam. Rats recovered to appear normal on the first day (low dose) and third day (high dose) after exposure. Normal body weight gain was noted during the study in the low-dose rats and in all high-dose rats except for two females. These showed slight decreases in

body weight on day 7, but had recovered by day 14. Autopsies performed on day 14 did not reveal any gross abnormalities.

The 4-hour acute inhalation median lethal concentration (LC_{50}) of thiamethoxam in rats was greater than 3.72 g/m^3, the highest technically achievable concentration (Shutoh, 1996).

(d) Dermal irritation

In a dermal irritancy study conducted according to Japanese Ministry of Agriculture, Forestry and Fisheries guideline 4200 and OECD guideline 404 (1992), six female Japanese White rabbits were exposed to 0.5 g of thiamethoxam (batch No. P.506006; purity 98.6%) under a semiocclusive dressing on the closely clipped dorsal skin for 4 hours. Dermal reactions were assessed at 1, 24, 48 and 72 hours after removal of the dressing. Dermal irritation was graded according to the Draize scoring method.

No dermal irritation was observed during the study. The mean irritation score over 24–72 hours was 0 for erythema and 0 for oedema. Thiamethoxam was not irritating to rabbit skin (Shibata, 1996a).

(e) Ocular irritation

In an ocular irritation study conducted according to OECD guideline 405 (1992), nine female Japanese White rabbits were exposed to 0.1 g of thiamethoxam (batch No. P.506006; purity 98.6%) instilled into the conjunctival sac of the left eye on day 1. Ocular reactions were assessed at 1, 24, 48 and 72 hours and 7 days after treatment. The eyes of three rabbits were rinsed 2–3 minutes after instillation. Twenty-four hours after application, a drop of 2% fluorescein (sodium salt) solution was instilled into the eyes and immediately rinsed with water in order to assist in the recognition of corneal damage. Ocular irritation was graded according to the Draize scoring method.

All rabbits survived, and there were no clinical signs of toxicity. Minimal (grade 1) erythema and oedema of the conjunctivae occurred at 1 hour in the unwashed eye of all rabbits and in the washed eye of two of three rabbits. The other rabbit in the washed group developed minimal conjunctival oedema only. Transient eye closure and discharge were observed in all rabbits immediately after application. No signs of eye irritation were present at 24, 48 and 72 hours in unwashed or washed eyes.

Thiamethoxam was transiently minimally irritating to the rabbit eye (Shibata, 1996b).

(f) Dermal sensitization

In a skin sensitization study conducted according to Japanese Ministry of Agriculture, Forestry and Fisheries guideline 4200 and OECD guideline 406 (1992), skin hypersensitivity in guinea-pigs exposed to thiamethoxam (batch No. P.506006; purity 98.6%) was assessed by the Magnusson & Kligman maximization method. Based on the findings of a preliminary study, the closely clipped dorsa of groups of 10 male and 10 female Pirbright White guinea-pigs were given, on day 1, 0.1 ml intradermal injections of 1% thiamethoxam in peanut oil, 1% thiamethoxam in Freund's complete adjuvant/saline or adjuvant/saline alone (test groups); 0.5% mercaptobenzothiazole in peanut oil alone or 0.5% mercaptobenzothiazole in adjuvant/saline alone (positive control groups); or peanut oil alone, adjuvant/saline mixture or adjuvant/saline mixture with peanut oil (negative control groups). One week later, the same areas of skin were treated by topical application of 30% w/v thiamethoxam in petroleum jelly, 50% mercaptobenzothiazole in petroleum jelly or petroleum jelly alone, respectively, and covered by an occlusive dressing for 48 hours. Two weeks after the topical induction, all guinea-pigs were challenged by occluded application of 10% thiamethoxam in petroleum jelly (test and negative control groups) or 10% mercaptobenzothiazole in petroleum jelly. The occlusive dressings were removed on the following day, and the condition of the test sites was assessed approximately 24 and 48 hours later and scored according to the Draize scale.

A skin reaction (erythema reaction with Draize score 1) was observed in one male (corresponding to a reaction rate of 5%) from the test group guinea-pigs after 48 hours only. No irritant skin reactions occurred in the negative control group. In contrast, the positive control group showed positive reactions (Draize scores 1–3) in 17 of 20 animals after 24 and 48 hours, corresponding to a sensitization rate of 85%. No effect on body weights occurred during the experimental period.

Thiamethoxam produced a skin reaction incidence of 5% and on this basis is judged not to be a skin sensitizer in this guinea-pig Magnusson & Kligman test (Winkler, 1995).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day dietary toxicity study in mice performed according to OECD guideline 408 (1987), groups of 10 male and 10 female mice (Tif:MAGf, SPF strain) were administered thiamethoxam (batch No. KI-4654/18; purity 98.4%) in the diet at a nominal concentration of 0, 10, 100, 1250, 3500 or 7000 ppm, providing measured intakes equal to 0, 1.41, 14.3, 176, 543 and 1335 mg/kg bw per day for males and 0, 2.01, 19.2, 231, 626 and 1163 mg/kg bw per day for females. Mortality and clinical signs were checked daily, and feed/water consumption and body weight were recorded weekly. Haematology was performed on all animals at the end of the treatment period. All mice were killed at termination and subjected to postmortem examination, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

Preliminary diet analyses demonstrated the stability at room temperature of thiamethoxam in diet. Analysis of representative test diets demonstrated achieved concentrations to be in the range 94.2–101% of nominal concentrations.

No treatment-related deaths occurred during the study, but one male at 7000 ppm died during blood sampling. Clinical signs of an adverse effect of treatment were restricted to transient respiratory sounds, without dyspnoea, at 3500 and 7000 ppm. Body weight gain was markedly reduced in males and to a lesser extent in females at 7000 ppm and slightly reduced in males at 3500 ppm. In females, feed consumption was reduced at 7000 ppm and to a lesser extent at 3500 ppm. It was not possible to evaluate any effect on feed consumption in the males due to spillage.

Trend tests on the haematological data indicated anaemia associated with a tendency to hyperchromasia and macrocytosis manifest as significant reductions in erythrocyte count, haemoglobin concentration and haematocrit in males and females of the 7000 ppm group. In addition, in females of the 1250, 3500 and 7000 ppm groups, there were minimal, but statistically significant, elevations in platelet numbers.

No treatment-related changes were observed at autopsy. Statistically significant increases were recorded in absolute and relative liver weights of female mice at 3500 and 7000 ppm and in the relative liver weight of male mice at 7000 ppm (Table 10). Ovary and spleen weights of females were significantly reduced at 3500 and 7000 ppm. Marked depressions of carcass weight at 7000 ppm in both males (32.5%) and females (14.9%) were associated with changes in the absolute and/or relative weights of the heart, liver, kidneys, adrenal glands, thymus (males only), gonads, spleen and thyroid gland, but in most cases there were no histological correlates for these changes. Histological correlates were identified for the liver and ovarian weight changes. These consisted of hepatocellular hypertrophy, single-cell necrosis, pigment accumulation in Kupffer cells and an increase in lymphocytic infiltration of the liver. Ovarian weight loss was associated with atrophy.

Table 10. Organ weight changes in mice treated with thiamethoxam in the diet for 90 days

	Organ weight											
	Male						Female					
	Dietary concentration (ppm)											
	0	10	100	1250	3500	7000	0	10	100	1250	3500	7000
Carcass (g)	47.48	47.76	46.62	48.61	45.30	32.01*$^{-}$	30.10	30.84	30.58	31.89	30.26	25.63*
Liver (g)												
- Absolute	2.774	2.636	2.502	2.641	2.738	2.138	1.787	1.698	1.732	2.005	2.108^{+}	1.822^{+}
- Relative[a]	58.13	55.13	53.40	54.26	60.32	66.85^{+}	59.36	55.15	56.70	62.82	69.57*	71.12*
Ovary (mg)												
- Absolute	—	—	—	—	—	—	48.10	50.07	41.26	43.21	38.71	31.64*$^{-}$
- Relative[a]	—	—	—	—	—	—	1.598	1.632	1.354	1.354	1.288^{-}	1.235^{-}
Spleen (g)												
- Absolute	0.093	0.090	0.087	0.092	0.88	0.097	0.097	0.098	0.104	0.092	0.087	0.085^{-}
- Relative[a]	1.961	1.886	1.868	1.896	1.936	3.105	3.216	3.180	3.398	2.904	2.874	3.406

From Bachmann (1996a)

* $P < 0.01$ (Lepage); −/+ negative/positive trend (Jonckheere)

[a] Relative to body weight.

Table 11. Significant microscopic changes in mice treated with thiamethoxam in the diet for 90 days

	Incidence of finding											
	Male						Female					
	Dietary concentration (ppm)											
	0	10	100	1250	3500	7000	0	10	100	1250	3500	7000
No. examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Liver												
Lymphocytic infiltration	0	0	1	1	4	5	1	1	0	0	5	3
Fatty change	9	8	4	9	10	5	9	9	8	10	9	10
Kupffer cell pigment	0	0	0	0	1	10	0	0	0	0	2	9
Hepatocyte: necrosis	0	0	0	0	0	8	0	0	0	0	3	10
Hepatocyte: hypertrophy	0	0	8	9	10	10	0	0	0	2	10	10
Ovaries												
Atrophy	—	—	—	—	—	—	0	0	1	1	5	10

From Bachmann (1996a)

Minimal to marked hypertrophy of centrilobular hepatocytes, minimal pigmentation of Kupffer cells and an increased incidence of minimal lymphocytic infiltration of the parenchyma were present in the livers of males and females at 3500 and 7000 ppm (Table 11). At 7000 ppm, minimal to moderate necrosis of single hepatocytes was also present. Both sexes at 1250 ppm also showed minimal centrilobular hypertrophy. Males at 100 ppm showed minimal hypertrophy as an isolated change in the liver, without the occurrence of other progressive hepatic alterations noted at higher dose levels. The ovaries of females at 3500 and 7000 ppm showed minimal to moderate atrophy in the form

of reduced numbers of corpora lutea. These effects on the ovary are most likely due to the growth retardation seen at these dose levels, rather than a direct effect of thiamethoxam. There were no other treatment-related morphological findings (Bachmann, 1996a).

The no-observed-adverse-effect level (NOAEL) in the 90-day dietary study in mice with thiamethoxam was 100 ppm, equal to a dose level of 14.3 mg/kg bw per day (males) and 19.2 mg/kg bw per day (females), based on the finding of liver hypertrophy at 1250 ppm, equal to 176 mg/kg bw per day in males, and liver hypertrophy and raised platelet counts at 1250 ppm, equal to 231 mg/kg bw per day in females. A minimal hepatocyte hypertrophy was observed in males only at 100 ppm, but in the absence of any other hepatic changes, this was considered an adaptive response.

Rats

In an oral study of toxicity, groups of five male and five female Tif:RAIf (SPF) rats were administered thiamethoxam (batch No. KGL4654/12; purity 95.0%) in the diet at a dose level of 0, 100, 1000, 2500 or 10 000 ppm, equal to 0, 8.0, 81.7, 198.6 and 710.6 mg/kg bw per day in males and 0, 8.7, 89.3, 210.6 and 762.6 mg/kg bw per day in females, for 28 consecutive days. Mortality and clinical signs were checked daily, and feed/water consumption and body weight were recorded weekly. Haematological, blood chemistry and urinalysis investigations were performed on all rats towards the end of the treatment period. All rats were killed at termination and subjected to postmortem examination, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically. In a separate investigation, measurement of replicating deoxyribonucleic acid (DNA) synthesis was performed on liver samples from the male animals. The results of this separate investigation are presented below (see Table 54).

Preliminary analysis of diets demonstrated the stability and homogeneity of the distribution of thiamethoxam. Analysis of test diets demonstrated achieved concentrations to be in the range 98–104% of nominal concentrations.

No deaths occurred and no clinical signs of a reaction to treatment were evident during the administration period. Body weight gain was reduced by 29% in males at 10 000 ppm, but females at this level were not affected. Minimal retardation of body weight gain occurred in males at 2500 ppm. Feed consumption was markedly reduced at 10 000 ppm in males throughout the treatment period, but in females, it was only slightly reduced during the first week.

There were no treatment-related effects on haematological profiles at dose levels up to and including 10 000 ppm. Isolated statistically significant differences from the controls, having no relationship to dose, are considered incidental to treatment. Platelet counts at 10 000 ppm were higher in both males (23%) and females (18%), but remained within the historical control range and were considered to be of no toxicological significance. Plasma cholesterol concentrations were significantly higher in males of the 1000 ppm (22%), 2500 ppm (26%) and 10 000 ppm (43%) groups and in females of the 10 000 ppm (70%) group. Other treatment-related effects were confined to this 10 000 ppm group and are considered to be minor consequences of renal and hepatic morphological changes. The statistically significant plasma changes at the highest dose consisted of a 35% increase in aspartate aminotransferase (AST) activity in males but not in females, a 6% reduction in albumin concentration in males but not in females, a 20% increase in urea level in females (and a non-significant 19% increase in males) and very small, yet significant, reductions in sodium concentrations of 1.7% in males and 0.5% in females. There were no effects on any urinary physiological parameters or on cellular and chemical constituents.

Treatment-related effects on organ weights occurred in both males and females at 1000 ppm and above (Table 12). At 10 000 ppm, absolute liver weights were increased in females and relative (to body weight) liver weights were increased in males and females. Relative kidney weights were increased at 2500 ppm in males, whereas in females, both absolute and relative kidney weights were increased at 1000 ppm, but not at 2500 ppm, and only relative kidney weights were increased at

Table 12. Organ weights in rats treated with thiamethoxam in the diet for 28 days

Parameter	Dietary concentration (ppm)				
	0	100	1000	2500	10 000
Males					
Body weight (g)	314.2	314.7	316.4	303.6	250.1*$^{-}$
Liver					
- Absolute (g)	14.95	14.92	14.87	14.89	16.07
- Relative (% body weight × 10)	47.54	47.47	47.00	49.02	64.28*$^{+}$
Kidneys					
- Absolute (g)	2.260	2.359	2.470	2.524	1.918
- Relative (% body weight × 10)	7.209	7.464	7.812	8.306*$^{+}$	7.670
Adrenals					
- Absolute (mg)	81.46	73.86	72.02	78.18	68.38*
- Relative (% body weight × 10)	0.261	0.236	0.227	0.256	0.274
Thymus					
- Absolute (mg)	712.0	643.8	675.9	598.5	541.1*
- Relative (% body weight × 10)	2.263	2.027	2.133	1.974	2.166
Females					
Body weight (g)	199.2	211.7	215.9	211.5	184.4
Liver					
- Absolute (g)	8.476	9.285	9.695	8.969	10.41*
- Relative (% body weight × 10)	42.42	43.77	44.87	42.39	56.37*
Kidneys					
- Absolute (g)	1.668	1.810	1.984*$^{+}$	1.826	1.757
- Relative (% body weight × 10)	8.390	8.536	9.204*$^{+}$	8.658	9.575*
Adrenals					
- Absolute (mg)	85.46	88.72	93.60	90.96	93.22
- Relative (% body weight × 10)	0.428	0.420	0.435	0.433	0.506
Thymus					
- Absolute (mg)	448.2	484.1	469.5	508.0	426.6
- Relative (% body weight × 10)	2.255	2.291	2.178	2.397	2.300

From Bachmann (1995)
* $P < 0.05$ (Wilcoxon); −/+ negative/positive trend (Jonckheere)

10 000 ppm. Other statistically significant organ weight changes were reductions in absolute adrenal and thymus weights of male but not female rats at 10 000 ppm.

At autopsy, dilatation of the renal pelvis was observed in two male rats of the 10 000 ppm group, but no other treatment-related gross lesions were recorded. Treatment-related microscopic changes were observed in males treated at 1000 ppm and above and in females at 2500 ppm and above (Table 13). The organs affected were liver, kidneys, adrenal glands and thyroid glands. Minimal to marked hypertrophy of centrilobular hepatocytes occurred particularly in males, but also in females, at 2500 and 10 000 ppm. Glycogen depletion was observed in males at 10 000 ppm, but it is not clear whether other hepatic lesions recorded were the result of treatment (i.e. glycogen depletion in females and cholangiofibrosis in males and females). In the kidneys, minimal to moderate hyaline change to the tubule epithelium occurred in males at 1000 ppm (3/5) and 2500 ppm (5/5), but not at

Table 13. Selected histopathology from rats treated with thiamethoxam for 28 days

Organ/finding	Incidence of finding									
	Males					Females				
	Dietary concentration (ppm)									
	0	100	1000	2500	10 000	0	100	1000	2500	10 000
Number of animals	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*
Liver										
Intrahepatic bile duct: cholangiofibrosis	2	0	1	1	2	0	0	0	0	2
Hepatocytes: glycogen deposition	4	3	2	3	0	2	2	0	1	0
Hepatocytes: hypertrophy	0	0	0	4	5	0	0	0	1	5
Kidneys										
Cyst	0	0	0	1	0	0	0	0	0	0
Lymphohistiocytic infiltration	0	0	0	1	0	0	0	0	0	0
Acute tubular lesion	0	0	0	2	0	0	0	0	0	0
Renal cortex: calcification	0	0	1	0	0	0	0	0	0	0
Renal tubule: hyaline change	0	0	3	5	0	0	0	0	0	0
Tubular basophilic proliferation	1	4	5	5	2	3	3	3	4	4
Renal pelvis: dilatation	0	0	1	0	3	0	0	0	0	0
Pelvic epithelium hyperplasia	0	0	1	0	1	0	0	0	0	1
Adrenal gland										
Cortical fatty change	0	0	0	0	3	0	0	0	0	2
Thyroid gland										
Follicular epithelial hypertrophy	1	1	1	2	3	0	0	0	0	2

From Bachmann (1995)

any other dose level, including 10 000 ppm, and hyperplasia of the pelvic epithelium occurred in one rat of each sex at 10 000 ppm. However, this latter lesion was also recorded for one male rat of the 1000 ppm group, so its relation to treatment is unclear. The hyaline (eosinophilic) change was later demonstrated to be the outcome of α_2-urinary microglobulin (α_{2u}-globulin) deposition (see Weber, 2000a,b,c,d). Many of the affected male rats also showed one or more additional kidney lesions (focal calcification, pelvic dilatation, renal cyst, basophilic proliferation, lymphohistiocytic infiltration or an acute tubular lesion), but with little or no evidence of a treatment relationship. A common lesion recorded in both males and females, tubular basophilic proliferation, may be the beginnings of chronic progressive nephropathy development, which is not a toxic response but a naturally occurring pathology specific to rats and with no human counterpart. There was no indication of a dose–response relationship for basophilic proliferation in females, of which the incidences in the 0, 100, 1000, 2500 and 10 000 ppm groups, respectively, were 3/5, 3/5, 3/5, 4/5 and 4/5. In males, the respective incidences were 1/5, 4/5, 5/5, 5/5 and 2/5. In the adrenal cortex, minimal to moderate fatty change mainly of the zona fasciculata was observed in both males and females of the 10 000 ppm group, but in no

other group. In the thyroid gland, the incidence of follicular cell hypertrophy was increased in males, combined with a moderately increased severity, at 10 000 ppm. In females, this observation was also made in two females, with minimal severity. No other treatment-related microscopic lesions were observed (Bachmann, 1995). There was no effect on replicating DNA synthesis in hepatocytes of male rats at dose levels up to 10 000 ppm (Persohn, 1995).

The NOAEL in the 28-day dietary study in rats was 100 ppm, equal to 8.0 mg/kg bw per day, based on increased kidney weights in both sexes (although these were not clearly dosed related) and hyaline changes in the renal tubule epithelium of males at a dose level of 1000 ppm, equal to 81.7 mg/kg bw per day. As the latter, male rat–specific kidney effects have no human relevance, they are not considered as contributing to the identification of a relevant NOAEL. The NOAEL of human relevance in the 28-day dietary study in rats was 100 ppm, equal to 8.0 mg/kg bw per day, based on increased plasma cholesterol concentrations at a dose level of 1000 ppm, equal to 81.7 mg/kg bw per day (Backmann, 1995).

In a 90-day dietary toxicity study, groups of 10 male and 10 female Tif:RAIf strain Sprague-Dawley-derived rats were administered thiamethoxam (batch No. KI-4654/18; purity 98.4%) in the diet at a concentration of 0, 25, 250, 1250, 2500 or 5000 ppm, equal to 0, 1.74, 17.6, 84.9, 168 and 329 mg/kg bw per day in males and 0, 1.88, 19.2, 92.5, 182 and 359 mg/kg bw per day in females, for 13 weeks. Rats were observed daily for clinical signs, and body weight and consumption of feed and water were measured weekly. Eye examinations were conducted before the start and at week 13 of treatment. Haematology, blood chemistry and urinalysis were performed towards the end of the study. At autopsy, the weights of selected organs were recorded for all rats. All major organs and tissues were examined microscopically.

Preliminary diet analyses demonstrated the stability at room temperature of thiamethoxam in diet. Representative analyses of test diets demonstrated achieved concentrations to be in the 94.2–104.6% range of nominal concentrations.

There were no treatment-related deaths or clinical signs of an adverse reaction to treatment, although one female at 1250 ppm died on day 57 with no histopathological treatment-related findings. Ophthalmoscopic examinations revealed no evidence of ocular toxicity. In the groups of male rats, body weight gains were statistically significantly retarded at 1250, 2500 and 5000 ppm by 22%, 19% and 27%, respectively, and the feed consumption in these groups was reduced (non-significantly) relative to the controls by 11%, 7% and 14%, respectively. Weight gains and feed consumption of all female groups and the male groups at dietary concentrations up to 250 ppm were unaffected by treatment. Water consumption in both sexes at 5000 ppm was slightly increased.

The only haematological effect was a 12% increase in the number of circulating platelets in males at 5000 ppm. Other, minimal differences between control and high-dose haematological values that were statistically significant or showed a positive trend were considered to reflect physiological variation, as individual values were within reference ranges. Minor treatment-related effects on blood chemistry occurred in males at 1250 ppm and above and in females at 2500 ppm and above. The effects included higher plasma urea concentrations in both sexes that were increased statistically significantly (24%) in males of the 5000 ppm group and increased plasma creatinine concentrations in males only at 1250 ppm and above, reaching 22% at 5000 ppm. In addition, plasma glucose concentrations were reduced in male rats at 1250 ppm and above, whereas plasma cholesterol concentrations were raised in both males (22%) and females (24%), but only at 5000 ppm. Minimal changes in the electrolyte balance and phosphate levels of both sexes are considered to be of no toxicological consequence. There were no treatment-related effects on urinary physiological parameters or cellular and chemical constituents of urine.

Gross examination at autopsy revealed no treatment-related effects on any tissue or organ examined. There appeared to be no statistically significant changes in the absolute weights of any

organs; however, the relative weights of the adrenals (44%), liver (21%), kidneys (22%), heart (15%) and spleen (27%) were increased in males at 5000 ppm. In male rats at 2500 ppm, there were also marginally increased relative liver (6%) and kidney (11%) weights. There was no histopathological correlation to the increased heart weight of males at 5000 ppm. Treatment-related effects in females were confined to a 16% reduction in thymus weights at 5000 ppm.

On the basis of histopathology, the liver, kidney, spleen and adrenal glands were identified as target organs for thiamethoxam (Table 14). Changes in liver morphology at 2500 and 5000 ppm were minimal to moderate centrilobular hypertrophy and an increased incidence of lymphohistiocytic infiltration of the parenchyma and, in the males only, an increased incidence of cholangiofibrosis of bile ducts. Females at 5000 ppm also showed minimal pigmentation of Kupffer cells. Changes in kidney morphology occurred at 250 ppm and above in males and 2500 ppm and above in females. Lesions observed in males were minimal to marked hyaline change in the tubular epithelium, acute and chronic tubular lesions and an increased incidence of tubular basophilic proliferation at 1250 ppm and above. Increased incidences of pelvic dilatation and epithelial hyperplasia as well as tubular cast formation were also evident. Renal lesions in females were confined to an increased incidence of chronic tubular lesions at 250 ppm and above and an increase in the severity of nephrocalcinosis (which occurred in all females of all groups). The pattern of effects in male rat kidneys at 250 ppm and above is consistent with α_{2u}-globulin nephropathy and chronic progressive nephropathy. Immunohistochemical studies have demonstrated the occurrence of α_{2u}-globulin nephropathy following thiamethoxam treatment (Weber, 2000b; see Table 54 below). α_{2u}-Globulin nephropathy is generally accepted to be unique to male rats, and male rats also have a tendency to develop chronic progressive nephropathy more than female rats. Neither process has any relevance in human risk assessment. There was an increased incidence of fatty change in the adrenal cortex in males at 1250 ppm and above and in females at 2500 ppm and above. In the spleen, there were increases in the incidence and severity of extramedullary haematopoiesis and haemosiderosis in males at 5000 ppm and an increase in the severity of haemosiderosis in females at 2500 and 5000 ppm.

Rat-specific pathology (α_{2u}-globulin nephropathy) was observed in males, but as this has no human relevance, it was not expected to form the basis for a NOAEL. There is no equivalent explanation available for the dose-related increase in chronic tubular lesions in female rats in the 3-month study, in which there was an increased incidence of chronic tubular lesions at 250 ppm, equal to 19.2 mg/kg bw per day; however, while this description presents a strict account of the observations within this particular study, it is significant that there was no similar increased incidence of kidney lesions in female rats after 12 months or 24 months (see below). It is concluded that these observations of renal tubule pathology in female rats represent an incidental finding that was not replicated in studies of longer-term duration.

The NOAEL of human relevance in the 90-day dietary study in rats was 250 ppm, equal to 17.6 mg/kg bw per day, based on reduced body weight gain and increased incidence of fatty change in the adrenals in males at 1250 ppm, equal to 84.9 mg/kg bw per day (Bachmann, 1996b).

Dogs

In a 90-day oral toxicity study in dogs, groups of four male and four female purebred Beagle dogs were administered thiamethoxam (batch No. P.506006; purity 98.6%) in the diet at a concentration of 0, 50, 250, 1000 or 2500/2000 ppm, equal to 0, 1.58, 8.23, 32.0 and 54.8 mg/kg bw per day in males and 0, 1.80, 9.27, 33.9 and 50.5 mg/kg bw per day in females, for 13 weeks. Dogs were observed daily for clinical signs of toxicity; feed consumption was measured daily, and body weight was recorded weekly. Eye examinations were conducted before the start and at week 13 of treatment. Haematology, blood chemistry and urinalysis were performed before exposure and at weeks 7 and 13. At autopsy, the weights of selected organs were recorded for all dogs. All major organs and tissues were examined microscopically.

Table 14. Histopathology of rats treated with thiamethoxam in the diet for 90 days

	Incidence of finding					
	Dietary concentration (ppm)					
	0	25	250	1250	2500	5000
Number of animals	*10*	*10*	*10*	*10*	*10*	*10*
Males						
Liver						
- Hepatocellular hypertrophy	0	0	0	0	6	10
- Lymphohistiocytic infiltration	3	4	6	5	4	9
- Cholangiofibrosis	2	4	2	4	5	6
- Pigmentation of Kupffer cells	0	0	0	0	0	0
Kidneys						
- Hyaline change	1	0	4	8	10	10
weighted grade	*1.0*	*0*	*1.3*	*1.5*	*1.7*	*2.3*
- Chronic tubular lesion	0	1	3	6	10	9
- Acute tubular lesion	0	0	0	3	8	9
- Lymphohistiocytic infiltration	2	2	1	3	6	3
- Basophilic proliferation	2	1	2	4	6	10
- Dilatation of renal pelvis	1	0	2	1	5	1
- Hyperplasia of pelvic epithelium	0	0	0	1	3	0
- Cast formation	1	3	2	3	3	5
- Calcification of renal cortex	0	0	0	0	0	1
- Nephrocalcinosis	0	0	0	0	0	0
Spleen						
- Haemosiderosis incidence	7	8	9	7	9	10
weighted grade	*1.0*	*1.9*	*1.4*	*1.6*	*1.7*	*2.0*
- Extramedullary haematopoiesis	3	2	3	5	2	5
weighted grade	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.8*
Adrenal gland						
- Fatty change	4	5	5	7	7	8
Females						
Liver						
- Hepatocellular hypertrophy	0	0	0	0	0	8
- Lymphohistiocytic infiltration	4	5	6	7	9	10
- Cholangiofibrosis	2	1	2	1	3	1
- Pigmentation of Kupffer cells	0	0	0	0	0	6
Kidneys						
- Hyaline change	0	0	0	0	0	0
- Chronic tubular lesion	4	5	7	7	9	10
- Acute tubular lesion	0	0	0	0	0	0
- Lymphohistiocytic infiltration	0	1	0	0	0	0
- Basophilic proliferation	2	1	1	2	0	1
- Dilatation of renal pelvis	2	0	1	1	0	0
- Hyperplasia of pelvic epithelium	0	0	0	0	0	0

Table 14 (continued)

	Incidence of finding					
	Dietary concentration (ppm)					
	0	25	250	1250	2500	5000
- Cast formation	0	1	0	0	2	0
- Calcification of renal cortex	0	0	0	0	0	1
- Nephrocalcinosis	10	10	10	10	10	10
weighted grade	*1.6*	*1.8*	*1.9*	*1.7*	*2.4*	*2.5*
Spleen						
- Haemosiderosis incidence	9	10	10	10	10	10
weighted grade	*1.7*	*2.3*	*2.1*	*1.9*	*2.6*	*2.7*
- Extramedullary haematopoiesis	4	6	6	7	2	3
Adrenal gland						
- Fatty change	0	0	2	1	4	6

From Bachmann (1996b)

Preliminary diet analyses demonstrated the stability of thiamethoxam in diet at room temperature. Representative analyses of test diets demonstrated achieved concentrations to be in the 91.4–97.8% range of nominal concentrations.

All dogs survived the scheduled treatment period, and there were no clinical signs of an adverse effect of treatment. Seven dogs in the highest dose group (receiving 2500 ppm) lost weight during the first 2 weeks, necessitating a dose reduction to 2000 ppm. Three of these dogs continued to show markedly depressed weight gain, whereas weight gain of the remaining animals was unaffected by treatment. In female dogs of this 2000 ppm group, there were group mean body weight losses at weeks 4 and 8 and a modest cumulative weight gain at week 13. Weight gains of the dogs receiving diets containing up to 1000 ppm thiamethoxam were unaffected at any time. Feed consumption was depressed at 2500 ppm and to a lesser extent when the dose level was reduced to 2000 ppm. Feed consumption was also slightly depressed in females at 1000 ppm. There was no evidence of ocular toxicity at any dose level.

Treatment-related haematology and blood chemistry effects occurred at 1000 and 2000 ppm (Table 15). Slight anaemia, associated with a tendency to hypochromasia, anisochromasia and microcytosis, had developed by week 13 in females at 2000 ppm. Reduced white cell counts (total, neutrophils, monocytes and lymphocytes) also occurred in females. Males at 2000 ppm showed reduced monocyte counts and a tendency to hypochromasia and anisochromasia of red blood cells. Eosinophilia occurred in some dogs at this dose level. Some dogs in the 1000 and 2000 ppm groups had prolonged thromboplastin (prothrombin) times. Consideration of the development of thromboplastin times within the individual dogs showed that, when compared with their own control (i.e. pretreatment) values, there were effects in males, with a NOAEL of 250 ppm, but no effect in females.

Lower plasma calcium concentration and alanine aminotransferase (ALT) activity were observed in both sexes at 1000 and 2000 ppm (Table 16). The latter was not considered to be an adverse effect, as no accompanying hepatocellular changes were observed microscopically. Furthermore, consideration of the development of plasma calcium concentrations within the individual dogs showed that, when compared with their own control (i.e. pretreatment) values, there were no effects in males at any dose level, but there was an effect in females, with a NOAEL of 1000 ppm.

Minimally reduced plasma albumin levels in both sexes at 1000 and 2000 ppm and minimally lower cholesterol and phospholipid levels in males at 2000 ppm are considered to be physiological adaptive responses rather than primary effects of thiamethoxam. There were no treatment-related effects on urinary physiological parameters or cellular and chemical constituents of urine.

Table 15. Selected haematology of dogs treated with thiamethoxam in the diet for 90 days

Parameter (unit)	Week	Dietary concentration (ppm)				
		0	50	250	1000	2000
Males						
White blood cells (10^9/l)	7	10.77	10.91	13.20	9.352	7.985
	13	9.233	10.41	10.65	9.548	10.24
Neutrophils (10^9/l)	7	5.940	6.095	8.334	4.950	3.418
	13	5.035	6.150	6.675*	5.998	6.138
Basophils (10^9/l)	7	0.055	0.045	0.046	0.043	0.039
	13	0.068	0.043	0.045*	0.043	0.035*
Monocytes (10^9/l)	7	0.550	0.385	0.561	0.364	0.126*
	13	0.328	0.368	0.335	0.340	0.058*
Red blood cells (10^{12}/l)	7	6.470	6.300	5.885	6.357	6.415
	13	6.625	6.163*	6.218	6.550	6.446
Haemoglobin (mmol/l)	7	8.575	8.425	8.038	8.400	8.525
	13	8.975	8.400	8.575	8.650	8.450
Haematocrit (l)	7	0.421	0.420	0.390*	0.413	0.416
	13	0.444	0.409	0.417	0.420	0.416
Thromboplastin (prothrombin) time (s)	7	33.64	33.68	32.11	39.43*	37.24
	13	36.18	35.82	36.63	41.61	41.16$^+$
Females						
White blood cells (10^9/l)	7	9.155	9.895	10.99	11.24*	4.581*
	13	8.223	8.590	10.08*	9.905	4.156*
Neutrophils (10^9/l)	7	5.138	5.413	6.040	6.125	1.701*
	13	4.958	4.808	6.235	5.745	1.631*
Monocytes (10^9/l)	7	0.375	0.408	0.445	0.445	0.084*
	13	0.183	0.198	0.225	0.303	0.093
Red blood cells (10^{12}/l)	7	6.330	6.083	6.618	6.078	6.036
	13	6.688	6.348	6.573	6.703	5.971*
Haemoglobin (mmol/l)	7	8.875	8.200	8.825	8.100	7.963
	13	9.400	8.550	8.850	8.850	7.813*$^-$
Haematocrit (l)	7	0.433	0.393*	0.428	0.390	0.377*
	13	0.456	0.415*	0.434	0.431	0.381*$^-$
MCV (fl)	7	86.33	64.53*	64.55*	64.10*	62.61*$^-$
	13	68.28	65.43	65.98	64.40*	63.86
MCH (fmol)	7	1.403	1.345	1.333	1.333*	1.323*
	13	1.408	1.348	1.345	1.323*	1.305*
Thromboplastin (prothrombin) time (s)	7	31.26	35.95*	34.66	39.33*	36.59
	13	35.37	37.88	38.29	44.26*$^+$	41.92$^+$

From Altmann (1996)

MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; * $P \leq 0.05$ by Dunnett's *t*-test (two-tailed); −/+ negative/positive trend (Jonckheere)

Table 16. Selected blood chemistry parameters of dogs treated with thiamethoxam in the diet for 90 days

Parameter (unit)	Week	Dietary concentration (ppm)				
		0	50	250	1000	2000
Males						
ALT (U/l)	7	35.50	41.13	29.85	25.00*	18.13*–
	13	81.03	49.00	35.08	25.40*–	13.10*–
Albumin (g/l)	7	31.89	32.13	31.88	30.65	30.02*
	13	33.10	32.85	33.69	31.18	30.31
Ca^{2+} (mmol/l)	7	2.803	2.758	2.788	2.678	2.710*
	13	2.743	2.755	2.810	2.698	2.698
Phospholipid (mmol/l)	7	4.465	4.160	4.285	4.208	3.703*
	13	4.455	4.235	4.108	4.068	3.698*
Cholesterol (mmol/l)	7	3.933	3.698	3.730	3.663	3.308
	13	3.930	3.853	3.633	3.753	3.368
Females						
ALT (U/l)	7	50.20	43.35	37.70*	26.63*–	17.41*–
	13	53.83	42.50	40.73	25.23*–	13.10*–
Albumin (g/l)	7	33.65	32.95	33.28	30.83	30.66–
	13	34.76	33.81	33.90	31.96*	30.15*–
Ca^{2+} (mmol/l)	7	2.798	2.723	2.840	2.689	2.640*
	13	2.783	2.735	2.815	2.695	2.663*
Cholesterol (mmol/l)	7	3.698	3.523	3.225	3.150	3.650
	13	3.733	3.665	3.170	3.175	3.488
Phospholipid (mmol/l)	7	4.223	4.193	3.875	3.775	4.028
	13	4.210	4.265	3.720*	3.678	3.815

From Altmann (1996)

U, units; * $P \leq 0.05$ by Dunnett's *t*-test (two-tailed); –/+ negative/positive trend (Jonckheere)

No treatment-related macroscopic changes were evident at necropsy. Testis and ovary weights were reduced at 2000 ppm by 43% and 35%, respectively, and histological correlates were identified. Slightly reduced heart, liver and kidney weights in females at 2000 ppm and increased thyroid weights in males at 50, 1000 and 2000 ppm were not associated with histological changes and are considered incidental to treatment with thiamethoxam. Minimal to marked reductions in spermatogenesis and increased incidences of spermatic giant cells occurred in the testes of all males at 2000 ppm (Table 17). One male also showed moderate tubular atrophy. These changes represent an immature histological appearance of the testis. Immature ovaries, consistent with retarded maturation, were observed in three females at 2000 ppm. The three dogs affected included two that failed to gain weight throughout the 13-week period and one that gained weight only modestly. The fourth female, which was least affected by general body weight effects, also did not demonstrate retarded ovarian development. The maturity of the uterus in two of these dogs reflected the immature stage of ovarian development. These delays in sex organ development are likely to have been a consequence of the general delay in growth and development resulting from thiamethoxam treatment and not a specific effect of thiamethoxam on the ovary. It is noted that the ages of these dogs at the beginning of treatment ranged from 23 to 27 weeks for males and from 22 to 30 weeks for females.

Table 17. Histopathology of selected sex organs of dogs treated with thiamethoxam in the diet for 90 days

Organ and finding	Males					Females				
	Dietary concentration (ppm)									
	0	50	250	1000	2000	0	50	250	1000	2000
Number of animals	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
Testes										
Tubular atrophy	0	0	0	0	1	—	—	—	—	—
Spermatogenesis reduced	0	1	0	0	4	—	—	—	—	—
Spermatic giant cells	1	1	0	1	4	—	—	—	—	—
Uterus										
Immature	—	—	—	—	—	0	0	0	0	2
Ovary										
Immature	—	—	—	—	—	0	0	0	0	3

From Altmann (1996)

The NOAEL in the 90-day oral toxicity study in dogs was 250 ppm, equal to 8.23 mg/kg bw per day, based on prolonged thromboplastin times at 1000 ppm, equal to 32.0 mg/kg bw per day (Altmann, 1996).

In a 52-week oral toxicity study, groups of four male and four female purebred Beagle dogs were administered thiamethoxam (batch No. P.506006; purity 98.6%) in the diet at a concentration of 0, 25, 150, 750 or 1500 ppm, equal to 0, 0.70, 4.05, 21.0 and 42.0 mg/kg bw per day in males and 0, 0.79, 4.49, 24.6 and 45.1 mg/kg bw per day in females. Dogs were observed twice daily for mortality and clinical signs of toxicity; feed consumption was measured daily, and body weight was recorded weekly. Eye examinations were conducted before the start and at the end of treatment. Haematology, blood chemistry and urinalysis were performed before exposure and at weeks 13, 26 and 52. At autopsy, the weights of selected organs were recorded for all dogs. All major organs and tissues were examined microscopically.

Analysis of diet samples was performed on the 13 diet mixes prepared throughout the study. The first mix was also analysed for homogeneity and stability. Analysis demonstrated thiamethoxam to be stable in the diet for at least 7 weeks at room temperature and to be homogeneously mixed. Achieved concentrations ranged from 86.3% to 107.4% of nominal concentrations. The overall mean achieved concentrations were 23.8, 140.6, 702 and 1433 ppm, in order of increasing nominal concentration.

No deaths or treatment-related clinical signs occurred during the study. The overall body weight gain of males at 1500 ppm was reduced by 26% during the study. Body weight gain was transiently lower among male dogs of the 25, 150 and 750 ppm groups at week 13 by 36%, 50% and 50%, respectively, but had recovered by week 26, so that cumulative weight gains were similar to those of the controls in all groups of males up to and including the 750 ppm group. Transient body weight loss occurred in females at 1500 ppm at the study start, but body weight subsequently increased and overall weight gain was comparable to that of the controls by week 26. Mean weight gain in 750 ppm females was depressed due to two animals that lost weight during the treatment period in spite of normal feed intake. As weight gain was not substantially affected at 1500 ppm, this finding is considered incidental to treatment. Mean feed consumption and conversion ratios were unaffected by treatment in male groups but were slightly reduced in females at 750 and 1500 ppm at the start of treatment. No ocular toxicity was evident at week 52.

Table 18. Selected blood chemistry parameters of dogs treated with thiamethoxam in the diet for up to 52 weeks

	Week	Dietary concentration (ppm)				
		0	25	150	750	1500
Males						
Urea (mmol/l)	−1	3.23	3.99	4.16	4.20	4.43
	13	3.09	3.75*	4.08	4.13*	5.53*$^{+}$
	26	3.85	4.50	4.94	4.89*	5.30
	52	3.79	4.78	4.65	4.83	4.97*
Creatinine (µmol/l)	−1	60.48	61.70	62.65	62.60	64.35
	13	61.95	65.44	65.73	68.93	85.23*
	26	60.63	65.34	66.05	71.15*	81.58*$^{+}$
	52	66.53	75.10	72.88	77.73*	85.95*$^{+}$
ALT (U/l)	−1	52.60	52.23	65.90	51.88	64.28
	13	49.10	53.28	49.73	33.38	22.05*$^{-}$
	26	49.40	45.80	49.90	32.60	23.65*$^{-}$
	52	54.10	45.40	53.05	33.95	30.53*$^{-}$
Females						
Urea (mmol/l)	−1	4.21	3.86	5.09	3.96	4.22
	13	3.82	3.92	4.23	5.35^{+}	5.08
	26	3.67	4.07	4.40	5.06*$^{+}$	4.21
	52	4.99	4.60	4.40	5.65	4.68
Creatinine (µmol/l)	−1	64.05	60.48	67.20	64.78	65.28
	13	62.63	65.53	71.38	79.15*	81.04*$^{+}$
	26	64.48	62.66	70.63	77.14	82.25*$^{+}$
	52	71.98	72.35	78.95	86.29	86.45
Albumin (g/l)	−1	35.32	35.15	33.42*	35.04	33.80
	13	35.48	35.60	34.14	33.55	31.90
	26	35.35	34.94	33.36	33.32	32.30*$^{-}$
	52	35.00	36.03	32.13	32.34	30.61
Albumin/globulin ratio	−1	1.50	1.51	1.59	1.57	1.44
	13	1.43	1.45	1.27	1.26	1.11^{-}
	26	1.38	1.36	1.27	1.31	1.19*
	52	1.27	1.35	1.25	1.18	1.10

From Altmann (1998)

U, units; * $P \leq 0.04$, Wilcoxon; −/+ negative/positive trend ($P \leq 0.01$, Jonckheere)

There were no treatment-related changes in haematological profiles at any dose level, except for prothrombin times, which were lower in both sexes at 1500 ppm throughout treatment compared with the controls.

There was a dose-related, minimal to slight increase in plasma creatinine and a tendency to higher plasma urea levels throughout the treatment period in both sexes at 750 and 1500 ppm (Table 18). A slight to moderate decrease in ALT activity, relative to pretest values, occurred in males at 750 and 1500 ppm during treatment, but could not be correlated with histological changes in the liver. Therefore, a reduction in the activity of this enzyme is considered not to represent an adverse

Table 19. Weights of selected organs in dogs treated with thiamethoxam in the diet for 52 weeks

	Males					Females				
	Dietary concentration (ppm)									
	0	25	150	750	1500	0	25	150	750	1500
Carcass										
- Absolute (kg)	11.46	11.43	11.80	10.95	11.17	10.56	10.27	10.38	9.61	10.46
Heart										
- Absolute (g)	112.7	112.8	114.0	112.4	123.1	102.8	101.2	88.9	98.9	109.1
- Relative (% body weight × 10)	9.85	9.87	9.65	10.25	11.03	9.78	9.86	8.58	10.52	10.48
Testes/ovaries										
- Absolute (g)	19.07	20.48	19.83	20.65	16.06	1.51	1.10	1.66	1.06	1.03
- Relative (% body weight × 10)	1.66	1.79	1.69	1.89	1.44	0.14	0.11	0.15	0.12	0.10
Spleen										
- Absolute (g)	59.59	47.05	37.17	29.92^{-}	47.40	73.58	66.69	50.89	46.52	47.37
- Relative (% body weight × 10)	5.24	4.12	3.15	2.72^{-}	4.22	6.91	6.38	4.82	5.26	4.49
Thyroid gland										
- Absolute (g)	1.17	1.08	0.99	1.24	1.18	1.08	1.06	1.14	2.38	1.22
- Relative (% body weight × 10)	0.10	0.09	0.08	0.11	0.11	0.10	0.10	0.11	0.21	0.12

From Altmann (1998)

$^{-}$ negative trend ($P \leq 0.01$, Jonckheere)

effect. Females at 1500 ppm had minimally lower albumin levels, associated with lower albumin to globulin ratios. No other treatment-related effects on blood chemistry occurred. There were no treatment-related quantitative or qualitative effects on urinary composition at any dose level.

Analysis of absolute and relative organ weights indicated that there was a minimal decrease in absolute and relative testis weights in 1500 ppm dogs, due mainly to the low testis weights of two animals (Table 19). Higher heart and thyroid weights in 1500 ppm males and lower ovary and spleen weights in females at 750 and 1500 ppm did not have histopathological correlates and are therefore considered incidental.

At autopsy, pulmonary nodules or mottled lungs were observed in several control and treated animals of both sexes. Microscopically, these findings represented various inflammatory or post-inflammatory changes that commonly occur in control animals from this source and are considered incidental to treatment. Microscopic examination of tissues/organs revealed a higher incidence of tubular atrophy in the testes at 750 and 1500 ppm; as this observation was also made in control animals, it is not clearly associated with treatment (Table 20). At 1500 ppm, the observation correlated with reduced testis weights in two animals. Although not clearly treatment related, this increase in the recorded incidence of testicular atrophy is consistent with the observation of an immature testis. Together with the lower testis weights in two animals, this may represent delayed testicular development, most likely as a consequence of the general growth and developmental delay resulting from treatment at the higher dose levels. Other microscopic alterations observed, particularly nonspecific inflammatory changes, thymic atrophy and fatty change of adrenal cortical cells, occur commonly in this colony of dogs, and the morphology and severity did not indicate an effect of treatment. Significantly, in comparison with the 90-day study in dogs, there was no evidence for developmental delay in female sex organs in this longer-term study.

Table 20. Histopathology of selected organs of dogs treated with thiamethoxam in the diet for 52 weeks

	Males					Females				
	Dietary concentration (ppm)									
	0	25	150	750	1500	0	25	150	750	1500
Number of animals examined	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
Adrenal glands										
Cortical fatty change	0	0	0	1	0	1	3	3	3	3
Lung										
Fibrosis	1	0	1	0	1	3	2	1	1	2
Bronchial-alveolar hyperplasia	0	0	1	0	0	1	1	0	0	0
Chronic inflammation	3	2	2	3	4	4	4	4	4	2
Osseous metaplasia	0	0	0	0	0	0	0	0	1	0
Testes										
Tubular atrophy	1	1	1	2	2	—	—	—	—	—
Spermatid giant cells	2	1	1	0	1	—	—	—	—	—
Inflammatory cell infiltration	0	0	1	0	0	—	—	—	—	—
Thymus										
Atrophy	3	4	3	3	4	3	2	2	4	3
Developmental cyst	2	3	1	2	1	—	3	2	3	1
Granuloma	0	0	0	0	0	0	0	0	1	0
Phagocytic cells	0	0	0	0	0	1	0	0	0	0

From Altmann (1998)

The NOAEL in the 52-week oral toxicity study in dogs was 750 ppm, equal to 21.0 mg/kg bw per day, based on prolonged thromboplastin times and reduced testis weights at 1500 ppm, equal to 42.0 mg/kg bw per day (Altmann, 1998).

(b) Dermal application

In a 28-day dermal toxicity study, groups of five male and five female Sprague-Dawley-derived (Tif:RAIf, SPF strain) rats were administered thiamethoxam (batch No. P.506006; purity 98.6%) at a dose level of 0, 20, 60, 250 or 1000 mg/kg bw per day applied under a semiocclusive dressing to intact shaven dorsal skin for 6 hours per day, 5 days per week, for 4 weeks. Thiamethoxam was formulated as a suspension in aqueous 1% carboxymethylcellulose/0.1% Tween 80 at nominal concentrations of 0, 10, 30, 125 and 500 mg/ml in order of increasing dose level. Mortality and clinical signs were checked daily, and individual body weights and feed and water consumption were recorded weekly. Haematological and blood chemistry investigations were performed on all rats towards the end of the treatment period. All animals were subjected to autopsy and postmortem examination, major organs were weighed and selected tissues were examined microscopically.

There were no deaths or clinical signs of toxicity during the study. Body weight gains in males of the 1000 mg/kg bw per day group were retarded during the first 2 weeks of treatment, but not at lower dose levels or in females at any dose level. Feed consumption was not affected in any group. Haematological profiles were unaffected by treatment at all dose levels. All statistically significant values showed no dose–response relationship, or the recorded values were within historical ranges. Females at 250 and 1000 mg/kg bw per day had slightly raised plasma glucose concentrations and minimally raised alkaline phosphatase activities (Table 21). Triglyceride concentrations were also

Table 21. Selected blood chemistry parameters in rats treated with thiamethoxam by skin application for 28 days

Parameter	Males					Females				
	Dose (mg/kg bw per day)									
	0	20	60	250	1000	0	20	60	250	1000
Glucose (mmol/l)	7.528	7.786	7.886	7.592	6.622	6.390	6.126	6.159	9.592*	9.739*+
Alkaline phosphatase (U/l)	171.5	140.0	144.7	155.2	135.6	108.3	95.42	95.62	174.5*	139.8+
Triglycerides (mmol/l)	0.501	0.623	0.688	0.708	0.516	0.515	0.506	0.438	0.700	1.091*+

From Gerspach (1996)

U, units; * $P < 0.05$ (Wilcoxon); + positive trend ($P < 0.01$, Jonckheere)

slightly raised in females at 1000 mg/kg bw per day. Significantly elevated plasma potassium concentrations in all groups of male rats were considered not to have been influenced by thiamethoxam, as control values were unusually low. Other differences from the controls in blood chemistry are not considered to be treatment related because they were too small to be of toxicological relevance (e.g. inorganic phosphate levels in males at 1000 mg/kg bw per day) or their occurrence showed no relationship to dose level.

No anomalies were observed at autopsy, and all absolute organ weights and their ratios to body weight were unaffected by treatment. Morphological changes in the skin at the application site occurred at low incidence in both treated and control animals and are considered to have been induced by the treatment procedure rather than by a response to thiamethoxam. The liver, kidneys and adrenal glands were identified as target organs on the basis of histological findings (Table 22). An increased incidence of minimal inflammatory cell infiltration and necrosis of single hepatocytes occurred in females at 250 and 1000 mg/kg bw per day; the elevated incidence of this lesion at 60 mg/kg bw per day and higher was considered to fall within the limits of normal variation for this strain of rat. Effects on liver morphology were absent in the males. In the kidneys, minimal tubular hyaline change in males and minimal chronic tubular lesions in females were apparent at 1000 mg/kg bw per day. A single female at this dose level also showed minimal basophilic cell infiltration in the renal tubules. Lower dose levels were unaffected by these renal changes. In the adrenal glands, minimal inflammatory cell infiltration was evident in the cortex of females at 1000 mg/kg bw per day. No other groups were affected.

The NOAEL in the 28-day dermal toxicity study in rats was 60 mg/kg bw per day, based on the blood chemistry changes (increased glucose and alkaline phosphatase levels) in females at 250 mg/kg bw per day. In view of the low order of toxicity by the percutaneous route in this study, a 90-day dermal study is not required (Gerspach, 1996).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 60 male and 60 female albino mice (Tif:MAGf, SPF strain) were administered thiamethoxam (batch No. P.506006; purity 98.6%) orally for 78 weeks in the diet at a concentration of 0, 5, 20, 500, 1250 or 2500 ppm, equal to 0, 0.65, 2.63, 63.8, 162 and 354 mg/kg bw per day in males and 0, 0.89, 3.68, 87.6, 215 and 479 mg/kg bw per day in females. Ten mice of each sex per group were designated for clinical laboratory investigations, and 50 mice of each sex per group for the oncogenicity study. Additional groups of 10 mice of each sex in the control and high-dose groups were similarly treated with thiamethoxam for 35 weeks and then killed for interim evaluation. Clinical

Table 22. Selected histopathological findings in rats treated with thiamethoxam by skin application for 28 days

Organ/finding	Incidence of finding									
	Males					Females				
	Dose (mg/kg bw per day)									
	0	20	60	250	1000	0	20	60	250	1000
Number of animals examined	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*
Adrenal glands										
Cortical fatty change	4	4	3	3	4	1	0	1	1	1
Inflammatory cell infiltration	0	0	0	0	0	0	1	0	1	3
Kidneys										
Chronic progressive nephropathy	0	0	0	0	1	0	1	0	0	0
Glomerulosclerosis	0	1	0	0	0	0	0	0	0	0
Chronic tubular lesion	1	0	1	1	1	1	2	2	1	4
Nephrocalcinosis	1	0	0	1	0	3	5	5	5	5
Renal tubular cast	0	0	1	0	1	0	1	0	1	0
Hyaline change	1	2	1	1	3	0	0	0	0	0
Calcification	0	1	0	0	0	0	0	0	0	0
Basophilic infiltration	2	2	0	2	1	0	0	0	0	1
Liver										
Inflammatory cell infiltration	3	4	4	3	2	2	2	4	5	5
Intrahepatic bile duct cholangiofibrosis	1	0	0	1	1	0	0	0	0	0
Extramedullary haematopoiesis	0	0	0	0	0	1	0	0	0	1
Kupffer cell hyperplasia	0	0	0	0	0	1	0	1	0	0
Hepatocyte necrosis	0	0	0	0	0	2	0	2	3	4

From Gerspach (1996)

observations were made daily, and body weights and feed consumption were recorded weekly for 13 weeks and monthly thereafter. Haematological investigations were performed at weeks 53 and 78 on samples from 10 mice of each sex per group. All mice were subjected to detailed autopsy and postmortem examination. Organ weights of all mice that survived to the end of their scheduled dosing period were recorded. Tissue/organ samples from all mice killed at 35 weeks, those that died or were killed during the study and all mice killed after 78 weeks were fixed and preserved. Microscopic examination of tissues was performed on all mice of the oncogenicity subgroup. The livers of the additional 10 mice of each sex in the control and high-dose groups were also examined microscopically after haematoxylin–eosin staining and appropriate special stains. In-life and organ weight data were statistically analysed by a univariate technique, using non-parametric methods where appropriate. Survival analysis was by Cox's regression model. Neoplastic lesions were analysed using Peto's mortality prevalence test, and non-neoplastic lesions by the Cochran-Armitage linear trend test.

Diet analyses demonstrated thiamethoxam to be stable in feed for at least 5 weeks at room temperature, whereas fresh diets were prepared monthly. Analysis of diet samples demonstrated a homogeneous distribution of thiamethoxam. The overall mean analytically determined concentrations were 4.9, 20.3, 502, 1251 and 2598 ppm.

Survival incidence and the incidences of single/multiple palpable masses were not affected by treatment in either sex. Clinical signs were restricted to a slightly increased incidence of abdominal distension in females at 1250 ppm and in males at 1250 and 2500 ppm. This finding correlated histologically, in most cases, with benign or malignant liver tumours. There was a slightly increased incidence of hypoactivity in females at 2500 ppm, which correlated with perimortal findings in unscheduled deaths. At 2500 ppm, body weight gain was retarded in males from week 7 and in females from week 38, resulting in an overall depression of weight gains of 18% and 14%, respectively. Feed consumption was unaffected by treatment in both sexes at all dose levels.

There were no treatment-related changes in haematological profiles at 53 and 78 weeks at any dose level. Significantly higher mean corpuscular haemoglobin occurred in males at 2500 ppm at 53 and 78 weeks, but as erythrocyte numbers, haemoglobin concentration and haematocrit values were unaffected by treatment, the differences were considered to have no toxicological significance. Occasional statistically significant differences from the controls occurred in some haematological parameters but were considered not to be toxicologically significant because there was no dose–response relationship or the changes were too small to be of biological relevance. Lymphatic leukaemia occurred in two control females, one female at 5 ppm, one female and two males at 500 ppm and one female at 1250 ppm. Females with leukaemia contributed to higher mean white blood cell counts and unstained cell counts in control and 500 ppm animals at week 78. No toxicological significance was attributed to these findings.

At week 35, the only effects on organ weights attributable to treatment were increased liver and adrenal weights in females treated at 2500 ppm. At week 79, mean carcass weight was reduced in males at 2500 ppm. Liver weights were increased dose-dependently in both sexes at 500, 1250 and 2500 ppm (Table 23). Other significant differences at 2500 ppm are considered to be incidental to treatment because of an effect on body weight (male brain weight), no histological correlate (male kidney weight) or aberrant mean value in the control group due to the presence of tumours (female spleen weight).

There were no treatment-related gross lesions at autopsy in week 35. Gross examination at week 79 revealed an increased incidence of masses and nodules in the liver at doses of 500 ppm and above, particularly in male groups. Thickening of the stomach in three males at 2500 ppm, a decreased incidence of enlarged seminal vesicles at 1250 and 2500 ppm and a decreased incidence of enlarged spleen in females at 2500 ppm are considered to be treatment related, as histological correlates were noted. A slight increase in the incidence of uterine masses at 2500 ppm was not supported by microscopic findings, and this was therefore considered incidental to treatment.

Liver histopathology performed at week 35 revealed non-neoplastic lesions only. A treatment-related increase occurred in the incidences of inflammatory cell infiltration, necrosis of single hepatocytes, Kupffer cell pigmentation and hepatocyte hypertrophy in both sexes at 2500 ppm (Table 24). The severity ranged from minimal to moderate. At week 79, treatment-related, non-neoplastic lesions in the liver occurred at 500 ppm and above, comprising increased incidences of focal cellular alteration (mainly eosinophilic), inflammatory cell infiltration, necrosis of single hepatocytes, hepatocellular hypertrophy, increased mitotic activity, deposition of pigments (lipofuscin and haemosiderin) and hyperplasia of Kupffer cells. The lesions ranged in severity from minimal to marked, and their incidences generally showed a positive dose–response relationship.

Treatment-related, non-neoplastic effects in tissues other than liver were increased incidences and severity of splenic extramedullary haematopoiesis and gastric mucosal epithelial hyperplasia in both sexes at 2500 ppm. The latter lesion was frequently accompanied by mild inflammatory cell infiltration and, in males, could be correlated with grossly observable thickening of the stomach wall. The incidences of some degenerative and inflammatory lesions were decreased at 2500 ppm and occasionally at 500 and 1250 ppm. Effects observed at reduced incidences included adrenal cortical hyperplasia, cataract formation, chronic nephropathy in males, pancreatic islet cell hyperplasia in

Table 23. Treatment-related organ weight changes in mice treated with thiamethoxam in the diet for 35 or 79 weeks

Organ	Dietary concentration (ppm)	Males				Females			
		Week 35		Week 79		Week 35		Week 79	
		Absolute weight	Relative weight[a]	Absolute weight	Relative weight[a]	Absolute weight	Relative weight[a]	Absolute weight	Relative weight[a]
Carcass (g)	0	53.93	—	53.15	—	35.84	—	40.57	—
	5	—	—	53.34	—	—	—	38.48	—
	20	—	—	52.17	—	—	—	38.83	—
	500	—	—	53.48	—	—	—	40.17	—
	1250	—	—	51.65	—	—	—	40.82	—
	2500	48.88	—	48.62*$^{-}$	—	35.69	—	37.62	—
Liver (g)	0	3.02	55.62	2.97	55.73	1.88	53.35	2.34	57.87
	5	—	—	3.08	58.67	—	—	2.19	57.44
	20	—	—	2.92	55.91	—	—	2.29	59.37
	500	—	—	3.20	60.02	—	—	2.56	63.79*$^{+}$
	1250	—	—	3.79*$^{+}$	73.75*$^{+}$	—	—	2.67+	65.55*$^{+}$
	2500	2.69	54.81	5.18*$^{+}$	106.4*$^{+}$	2.30	64.44*	2.97*$^{+}$	78.54*$^{+}$
Adrenals (mg)	0	9.81	0.18	10.02	0.19	20.36	0.58	19.35	0.49
	5	—	—	9.27	0.18	—	—	19.09	0.50
	20	—	—	8.80*	0.17	—	—	17.56	0.46
	500	—	—	9.11*	0.17	—	—	20.84	0.53
	1250	—	—	9.50	0.19	—	—	18.88	0.48
	2500	9.95	0.21	10.02	0.21	25.58	0.72	18.44	0.50

From Bachmann (1998b)

* $P \leq 0.01$ (LePage); −/+ negative/positive trend ($P \leq 0.01$, Jonckheere)

[a] % body weight × 10.

Table 24. Incidences of non-neoplastic hepatic lesions in mice treated with thiamethoxam in the diet for 35 or 79 weeks

	Incidence of lesion											
	Males						Females					
	Dietary concentration (ppm)											
	0	5	20	500	1250	2500	0	5	20	500	1250	2500
Week 35												
Number examined	*10*	*0*	*0*	*0*	*0*	*10*	*10*	*0*	*0*	*0*	*0*	*10*
Inflammatory cell infiltration	1	—	—	—	—	8	3	—	—	—	—	8
Single-cell necrosis	0	—	—	—	—	10	0	—	—	—	—	9
Kupffer cell pigmentation	0	—	—	—	—	8	0	—	—	—	—	4
Hepatocyte hypertrophy	0	—	—	—	—	9	0	—	—	—	—	10
Week 79												
Number examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Deposition of pigment	2	2	3	13	33	44	6	5	3	5	14	30

Table 24 (continued)

	Incidence of lesion											
	Males						Females					
	Dietary concentration (ppm)											
	0	5	20	500	1250	2500	0	5	20	500	1250	2500
Focus of alteration	7	4	4	11	22	32	2	2	2	2	14	37
Hepatocellular hypertrophy	8	11	6	41	40	45	3	2	3	19	39	45
Increased mitotic activity	0	0	0	1	10	8	1	1	0	4	5	4
Inflammatory cell infiltration	13	9	13	33	41	43	18	20	20	24	33	45
Kupffer cell hyperplasia	0	0	1	0	0	10	2	1	0	0	1	2
Single-cell necrosis	5	3	5	40	40	46	3	2	5	18	36	46

From Bachmann (1998b)

Table 25. Incidences of neoplastic hepatic lesions in mice treated with thiamethoxam in the diet for 79 weeks

	Malignant or benign	Incidence of lesion											
		Males						Females					
		Dietary concentration (ppm)											
		0	5	20	500	1250	2500	0	5	20	500	1250	2500
Number examined		*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Hepatocellular adenocarcinoma	m	3	3	2	4	4	16	0	0	0	0	2	3
1st hepatocellular adenoma	b	9	5	8	17	21	39	0	0	0	5	8	28
2nd hepatocellular adenoma	b	3	2	3	3	12	31	0	0	0	0	5	14
Multiple hepatocellular adenomas	b	0	1	0	0	6	14	0	0	0	0	2	7
Metastatic carcinoma	m	0	0	0	0	1	0	1	0	0	0	1	0
Haemangioma	b	0	1	1	0	0	0	2	1	0	0	0	0
1st haemangiosarcoma	m	1	0	0	0	1	0	1	0	0	0	1	0
2nd haemangiosarcoma	m	1	0	0	0	1	0	0	0	0	0	0	0
Hepatoblastoma	m	0	0	0	0	1	0	0	0	0	0	0	0
Metastatic osteosarcoma	m	0	0	0	0	0	0	0	0	1	0	0	0
Any hepatic neoplastic lesion		11	7	10	19	22	44	0	0	0	5	9	29

From Bachmann (1998b)
b, benign; m, malignant

males, lymphocytic infiltration of the salivary gland in females, chronic inflammation and dilatation of the seminal vesicles (correlated with reduced incidence of enlarged seminal vesicles at necropsy), splenic white pulp hyperplasia (correlated in females with decreased incidence of enlarged spleen at necropsy) and testicular tubular atrophy. Particularly in males, reduced incidences are considered to be associated with depressed body weight gain and are not regarded as adverse effects.

At 79 weeks, the incidence of hepatocellular adenoma was significantly greater than concurrent and historical control levels in both sexes at 500 ppm and above. The incidence of hepatocellular adenocarcinoma was also significantly greater than concurrent and historical control levels in females at 1250 ppm and in both sexes at 2500 ppm (Table 25). No increased incidences of neoplasms in other organs were observed. Decreased incidences of systemic malignant lymphomas in females were consistent with significantly lower spleen weights.

The NOAEL in the 78-week dietary study in mice was 20 ppm, equal to 2.63 mg/kg bw per day, based on increased liver weights, hepatocellular hypertrophy, pigment deposition, inflammatory cell infiltration and single-cell necrosis at 500 ppm, equal to 63.8 mg/kg bw per day. Thiamethoxam was tumorigenic in mice and induced hepatocellular adenomas in male and female mice at a dose level of 500 ppm, equal to 63.8 mg/kg bw per day in male mice and 87.6 mg/kg bw per day in female mice, and hepatocellular adenocarcinomas in male mice at 2500 ppm, equal to 354 mg/kg bw per day. A mouse-specific mode of (toxic and carcinogenic) action was proposed through which it was clear that hepatotoxicity and hepatocarcinogenicity were unlikely to occur at human levels of dietary exposure (Bachmann, 1998b). A proposed mode of carcinogenic action of thiamethoxam in mice is evaluated in Appendix 1.

The NOAEL for non-hepatic effects was 1250 ppm (equal to 162 mg/kg bw per day), based on reductions in body weight and effects on spleen and stomach at 2500 ppm (equal to 354 mg/kg bw per day).

Rats

Groups of 70 male and 70 female Sprague-Dawley rats (strain Tif:RAIf, SPF) were administered thiamethoxam (batch No. P.506006; purity 98.6%) orally for 104 weeks in the diet at a concentration of 0, 10, 30, 500 or 1500 ppm, equal to 0, 0.41, 1.29, 21.0 and 63.0 mg/kg bw per day, in males and 0, 10, 30, 1000 or 3000 ppm, equal to 0, 0.48, 1.56, 50.3 and 155 mg/kg bw per day, in females. Twenty rats of each sex per group were designated for clinical laboratory investigations, and 50 rats of each sex per group for the carcinogenicity study. Additional groups of 10 rats of each sex per group were similarly treated with thiamethoxam for 52 weeks and then killed for interim evaluation. Clinical observations were made daily, body weights and feed consumption were recorded weekly for 13 weeks and monthly thereafter, and water consumption was recorded monthly. The eyes of all rats in the carcinogenicity subgroup were examined before dosing and at 104 weeks. Control and high-dose rats were also examined at weeks 26, 52 and 77. Laboratory investigations were performed at weeks 13, 27, 53 and 78 and at the end of the treatment period on 20 rats of each sex per group for haematology and on 10 rats of each sex per group for blood chemistry and urinalysis. All rats were subjected to detailed autopsy and postmortem examination. Organ weights of all rats that survived to the end of their scheduled dosing period were recorded. Tissue/organ samples from all rats killed at 52 weeks, those that died or were killed during the study and all rats killed at the end of the study were fixed and preserved. Microscopic examination of tissues was performed on all rats of all treatment and control groups, with the exception of the 20 rats of each sex per group designated for laboratory investigations. In-life and organ weight data were statistically analysed by a univariate technique, using non-parametric methods where appropriate. Survival analysis was by Cox's regression model. Neoplastic lesions were analysed using Peto's mortality prevalence test, and non-neoplastic lesions by the Cochran-Armitage linear trend test.

Diet analyses demonstrated thiamethoxam to be stable in feed for at least 7 weeks at room temperature, whereas fresh diets were prepared monthly. Analysis of diet samples 13 times demonstrated a homogeneous distribution of thiamethoxam and achieved concentrations in the range 89.3–107.6% of nominal during the 4-week use period. The overall mean analytically determined concentrations were 10, 31, 507, 1011, 1502 and 3033 ppm.

Survival incidence was not affected by treatment, there were no clinical signs of an adverse effect of treatment and the incidences of single and multiple palpable masses were unaffected by treatment at any dose level. Body weight development in all male groups was unaffected by treatment, but females at 3000 ppm had slightly depressed body weight gain from week 3 until termination, at which time cumulative weight gain was depressed by 12.6%. Feed consumption was unaffected by treatment in both sexes at all dose levels, but water consumption of males at 1500 ppm was raised by 13%. No effect on water consumption occurred in other groups. Eye examinations revealed no evidence of ocular toxicity at any time.

There were no treatment-related changes in haematology, blood chemistry or urinalysis parameters. Occasional statistically significant differences from the controls, or trends, occurred in these parameters, but were considered not to be toxicologically significant because there was no consistency between sampling intervals, there was no dose–response relationship or the changes were too small to be of biological relevance. A myeloid leukaemia was identified in one male rat at 1500 ppm in week 53. This type of neoplasm occurs spontaneously at low incidence in rats of this source and strain, and it was considered unrelated to treatment.

There were no treatment-related effects on organ weights at any dose level at either 52 or 104 weeks. At 104 weeks, mean carcass weight in high-dose males and females was reduced by 4% and 8%, respectively. There were no significant differences from control values in absolute and relative organ weights among the males, with the exception of a minor negative trend in the relative thyroid weight of males at 1500 ppm. The trend was considered not to be of toxicological significance because the mean value was similar to historical control values and no related histological changes were evident. Mean absolute adrenal weights appeared elevated in males at 10, 500 and 1500 ppm, but not at the intermediate dose of 30 ppm. The highest mean value was for the 10 ppm dose in both males and females. These are clearly erratic results and include some particularly high individual adrenal weights in the 10 and 1500 ppm group males. The variations in adrenal weights cannot be interpreted with justification as being due to thiamethoxam. Female mean relative thyroid weights were higher than control values in all treated groups, but remained within the historical control range. In the absence of corroborative clinical chemistry or histological changes, the differences were judged to be without toxicological significance.

Postmortem examination of rats sacrificed after 52 and 104 weeks and unscheduled deaths between 52 and 104 weeks revealed no treatment-related lesions. Most gross lesions occurred at comparable frequencies in the control and treated groups. Morphological correlates of gross lesions showing intergroup differences in incidence did not indicate an effect of treatment. The nature of the lesions was similar to those occurring spontaneously in this strain of rat.

No treatment-related neoplastic lesions occurred in rats that were killed at 52 weeks or that died during the first 52 weeks. Three malignant and seven benign neoplasias were identified in total. All were diagnosed as neoplasms that occur spontaneously in this strain of rat, and only pituitary adenomas occurred in more than one individual. The distribution between the control and treated groups did not indicate an effect of treatment. Non-neoplastic, treatment-related lesions at 52 weeks were increased incidences of renal tubular regenerative changes, chronic tubular lesion and tubular basophilic proliferation in males at 500 and 1500 ppm. Minimally increased incidences of renal tubular and pelvic lymphocytic infiltration also occurred at 1500 ppm, but without tubular hyaline change. These alterations are considered to represent the sequelae of α_{2u}-globulin-mediated nephropathy. The kidneys of females at all dose levels were unaffected. There was a minimal increase in the severity of splenic haemosiderosis in females at 3000 ppm from a mean score of 2.4 in the controls to 3.0 in the 3000 ppm group. In the liver, there were increased incidences of cholangiofibrosis in males at 10 ppm (5/10), 500 ppm (6/10) and 1500 ppm (4/10) compared with the controls (2/10) and an increased incidence of inflammatory cell infiltration in males at 10 ppm (4/10), 30 ppm (3/10) and 500 ppm (5/10) relative to controls (1/10). These findings, which were not apparent after 104 weeks of treatment, are considered incidental to treatment because of the lack of a dose–response relationship. Other non-neoplastic lesions occurred in animals that died during the first year or that were sacrificed at 52 weeks, but all commonly occur in this strain of rat, and the incidence, distribution and morphology did not indicate an effect of treatment.

In rats that were killed at 104 weeks or that died between weeks 53 and 104, treatment-related, non-neoplastic changes in the kidneys and liver were observed. In the kidneys, a slightly increased incidence of slight/moderate chronic nephropathy occurred in males at 1500 ppm (Table 26), accompanied by a minimal increase in the incidence of lymphocytic infiltration of the renal cortex at 1500

Table 26. Incidences of non-neoplastic renal hepatic lesions in rats treated with thiamethoxam in the diet for 104 weeks

	Incidence of lesion									
	Males					Females				
	Dietary concentration (ppm)									
	0	10	30	500	1500	0	10	30	1000	3000
Kidneys										
No. examined	*50*	*50*	*49*	*50*	*50*	*50*	*50*	*49*	*50*	*50*
Lymphocytic infiltration	10	10	7	14	17	2	3	4	2	2
Average grade	*1.6*	*1.7*	*1.4*	*1.9*	*1.7*	*1.5*	*2.0*	*1.5*	*1.5*	*2.0*
Chronic nephropathy	30	35	32	37	42	12	10	8	6	10
Average grade	*2.4*	*2.6*	*2.4*	*2.5*	*2.7*	*2.8*	*2.9*	*2.0*	*2.2*	*2.5*
Tubular hyaline change	0	1	1	0	2	2	1	0	0	0
Liver										
No. examined	*50*	*50*	*49*	*50*	*50*	*50*	*49*	*49*	*50*	*50*
Focus of cellular alteration	20	21	15	21	20	10	21	12	15	26
Average grade	*2.2*	*2.3*	*1.6*	*2.0*	*2.0*	*1.9*	*1.6*	*1.5*	*1.4*	*2.1*
Subtype:										
- Amphophilic	3	3	1	5	1	3	2	1	1	3
- Basophilic	1	2	0	2	0	4	6	5	5	6
- Clear cell	13	10	11	11	14	3	5	2	5	15
- Eosinophilic	3	5	2	3	5	0	8	4	4	2
- Mixed	0	1	1	0	0	0	0	0	0	0

From Bachmann (1998a)

ppm. Two animals also showed tubular hyaline change. In the liver, a treatment-related increase occurred in the incidence of slight/moderate focal cellular alteration, generally of the clear cell subtype, in females at 3000 ppm. All other non-neoplastic findings in the liver and kidneys were of a similar nature, severity and incidence in both treated and control groups. In other tissues, some non-neoplastic lesions occurred at slightly higher incidences in treated groups than in controls, but in all instances, these are considered unrelated to treatment with thiamethoxam, as their occurrence is not dose related, the incidence is within the historical control range or the lesions are known to occur spontaneously in aged rats of this strain.

All neoplastic findings, both malignant and benign, occurring at 104 weeks are considered incidental to treatment with thiamethoxam, as the incidences in treated and control groups are similar, the intergroup distribution shows no relationship to dose level, or the incidences are within historical control ranges and the lesions are known to occur spontaneously in aged rats.

The NOAEL in the 104-week dietary study in rats was 30 ppm, equal to 1.3 mg/kg bw per day in males, based on increased incidences of renal chronic tubular lesions and basophilic proliferation in males at 500 ppm, equal to 21.0 mg/kg bw per day. This renal lesion was considered to be α_{2u}-globulin mediated and therefore has no human relevance. The relevant NOAEL in rats in the 104-week dietary study was 1000 ppm, equal to 50.3 mg/kg bw per day in females, based on foci of cellular alteration in the liver and increased severity of splenic haemosiderosis at 3000 ppm, equal to 155 mg/kg bw per day (Bachmann, 1998a).

Table 27. Results of studies of genotoxicity with thiamethoxam

End-point	Test object	Concentration/dose[a] (LED/HID)	Batch No.; purity (%)	Result	Reference
In vitro					
Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2uvrA (± Aroclor 1254–treated rat liver S9), standard plate test	5000 µg/plate	P.506006; 98.6	Negative	Hertner (1995a)
Gene mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 (± thiamethoxam-treated mouse liver S9), standard plate test	5000 µg/plate	P.506006; 98.6	Negative	Deparade (1999)
Gene mutation	Chinese hamster lung V79 cells *hprt* locus	2220 µg/ml −S9 3330 µg/ml +S9	P.506006; 98.6	Negative Negative	Ogorek (1996a)
Chromosomal aberration	Chinese hamster ovary cells	2270 µg/ml −S9 4540 µg/ml +S9	P.506006; 98.6	Negative Negative	Zeugin (1996)
Unscheduled DNA synthesis	Primary cultures of male Tif:RAIf rat hepatocytes	1665 µg/ml	P.506006; 98.6	Negative	Ogorek (1996b)
Unscheduled DNA synthesis	Primary cultures of male Tif:MAG mouse hepatocytes	235 µg/ml	P.506006; 98.6	Negative	Ogorek (2000)
In vivo					
Micronucleus formation	Male and female Tif:MAGf mice, bone marrow cells	1000 mg/kg bw (males), 1250 mg/kg bw (females) × 1 po, observations on males at 16, 24 and 48 h and on females at 16 h after dosing	OP235005; 99.4	Negative	Hertner (1995b)

HID, highest ineffective dose; LED, lowest effective dose; po, by mouth (per os); S9, 9000 × *g* rat or mouse liver supernatant

2.4 Genotoxicity

Thiamethoxam was tested for genotoxicity in a range of assays, both in vitro and in vivo (Table 27; all study references are listed in this table). There was no evidence of mutagenic activity in two bacterial reverse mutation assays performed over dose ranges of up to 5000 µg/plate. One of these assays was performed in the presence and absence of an exogenous metabolic activating system (S9 mix) prepared in the standard way from male rats and using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA. The other assay used *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of S9 mix prepared from mice treated with three dose levels of thiamethoxam for 14 days. Also, no mutagenic activity was observed at the *hprt* locus in a single study in the presence and absence of S9 mix with Chinese hamster lung V79 cells, and no clastogenic activity was observed in a single study in the presence and absence of S9 mix with Chinese hamster ovary cells. Unscheduled DNA synthesis activity was measured in two studies with primary cultures of hepatocytes, one from rats and one from mice. No increased DNA repair synthesis was observed in either study.

A single in vivo study was conducted for micronucleus induction in polychromatic erythrocytes from the bone marrow of male and female Tif:MAGf mice treated with thiamethoxam orally by gavage on a single occasion. No increased frequencies of micronucleated cells from thiamethoxam-treated mice were observed.

It is concluded from this battery of tests that thiamethoxam demonstrated no genotoxic or mutagenic activity and is considered unlikely to present a genotoxic hazard to humans.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Two multigeneration studies were conducted on thiamethoxam, the first to comply with regulations in Japan, the second to comply with regulations in the United States of America (USA).

In a two-generation study of reproduction in rats, groups of male and female Sprague-Dawley-derived Tif:RAIf, SPF strain rats were administered thiamethoxam (batch No. P.506006; purity 98.6%) continuously in the diet at a dose level of 0, 10, 30, 1000 or 2500 ppm throughout the two generations. These dietary concentrations were equal to 0, 0.4–1.5, 1.4–4.3, 45.6–144.0 and 117.6–362.9 mg/kg bw per day for males and 0, 0.6–2.1, 1.8–6.4, 59.3–219.6 and 14.8–541.3 mg/kg bw per day for females.

The F_0 generation comprised 30 males and 30 females in each dose group. They received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation for two litters per generation. Litters were culled to four pups of each sex, where possible, on day 4 post-partum. The F_1 generation was selected from the first litters of the F_0 generation. Clinical signs, body weights, feed consumption, mating, gestation and parturition parameters, pup survival and developmental or behavioural landmarks were recorded. A gross necropsy examination was performed on all pups not selected for mating. All parental animals were necropsied after weaning of the second litters and subjected to macroscopic examination and histopathology of the sex and target organs. Sperm analysis (motility, morphology and spermatid counts) was performed on 15 males per group of the F_0 and F_1 parental generations. Male and female mating and fertility indices, female gestation and parturition indices, and litter live birth, viability and lactation indices were calculated. Continuous data were analysed statistically using analysis of variance (ANOVA) and Dunnett's *t*-test, quantal data by the chi-squared test followed by Fisher's exact test, and non-parametric data by Kruskal-Wallace non-parametric ANOVA followed by the Dunnett test.

In an ancillary sperm cell parameter study, groups of 30 previously unmated male Sprague-Dawley-derived rats (Tif:RAIf, SPF strain, age 6–7 weeks) were fed thiamethoxam in the diet for 10 weeks at a nominal concentration of 0, 10, 30, 1000 or 2500 ppm. Body weight, feed consumption and clinical signs were recorded. After 10 weeks, the animals were killed and necropsied, the testes, epididymides, prostate and seminal vesicles (with coagulating glands) were weighed, and epididymis sperm cells and/or testis spermatids were evaluated for number, motility and morphology. Changes in procedure from the first study were implemented to reduce and standardize the time for sperm collection, to refine the technique for opening the cauda epididymis and to randomize the order in which sperm evaluations were performed to minimize between-day bias.

Analysis of representative diet samples demonstrated that thiamethoxam was stable in the diet for at least 5 weeks at room temperature. Homogeneity varied in the range −4% to +5% of nominal. Analysis of representative diet samples from 7 of 12 mixes showed the overall mean concentrations to be 9.6, 29.1, 1022 and 2590 ppm, in order of increasing concentration.

There were no treatment-related deaths or clinical signs of an adverse reaction to treatment. Body weight gain was significantly reduced by 10.4% throughout the study in males at 2500 ppm. There was no significant effect on weight gain in the other male groups or in any female group, either before mating or during gestation and lactation. Feed consumption was transiently reduced by 7.5%

in males at 2500 ppm during the first 2 weeks of the first premating treatment period, but not later. No other groups of either sex were affected.

There were no treatment-related effects on absolute or relative organ weights at any dose level. Statistically significant, slightly higher relative weights of spleen, heart and liver in males at 2500 ppm are considered to be related to slightly lower terminal body weights and not a direct effect of treatment.

At autopsy, there was no effect on the gross appearance of organs in male or female F_0 parental animals at any dose level. There were no effects on the microscopic appearance of the reproductive organs of F_0 males and females at 2500 ppm or in non-pregnant females and males that failed to mate. An increased incidence of minimal to marked hyaline change in renal tubule cells was observed in male rats at 1000 and 2500 ppm, and a slightly increased incidence of renal tubular casts was observed in males at 2500 ppm. Both findings were attributed to the treatment.

Sperm analyses revealed no treatment-related changes in the concentration of spermatids in testes at any dose level. Sperm morphology was unaffected by treatment.

Sperm motility in all thiamethoxam-treated groups was reduced significantly by approximately 20%. However, variability, reflected in large standard deviations, was high in all groups, indicating probable technical flaws. In view of the absence of effects on spermatid concentration, histological morphology, sperm morphology and fertility during both pairings and the absence of a dose–response relationship for reduced motility, the toxicological relevance of these results is considered equivocal. One technical flaw considered to have contributed to the variability between samples was poor standardization of the interval between sacrifice and sperm evaluation. A flaw probably accounting for the significant differences among groups was the conduct of sperm evaluations in group order rather than in randomized order. In a second sperm analysis study, reported below, procedural changes were implemented to reduce and standardize the time for sperm collection, to refine the technique for opening the cauda epididymis and to randomize the order in which sperm evaluations were performed to minimize between-day bias. This second sperm analysis study showed no effect of treatment on any sperm parameters, suggesting that these apparent changes in sperm motility were not an effect of treatment.

There were no treatment-related differences in reproductive parameters among the groups (Table 28). The number of animals that mated, the pregnancy incidence, the mean time to mating and the number of males and females that failed to mate or mated unsuccessfully were comparable in all treated and control groups for both the first and second matings, with the exception of the group treated at 1000 ppm, which showed slightly lower insemination and pregnancy incidences on both occasions. There was no effect of treatment at any dose level, at either mating, on the duration of gestation or on gestation and parturition indices.

In both F_{1a} and F_{1b}, the numbers of litters were lower in the 1000 ppm group, but were unaffected in either the lower or 2500 ppm dose groups, indicating that this was not a treatment-related response. A consequence was that a lower total number of pups was born, whereas the mean litter sizes were unaffected at the 1000 ppm dose level. Mean litter sizes at birth were slightly, but not significantly, lower than the control group at 2500 ppm in the F_{1a} (13.6 versus 11.4) and F_{1b} (13.8 versus 12.1) generations. In addition, the loss of one litter (nine stillborn pups) was responsible for an increased number of stillborn pups in the F_{1b} 1000 ppm group. No similar observation was made in the F_{1a} 1000 ppm group or in any other group in this study. Mean birth weight was unaffected by treatment at any dose level, but slightly reduced pup weight gain occurred in both F_{1a} and F_{1b} at 2500 ppm during the last 2 weeks of lactation. Sex ratios, viability and lactation indices, clinical signs, achievement of physical/behavioural developmental landmarks and macroscopic findings at necropsy were unaffected by treatment.

There were no treatment-related deaths or clinical signs in the F_1 parental animals, although two rats at 2500 ppm and one at 10 ppm were killed in a moribund condition. The overall weight gain

Table 28. Reproductive parameters: F_0 parental animals

	Dietary concentration (ppm)				
	0	10	30	1000	2500
First mating					
Females placed with males (*n*)	30	30	30	30	30
Males placed with females (*n*)	30	30	30	30	30
No. of days until evidence of mating	5.3	4.9	4.0	3.7	4.4
Total inseminated (*n*)	28	29	29	26	29
Total pregnant (*n*)	25	28	28	23	27
Pregnant females died or sacrificed moribund (*n*)	0	0	1	0	0
Duration of gestation (days)	22.1	21.8	22.2	22.0	22.0
Total delivering with liveborn pups (*n*)	25	28	27	23	27
Second mating					
Females placed with males (*n*)	30	30	29	30	30
Males placed with females (*n*)	30	30	29	30	30
Days until evidence of mating	3.3	3.0	4.3	4.1	3.2
Total inseminated (*n*)	28	29	29	25	29
Total pregnant (*n*)	24	26	27	22	27
Duration of gestation (days)	22.2	22.0	22.1	21.9	22.2
Pregnant females died or sacrificed moribund (*n*)	0	0	0	0	0
Total delivering with liveborn pups (*n*)	24	26	27	22	27

From Doubovetzky (1998)

of the selected F_1 parental rats was unaffected by treatment. The weight gain in male rats was reduced at the end of the first mating at 2500 ppm, but had recovered by the end of the second mating period. The early weight gain deficit reflects the lower starting weight in males of this group (Table 29). Feed consumption was also unaffected by treatment. Minimally higher apparent feed consumption at 2500 ppm, occasionally observed throughout the treatment period, is considered likely to reflect increased wastage.

Organ weights of F_1 parental rats are given in Table 30. Statistically significant increases in relative (to body weight) spleen (10.5%) and liver (9.1%) weights were observed in male rats, but not in females. Absolute spleen and liver weights were not significantly affected by treatment. Combined weights of the testes were significantly lower in 2500 ppm rats compared with controls, but microscopic examination showed no dose–response relationship in the incidences of tubular atrophy. Lower thymus weights occurred in females at 30, 1000 and 2500 ppm, but again there was no histological correlate, suggesting that the differences were incidental to treatment. The thymus histology of all female rats surviving to the study completion was subjected to morphometric assessment for atrophy and reported separately (Weber, 2000e). The analysis revealed mean percentages of thymic cortical area of approximately 65% in all dose groups. There was no statistically significant difference compared with control; in addition, there was no indication of an increasing or decreasing dose-related trend (Weber, 2000e).

The gross appearance of tissues and organs at autopsy in F_1 parental rats was unaffected by treatment. No treatment-related histological lesions were observed in the reproductive organs of parental rats at 2500 ppm or in non-pregnant females and males that failed to mate. Tubular atrophy of the testes was increased in the treated groups, but the incidences were not dose related and were

Table 29. Body weight development: F_1 parental animals

Day	Time point	Parameter	Dietary concentration (ppm)				
			0	10	30	1000	2500
Males							
134	Start premating	Mean body weight (g)	162.0	153.2	163.3	161.6	147.8
204	End 1st premating	Mean body weight (g)	470.2	469.5	456.9	453.6	437.9*
		Cumulative body weight gain (g)	308.2	316.3	293.6	292.0	290.1
		Body weight gain (% of control)	—	+2.6	−4.7	−5.3	−5.9
323	End 2nd postmating	Mean body weight (g)	603.8	612.2	598.2	588.8	589.2
		Cumulative body weight gain (g)	441.8	459.0	434.9	427.2	441.4
		Body weight gain (% of control)	—	+3.9	−1.6	−3.3	−0.1
Females							
134	Start premating	Mean body weight (g)	146.5	134.3	141.7	142.3	134.7
204	End 1st premating	Mean body weight (g)	294.1	281.4	284.4	288.9	279.0
		Cumulative body weight gain (g)	147.6	147.1	142.7	146.6	144.3
		Body weight gain (% of control)	—	−0.3	−3.3	−0.7	−2.2
334	End 2nd lactation	Mean body weight (g)	372.5	357.2	358.7	371.3	369.0
		Cumulative body weight gain (g)	226.0	222.9	217.0	229.0	234.3
		Body weight gain (% of control)	—	−1.4	−4.0	+1.3	+3.7

From Doubovetzky (1998)

* $P \leq 0.05$, ANOVA + Dunnett

Table 30. Organ weights: F_1 parental animals

Organ	Organ weight[a]									
	Males					Females				
	Dietary concentration (ppm)									
	0	10	30	1000	2500	0	10	30	1000	2500
Carcass										
- Absolute	582	593	578	575	573	350	335	341	342	339
Spleen										
- Absolute	0.88	0.86	0.87	0.85	0.96	0.63	0.62	0.61	0.65	0.64
- Relative	15.19	14.50	15.03	14.96	16.79**	17.99	18.65	18.07	18.96	18.82
Liver										
- Absolute	22.59	22.74	22.12	22.92	24.43	14.21	13.21	13.41	13.74	14.17
- Relative	388.54	382.82	382.77	396.94	423.72**	405.77	394.17	394.01	401.67	418.52
Testes/ovaries										
- Absolute	4.48	4.42	4.24	4.41	4.08*	0.23	0.20**	0.22	0.22	0.23
- Relative	77.36	75.31	74.09	77.30	72.43	6.67	6.11	6.37	6.43	6.77
Thymus										
- Absolute	0.45	0.44	0.40	0.41	0.41	0.32	0.29	0.28*	0.27**	0.26**
- Relative	7.72	7.41	6.98	7.22	7.18	9.19	8.77	8.08	7.80*	7.65**

From Doubovetzky (1998)

* $P \leq 0.05$; ** $P \leq 0.01$, ANOVA + Dunnett

[a] All absolute weights in grams; relative organ weights are % of body weight × 100.

considered not to be of toxicological significance. Treatment of males at 1000 or 2500 ppm produced increased incidences of casts and minimal to marked hyaline changes in the renal tubule similar to those observed in F_0 male rats, which have no human significance.

In view of the potential importance of tubular atrophy, this lesion was re-examined. The re-examination involved additional histopathology of the testes from the F_0 and F_1 generation rats in order to discriminate between the minute focal tubular changes and diffuse tubular atrophy. In F_0 rats, the incidence and severity of both findings were similar in control and treated groups. In F_1 rats, there was an increase in the incidence and severity of diffuse tubular atrophy in the group fed 1000 ppm, but not at higher or lower dose levels. The diffuse tubular atrophy of the testes at 1000 ppm, but not at higher or lower dose levels, is unlikely to be treatment related (Doubovetzky, 1999).

Sperm analyses revealed no treatment-related changes at any dose level in morphology or the concentration of spermatids in testes. Sperm motility at 10 and 2500 ppm was reduced significantly by approximately 18%; however, variability, as reflected by large standard deviations, was high in all groups and was probably attributable to technical flaws in the method of sperm analysis, an issue that has been discussed above. In view of the absence of effects on spermatid concentration, histological morphology, sperm gross morphology and fertility during both pairings, the toxicological relevance of these results is considered equivocal, but led to a specific sperm analysis study, reported below. This study revealed no effect of treatment on any sperm parameters.

There were no treatment-related effects on reproductive parameters at any dose level in F_1 parental rats. The number of rats that mated, the pregnancy incidence, the mean time to mating and the number of males and females that failed to mate or mated unsuccessfully were comparable in all treated and control groups at both mating periods, with the exception of the group treated at 30 ppm, which showed slightly lower insemination and pregnancy incidences on both occasions. There was no effect of treatment at any dose level, at either mating, on the duration of gestation or on gestation and parturition indices.

Although F_2 birth weights were unaffected, the weight gain of the F_{2a} pups was slightly reduced throughout lactation at 2500 ppm and minimally reduced during the latter part of lactation for F_{2a} and F_{2b} pups at 1000 and 2500 ppm (Table 31). The observed reduction in pup weight gain was at day 14 after birth, there being no significant differences at day 7. The pups start to consume the same diet as the dam from around day 11 after birth. This indicates that the effect is most likely occurring as a result of direct exposure of the pups to thiamethoxam via diet, rather than via milk while suckling. The effects represent general toxicity associated with the much higher dose (per unit body weight) received by pups during this period compared with that received by the adults and is thus interpreted as not indicative of a developmental effect.

Litter size at birth, mean birth weight, sex ratios, viability and lactation indices, clinical signs and macroscopic findings at autopsy were unaffected by treatment at all dose levels. Whereas achievement of physical/behavioural developmental landmarks was also unaffected by treatment in the F_{2a} groups, in the F_{2b} groups, the mean time to establishment of the surface righting reflex was slightly, but significantly, longer in pups at 2500 ppm (2.3 days) compared with the other groups, including the controls (2.1–2.2 days), possibly reflecting lower body weights in this group (Doubovetzky, 1998).

In the second sperm cell study, the homogeneity of representative diet samples was shown by analysis to be acceptable and in the range from −7% to +6% of nominal. Analysis of representative diet samples showed the overall mean concentrations to be 0, 9.7, 31.8, 1044 and 2540 ppm. Overall achieved dose levels were 0, 0.5–0.7, 1.6–2.3, 53.4–74.8 and 141.5–174.7 mg/kg bw per day, respectively.

There were no treatment-related deaths or clinical signs of an adverse effect of treatment. Significantly lower body weight gains occurred during weeks 1 through 3 at 2500 ppm, resulting in

Table 31. Litter data: F_{2a} and F_{2b} litters

Parameter	Dietary concentration (ppm)				
	0	10	30	1000	2500
F_{2a}					
Number of litters	28	27	25	26	27
Total pups born	387	364	348	359	351
Mean live litter size	13.8	13.3	13.6	13.6	13.0
Live birth index	99.7	98.4	98.0	98.6	100.0
Viability index	97.4	96.6	96.8	99.2	97.7
Sex ratio (% females day 0)	52.6	50.6	54.5	48.0	53.8
Mean pup weight (g)					
- Day 0	6.3	6.2	6.0	6.1	6.1
- Day 4 (pre-cull)	9.3	9.2	8.8	9.0	8.8
- Day 4 (post-cull)	9.5	9.4	9.0	9.1	8.9
- Day 7	15.5	15.0	14.5	14.7	14.4
- Day 14	31.5	30.5	30.2	30.0	28.9**
- Day 21	54.4	52.1	51.9	51.3*	48.7**
F_{2b}					
Number of litters	28	25	21	28	25
Total pups born	396	360	301	387	353
Mean live litter size	14.1	13.8	14.1	13.8	13.9
Live birth index	99.7	96.1	98.3	99.5	98.6
Viability index	97.0	94.8	98.6	93.5	95.1
Sex ratio (% females day 0)	49.6	48.8	53.0	55.3	47.7
Mean pup weight (g)					
- Day 0	6.3	6.1	6.1	6.0	6.2
- Day 4 (pre-cull)	9.2	9.2	9.2	8.8	8.6
- Day 4 (post-cull)	9.3	9.3	9.4	8.9	8.7
- Day 7	15.2	15.1	15.3	14.3	14.1
- Day 14	32.1	31.6	31.5	29.8	30.4
- Day 21	56.5	55.1	54.8	52.0*	52.0*

From Doubovetzky (1998)

* $P \leq 0.05$; ** $P \leq 0.01$ (ANOVA + Dunnett)

a 14% reduction in weight at the end of treatment. Overall weight gain was unaffected by treatment at lower dose levels, although at 1000 ppm, transiently reduced weight gain was observed in week 2 only. Feed consumption was slightly reduced at 2500 ppm during the first 6 weeks of treatment.

There were no treatment-related effects on sex organ weights. Higher mean relative testis and right cauda epididymis weights at 2500 ppm are attributed to the reduced mean exsanguinated body weights of this group.

There was no effect on the gross appearance of organs at autopsy. There were no effects on the proportion of motile sperm, sperm morphology, concentration of spermatids in the testes or number of sperm cells per milligram cauda epididymis fluid (Table 32). The standard deviations calculated for the number of motile epididymal sperm (4–12) were substantially lower than in the original study (14–21), suggesting that technical flaws were the cause of the apparently reduced numbers of motile

Table 32. Sperm parameters

Parameter	Dietary concentration (ppm)									
	0		10		30		1000		2500	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Number used for evaluation	*30*		*30*		*30*		*30*		*30*	
Total spermatids ($\times 10^6$/g testis)	59.6	9.6	58.2	10.7	55.0	10.8	57.5	10.0	55.8	12.9
% abnormal sperm	12.2	6.3	11.7	5.8	11.6	4.9	12.2	5.1	11.0	5.3
Total sperm/mg cauda liquid $\times 10^6$	2.37	0.67	2.67	0.83	2.31	0.88	2.43	0.83	2.39	0.73
% motile sperm cells	73.1	12	75.5	4	73.0	7	73.5	7	74.5	8

From Doubovetzky (1998)
SD, standard deviation

Table 33. Overall mean dose of thiamethoxam received by rats in a two-generation study of reproduction with thiamethoxam

Generation/sex	Time	Mean dose received (mg/kg bw per day)			
		Dietary concentration (ppm)			
		20	50	1000	2500
F_0 males	Weeks 1–27	1.2	3.0	61.7	155.6
F_1 males	Weeks 1–29	1.5	3.7	74.8	191.5
F_1 satellite males	Weeks 1–29	1.4	3.5	71.9	181.1
F_0 females	Weeks 1–10	1.7	4.3	84.4	208.8
F_1 females	Weeks 1–10	2.1	5.6	110.1	276.6
F_0 females	Gestation	1.2	3.1	62.2	158.9
F_1 females	Gestation	1.4	3.6	71.0	181.8
F_0 females	Postpartum	3.2	8.3	172.2	415.3
F_1 females	Postpartum	3.7	9.9	194.2	484.9

From Moxon (2004)

sperm in the multigeneration study. Sperm motility values were consistent with other published data (Chapin et al., 1992) from rats (Doubovetzky, 1998).

The NOAEL for parental toxicity in the multigeneration study in rats was 30 ppm, equal to 1.4 mg/kg bw per day for male rats, based on hyaline change and casts in renal tubules in males at 1000 ppm, equal to 45.6 mg/kg bw per day. This observation has no human relevance. The relevant NOAEL for parental toxicity was 1000 ppm, equal to 45.6 mg/kg bw per day, based on significantly reduced body weight gain at 2500 ppm, equal to 117.6 mg/kg bw per day, in F_0 generation males. The NOAEL for reproductive toxicity in the multigeneration study in rats was 2500 ppm, equal to 117.6 mg/kg bw per day for F_0 males, the highest dose tested. The overall NOAEL for pups and developing offspring was 30 ppm, equal to 1.4 mg/kg bw per day for males, based on reduced body weight gains of pups during F_1 lactation at 1000 ppm, equal to 45.6 mg/kg bw per day for males (Doubovetzky, 1998).

It is concluded that thiamethoxam was not a selective reproductive toxin in this study.

In the second two-generation study of reproduction in rats, groups of male and female Sprague-Dawley-derived Tif:RAIf, SPF strain rats were administered thiamethoxam (batch No. P.506006; purity 98.6%) continuously in the diet at dose levels of 0, 20, 50, 1000 or 2500 ppm throughout the two generations. These dietary concentrations were equal to the doses given in Table 33.

The F_0 generation comprised 26 males and 26 females in each dose group. The F_0 generation received the treated diet for 10 weeks before pairing. The rats were allowed to rear the ensuing F_{1A} litters to weaning. The breeding programme was repeated with F_1 parents selected from the F_{1A} pups to produce the F_{2A} litters, after a 10-week premating period of exposure to thiamethoxam. In addition, 14 males per group in the F_1 generation were selected from the remaining pups and retained to generate histological data on the testis only (and referred to as satellite males). The growth of both parental generations, reproductive function, mating behaviour, conception, gestation, parturition, lactation and weaning and the growth and development of the pups were monitored and recorded.

There was no effect of thiamethoxam on the clinical condition of the F_0 or F_1 parent animals or the F_1 satellite males.

Body weights were lower for the F_0 males given 2500 ppm in comparison with the control group. This effect was accompanied by a reduction in feed consumption and feed utilization during weeks 1–4. The lower body weights of the F_0 males given 1000 ppm were not statistically significantly different from the control body weights (with one exception at week 4) and were not associated with any reduction in feed consumption; they were therefore considered to be of no toxicological significance. No similar effects of 1000 or 2500 ppm thiamethoxam on body weight or feed consumption were observed in the F_1 males or in the F_0 and F_1 females during the premating period, gestation or postpartum. Nevertheless, feed consumption was statistically significantly reduced in the 2500 ppm group in comparison with the control group for week 3 postpartum for the F_0 females with litters. It was noted, however, that feed consumption was similar in the 2500 ppm and 20 ppm groups, suggesting that the reduction is more likely to reflect normal intergroup variation than to be an effect of treatment.

Study of reproductive function indicated that there was no effect of thiamethoxam in either the F_0 or F_1 females on mean cycle length, the number of cycles in a 25-day period, the precoital interval, length of the gestation period or mating success (i.e. the production of at least one live pup on the day of littering).

For the F_0 females, the proportion and percentage of successful matings were lower in the 20 and 2500 ppm groups (18/26) in comparison with the control group (21/26), but were not statistically significantly different. Also, there was no effect of thiamethoxam on the proportion of successful matings of the F_1 females.

Up to five whole-litter losses occurred in each group of the F_0 and F_1 generations. Four whole-litter losses occurred in the F_0 control group, whereas none occurred in the F_1 control group. The incidence of litters affected was not dose related and was considered to be incidental to treatment with thiamethoxam.

There was no effect of thiamethoxam on the proportion or percentage of liveborn pups or on the proportion of litters with all pups born alive for either the F_{1A} or F_{2A} litters. No treatment-attributable effect on mean litter size or pup survival on day 1, 5, 8, 15 or 22 was observed for either the F_{1A} or F_{2A} litters.

With the exclusion of whole-litter losses, the proportions of F_{1A} and F_{2A} pups surviving in the 50, 1000 and 2500 ppm groups were comparable with those of the control groups; the lowest percentages of pups surviving in each generation were recorded for the 20 ppm groups. With the inclusion of whole-litter losses, the proportions of pups surviving in the 50, 1000 and 2500 ppm groups were higher than that in the control group for the F_{1A} generation and lower than that in the control group for the F_{2A} generation (where there were no whole-litter losses in the control group). In the absence of a dose–response relationship, the lower pup survival in the 20 ppm groups was considered to be unrelated to treatment.

There was no effect of thiamethoxam on the sex ratio, body weights or clinical condition of the F_{1A} or F_{2A} pups.

On days 15 and 22, total litter weight of the F_{1A} pups was statistically significantly lower in the 2500 ppm group in comparison with the control group. This was due to a slightly smaller litter size in the 2500 ppm group combined with a slightly lower pup body weight (rather than a slightly higher pup body weight typically associated with smaller litters). As the effect was not also observed earlier in postnatal development, it was concluded that the slightly lower total litter weight was due to the direct consumption of diet containing 2500 ppm by the pups, rather than a developmental effect. However, there were no statistically significant differences in total litter weight for the F_{2A} pups.

There was no effect of thiamethoxam on the mean day of age when preputial separation or vaginal opening occurred.

Postmortem examination of organ weights of parental rats showed that there was no effect of thiamethoxam on the weight of the brain, right cauda epididymis, ovaries, prostate gland, seminal vesicles or uterus with cervix.

For the F_0 males, relative (to body weight) adrenal and kidney weights were statistically significantly greater in the 2500 ppm group in comparison with the control group; however, no statistically significant differences in these relative organ weights were observed for F_0 females, F_1 males or F_1 females in any of the thiamethoxam-treated groups.

Liver weights were significantly increased in F_0 male rats of the 1000 ppm group, and relative liver weights were significantly increased in F_1 male and F_1 female rats of the 2500 ppm group. No other statistically significant liver differences were recorded. Kidney weight relative to body weight was significantly increased in F_0 males of the F_0 generation, but not in the F_1 generation.

Pituitary weights were significantly increased in F_0 females of the 1000 and 2500 ppm groups, but not in any other group.

The mean spleen weight was notably higher for the F_0 males given 2500 ppm, but this was due to one male (number 120) with an enlarged spleen (weight 4.569 g). For the F_1 males given 2500 ppm, the relative (to body weight) spleen weight was statistically significantly greater than in the control group.

Absolute and relative (to body weight) weights of the epididymides were unaffected by treatment in the F_0 groups. In the F_1 generation, however, the absolute weight of the combined epididymides was statistically significantly greater in the 2500 ppm group than in the control group, and the relative weights of the combined epididymides were statistically significantly greater than in the control group in both the 1000 and 2500 ppm groups.

Testis weights were unaffected by treatment in the F_0 generation, but they were significantly increased, relative to body weight, in the F_1 20, 1000 and 2500 ppm groups, but not in the 50 ppm group. Comparison of the F_0 and F_1 control group testis weights would suggest that a reason for the significantly higher relative weights in the F_1 generation 20 and 1000 ppm groups was a particularly low control group testis weight (Table 34).

Sperm parameter data were analysed with and without the males listed in Table 35, which, upon gross examination, were found to have an abnormal testis and/or cauda epididymis and presented poor sperm samples. The data excluding the abnormal sperm samples are probably the more appropriate for evaluation, because the distribution of the males with gross abnormalities of these organs showed no dose–response relationship.

There was no effect of thiamethoxam on the number of sperm in the right testis of the F_0 males, but for the F_1 males given 50, 1000 or 2500 ppm, the total number of sperm and the number of sperm per gram of right testis were statistically significantly lower than in the control group.

There was no effect of thiamethoxam on the number of sperm in the right cauda epididymis of the F_0 males, but for the F_1 males given 2500 ppm, the total number of sperm and the number of sperm per gram of right cauda epididymis were statistically significantly higher than in the control group.

Table 34. Organ weights (adjusted for final body weight) in male rats of a two-generation dietary study of reproduction with thiamethoxam

Organ	Adjusted organ weight (g)				
	Dietary concentration (ppm)				
	0	20	50	1000	2500
F_0					
Kidney	3.00	3.03	3.01	2.95	3.11*
Testis	4.13	4.03	4.25	4.04	4.18
Epididymis	1.638	1.545	1.619	1.582	1.674
F_1					
Kidney	2.83	2.88	2.85	2.81	2.85
Testis	3.90	4.12*	4.01	4.13*	4.21**
Epididymis	1.584	1.617	1.615	1.656*	1.668*

From Moxon (2004)
* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

Table 35. Abnormal testis samples observed in a two-generation dietary study of reproduction in rats with thiamethoxam

Dietary concentration (ppm)	Male number	Comments
F_0		
0	8	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
20	34	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
	48	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
1000	80	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
	101	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
2500	109	Right cauda reduced, no sperm visible. Testis reduced, few sperm visible.
	117	Right cauda reduced, few sperm visible. Testis reduced, few sperm visible. Sperm abnormal.
	129	Mass on right cauda. Sperm abnormal.
F_1		
2500	122	Right cauda reduced. Sperm abnormal.

From Moxon (2004)

There was no effect of thiamethoxam on the percentage of motile sperm in any treatment group.

For the F_0 males, there was no effect of thiamethoxam on straight line, curvilinear or average path velocities. For the F_1 males given 2500 ppm, the straight line, curvilinear and average path velocities were statistically significantly lower in comparison with the control group (Table 36). There was no effect of thiamethoxam on straight path velocities.

Table 36. Sperm velocity in F_1 parent males of a two-generation study of reproduction in rats with thiamethoxam

Observation	Dietary concentration (ppm)				
	0	20	50	1000	2500
Curvilinear velocity	305.0	297.2	302.4	297.7	289.6**
Average path velocity	123.9	122.1	123.1	120.4	116.2**

From Moxon (2004)

** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

Gross postmortem examination revealed no abnormalities in any tissue from the F_0 or F_1 rats that could be attributed to treatment with thiamethoxam. Furthermore, there was no significant difference in the number of uterine implantation sites or postimplantation losses for either the F_0 or F_1 rats.

Microscopic examination of the kidneys revealed increased tubule cell hyaline droplet formation in the majority of F_0 males given 2500 ppm thiamethoxam and in a few given 1000 ppm. Similar findings were observed in F_1 males, except that the incidence in F_1 males given 1000 ppm (17/26) was much higher than in the F_0 generation (3/26). This type of change was not observed in males given 20 ppm or 50 ppm in either generation or in any females. Other minor (i.e. minimal or slight) kidney changes observed at an increased incidence in males given 2500 ppm were hyaline eosinophilic casts, tubular dilatation with granular eosinophilic casts at the cortico-medullary junction, tubule cell basophilia and interstitial mononuclear cell infiltration. Slight increases in the incidences of hyaline eosinophilic casts and tubule cell basophilia compared with controls were also observed in F_1 males given 1000 ppm.

Germ cell loss, recorded under four morphologies, according to degree and distribution, was observed in the testis of some treated and control males. As the four categories clearly represented variable degrees of a single pathological process, they were merged to a single finding (i.e. germ cell loss/disorganization with or without Sertoli cell vacuolation) for the purposes of tabulation and evaluation.

An increased incidence (14/26) of minimal germ cell loss/disorganization with or without Sertoli cell vacuolation was observed in the testis of F_1 males given 2500 ppm compared with the control and lower dose groups (1/26 – 3/26). The change was extremely minor, affecting a few scattered tubules, minimal change being defined as affecting an average of not more than 10 tubules per section for the four sections examined from each testis. It was characterized by variable germ cell loss ranging from complete depletion (i.e. Sertoli cell–only tubules) to minimal loss of a few germ cells in one segment of a tubule cross-section. In some instances, partial germ cell loss was associated with germ cell disorganization and Sertoli cell vacuolation, indicating a degenerative process. The change was not associated with any discernible reduction in epididymal sperm or increase in epididymal desquamated germ cells. The average total number of tubule cross-sections affected in all four sections per testis examined from affected males receiving 2500 ppm was 10.2, which represents approximately 0.4% of tubule cross-sections in four average testis sections. This calculation is based on an approximate count of tubule cross-sections in the four standard testis sections from a representative control male conservatively estimated at 2500. The change was most frequently observed in regions adjacent to the rete testis.

A small number of F_0 males given 20 ppm (2/26) or 2500 ppm (3/26) showed severe germ cell loss, affecting nearly all tubules, which amounted to nearly total atrophy of the tubules. These were considered to be spontaneous lesions, unrelated to treatment, because the incidence was low, unrelated to dose and not repeated in the F_1 generation and the changes were identical to those observed in control rats of this strain (quoted in Moxon, 2004).

Table 37. Incidence of rats with testicular tubules showing germ cell loss or disorganization, with or without Sertoli cell vacuolation

	Dietary concentration (ppm)				
	0	20	50	1000	2500
Total number of F_1 males (main study + satellites)	40 (26 + 14)	40 (26 + 14)	40 (26 + 14)	40 (26 + 14)	40 (26 + 14)
Main study: unilateral/bilateral status unknown	3/26	1/26	1/26	3/26	15/26
Satellites: unilateral	1/14	4/14	2/14	3/14	0/14
Satellites: bilateral	1/14	0/14	0/14	1/14	5/14
Total incidence	5/40	5/40	3/40	7/40	20/40

From Moxon (2004)

For the main study males, only one testis was available for histological examination, and so it was not possible to determine whether changes were unilateral or bilateral. For this reason, a satellite group of F_1 males was used from which both testes were available. Minimal testicular tubule changes were observed as on the main study. However, a treatment-related increase in incidence of germ cell loss/disorganization with or without Sertoli cell vacuolation was confined to minimal bilateral change at 2500 ppm only. The average number of tubule cross-sections affected per testis in the four sections per testis examined from satellite males receiving 2500 ppm thiamethoxam that showed bilateral change was 9.1—that is, similar to the corresponding figure (10.2) from the main study F_1 males and again representing approximately 0.4% of tubule cross-sections in the four sections examined.

Unilateral change (in the satellite groups) was observed at a low incidence and unrelated to dose in control and treated groups, suggesting that, in contrast to the bilateral change, this was a spontaneous lesion. As mentioned above, it was not possible to distinguish between unilateral and bilateral changes in the main study rats because only one testis was available for examination. Therefore, the incidence of lesions reported for the main study rats represents a summation of spontaneous and treatment-related lesions.

The testicular histology data from main study F_1 and satellite F_1 males are summarized in Table 37 and show the overall incidence of germ cell loss/disorganization with or without Sertoli cell vacuolation in both groups. When the incidences of change in both groups are added together, regardless of unilateral or bilateral status, the data confirm that there is an increase at 2500 ppm thiamethoxam, with a clear no-effect level at 1000 ppm. Although the incidence was clearly increased at 2500 ppm, the nature and severity of the lesions were identical to those observed at a low incidence in control and lower dose group F_1 animals and in all groups of F_0 males.

No changes were detected in any other tissue from F_0 or F_1 rats that could be attributed to treatment with thiamethoxam. Also, the numbers of small follicles in the ovaries of F_1 females receiving 2500 ppm thiamethoxam were comparable to the numbers observed in control females.

Postmortem examination of the F_{1A} or F_{2A} pups did not reveal any gross morphological changes or brain, spleen or thymus weight changes that could be ascribed to thiamethoxam treatment.

The NOAEL for parental toxicity in the second multigeneration study in rats was 50 ppm, equal to 3.0 mg/kg bw per day for male rats, based on hyaline change and casts in renal tubules in males at 1000 ppm, equal to 61.7 mg/kg bw per day. This observation has no human relevance. The relevant NOAEL for parental toxicity was 1000 ppm, equal to 61.7 mg/kg bw per day, based on significantly reduced body weight gain at 2500 ppm, equal to 155.6 mg/kg bw per day, in F_0 generation males.

The NOAEL for reproductive toxicity in the multigeneration study in rats was 1000 ppm, equal to 74.8 mg/kg bw per day for F_1 males, based on minimal testicular germ cell loss or disorganiza-

tion, with or without Sertoli cell vacuolation (and unaccompanied by any reduction in epididymal sperm numbers), at 2500 ppm, equal to 191.5 mg/kg bw per day. These effects were not observed in the earlier study (Doubovetzky, 1998, 1999), a difference that could be attributed to a refinement of the methods used in Moxon (2004) for opening the cauda epididymis and randomizing the order in which sperm observations were performed, in order to minimize between-day bias in the ancillary sperm cell parameter study, in which only F_0 males were investigated.

The NOAEL for pups and developing offspring was 1000 ppm, equal to 74.8 mg/kg bw per day for males and 110.1 mg/kg bw per day for females, based on reduced body weight gains of pups during F_1 lactation at 2500 ppm, equal to 191.5 mg/kg bw per day for males and 276.6 mg/kg bw per day for females (Moxon, 2004).

It is concluded that thiamethoxam was not a selective reproductive toxin in this study.

(b) Developmental toxicity

Rats

Groups of 24 mated female Sprague-Dawley rats (Tif:RAIf, SPF strain, at least 8 weeks old, supplied by Ciba-Geigy Ltd, Switzerland) were treated orally, by gavage, with thiamethoxam (batch No. P.506006; purity 98.6%) in 0.5% aqueous carboxymethylcellulose at a dose level of 0, 5, 30, 200 or 750 mg/kg bw from day 6 through 15 of gestation (day 0 = plug or sperm observed in vaginal smear). Mortality was recorded twice daily, and clinical signs and body weight were recorded daily. Feed consumption was measured throughout gestation. Dams were killed on day 21 and the fetuses delivered by caesarean section. The uterine tract and contents were removed and weighed. Gross examination was made of main organs of the thoracic and abdominal cavities, and ovaries (including corpora lutea count), uteri (implantation site count) and placentae were examined macroscopically. Fetuses were sexed, examined for external malformations and variations and then weighed. Approximately one half of the fetuses from each litter were examined for soft tissue malformations and variations after fixation in Bouin's solution and micro-dissection of the head, thoracic and abdominal viscera. The remaining fetuses were subjected to skeletal evaluation using Dawson's technique and examined for skeletal malformations, anomalies and variations. A malformation was defined as a very rare, permanent structural change that may adversely affect fetal survival, development or function. A distinction was made between skeletal anomalies and skeletal variations. A skeletal anomaly is a rare, slight to moderate, permanent or reversible structural change that is not considered to impair fetal survival, development or function. A variation is a relatively frequent, transient structural deviation from normal development that is considered not to have any detrimental effect on fetal survival, development or function. Variations occur regularly in fetuses from untreated rats. Continuous data were analysed statistically using ANOVA and Dunnett's *t*-test, quantal data by the chi-squared test followed by Fisher's exact test, and non-parametric data by Kruskal-Wallace non-parametric ANOVA followed by the Mann-Whitney U test.

Analysis of formulations demonstrated that thiamethoxam was stable in the vehicle and was homogeneously distributed (relative standard deviations in the range −4.0% to +5.0%). Analysis showed mean achieved concentrations to be 98.1%, 94.7%, 95.0% and 99.5% of the nominal values, in order of increasing dose level.

No treatment-related deaths occurred, but one dam at 750 mg/kg bw per day was killed on day 9 for humane reasons. Transient hypoactivity, piloerection and, in two females, regurgitation of dosing material were the only clinical findings related to treatment. Maternal body weight gain (uncorrected for the uterus and its contents) was moderately depressed during the treatment period at 750 mg/kg bw per day, and body weight change was decreased (−46.5%) at this dose and minimally decreased (−15.8%) at 200 mg/kg bw per day over treatment days 6–15. No effects on body weight were observed at lower dose levels. Also, no significant body weight reductions were observed when

Table 38. Intrauterine data from rats treated with thiamethoxam in a developmental toxicity study

	Dose (mg/kg bw per day)				
	0	5	30	200	750
Dams with implants	22	23	23	22	23
Dams with viable fetuses	22	23	23	22	22
Dams with resorptions (%)	8 (33)	9 (38)	6 (25)	5 (21)	6 (25)
Dams with affected implants[a] (%)	8 (33)	9 (38)	6 (25)	5 (21)	7 (29)
Corpora lutea/dam	15.5 ± 2.2[b]	14.7 ± 2.8	15.0 ± 2.2	16.0 ± 1.7	15.4 ± 2.8
Implants/dam	14.5 ± 3.2	13.5 ± 2.6	14.4 ± 2.1	14.9 ± 1.4	14.3 ± 3.9
Preimplantation loss[c] (%)	7.1 ± 16.5	8.3 ± 7.0	3.5 ± 6.8	6.7 ± 7.6	9.3 ± 19.5
Postimplantation loss[d] (%)	3.3 ± 4.9	3.1 ± 4.2	2.9 ± 5.3	2.2 ± 4.4	2.8 ± 5.3
Live fetuses/dam	14.0 ± 3.1	13.1 ± 2.7	14.0 ± 2.0	14.6 ± 1.6	13.9 ± 4.0
Dead fetuses/dam	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Resorptions/dam: early	0.5 ± 0.7	0.4 ± 0.5	0.4 ± 0.8	0.3 ± 0.6	0.4 ± 0.7
Resorptions/dam: late	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0
Malformed live fetuses[e] (%)	0.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
Affected implants[f] (%)	3.7 ± 4.9	3.1 ± 4.2	3.6 ± 5.4	2.2 ± 4.4	2.8 ± 5.3
Sex ratio (% females)	48.9	57.0 ± 0.2	51.9 ± 1.0	46.4 ± 1.0	52.8 ± 1.0
Mean pup body weight (g)	5.3 ± 0.3	5.3 ± 0.3	5.2 ± 0.3	5.2 ± 0.3	4.8** ± 0.3
Reproductive tract weight (g)	99.0 ± 21.8	93.7 ± 15.8	98.3 ± 13.6	102.6 ± 11.4	90.2 ± 24.2
Uncorrected body weight (g)	360.1	358.9	358.5	354.8	335.4*
Corrected body weight[g] (g)	261.1	265.2	260.2	252.2	245.2*
Corrected body weight change: gestation[h] (g)	63.3	68.8	64.2	55.7	49.2
Uncorrected body weight change: treatment (g)	63.4	62.0	63.8	53.4**	33.9**
Corrected body weight change: treatment[i] (g)	33.2	37.3	33.7	26.1	31.0

From Winkler (1996a)

* $P \leq 0.05$; ** $P \leq 0.01$ (two-tailed)

[a] Affected implants include dead fetuses, resorptions and malformed live fetuses.

[b] Mean ± standard deviation. Mean value determined for each female.

[c] 100 minus the percentage of corpora lutea that are reflected in the implantation sites.

[d] 100 minus the percentage of implants that are viable at the time of intrauterine inspection.

[e] (Number of malformed live fetuses/total live fetuses) × 100.

[f] [(Dead fetuses + resorptions + malformed live fetuses)/total implants] × 100.

[g] Body weight (gestation day 21) − weight of reproductive tract plus contents.

[h] Body weight (gestation day 21 − gestation day 0) − weight of reproductive tract plus contents.

[i] Body weight (gestation day 21 − gestation day 7) − weight of reproductive tract plus contents.

maternal body weights corrected for uterine contents were compared for the treatment period. Feed consumption was depressed during the treatment period at 750 mg/kg bw per day.

Pregnancy incidence, mean number of corpora lutea, preimplantation loss, number of implantation sites, early and late postimplantation losses, mean numbers of live fetuses and the sex ratio were unaffected by treatment at all dose levels (Table 38). A significant reduction in the mean body weight of live fetuses occurred at 750 mg/kg bw per day. This was attributed to the maternal toxicity (reduced body weight gain) observed at this dose level.

In view of the possibly treatment-related thymus atrophy in the F_1 females in one of the two-generation rat studies described above (Doubovetzky, 1998; Weber, 2000e), the thymus weights of

Table 39. Incidence of malformations and treatment-related anomalies and variants in rats treated with thiamethoxam in a developmental toxicity study

	Dose (mg/kg bw per day)				
	0	5	30	200	750
Litters evaluated	22	23	23	22	22
Fetuses evaluated	309	302	322	321	305
Malformations					
External malformations					
- Runt fetus	1	0	2	0	0
- Mouth: agnathia	0	0	1	0	0
- Skin: generalized oedema	1	0	0	0	0
Visceral malformations					
- Diaphragmatic hernia	0	0	0	0	1
Skeletal malformations					
- Unossified mandibula and os pubis	0	0	1	0	0
Total no. of fetuses with any malformation	1	0	2	0	1
Skeletal anomalies					
Total no. (%) of fetuses with any anomaly	23 (14.9)	14 (9.3)	16 (10.1)	12 (7.2)	40 (26.7)
No. (%) of fetuses with:					
- Asymmetric sternebra 6	4 (2.6)	3 (2.0)	2 (1.3)	3 (1.8)	11 (7.3)
- Irregular/absent ossification of occipital bone	3 (1.9)	0	2 (1.3)	2 (1.2)	12 (8.0)*
Skeletal variants					
Total no. (%) of fetuses with any variant	154 (100)	150 (100)	158 (100)	166 (100)	150 (100)
No. (%) of fetuses with:					
- Poor ossification of sternebra 5	0	0	1 (0.6)	0	10 (6.7)**
- Shortened 13th rib	13 (8.4)	5 (3.3)	4 (2.5)*	21 (12.7)	27 (18.0)*
- Absent ossification of metatarsal 1	15 (9.7)	31 (20.7)*	24 (15.2)	16 (9.6)	51 (34.0)**

From Winkler (1996a)
* $P < 0.05$; ** $P \leq 0.01$

the fetuses were recorded (Doubovetzky, 2000). No specific effect on the prenatal development of the thymus was identified.

The incidence and type of external, visceral and skeletal malformations were not affected by treatment at any dose level (Table 39). Increased incidences of skeletal anomalies and variants occurred at 750 mg/kg bw per day. Treatment-related anomalies were asymmetric sternebrae and irregular, poor or absent ossification of the occipital bone. Other commonly occurring skeletal anomalies were present at similar incidences in all treated and control groups. Skeletal variants were recorded in all fetuses from all dose groups. Treatment-related increases in the incidence of skeletal variants occurring at 750 mg/kg bw per day were poor ossification of sternebra 5, shortened 13th rib, absent ossification of metatarsal 1, and poor or absent ossification of one or more phalanges (commonly absence of proximal phalanges, incidences not shown in Table 39). These findings are considered to represent a treatment-related delay of ossification, secondary to reduced pup weight, in turn a reflection of maternal toxicity. The incidences of skeletal variants were unaffected by treatment at dose levels lower than 750 mg/kg bw per day (Winkler, 1996a).

The NOAEL for maternal toxicity in rats was 30 mg/kg bw per day, based on slightly decreased body weight gain in dams, providing a lowest-observed-adverse-effect level (LOAEL) of 200 mg/kg

bw per day. The NOAEL for fetotoxicity was 200 mg/kg bw per day, based on mild reduction in mean fetal body weight at 750 mg/kg bw per day. Further evidence of fetotoxicity at this dose was increased incidences of skeletal anomalies (irregular or absent ossification of the occipital bone) and skeletal variants (poor ossification of sternebra 5, shortened 13th rib and non-ossification of metatarsal 1). Thiamethoxam was not teratogenic in the developmental toxicity study in rats (Winkler, 1996a).

Rabbits

Groups of 19 mated female rabbits (Russian Chbb:HM strain, at least 3 months old) were treated orally, by gavage, with thiamethoxam (batch No. P.506006; purity 98.6%) in 0.5% aqueous carboxymethylcellulose from day 7 through day 19 of gestation (day 0 = plug or sperm in smear) at a daily dose level of 0, 5, 15, 50 or 150 mg/kg bw per day. Females were observed twice daily for mortality and once daily for clinical signs. Body weights were recorded daily. Feed consumption was recorded on days 4, 7, 12, 16, 20, 24 and 29. Dams were killed on day 29 and the reproductive tract plus contents removed by hysterectomy and weighed. Examinations were made of the main organs of the thoracic and abdominal cavities as well as ovaries, uteri and placentae, and the numbers of corpora lutea were counted. In dams that died or were killed before scheduled autopsy, the number and location of implantation and/or abortion sites in the uterus were recorded. In dams killed at scheduled autopsy, the number and location of live and dead fetuses or early and late (embryonic or fetal) losses were recorded. Fetuses were numbered, tagged individually, weighed, sexed and examined for external malformations and variations. All fetuses were examined for thoracic/abdominal soft tissue or skeletal malformations, anomalies and variations. A distinction was made between skeletal anomalies and skeletal variations, as defined above (see section on rats). Approximately one half of the fetuses were examined for cranial soft tissue malformations. All fetal trunks and approximately half of the fetal heads per litter were assigned to skeletal assessment according to the staining technique of Dawson. Continuous data were analysed statistically using ANOVA and Dunnett's *t*-test, quantal data by the chi-squared test followed by Fisher's exact test, and non-parametric data by Kruskal-Wallace non-parametric ANOVA followed by the Mann-Whitney U test.

Analysis of dose formulations showed that the test article was stable in the vehicle and homogeneously dispersed (−2% to +3% mean concentration). Overall mean achieved concentrations were within the range 97.2–101.4% of the nominal value.

Three rabbits treated at 150 mg/kg bw per day died or were killed for humane reasons at the end of the treatment period or shortly thereafter. Treatment-related clinical signs at 150 mg/kg bw per day comprised a bloody discharge in the perineal area of 13 rabbits, including all those that died or were killed. Deaths and clinical signs of adverse effects of treatment were not apparent at lower dose levels. Overall mean body weight was markedly reduced by treatment at 150 mg/kg bw per day, due primarily to weight loss during the early part of the treatment period (Table 40). Weight gain reduction at 50 mg/kg bw per day during treatment was minimal and non-significant. Weight gain recovery at 150 mg/kg bw per day was rapid in the post-treatment period on days 20–29 of gestation. Feed consumption was significantly and dose-dependently reduced in the 50 and 150 mg/kg bw groups during the treatment period (days 7–12 and 12–16) and from days 16 to 20 in the 150 mg/kg bw per day group. A significant, compensatory increase in feed consumption was recorded in the 150 mg/kg bw per day group in the post-treatment period (Table 40). Feed consumption and body weight gain were unaffected by treatment at lower dose levels.

At autopsy, the uterine contents of the three rabbits that died or were killed before schedule in the 150 mg/kg bw per day group were haemorrhagic, and one rabbit also showed a haemorrhagic vagina. There were no other treatment-related autopsy findings.

Total resorption occurred in three rabbits at 150 mg/kg bw per day and in one rabbit at 50 mg/kg bw per day. Therefore, there was a treatment-related reduction in the number of rabbits with live fetuses and a concomitant increase in postimplantation loss at 150 mg/kg bw per day. The mean pup

Table 40. Mean body weight gain and feed consumption in rabbits treated with thiamethoxam in a developmental toxicity study

	Dose (mg/kg bw per day)				
	0	5	15	50	150
Mean body weight (g)					
- Day 0	2733	2833	2840	2833	2718
- Day 7	2726	2854	2834	2819	2740
- Day 20	2778	2899	2873	2838	2667
- Day 29	2840	2998	3001	2952	2777
Mean body weight gain (g)					
- Days 0–6 (pretreatment)	−7	21	−6	−14	22
- Days 7–12 (treatment)	−26	−23	−17	−30	−105**
- Days 12–16 (treatment)	57	69	73	47	38
- Days 16–20 (treatment)	21	−2	−17**	3	−6
- Days 20–24 (post-treatment)	−2	19	20	24	28
- Days 24–29 (post-treatment)	64	81	108	91	83
- Overall (gestation period)	107	165	161	119	59
Feed consumption (g/day)					
- Days 0–4 (pretreatment)	93.5 ± 14.4[a]	94.1 ± 19.1	91.6 ± 17.4	95.4 ± 10.0	98.3 ± 11.4
- Days 4–7 (pretreatment)	88.6 ± 11.2	97.9 ± 20.0	95.3 ± 12.6	92.0 ± 13.2	100.4 ± 17.3
- Days 7–12 (treatment)	88.7 ± 14.1	92.4 ± 15.1	88.5 ± 15.8	69.2** ± 9.9	21.4** ± 9.4
- Days 12–16 (treatment)	86.7 ± 13.6	91.2 ± 17.4	79.1 ± 20.7	64.2** ± 19.1	38.5** ± 21.0
- Days 16–20 (treatment)	96.3 ± 21.7	95.9 ± 20.2	88.5 ± 22.0	80.6 ± 20.6	56.8** ± 27.7
- Days 20–24 (post-treatment)	96.3 ± 28.7	99.2 ± 21.1	93.1 ± 17.2	100.9 ± 21.2	125.1* ± 16.6
- Days 24–29 (post-treatment)	93.4 ± 26.1	96.8 ± 20.4	100.3 ± 14.5	103.3 ± 23.9	121.6* ± 17.2

From Winkler (1996b)

* $P \leq 0.05$; ** $P \leq 0.01$

[a] ± standard deviation.

weights of both sexes were reduced as a result of treatment at 150 mg/kg bw per day. Gravid uterus weight, number of corpora lutea, preimplantation loss, number of implantation sites and number of dead fetuses were unaffected by treatment (Table 41).

No external or skeletal fetal malformations occurred in the study, and no treatment-related visceral malformations were evident. In the 0, 5, 15, 50 and 150 mg/kg bw per day groups, respectively, there were 0, 1, 2, 0 and 1 fetuses with flexure of the forepaw, probably due to restriction of movement within the uterus; it is not a malformation, and the incidence was not dose related. The distribution of visceral malformations did not indicate an effect of treatment at any dose level. Small gall bladder was noted in some groups, but the incidence was haphazard and did not show any dose–response relationship. The fetal incidence of the skeletal anomaly fused sternebrae was increased at 150 mg/kg bw per day, but not at lower dose levels. This is considered to be treatment related as a consequence of reduced birth weight, which is in turn a reflection of maternal toxicity. The distribution of other skeletal anomalies did not indicate an effect of treatment. Skeletal variants occurred in a majority of fetuses from most litters in all treatment groups. The overall fetal incidences were 70.9%, 78.4%, 79.4%, 79.5% and 75.6% in order of increasing dose level. One skeletal variation, absent ossification of the medial phalanx of anterior digit 5, occurred at a fetal incidence of 8.9% at 150 mg/kg bw per day, whereas the control group incidence was 0%. Although the observed incidence was

Table 41. Intrauterine data from rabbits treated with thiamethoxam in a developmental toxicity study

	Dose (mg/kg bw per day)				
	0	5	15	50	150
Does with implants	15	19	19	19	18
Does with live fetuses	15	19	19	18	12
Does with resorptions (%)	9 (47)	4 (21)	9 (47)	8 (42)	13 (68)
Does with affected implants[a] (%)	10 (53)	8 (42)	10 (53)	10 (5.3)	16 (84)
Corpora lutea/doe	6.7 ± 1.2[b]	7.2 ± 1.2	7.2 ± 1.4	6.9 ± 1.3	7.1 ± 1.6
Implants/doe	4.7 ± 2.1	5.4 ± 1.2	5.9 ± 1.7	5.4 ± 2.0	5.4 ± 2.1
Preimplantation loss[c] (%)	32.6 ± 25.2	23.1 ± 21.7	17.5 ± 12.2	23.7 ± 18.4	25.1 ± 19.0
Postimplantation loss[d] (%)	21.0 ± 24.3	6.3 ± 16.1	9.9 ± 12.8	16.3 ± 25.9	45.6[§] ± 35.4
Live fetuses/doe	3.7 ± 2.2	5.1 ± 1.5	5.4 ± 1.7	4.6 ± 2.2	3.0 ± 2.4
Dead fetuses/doe	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Resorptions/doe: early	1.0 ± 1.1	0.3 ± 0.6	0.5 ± 0.7	0.6 ± 1.0	2.4 ± 2.7
Resorptions/doe: late	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.5	0.0 ± 0.0
Malformed live fetuses[e] (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Affected implants[f] (%)	1.0 ± 1.1	0.3 ± 0.6	0.6 ± 0.7	0.8 ± 1.1	2.4 ± 2.7
Sex ratio (% females)	43.6	50.5	54.9	46.5	51.1
Mean pup body weight (g)					
- All	44.0 ± 4.3	41.5 ± 3.2	41.7 ± 2.9	42.1 ± 3.9	37.5** ± 4.5
- Males	44.4 ± 4.9	41.7 ± 3.6	43.0 ± 3.3	42.2 ± 4.1	38.8** ± 4.9
- Females	41.8 ± 2.0	40.9 ± 3.2	40.8 ± 3.2	41.1 ± 4.3	36.6** ± 5.4
Reproductive tract weight (g): all	226 ± 115	303 ± 82	316 ± 80	294 ± 99	210 ± 100
Corrected body weight[g]	2614	2695	2685	2658	2567
Corrected body weight change: gestation[h]	−119	−138	−155	−175	−151
Corrected body weight change: treatment[i]	−112	−159	−149	−161	−173

From Winkler (1996b)

[§] Not significant ($P = 0.386$); ** $P \leq 0.01$

[a] Affected implants include dead fetuses, resorptions and malformed live fetuses.

[b] Mean ± standard deviation. Mean value determined for each female.

[c] 100 minus the percentage of corpora lutea that are reflected in the implantation sites.

[d] 100 minus the percentage of implants that are viable at the time of intrauterine inspection.

[e] (Number of malformed live fetuses/total live fetuses) × 100.

[f] [(Dead fetuses + resorptions + malformed live fetuses)/total implants] × 100.

[g] Body weight (gestational day 29) − weight of reproductive tract plus contents.

[h] Body weight (gestational day 29 − gestational day 0) − weight of reproductive tract plus contents.

[i] Body weight (gestational day 29 − gestational day 7) − weight of reproductive tract plus contents.

within the historical control range of 0–9.2%, the finding may be an indication of treatment-related delayed ossification (Table 42) (Winkler, 1996b).

The NOAEL for maternal toxicity in rabbits was 15 mg/kg bw per day, based on reduction in body weight gain and feed consumption during the treatment period in dams at 50 mg/kg bw per day. The NOAEL for fetotoxicity in rabbits was 50 mg/kg bw per day, based on increased postimplantation loss and reduction in fetal body weights at 150 mg/kg bw per day. Additional evidence of fetotoxicity at this dose was increased incidences in fetuses of delayed ossification, absent ossification and anomalies (fused sternebrae) (Winkler, 1996b).

Table 42. Fetal incidence and nature of malformations and treatment-related skeletal anomalies and variations in rabbits treated with thiamethoxam in a developmental toxicity study

	Dose (mg/kg bw per day)				
	0	5	15	50	150
Litters evaluated	15	19	19	18	12
Fetuses evaluated	55	97	102	88	45
Visceral malformations					
Renal aplasia	1	0	0	0	0
Uretral aplasia	1	0	0	0	0
Gall bladder aplasia	0	1	0	0	0
Diaphragmatic hernia	0	0	0	1	0
Skeletal malformations	0	0	0	0	0
External malformations	0	0	0	0	0
Total no. of malformed fetuses (% fetal incidence)	1 (1.8)	1 (1.0)	0 (0)	1 (1.1)	0 (0)
(% litter incidence)	(6.7)	(5.3)	(0)	(5.6)	(0)
Visceral anomalies					
Testicular hypoplasia, right	0	0	0	1	0
Small gall bladder	0	6	1	0	3
Skeletal anomalies					
Total no. (%) of fetuses with any skeletal anomaly	8 (14.5)	9 (9.3)	7 (6.9)	5 (5.7)	11 (24.4)
No. (%) of fetuses with:					
- Fused sternebrae 2 and 3	0	0	1 (1.0)	0	3 (6.7)
- Fused sternebrae 3 and 4	0	0	2 (2.0)	1 (1.1)	5 (11.1)*
- Fused sternebrae 4 and 5	1 (1.8)	0	3 (2.9)	1 (1.1)	2 (4.4)
Skeletal variations					
Total no. (%) of fetuses with any skeletal variation	39 (70.9)	76 (78.4)	81 (79.4)	70 (79.5)	34 (75.6)
Absent ossification of the medial phalanx	0	1 (1.0)	0	0	4 (8.9)

From Winkler (1996b)

* $P \leq 0.05$

2.6 Special studies

(a) Neurotoxicity

In a single-dose neurotoxicity study, groups of fasted Sprague-Dawley rats (Crl:CD® BR strain, 10 of each sex per group, approximately 7 weeks old at dosing) were administered a single dose orally by gavage of thiamethoxam (batch No. 9600110; purity 98.7%) in 0.5% methylcellulose solution at a dose level of 0, 100, 500 or 1500 mg/kg bw. Records were made of morbidity and mortality twice daily, clinical observations daily and detailed physical examinations weekly. Body weights were recorded on the day prior to treatment and weekly thereafter. The rats were subjected to neurological function tests and observations (functional observational battery [FOB]) and assessment of locomotor activity prior to treatment, on day 1 (approximately 2–3 hours after dosing) and 1 and 2 weeks later. The tests were performed blind, under red light/white noise conditions. The FOB included home cage/hand-held and open-field observations, response observations in a testing arena and performance measures (including grip strength, foot splay on landing, rectal temperature, tail flick latency and auditory startle response). At termination on day 16, whole-body perfusion fixation was performed on six rats of each sex per group, and central and peripheral nervous system tissues

(brain, four levels of spinal cord, pituitary, cervical and lumbar dorsal root ganglia, cervical and lumbar dorsal and ventral root nerves, Gasserian ganglia [or trigeminal ganglia, important in rats as the first part of the pathway from whiskers to the brain], sciatic, tibial and sural nerves and eyes with optic nerves) were fixed. Anterior tibialis and gastrocnemius skeletal muscles and gross lesions were also fixed. The central and peripheral nervous system tissues from control and high-dose rats were prepared for microscopic evaluation. The remaining four rats of each sex per group were subjected to autopsy, their tissues and organs examined postmortem and the tissues discarded. Sections of all nerve tissues collected from control and high-dose groups were examined microscopically. As there was no histological evidence of neurotoxicity in high-dose rats of either sex, the intermediate- and low-dose groups were not examined.

Three females at 1500 mg/kg bw died within 24 hours of dosing. Treatment-related clinical signs were observed at 1500 mg/kg bw on the day of dosing only. The signs were partial closure of eyes in some rats of both sexes and, in females only, tremors, hypoactivity and coldness to the touch. All other rats survived to the end of the study without clinical signs of an adverse reaction to treatment. Body weight gain was significantly reduced in males at 1500 mg/kg bw during the first week after dosing, but the animals gained more weight than the controls during the remainder of the study.

Treatment-related effects in the FOB/locomotor activity tests were limited to the evaluation performed 2–3 hours after dosing. There were no effects at any dose level during the week 1 and week 2 evaluations. At 2–3 hours after dosing, results of the home cage/hand-held observations revealed an increased incidence of moderate ease of removal from the home cage, active or rigid handling/body tone, tremors and ptosis at 1500 mg/kg bw. In addition, a slightly drooped palpebral closure was noted at 500 and 1500 mg/kg bw. Slight lacrimation occurred in females at 1500 mg/kg bw, and mildly impaired respiration was observed in 1500 mg/kg bw rats and in one male at 500 mg/kg bw. A dark-coloured nasal discharge was seen in one male at 500 mg/kg bw and a further male at 1500 mg/kg bw. Open-field observations on the 1500 mg/kg bw rats revealed a longer latency to first step, an increased incidence of a crouched-over posture in females, a mild to moderate impairment of gait, tremors and hypoarousal, and reduced rearing behaviour. A mild impairment of gait also occurred in one male at 500 mg/kg bw. Response and performance observations showed an increased incidence of uncoordinated landing in the righting reflex test, lower mean rectal temperatures and significantly higher mean forelimb grip strength values for males at 500 and 1500 mg/kg bw. The auditory startle response test required a significantly higher mean input stimulus value for males at 1500 mg/kg bw. Automated measurement of locomotor activity showed significantly lower activity during the first 15–20 minutes of testing at 500 and 1500 mg/kg bw.

Gross pathological findings in the three females that died prior to the scheduled end of the experiment revealed that all had dark-brown lobes of the liver and one had mottled dark-red lobes of the lung. In rats that survived to the end of the study, there were no treatment-related postmortem findings at any dose level. Microscopic examination of tissues from whole-body perfused rats revealed no treatment-related histological alterations in the tissues of the central and peripheral nervous systems. The lesions observed were of the type, incidence and severity anticipated in rats of this age and strain and occurred in both the control and high-dose groups.

In the absence of treatment-related neurohistological changes and persistent functional changes, the transient functional effects observed are considered to be signs of overt toxicity and/or signs of pharmacological overstimulation.

The NOAEL in the single-dose neurotoxicity study in rats was 100 mg/kg bw, based on transient behavioural changes at 500 mg/kg bw (Minnema, 1997).

In a subchronic neurotoxicity study, five groups of 10 male and 10 female Sprague-Dawley rats (Crl:CD® BR strain, approximately 6 weeks old) were administered thiamethoxam (batch No. 9600110; purity 98.7%) orally, in the diet, at concentrations of 0, 10, 30, 500 or 1500 ppm (males)

and 0, 10, 30, 1000 or 3000 ppm (females) for 13 weeks. These concentrations delivered mean doses equal to 0, 0.7, 1.9, 31.8 and 95.4 mg/kg bw per day (males) and 0, 0.7, 2.1, 73.2 and 216.4 mg/kg bw per day (females). Records were made of morbidity and mortality twice daily, clinical observations daily and detailed physical examinations weekly. Body weights were recorded 1 day before treatment commenced and weekly thereafter. Feed consumption was measured weekly. Ophthalmoscopic examinations were performed prior to treatment and during week 13. The rats were subjected to a battery of neurological function tests and observations (FOB) and assessment of locomotor activity prior to treatment and during weeks 4, 8 and 13. The tests were performed blind, under red light/white noise conditions. The FOB included home cage/hand-held and open-field observations, response observations in a testing arena and performance measures (including grip strength, foot splay on landing, rectal temperature, tail flick latency and auditory startle response). After at least 13 weeks of treatment, whole-body perfusion was performed on the first six rats of each sex per group. Central and peripheral nervous system tissues (brain, four levels of spinal cord, pituitary, cervical and lumbar dorsal root ganglia, cervical and lumbar dorsal and ventral root nerves, Gasserian ganglia, sciatic, tibial and sural nerves, eyes with optic nerves) were fixed in formalin. Anterior tibialis and gastrocnemius skeletal muscles, kidneys and gross lesions were also fixed. The central and peripheral nervous system tissues from control and high-dose animals were prepared for histological evaluation as paraffin-embedded 5 µm sections and/or plastic-embedded 1 µm sections, as appropriate. As there was no histological evidence of neurotoxicity in the high-dose rats of either sex, tissues of the low- and intermediate-dose groups were not examined. All remaining rats were subjected to autopsy. Kidneys and gross lesions were fixed from non-perfused rats, but all other tissues were discarded.

Analysis of representative diet samples demonstrated acceptable homogeneity (variation between samples up to 14%), acceptable stability (stable for 7 days at room temperature) and acceptable achieved concentrations (all within 10% of nominal concentrations, with some minor exceptions).

No deaths occurred, there were no treatment-related clinical signs of an adverse effect of treatment, and body weight development and feed consumption throughout the study were unaffected by treatment at all dose levels. After 13 weeks of treatment, there was no evidence of ocular toxicity at any dose level.

Neurobehavioural assessment of animals at all dose levels and at all time points revealed no treatment-related effects on any of the home cage/hand-held and open-field observations, response observations in the testing arena or performance measures (grip strength, foot splay on landing, rectal temperature, tail flick latency and auditory startle response). Locomotor activity was also unaffected by treatment at all dose levels and at all time points.

There were no treatment-related gross pathological findings. Microscopic examination of central and peripheral nervous system tissues indicated that no treatment-related alterations were present in rats of the high-dose groups. Lesions that were observed were of the type, incidence and severity anticipated in rats of this age and strain, and they occurred in both the control and high-dose groups.

The NOAEL for systemic toxicity and neurotoxicity in the 13-week neurotoxicity study was 1500 ppm, equal to 95.4 mg/kg bw per day, in males and 3000 ppm, equal to 216.4 mg/kg bw per day, in females, the highest doses tested, based on the absence of treatment-related effects at these doses (Minnema, 1998).

(b) Developmental neurotoxicity study in rats

In a postnatal developmental neurotoxicity study conducted according to OECD guideline 426 (2003 draft), groups of 30 time-mated, pregnant female Alpk:APfSD (Wistar-derived) rats received thiamethoxam (batch No. 506006; purity 98.8%) in the diet at a concentration of 0, 50, 400 or 4000 ppm, respectively, from gestation day 7 through postnatal day 22. These dietary concentrations delivered doses equal to 0, 4.3, 34.5 and 298.7 mg/kg bw per day during gestation and 0, 8.0, 64.0 and

593.5 mg/kg bw per day postpartum, assuming that the feed consumption of pups was zero. While this assumption is correct for postpartum life up to about day 11, it is not correct for days 11–22, during which solid food is taken. Pups were allocated to the F_1 phase of the study on postpartum day 5, separated from the dam on day 29 and allowed to grow to adulthood.

Observations made on maternal rats were for clinical signs twice daily and for body weights and feed consumption immediately prior to administration of test diets on day 7, on days 15 and 22 of gestation and on days 1, 5, 8, 12, 15 and 22 postpartum. Additionally, feed consumption was measured on day 1, and body weights were measured on the day of termination.

The maternal rats were subjected to a battery of neurological function tests (FOB) conducted blind on days 10 and 17 of gestation and days 2 and 9 of lactation The FOB included, but was not limited to, the following:

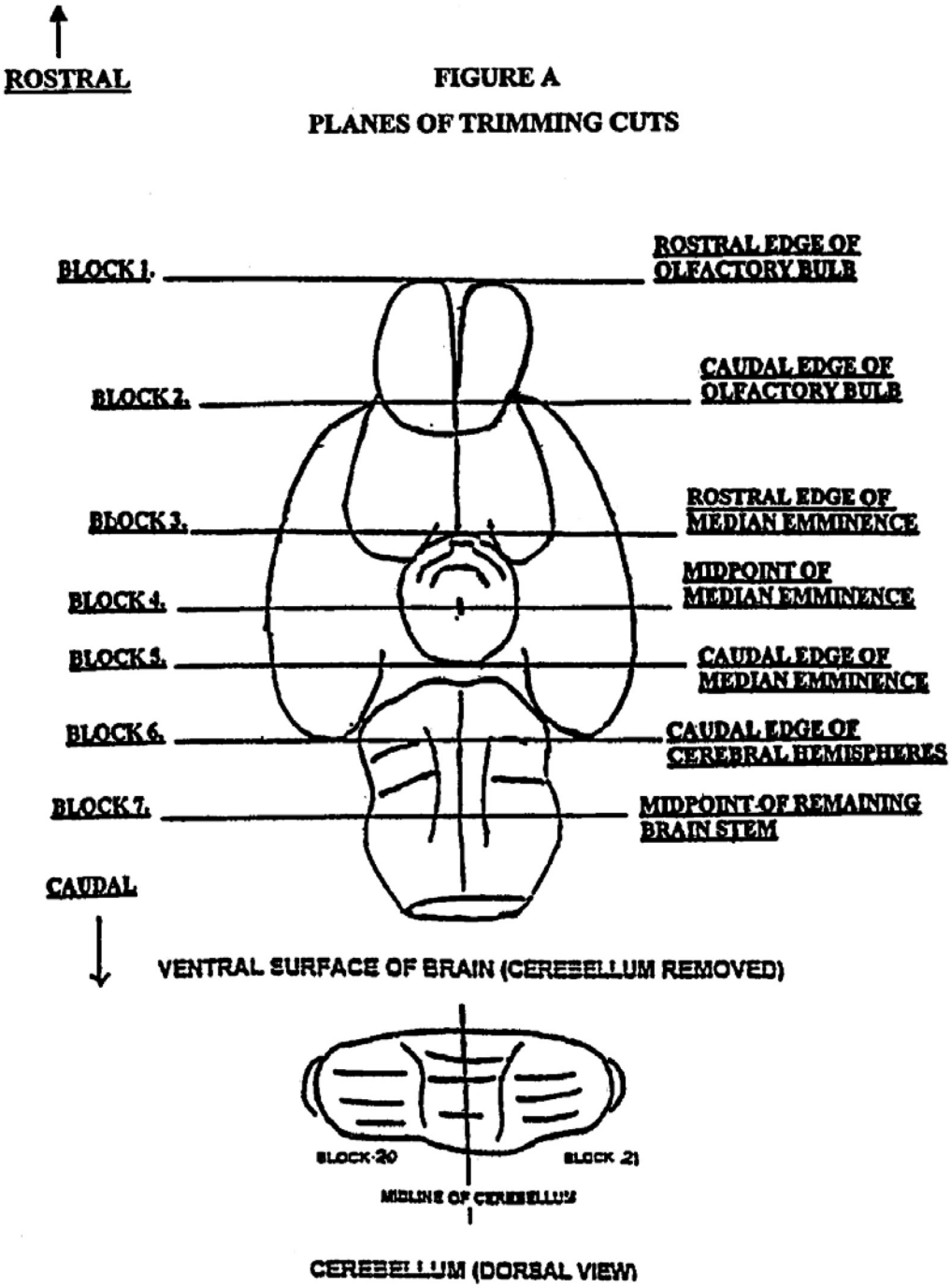
- assessment of signs of autonomic function (e.g. lacrimation, ptosis, pupillary function, exophthalmus, salivation, piloerection, urination and defecation);
- description, incidence and severity of any convulsions, tremors or abnormal movements;
- description and incidence of posture and gait abnormalities;
- description and incidence of any unusual or abnormal behaviour, excessive or repetitive actions and general signs of toxicity, including thin, dehydrated, altered muscle tone and altered fur appearance (including staining around the nose or mouth).

The offspring in each litter were examined as soon as possible after parturition was complete (day 1 postpartum) and always within 24 hours. On days 1 and 5 postpartum, the sex, weight and clinical condition of each pup were recorded, after which litters were standardized to eight randomly selected pups, although sexes were represented as equally as possible. Litters of seven or eight pups with at least three male and three female pups were used for selection of the F_1 generation. Pups not selected for the F_1 generation on day 5 postpartum were killed and discarded.

Postpartum observations for clinical signs were made on F_1 pups at least once a day and any significant findings recorded until day 5, after which detailed clinical observations and body weights were recorded for the selected F_1 rats on days 5, 12, 18, 22, 29, 36, 43, 50 and 57 postpartum and prior to termination on day 63 postpartum. The selected F_1 pups were examined for specific developmental landmarks that included day of age on which vaginal opening occurred in females, from day 29, and when preputial separation occurred in males, from day 41. Body weights were recorded on these days. In addition, the selected pups were designated for FOB tests, as described for the maternal rats above, on days 5, 12, 22, 36, 46 and 61, motor activity testing on days 14, 18, 22 and 60 and auditory startle reflex on days 23 and 61. The pups were also assessed for learning and memory. The test used a "Y"-shaped water maze with one escape ladder. The time taken by the pup to find the escape ladder was recorded for each trial. The rats were given six trials on either day 21 or day 59. In addition, a straight "maze" (channel) was used to evaluate swimming speed. Each pup completed one trial in the straight channel immediately following the six trials in the "Y"-shaped water maze. Three days later, on day 24 or day 62, the pups were retested using the same procedures.

Gross postmortem examinations were conducted on parental females that failed to produce litters, females with litters not required for selection and, on day 29 postpartum, the parental female rats whose litters had been selected. Females with total litter loss were killed and discarded without examination. Gross postmortem examinations were also conducted on pups (one of each sex per litter, to give at least 10 of each sex per group) on day 12 and day 63. The brains of these rats were weighed, fixed and stored. In addition, at least 10 rats of each sex per group were deeply anaesthetized by intraperitoneal injection of sodium pentobarbitone and killed by perfusion fixation with formol saline. The rats were perfused with a volume of fixative approximately equivalent to their estimated body

Figure 3. Planes of trimming cuts



weight. Organs and tissues selected and fixed, processed and examined from these rats were brain, eye (with optic nerve and retina), spinal cord (including cervical and lumbar swellings), spinal nerve roots (dorsal and ventral root fibres) at cervical and lumbar swellings, dorsal root ganglia at cervical and lumbar swellings, proximal sciatic nerve, proximal tibial nerve, distal tibial nerve (tibial nerve calf muscle branches) and gastrocnemius muscle. In the first instance, blocks from the control and high-dose rats were sectioned and stained. Subsequently, selected areas of the dorsal cortex, thalamus and hippocampus were processed from animals in the intermediate-dose groups.

Brain morphometric measurements were made using an image analyser at levels 2–5 in the brain and a section of cerebellum (Figure 3).

Table 43. Distribution of parental females killed ahead of schedule in a developmental neurotoxicity study in rats treated with thiamethoxam

	Dietary concentration (ppm)			
	0 (control)	50	400	4000
Failed to litter		0	0	1
Total litter loss	—	1	1	1
Insufficient pups[a]	8	6	2	2

From Brammer (2003)

[a] Sufficient pups = at least three males and three females in a litter of at least seven pups.

Table 44. Intergroup comparison of maternal body weights during gestation (selected time points; adjusted mean values shown for day 15 onwards) in a developmental neurotoxicity study in rats treated with thiamethoxam

Day	Dietary concentration (ppm)			
	0 (control)	50	400	4000
1	262.0	259.1	261.9	257.9
15	335.3	335.1	334.4	320.7**
22	417.0	415.6	417.8	394.9**

From Brammer (2003)

** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

All analyses were carried out in SAS.[1] Fisher's exact tests were used for the proportion of whole-litter losses, proportion of pups born alive, proportion of pups surviving, proportion of litters with all pups alive, proportion of litters with all pups surviving, proportion of male pups and the developmental landmarks; ANOVA was used for most body weight analyses and for feed consumption data, motor activity measurements, startle response tests, swimming times, time to preputial separation or vaginal opening, brain weights and brain morphometry measurements; and analyses of covariance were used for mean pup body weights subsequent to day 1, litter-based body weights (separately for males and females) subsequent to day 5 and brain weight as a covariant of final body weight.

Among the parental rats, a number of females in each group were killed prior to the scheduled termination for the reasons given in Table 43. The female that failed to litter was found not to be pregnant.

No treatment-related clinical signs were seen either during general observations of the females or during the FOBs on days 10 and 17 of gestation and days 2 and 9 of lactation.

The body weights of the dams fed 4000 ppm thiamethoxam were statistically significantly lower than those of controls on days 15 and 22 of gestation, by 4–5% (equivalent to approximately 18% lower body weight gain from days 7 to 22 of gestation) (Table 44). This lower body weight was maintained from days 1 to 22 postpartum, although there was no effect of treatment on postpartum weight gain (Table 45). Feed consumption of the dams fed 4000 ppm thiamethoxam was also statistically significantly lower than that of controls (by 17–20%) on days 7–22 of gestation, but this reduced feed consumption continued throughout the postpartum period.

[1] SAS Institute Inc. SAS/STAT 9.1 user's guide. Cary, NC, USA, SAS Institute Inc., 2004.

Table 45. Intergroup comparison of maternal body weights postpartum (selected time points; adjusted mean values shown for day 15 onwards) in a developmental neurotoxicity study in rats treated with thiamethoxam

Day	Dietary concentration (ppm)			
	0 (control)	50	400	4000
1	314.6	313.1	313.2	291.7**
15	369.1	362.5	362.0	344.9**
29	353.1	350.2	353.2	352.4

From Brammer (2003)

* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

Table 46. Litter size and viability in a developmental neurotoxicity study in rats treated with thiamethoxam

Observation	Dietary concentration (ppm)			
	0 (control)	50	400	4000
Proportion of litters with all pups born live	28/30	25/30	27/30	27/29
Proportion of male pups (day 1)	161/358	179/363	217/386**	189/380
% of male pups (day 1)	44.8	50.4	56.6**	50.0
Proportion of male pups (day 5)	158/344	166/336	204/362**	181/354
% of male pups (day 5)	45.7	50.8	56.7*	51.3
Mean litter size (day 1)	11.9	12.0	12.8	13.1
Mean litter size (day 5)	11.5	11.6	12.5	12.6
Proportion of pups surviving (days 1–5)	344/358	336/347	362/372	354/367

From Brammer (2003)

* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

There was no effect of treatment with thiamethoxam on the duration of gestation (mean of 22 days in all groups) or on the proportion of male pups; the statistically significant difference from control in the 400 ppm group was within the range of typical variability and was clearly unrelated to treatment (Table 46). There was no effect of maternal treatment with thiamethoxam on the clinical observations made on pups. The proportion of pups born live was comparable for all groups, and there was no effect of treatment on litter size on day 1. The whole-litter losses (in one female from each thiamethoxam-treated group) were considered to be unrelated to treatment.

The body weights of male and female pups born to dams fed 4000 ppm thiamethoxam were statistically significantly lower than those of controls on day 1 (by approximately 8%) and day 5 (pre-cull) (by approximately 12%), but there were no treatment-related effects on pup weights at lower doses or on total litter weight on day 1 or day 5 (pre-cull) at any dose.

In the selected F_1 rats, no treatment-related clinical signs were observed, and there were no effects of treatment detected in the FOBs on days 5, 12, 22, 36, 46 and 61. Three rats were killed intercurrently due to clinical signs, all of which were considered to be incidental to treatment with thiamethoxam. Body weights in the 4000 ppm group were statistically significantly lower than those of controls on day 5 (by approximately 12%) and remained lower than those of controls throughout the study (with a maximum effect on day 18 of 13%).

Preputial separation was delayed by an average of 1.5 days relative to the controls in the 4000 ppm males, and the body weight on the day of preputial separation was lower than that of controls in this group, whereas there was no treatment-related effect on the day of vaginal opening.

Table 47. Intergroup comparison of startle amplitude: selected time points in a developmental neurotoxicity study in rats treated with thiamethoxam

	Startle amplitude (V)							
	Males				Females			
	Dietary concentration (ppm)							
	0	50	400	4000	0	50	400	4000
Day 23 repetitions 1–10	604.0	382.9*	405.3*	429.6	600.8	450.2	533.9	377.1*
Day 61 repetitions 11–20	715.8	846.2	1048.1*	930.9	896.8	932.7	844.8	810.0
Day 61 repetitions 21–30	664.6	746.2	970.5*	858.5	803.2	700.5	778.3	668.4
Day 61 repetitions 31–40	596.5	731.5	931.8**	761.5	685.0	748.3	766.5	626.8

From Brammer (2003)

* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

Table 48. Intergroup comparison of startle response: selected time points in a developmental neurotoxicity study in rats treated with thiamethoxam

	Time to maximum amplitude (ms)							
	Males				Females			
	Dietary concentration (ppm)							
	0	50	400	4000	0	50	400	4000
Day 61 repetitions 21–30	25.0	22.2*	22.6	21.8*	22.5	22.7	23.4	23.7
Day 61 repetitions 41–50	23.5	23.3	24.2	23.6	21.7	22.3	22.5	24.2*

From Brammer (2003)

* $P < 0.05$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

Locomotor activity was unaffected by treatment. A statistically significant difference from control values observed only on day 14 of the 50 ppm group was clearly not dose related.

There was no effect of maternal treatment with thiamethoxam on startle amplitude or the time to maximum amplitude of the F_1 rats on day 23 or 61. Some statistically significant differences from control (especially in the 400 ppm group) provided no evidence for any treatment-related effects (Tables 47 and 48).

There was no effect of maternal treatment with thiamethoxam on the ability to learn or on memory on days 21 and 24 and on days 59 and 62. Learning in all groups at both time points was demonstrated by a decreased time taken to complete the "Y"-shaped water maze task between trials 1 and 6 in the learning phase (on day 21 or 59). Typically, the time taken to complete trial 6 was less than half the time taken on trial 1. Memory was demonstrated by the difference in time taken to complete trial 1 between the learning and memory phases. For all groups at all time points, group mean time for trial 1 (memory phase) was much less than that for trial 1 (learning phase). A small number of isolated statistically significant differences from the control group showed no consistent pattern and were therefore considered incidental to treatment.

Absolute (fresh) brain weight was lower than that of controls on days 12 and 63 in males and females of dams fed 4000 ppm thiamethoxam. There were no differences from control following adjustment for terminal body weight (Table 49). Post-perfusion on day 63, brain weight was lower than that of controls in males and females of dams fed 4000 ppm thiamethoxam and in females of dams fed 400 ppm thiamethoxam, but again, this effect was not apparent following adjustment for terminal body weight.

At autopsy, no gross effects of treatment were observed.

Table 49. Intergroup comparison of brain weights: F_1 rats in a developmental neurotoxicity study in rats treated with thiamethoxam

	Brain weight (g)							
	Males				Females			
	Dietary concentration (ppm)							
	0	50	400	4000	0	50	400	4000
Day 12								
Absolute brain weight	1.15	1.16	1.13	1.10*	1.11	1.09	1.10	1.06*
Brain weight adjusted for body weight	1.15	1.14	1.12	1.13	1.10	1.08	1.10	1.09
Day 63								
Absolute brain weight	2.07	2.02	2.04	1.99*	1.90	1.89	1.86	1.86
Brain weight adjusted for body weight	2.06	2.02	2.03	2.01	1.88	1.90	1.87	1.87

From Brammer (2003)

* $P < 0.05$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

A detailed microscopic examination of the nervous system revealed no effects of maternal treatment with thiamethoxam at doses up to 4000 ppm. Minimal demyelination was observed in the peripheral nerves in both control and treated rats. Although the incidence of this lesion showed both small increases and decreases in treated rats, this is a common spontaneous change in this age and strain of rat, and there were no consistent trends to suggest a relationship with treatment. The remaining microscopic findings were considered to be spontaneous and unrelated to treatment.

Brain morphometry at day 12 did not reveal any treatment-related effects. A small number of statistically significant differences in some morphometric parameters at 4000 ppm were not apparent after adjustment for body weight. These differences are therefore considered to be a consequence of the reduced birth weight and lower body weights seen in the 4000 ppm group. The only statistically significant difference after adjustment for body weight was an increase in the width of the thalamus at level 5 in females in the 4000 ppm group. However, in the absence of differences in the adjusted or unadjusted values in males or in the thalamus measurements at other brain levels, this is considered to be incidental to maternal treatment with thiamethoxam.

Brain morphometry at day 63 revealed a number of statistically significant differences in males and females in the 4000 ppm group in several parameters at levels 3–5 prior to adjustment for body weight. Many of these were not apparent after adjustment for body weight, suggesting that they are a consequence of the reduced birth weight and lower body weights seen at this dose level. Nevertheless, following adjustment for body weight, the statistically significant differences from control values described below remained.

At 4000 ppm in males, there were decreases at level 5 in the width of the thalamus and in the width of the hippocampus. In females at 4000 ppm, there were decreases at level 4 in the width of the thalamus and in the total brain width and at level 5 in the width of the thalamus. The decrease in total width at level 4 can be accounted for by the decrease in thalamus width. There were no statistically significant differences noted in these brain regions examined in the lower dose groups.

In all the male groups and females at 4000 ppm, a statistically significant difference from the control value was present, after adjustment for body weight, at level 3 in the height of the dorsal cortex parallel to the mid-sagittal line. The values for all treated groups were within the range of historical control mean values (Table 50). In addition, there was no dose–response relationship. These differences are therefore considered to be incidental to treatment.

There was a request to provide supplementary data on selected brain morphometry from pups of the 50 and 400 ppm dose levels of this experiment at days 12 and 63 (Brammer, 2007). This

Table 50. Brain morphometry at postnatal day 63 in a developmental neurotoxicity study in rats treated with thiamethoxam

	Brain morphometry (mm)							
	Males				Females			
	Dietary concentration (ppm)							
	0	50	400	4000	0	50	400	4000
Level 3 dorsal cortex 1 (3AB) mean	1.58	1.35*	1.37*	1.40*	1.51	1.50	1.48	1.46
Adjusted mean	1.58	1.35*	1.37*	1.40*	1.54	1.51	1.48	1.42*
Historical control values range								
High mean ± standard deviation	1.53 ± 0.11				1.46 ± 0.11			
Low mean ± standard deviation	1.25 ± 0.16				1.31 ± 0.07			

From Brammer (2003)

* $P < 0.05$

Table 51. Intergroup comparison of brain morphometry (selected measurements/adjusted mean): F_1 animals (day 12) in a developmental neurotoxicity study in rats treated with thiamethoxam

Sex	Parameter	Brain morphometry[a]			
		Dietary concentration (ppm)			
		0 (control)	50	400	4000
Males	Cerebellum length (8L)	4.40	4.11*	4.09**	4.34
Males	Cerebellum – prepyramidal fissure – thickness of molecular layer (8PPFM)	63.7	59.2	61.7	59.5
Females	Level 4 – thalamus – width (4E)	7.64	7.19**	7.34	7.29*

From Brammer (2007)

* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

[a] Units of measurement for brain morphometry are in millimetres (mm), except the cerebellum-preculminate fissure thickness and prepyramidal fissure thickness (8PCF1 to 8PPF0), which are in micrometres (μm).

involved examination of day 12 slides from brain level 4 (corpus callosum thickness and thalamus width) and the cerebellum (length and prepyramidal fissure – thickness of the molecular layer) and day 63 slides from brain levels 3 (dorsal cortex 2 thickness and piriform cortex thickness), 4 (dorsal cortex thickness, corpus callosum thickness, thalamus height and width, thalamus/cortex overall width and hippocampus, width of dentate gyrus) and 5 (dorsal cortex thickness and hippocampus overall width).

In day 12 males or females, a small number of brain morphometry parameters were statistically significantly different from control at 4000 ppm thiamethoxam.

At 4000 ppm thiamethoxam only, the thickness of the molecular layer of the cerebellar prepyramidal fissure was statistically significantly lower than control (by 12.2%) in day 12 males, but not statistically significant for adjusted mean (by 6.6%) (Table 51), and not in females. Adjusted mean cerebellum length was statistically significantly lower by 7.0% compared with control at 400 ppm in males, but not at 4000 ppm (1.4%). These measures of the cerebellum are clearly quite variable at this growth stage, but a treatment-related effect at 4000 ppm cannot be excluded.

Level 4 thalamus width was statistically significantly lower in all three treated groups for females (Table 51), but there was no coherent dose–response relationship, and the magnitude of the differences was small (maximum 5.9% at 50 ppm). There was no difference in this thalamus parameter in males at any dose level, and there were no differences in any related structures in the

females, including level 5 thalamus width, level 4 thalamus height and level 4 thalamus/cortex overall width. These small differences are therefore considered not to be treatment related.

Corpus callosum thickness was not statistically significant at any dose level. However, examination of this parameter in the intermediate-dose groups was requested, because the value in 4000 ppm females was 15.5% lower than in controls. There were no statistically significant differences from control in males at 4000 ppm or in the intermediate-dose groups on day 12. Considering the lack of any statistically significant differences and the small differences from control in all additional measurements, it was concluded that there was no treatment-related effect on day 12 corpus callosum thickness.

In the day 63 brain morphometry data, there were a number of statistically significant differences in males and females in the 4000 ppm group in several parameters at brain levels 3–5 prior to adjustment for body weight. Many of these were not apparent after adjustment for body weight, suggesting that they were a consequence of the reduced birth weight and lower body weights seen at this dose level (Brammer, 2003). Nevertheless, the overall pattern of lower values for a number of parameters (unadjusted for body weight) suggests that a treatment-related effect cannot be excluded at 4000 ppm. In contrast, additional analysis of the intermediate-dose groups (unadjusted for body weight) revealed only a very small number of differences from control, none of which was considered to be treatment related. Even after adjustment for body weight, most parameters were statistically significantly different from the controls in the 4000 ppm group, particularly among males, whereas statistically significant differences in the 400 and 50 ppm groups were few and not consistent with dose-related responses (Table 52).

In males, level 3 dorsal cortex 1 thickness was statistically significantly lower than control in all three treatment groups. However, comparison of concurrent with historical control data from other developmental neurotoxicity studies suggested that the concurrent control data in this study were somewhat high (Table 53). Of greater importance, there was no semblance of a dose–response relationship, there was no difference in females and the values for the treated groups (1.35, 1.37 and 1.40 mm for groups 2, 3 and 4) were all close to the mean of the historical controls for this parameter (1.34 mm).

Level 4 thalamus width values were statistically significantly lower than control values in males at 400 and 4000 ppm. Once again, it may be argued that the control group value in this experiment was high in comparison with control group values from other experiments. In addition, no changes to related parameters, such as level 4 thalamus height or level 5 thalamus width, were observed at 400 ppm in males. The level 4 thalamus width in females at 400 ppm, although statistically significantly different from the concurrent control, was higher than the control value, not lower. Therefore, the small difference in level 4 thalamus width in males at 400 ppm (4.5%) was considered not to be treatment related.

Level 4 thalamus/cortex overall width was statistically significantly lower than the control value at all three treatment levels in day 63 males. However, similar to the pattern described above for thalamus width, the concurrent control value was near the high end of the historical control data, and the mean of the historical control value (14.4 mm) was very close to the values for the 50 and 400 ppm groups (14.14 and 14.18 mm, respectively). The actual differences from control values were small (4.6%, 4.3% and 5.0%, respectively) and showed no dose-related response. The weight of evidence indicates that there was not a treatment-related effect for this parameter.

In females at day 63, level 3 dorsal cortex 2 thickness was statistically significantly lower than the control in the 50 ppm group. In the absence of any dose–response relationship, this also was not considered to be treatment related.

Table 52. Intergroup comparison of brain morphometry (selected measurements/adjusted mean): F_1 animals (day 63) in a developmental neurotoxicity study in rats treated with thiamethoxam

Parameter	Brain morphometry[a]			
	Dietary concentration (ppm)			
	0 (control)	50	400	4000
Males				
Level 3 – dorsal cortex 1 – thickness (3AB)	1.58	1.35**	1.37**	1.40**
Level 3 – dorsal cortex 2 – thickness (3BB)	1.90	1.88	1.87	1.72**
Level 3 – piriform cortex – thickness (3CB)	1.52	1.51	1.49	1.38**
Level 4 – dorsal cortex – thickness (4AB)	1.53	1.46	1.46	1.36**
Level 4 – corpus callosum – thickness (4C)	0.45	0.41	0.44	0.38*
Level 4 – thalamus – height (4D)	5.61	5.35	5.55	5.07**
Level 4 – thalamus – width (4E)	8.94	8.70	8.58*	8.46*
Level 4 – thalamus/cortex – overall width (4F)	14.81	14.12*	14.18*	14.12
Level 4 – hippocampus – width dentate gyrus (4HB)	0.64	0.60	0.61	0.59*
Level 5 – thalamus width (5C)	8.10	8.03	7.93	7.51**
Level 5 – hippocampus – overall width (5EB)	1.56	1.54	1.61	1.45*
Females				
Level 3 – dorsal cortex 1 – thickness (3AB)	1.54	1.51	1.48	1.41*
Level 3 – dorsal cortex 2 – thickness (3BB)	1.79	1.64**	1.71	1.70
Level 4 – dorsal cortex – thickness (4AB)	1.41	1.42	1.41	1.30*
Level 4 – thalamus – height (4D)	5.19	5.59*	5.40	5.25
Level 4 – thalamus – width (4E)	8.44	8.50	8.73*	8.04**
Level 4 – thalamus/cortex – overall width (4F)	14.44	14.40	14.73	13.55**
Level 5 – thalamus – width (5C)	7.86	7.64	7.74	7.31**

From Brammer (2007)

* $P < 0.05$; ** $P < 0.01$ (statistically significant difference from control group mean; Student's *t*-test, two-sided)

[a] Units of measurement for brain morphometry are in millimetres (mm), except the cerebellum-preculminate fissure thickness and prepyramidal fissure thickness (8PCF1 to 8PPF0), which are in micrometres (µm).

Table 53. Ranges and mean values for historical control data: selected brain morphometry measurements in a developmental neurotoxicity study in rats treated with thiamethoxam[a]

Parameter	Brain morphometry measurements (mm)			
	Minimum	Maximum	Mean	Standard deviation
Day 63 – males				
Level 3 – dorsal cortex 1 – thickness	1.22	1.53	1.34	0.09
Level 4 – corpus callosum – thickness	0.31	0.46	0.37	0.05
Level 4 – thalamus – width	8.27	8.86	8.59	0.18
Level 4 – thalamus/cortex – overall width	14.2	14.9	14.4	0.2
Day 63 – females				
Level 3 – dorsal cortex 1 – thickness	1.22	1.46	1.32	0.08
Level 4 – corpus callosum – thickness	0.30	0.45	0.36	0.05
Level 4 – thalamus – width	8.19	8.71	8.41	0.16
Level 4 – thalamus/cortex – overall width	13.6	14.6	14.1	0.3

From Brammer (2007)

[a] Ranges, means and standard deviations were calculated for those parameters where these historical control data are discussed in the text for males or females. Values are from control groups in developmental neurotoxicity studies conducted from 2001 to 2004 (Brammer, 2003, 2007).

Level 4 thalamus width in females was statistically significantly higher than the control value at 400 ppm and lower at 4000 ppm. At 400 ppm, this difference was small (3.2%) and opposite to the effect at the high dose and, as described for males, was considered not to be treatment related.

The NOAEL for maternal toxicity in this study of developmental neurotoxicity was 400 ppm, equal to 34.5 mg/kg bw per day, based on decreased body weight gain and feed consumption in dams throughout gestation and postpartum at 4000 ppm, equal to 298.7 mg/kg bw per day. The NOAEL for fetotoxicity in this study of developmental neurotoxicity was 400 ppm, equal to 64.0 mg/kg bw per day, based on reduced birth weight, reduced pup body weight gain and some evidence of delayed preputial separation at 4000 ppm, equal to 298.7 mg/kg bw per day. The NOAEL for developmental neurotoxicity was 4000 ppm, equal to 298.7 mg/kg bw per day, the highest dose tested, based on an absence of any quantitative histological or behavioural changes accompanying the small changes in brain morphometry (Brammer, 2007).

(c) Other special studies

Special studies on thiamethoxam were performed to investigate the etiology of adenoma and adenocarcinoma formation in mouse liver during the 18-month carcinogenicity study (Bachmann, 1998a). Special studies were also performed to investigate the renal tubule changes in rats. The results of these supplementary studies are summarized in Table 54.

(i) Mouse liver tumours

The incidences of any hepatic tumours in mice after 18 months of exposure to thiamethoxam at 0, 5, 20, 500, 1250 or 2500 ppm were, in males, 11, 7, 10, 19, 22 and 44 (out of 50) and, in females, 0, 0, 0, 5, 9 and 29 (out of 50) (Bachmann, 1998b). Most were hepatocellular tumours. The incidences of primary hepatocellular adenomas in these same dose groups of 50 mice of each sex, respectively, were, for males, 9, 5, 8, 17, 21 and 39, and, for females, 0, 0, 0, 5, 8 and 28. In addition, hepatocellular carcinomas were significantly increased in males of the highest-dose group, with incidences of 3, 3, 2, 4, 4 and 16, whereas in females, there were a few hepatocellular carcinomas in the two highest dose groups only: 0, 0, 0, 0, 2 and 3. Thus, any explanation for the induction of these tumours should account for their occurrence in both male and female Tif:MAGf(SPF) mice (but particularly in male mice) from a dietary exposure concentration of 500 ppm. The genetic toxicity database for this compound indicates that it is not a genetic toxin; therefore, other modes of action were to be investigated with, most probably, a dose below which tumours would not be expected to occur (i.e. a "threshold"). Modes of action are addressed in Appendix 1. Furthermore, there was no evidence for hepatocellular adenoma or carcinoma induction in rats receiving thiamethoxam in their diets at 0, 10, 30, 500 or 1500 ppm for 2 years (Bachmann, 1998a). The numbers of hepatocellular adenomas in these groups (out of 49 or 50 of each sex) were, for males, 0, 1, 0, 1 and 1, and, for females, 0, 2, 0, 2 and 2. The numbers of hepatocellular adenocarcinomas were also sporadic, being, for males, 0, 0, 1, 1 and 0; and for females, 0, 0, 1, 0 and 0. Thus, any mode of action should also account for the species specificity of the response.

A number of properties of thiamethoxam have been identified, some of which appear to be time- and dose-related key events that characterize the emergence of liver cancer in thiamethoxam-treated mice in a manner that resembles the cholesterol-lowering drugs, statins, which also induce liver tumours in mice. Thus, the mode of action of this pesticide may be thought to resemble that of the statins; however, the studies conducted so far provide no evidence for a statin-like inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, this being the rate-limiting enzyme in cholesterol biosynthesis (see below). Properties of thiamethoxam identified in mice include perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single-cell necrosis and as apoptosis) and a sustained increase in cell replication rates. These changes occur in a dose-dependent

Table 54. Summary of special studies conducted on thiamethoxam

Study; test system; dose levels	NO(A)EL	LO(A)EL	Main effects	Reference
In vivo liver biochemical parameters; male and female mice; 14 days of feeding; 0, 100, 500, 2500 ppm	100 ppm	500 ppm	Moderate induction of liver phase I and II xenobiotic metabolizing enzymes	Trendelenburg (1998)
In vivo hepatic cell proliferation; male and female mice; up to 59 days of feeding; 0, 100, 500, 2500 ppm	Proliferation: 100 ppm (males), 500 ppm (females) Hepatotoxicity: < 100 ppm (males), 500 ppm (females)	Proliferation: 500 ppm (males), 2500 ppm (females) Hepatotoxicity: 100 ppm (males), 2500 ppm (females)	2500 ppm: Hepatic glycogenesis and fatty change after 3+ days of treatment Increased liver weight at 2500 ppm after 7+ days of treatment Hepatic necrosis/apoptosis after 27+ days of treatment Hepatic deposition of lipogenic pigment after 59 days of treatment Increased hepatocyte proliferation after 3+ days of treatment	Weber (1998)
Changes in plasma cholesterol levels during dietary feeding studies Mice: 0, 50, 200, 500, 1250, 2500, 5000 ppm Rats: 0, 1000, 3000 ppm	200 ppm for cholesterol reduction in mice	Reduction in plasma cholesterol in mice at 500 ppm and above	Sustained and dose-dependent reduction in plasma cholesterol in mice but not in rats	Green (2003b)
Comparative hepatotoxicity in weanling and adult mice; 0, 500, 1250, 2500 ppm	Adults < 500 ppm; weanlings 500 ppm	Adults 500 ppm; weanlings 1250 ppm	Significant reduction in plasma cholesterol in adults at 500 ppm and above, in weanlings at 1250 ppm and above; internal dose in weanlings approximately 2× adults; thus, weanlings are less sensitive to the hepatic effects of thiamethoxam	Green (2003c)
Hepatic cell proliferation and apoptosis in male mice fed thiamethoxam for up to 50 weeks; 0, 50, 200, 500, 1250, 2500, 5000 ppm	200 ppm	500 ppm	General toxicity at 2500 and 5000 ppm; time- and dose-related hepatotoxicity; increased AST and ALT at 1250 ppm and above, increased apoptosis and necrosis at 500 ppm and above; increased cell proliferation at 1250 ppm and above	Weber (2003)
Comparative toxicity of thiamethoxam and metabolites in two strains of mouse; 2500 ppm thiamethoxam; 2000 ppm CGA 322704; 500 ppm CGA 265307	Highest dose tested	N/A	In contrast to the effects of thiamethoxam, CGA 322704 and CGA 265307 had no significant effects on liver; both strains responded similarly	Noakes (2003a)
Sublobular assessment of hepatic cell proliferation; 0, 200, 500, 1250 ppm	200 ppm	500 ppm	Thiamethoxam induces increases in hepatocellular labelling at 500 and 1250 ppm	Soames (2003)

Table 54 (continued)

Study; test system; dose levels	NO(A)EL	LO(A)EL	Main effects	Reference
Hepatotoxicity of metabolites fed for up to 50 weeks; 2500 ppm thiamethoxam; 2000 ppm CGA 322704; 500 ppm CGA 265307; 500, 1000 ppm CGA 330050	Thiamethoxam: N/A; CGA 330050: after 10 weeks, 500 ppm; CGA 322704 and CGA 265307: highest doses tested	Thiamethoxam: 2500 ppm, only concentration tested; CGA 330050: after 10 weeks, 1000 ppm	Thiamethoxam and CGA 330050 induced significant hepatic effects; CGA 322704 and CGA 265307 had no significant effects on liver; metabolite CGA 330050 has a key role in the mechanism of thiamethoxam hepatotoxicity	Green (2003d)
Role of nitric oxide in mouse hepatotoxicity	N/A	N/A	CGA 265307 inhibits nitric oxide synthase; this inhibition of the normal protective effect of nitric oxide exacerbates the liver toxicity induced by CGA 330050	Green (2003e)
Hepatic cell proliferation and apoptosis in female rats fed thiamethoxam for up to 50 weeks; 1000, 3000 ppm	3000 ppm, highest concentration tested	N/A	No effect on hepatocyte proliferation detected, as measured by increased S-phase DNA synthesis or apoptosis	Noakes (2003b)
Biochemical parameters in rat liver after 1 and 10 weeks; 1000, 3000 ppm thiamethoxam	N/A	N/A	No remarkable inducing effect on xenobiotic metabolizing enzymes; no effect on hepatic glutathione concentration or on activity of γ-glutamylcysteine synthetase	Waechter (2003)
Comparative metabolism in mice and rats in vivo and in mouse, rat and human liver fractions in vitro	N/A	N/A	Production of key metabolites in vivo much higher in mice than rats; induction of metabolism in mice over time, whereas reduction seen in rats; results in plasma concentrations of CGA 265307 in mice after 10 weeks that are 108-fold higher than in rats; a similar difference is seen in vitro, with rate conversions in mouse liver being much higher than those in rat liver fractions; the rate conversions in human liver were even lower than in rat liver, indicating that human metabolism of thiamethoxam to the key metabolites would be minimal	Green (2002)
Metabolism in rats and mice in dietary feeding studies 50 week mouse: 500, 1250, 2500 ppm thiamethoxam 50 week rat: 1000, 3000 ppm thiamethoxam 20 week mouse: 2500 ppm thiamethoxam, 200 ppm CGA 322704, 500 ppm CGA 265307 1 week mouse: 1000 ppm CGA 330050	N/A	N/A	Following administration of thiamethoxam, the plasma concentrations of parent were similar in rats and mice, but the concentrations of CGA 322704, CGA 265307 and CGA 330050 were much higher in mice than in rats, with evidence for induction of metabolism in mice, but not rats; following administration of CGA 322704 to mice, the only major metabolite detected was CGA 265307; as CGA 3222704 is not carcinogenic in the mouse, CGA 265307 is unlikely to be responsible for the carcinogenicity of thiamethoxam in the mouse;	Green (2003a)

Table 54 (continued)

Study; test system; dose levels	NO(A)EL	LO(A)EL	Main effects	Reference
			CGA 330050 is the only major metabolite identified that occurs in significantly greater quantities in mice than in rats and that is unique to thiamethoxam; it is therefore likely to have a key role in the carcinogenicity of thiamethoxam in the mouse	
In vivo hepatic cell proliferation; male rats; 28 days feeding; 0, 10 000 ppm	10 000 ppm (~710.6 mg/kg bw per day), highest dose tested	N/A	No effect on hepatocyte proliferation detected, as measured by increased S-phase DNA synthesis	Persohn (1995)
In vitro cytotoxicity; rat/mouse hepatocytes; 0, 10–5000 µmol/l (9 concentrations)	5000 µmol/l (rat and mouse), highest concentration tested	N/A	No cytotoxicity detected in either species	Bouis (1997)
In vivo acute hepatic cell damage; male mice; 500 mg/kg bw	N/A	500 mg/kg bw	Cytoplasmic condensation of mostly periportal hepatocytes at 6 h, suppression of hepatocellular mitotic activity at 24 h after dosing	Weber (1999a)
In vivo hepatic cell apoptosis; male mice; up to 59 days of feeding, 0, 100, 500, 2500 ppm; 9 months of feeding, 0, 2500 ppm	100 ppm (~15.8 mg/kg bw per day)	500 ppm	Increase in apoptotic activity after 59 days, still present after 9 months (data only at 2500 ppm)	Weber (1999b)
Immunohistochemical assessment of α_{2u}-globulin in the rat kidney upon administration of CGA 293343 for 28 days, 90 days, 12 months, 24 months	N/A	N/A	Renal tubular hyaline change	Weber (2000a,b,c,d)

LO(A)EL, lowest-observed-(adverse-)effect level; N/A, not applicable; NO(A)EL, no-observed-(adverse-)effect level

manner and are seen in their entirety only at the dose levels at which tumour incidences are increased. At least some of them may therefore constitute key events in a mode of (carcinogenic) action, although it is perhaps difficult to understand a key role for interference with cholesterol metabolism. An important question is whether they all occur at least at all dose levels (but not necessarily all examination times) at which tumour incidences are increased. It is, of course, possible that a key event acting in a minimal fashion at a lower dose level might be insufficient to trigger succeeding steps in the process. Generally, it is not possible to refer back to the standard toxicity studies on mice (3-month feeding study: Bachmann, 1996a; 18-month feeding study: Bachmann, 1998a) for support of the investigation results, because the required measurements and observations were not made. In spite of the statin-like properties, the inductions noted in liver phase I and phase II enzymes had similarities with those produced by phenobarbital (Trendelenburg, 1998). This similarity is possibly misleading, however, as the effect on these enzymes is small, and it has been suggested (Trendelenburg, 1998) that it is insufficient to account for the emergence of liver tumours.

Table 55. Successive changes in the livers of mice treated with thiamethoxam for up to 50 weeks

Study week	Observation
1–10	Liver dysfunction: Decreased protein synthesis, glycogen and lipid accumulation, reduced cholesterol, cytochrome P450 induction and hepatocellular hypertrophy
10–50	Hepatotoxicity: Increased ALT/AST, pigmentation, inflammatory cell infiltration, hepatocellular hypertrophy, single-cell necrosis and increased apoptosis
20–50	Sustained increase in cell replication rates

From Trendelenburg (1998); Weber (1998, 2003); Green (2003b,c); Noakes (2003a)

Several progressive steps in the hepatotoxicity of thiamethoxam in mice include an early-stage mild hepatic dysfunction caused by the metabolite CGA 330050 (characterized by lowering of plasma cholesterol, induction of cytochrome P450 isoenzymes, decreased protein synthesis and glycogen and lipid accumulation), leading to hypertrophy, lymphocytic infiltration and pigmentation of hepatocytes and Kupffer cells. These processes may be exacerbated by another specific thiamethoxam metabolite, CGA 265307. This metabolite does not seem to be hepatotoxic, but it is a competitive inhibitor with arginine, the natural substrate of nitric oxide synthase in vitro (Green, 2003e). Nitric oxide has a regulatory role in liver in modulating the adverse effects of tumour necrosis factor alpha (TNFα) released from endothelial cells during chemical-induced hepatotoxicity. Consequently, there is likely to be exacerbation of cytotoxicity, single-hepatocyte necrosis and apoptosis, which are believed to lead to increased cell proliferation. Much of this (but not all) could constitute key events in a mode of action that finally leads to the formation of hepatic tumours.

The hepatic changes preceding the emergence of tumours have been studied in experiments in which mice were fed diets containing 0–5000 ppm thiamethoxam for periods from 1 to 50 weeks (Trendelenburg, 1998; Weber, 1998, 2003; Green, 2003b,c; Noakes, 2003a). The results of these studies suggest the progression of changes shown in Table 55.

In this study of up to 50 weeks' duration, hepatocellular neoplasms did develop in small numbers, even in controls, in weeks 40–50, but the higher incidences were observed only in the 79-week carcinogenicity study.

The results from these six reports are summarized below.

1–10 weeks: Liver dysfunction

The earliest stages of thiamethoxam's effect on the mouse liver (within 1 week of dosing) are characterized by histological changes, including accumulation of glycogen and lipid in centrilobular regions of the liver. These histological changes are accompanied by several significant biochemical changes, including those described below.

Protein synthesis: A statistically significant reduction in plasma total protein was observed in male Tif:MAGf mice (females not studied) fed on diets containing 2500 ppm thiamethoxam for 2 and 11 weeks, but with signs of recovery at 21 weeks. This reduction was not due to reduced albumin concentration and was not induced by the major metabolites, CGA322704 at 2000 ppm or CGA265307 at 500 ppm (Noakes, 2003a).

Cytochrome P450 induction: A significant increase in hepatic cytochrome P450, principally of the 2B family, was seen in mice fed on diets containing 0, 100, 500 or 2500 ppm thiamethoxam for 14 days (Trendelenburg, 1998). Thiamethoxam was found to be a moderate (up to 9-fold) inducer of mouse CYP2B isoenzymes and both 7-pentoxyresorufin *O*-dependentylase (PROD) and 7-benzyloxyresorufin *O*-debenzylase (BROD) activities. However, these moderate increases alone were considered insufficient to be causally linked to the development of liver cancer. The enzymatic changes found are summarized in Table 56.

Table 56. Summary of enzyme activities

Assay	Result
Protein content	No effect in either sex at any dose in supernatant and cytosolic fractions
	24% increase in females at 2500 ppm in microsomal fraction
Cytochrome P450	59% increase in males at 2500 ppm
	52% increase in females at 2500 ppm
7-Ethoxyresorufin *O*-deethylase	No effect in males at all dose levels
	45% increase in females at 500 ppm
	157% increase in females at 2500 ppm
7-Pentoxyresorufin *O*-dependentylase	42% increase in males at 500 ppm
	725% increase in males at 2500 ppm
	51% increase in females at 100 ppm
	103% increase in females at 500 ppm
	711% increase in females at 2500 ppm
7-Benzyloxyresorufin *O*-debenzylase	72% increase in males at 500 ppm
	1021% increase in males at 2500 ppm
	53% increase in females at 100 ppm
	109% increase in females at 500 ppm
	782% increase in females at 2500 ppm
Coumarin 7-hydroxylase	55% increase in males at 2500 ppm
	No effect in females at any dose level
Regioselective and stereoselective hydroxylation of testosterone	Total oxidation rates increase in males by 41% due to increased hydroxylation at positions 2β, 6α, 6β, 15α and oxidation to androstenedione; total oxidation rates increase in females by 37% due to increased hydroxylation at positions 6α, 16α, 16β and oxidation to androstenedione
Lauric acid 11-hydroxylation	47% increase in males at 2500 ppm
	104% increase in females at 2500 ppm
Lauric acid 12-hydroxylation	No effect in males at any dose level
	No effect in females at any dose level
Uridine diphosphate-glucuronosyltransferase	33% increase in males at 2500 ppm
	31% increase in females at 2500 ppm
Glutathione *S*-transferase	61% increase in males at 2500 ppm
	198% increase in females at 2500 ppm
Epoxide hydrolase	50% increase in males at 2500 ppm
	133% increase in females at 2500 ppm
Cyanide-insensitive peroxisomal-β-oxidation	No effect in males at any dose level
	No effect in females at any dose level

From Trendelenburg (1998)

Differences in thiamethoxam metabolism in mice and rats in vivo and mice, rats and humans in vitro: Following dietary administration of thiamethoxam to mice and rats at 2500 ppm and 3000 ppm, respectively, for 1 or 10 weeks, quantitative differences were seen in the concentrations of metabolites in plasma that increased with time of exposure. Over the duration of the study, there was evidence of induction of the metabolism of thiamethoxam in mice, whereas a reduction in metabolism of thiamethoxam was observed in rats. After 10 weeks, the concentration of CGA 265307 in plasma was 108-fold higher in mice than in rats (Table 57).

Table 57. Concentration of thiamethoxam and its metabolites in plasma in rats and mice fed on diets containing thiamethoxam for up to 50 weeks

	Concentrations (µg/ml)					
	Mouse			Rat		
	Dietary concentration (ppm)					
	2500			3000		
	Weeks on diet					
	1	10	50	1	10	50
Thiamethoxam	11.82	14.86	9.71	7.01	19.21	7.91
CGA 322704	2.54	5.31	3.38	0.96	0.63	1.20
CGA 265307	1.98	7.05	4.20	0.09	0.05	0.05
CGA 330050	0.86	1.50	1.12	0.14	0.10	0.12

From Green (2003a)

The species differences seen in vivo were reflected in the in vitro rate comparisons (Figures 4–7).

The mean rate for the conversion of thiamethoxam to CGA 265307 via CGA 322704 was 54-fold lower in rat liver than in mouse liver, and via CGA 330050 was 87-fold lower in rat liver than in mouse liver.

The rates for each of the metabolic transformations of thiamethoxam by its major pathways were significantly lower in microsomal preparations from human liver than from mouse liver. The mean rate for the conversion of thiamethoxam to CGA 265307 via CGA 322704 was 371-fold lower in human liver than in mouse liver, and the conversion via CGA 330050 was 238-fold lower in human liver than in mouse liver. The remarkably high Michaelis-Menten constant (K_m) values seen in human livers for the metabolism of thiamethoxam to CGA 322704 and CGA 330050 suggest that metabolism of thiamethoxam by these pathways in humans would be minimal at expected exposure levels (Green, 2002). A proposed interconversion pathway for thiamethoxam and these metabolites is shown in Figure 8.

The trends in metabolism in mice and rats first observed over the early weeks of exposure to thiamethoxam persisted in subsequent studies in which the metabolism of thiamethoxam was investigated in blood and liver samples from mice and rats following dietary administration of thiamethoxam and its major metabolites CGA 322704, CGA 265307 and CGA 330050 for up to 50 weeks.

Following administration of thiamethoxam to mice at 500, 1250 or 2500 ppm, the dose–response relationship was approximately linear over the dose range tested, with no evidence of saturation of metabolic pathways at higher dietary dose levels (Table 58).

Following administration of thiamethoxam to mice at 2500 pm in the diet, the concentrations of thiamethoxam and its metabolites in plasma and liver were similar. Plasma concentrations are therefore a reasonable reflection of the concentrations in liver, the target organ for carcinogenicity in the mouse.

Following administration of thiamethoxam to mice and rats at 2500 ppm and 3000 ppm in the diet, respectively, the concentration of thiamethoxam in plasma was similar in the mouse and the rat, demonstrating that the internal dose of the test substance was similar. As the concentration of thiamethoxam in plasma reflects the concentration in liver, the absence of liver tumours in rats suggests that parent thiamethoxam is not responsible for the liver tumours seen in mice.

Figure 4. The metabolism of thiamethoxam to CGA 322704 in mouse, rat and human liver microsomes (Green, 2002)

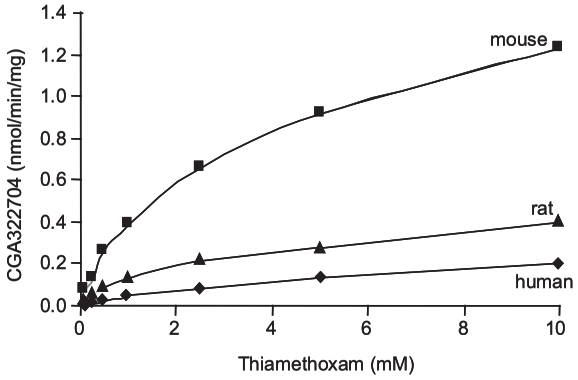


Figure 5. The metabolism of CGA 322704 to CGA 265307 in mouse, rat and human liver microsomes (Green, 2002)

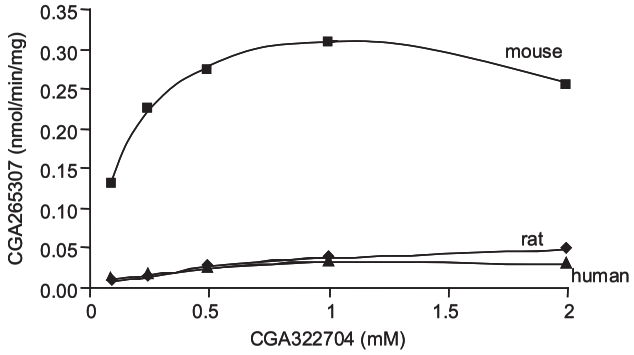


Figure 6. The metabolism of thiamethoxam to CGA 330050 in mouse, rat and human liver microsomes (Green, 2002)

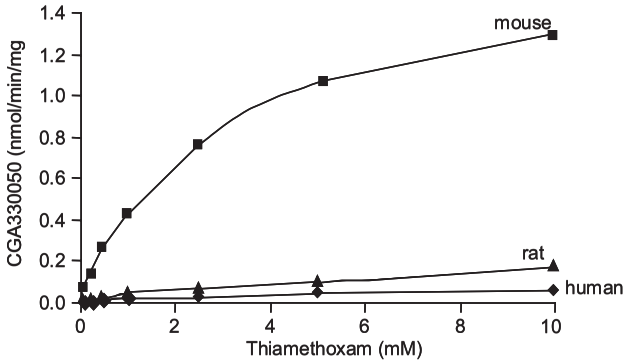


Figure 7. The metabolism of CGA 330050 to CGA 265307 in mouse, rat and human liver microsomes (Green, 2002)

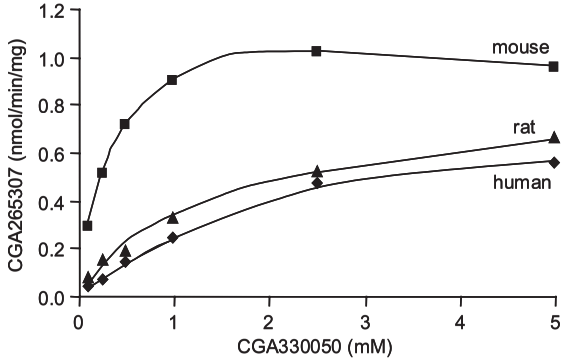


Figure 8. Proposed metabolic pathway of thiamethoxam in the mouse and rat (Green, 2002)

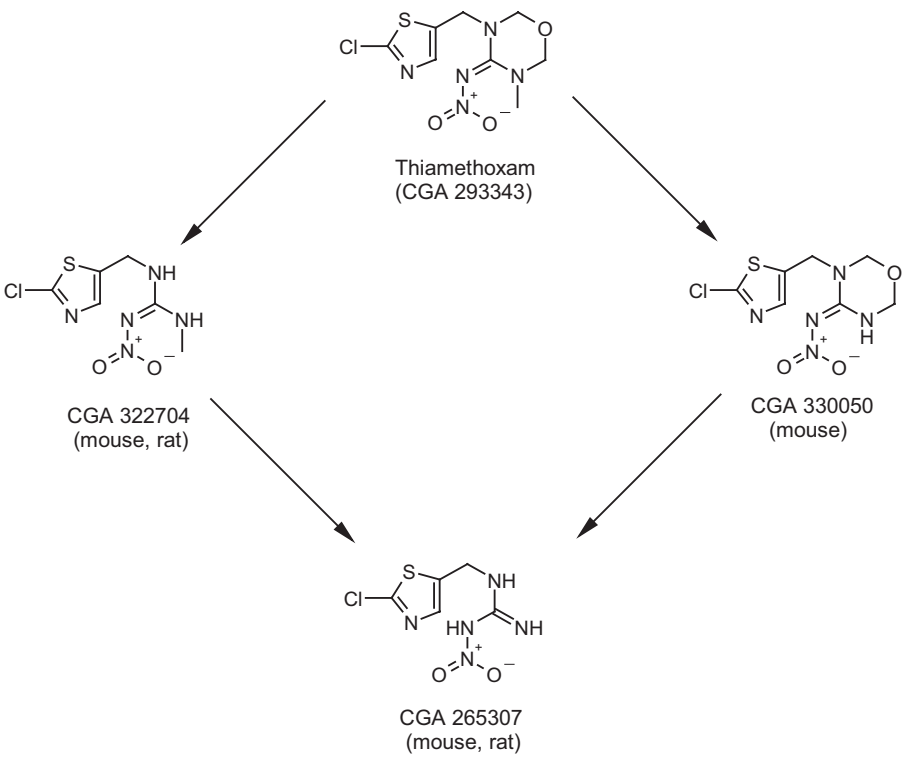


Table 58. Concentration of thiamethoxam and its metabolites in plasma in mice fed on diets containing thiamethoxam for 10 and 50 weeks

	Concentration (μg/ml)					
	Dietary concentration (ppm)					
	500	1250	2500	500	1250	2500
	Weeks on diet					
	10			50		
Thiamethoxam	3.78	8.63	14.86	2.26	5.54	9.71
CGA 322704	1.08	2.63	5.31	0.80	1.99	3.38
CGA 265307	1.68	3.62	7.05	1.48	3.31	4.20
CGA 330050	0.53	0.91	1.50	0.40	0.85	1.12

From Green (2003a)

The concentrations of the three metabolites CGA 322704, CGA 265307 and CGA 330050 in plasma were considerably higher (8-fold, 140-fold and 15-fold higher, respectively, after 10 weeks) in mice than in rats. The increase in plasma concentration of CGA 265307 after 1 week and 10 weeks in mice indicates that there is induction of the pathway(s) leading to the formation of this metabolite in this species. The differences in concentrations of CGA 265307 and CGA 330050 between mice and rats are sufficient to suggest that they may account for the species differences in carcinogenicity. These findings are also consistent with the study (above) in which the rate constants for this pathway were measured in vitro in rat, mouse and human liver fractions (Green, 2002).

Metabolite CGA 322704 (clothianidin) has been tested in rodent carcinogenicity studies; however, tumour incidences were not increased in either mice (CD-1 strain) or rats at levels up to 3000 ppm and 2000 ppm, respectively (Federal Register, 2003). The possibility that different mouse strains respond differently to thiamethoxam was investigated; however, no difference was seen in the metabolism of thiamethoxam, CGA 322704 or CGA 265307 in TifMAGf and CD-1 strains, so it is considered unlikely that the differences in the carcinogenicity of thiamethoxam and CGA 322704 were due to the use of different strains of mouse (Green, 2003a).

The evidence suggesting liver enzyme induction in mice, but not in rats, was investigated further. After treatment of female Tif:RAIf (SPF) rats with thiamethoxam, some of the investigated biochemical and immunochemical liver parameters were slightly increased, whereas others decreased. However, the observed changes gave no indication for any remarkable inducing effect of thiamethoxam on the hepatic phase I and phase II xenobiotic metabolizing enzymes. In addition, treatment did not affect the hepatic glutathione concentration or the activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis (Waechter, 2003). This is in contrast to similar studies in the mouse, in which thiamethoxam was a moderate inducer of hepatic xenobiotic metabolizing enzymes and caused an increased hepatic glutathione concentration as well as an increased activity of γ-glutamylcysteine synthetase (Trendelenburg, 1998).

Plasma cholesterol changes: The earliest and most significant change in mice fed on diets containing thiamethoxam is a marked reduction in plasma cholesterol levels. This was demonstrated in a study by Noakes (2003a), in which blood cholesterol levels were statistically significantly reduced by 4%, 38% and 35%, respectively, in male Tif:MAGf mice (females not studied) fed diets containing 2500 ppm thiamethoxam for 2, 11 and 21 weeks. The early reduction in plasma cholesterol level was confirmed in a separate experiment by Green (2003b), in which plasma cholesterol level was reduced 31% in mice receiving 2500 ppm thiamethoxam for 4 weeks. Return to control diet in this second Green (2003b) experiment for another 2 or 4 weeks non-significantly changed the cholesterol concentration in comparison with the controls by +19% and −8%, respectively. Even after just 1 week on diets containing thiamethoxam at 1250 and 2500 ppm, plasma cholesterol levels were reduced in weanling mice by 15% and 21%, respectively, but not at 500 ppm (+4.5%), whereas 1 week on the 500, 1250 or 2500 ppm diets reduced plasma cholesterol levels in adult mice by 22%, 32% and 28%, respectively (Green, 2003c). Other studies showed that the effect on cholesterol was persistent and occurred at all dietary concentrations associated with increases in liver tumour incidence in mice. Dietary administration of thiamethoxam to Tif:MAGf mice at 0, 50, 200, 500, 1250, 2500 or 5000 ppm for up to 50 weeks statistically significantly reduced plasma cholesterol levels at all sampling times (10, 20, 30, 40 and 50 weeks) in the 5000 ppm group and at most sampling times in the 2500 and 1250 ppm groups. Cholesterol level was also significantly reduced in the 500 ppm group at 10 and 20 weeks by 30% and 28%, respectively, but not at later times. Cholesterol was not reduced at any sampling time in the 200 or 50 ppm groups (Green, 2003b). The mechanism by which cholesterol is reduced in mice has not been established. Based on the assumption that thiamethoxam was acting like statins, the effect of thiamethoxam on 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis, was investigated, but the enzyme was not inhibited in mice treated with 2500 ppm thiamethoxam diets for 20 weeks, and neither thiamethoxam nor its metabolites CGA265307 and CGA330050 at 300 µmol/l for 20 minutes inhibited the enzyme in vitro (Green, 2003b). These concentrations were of the order 10-fold higher than the normal substrate concentrations.

In comparisons of the activity of thiamethoxam with those of some of its metabolites, it was found that the concentration of plasma cholesterol in mice was not induced by CGA 322704 at 2000 ppm or by CGA 265307 at 500 ppm given for up to 20 weeks, whereas 2500 ppm thiamethoxam reduced the values at 1, 10 and 20 weeks by 19%, 38% and 35%, respectively. In contrast, the metabolite CGA 330050 reduced plasma cholesterol levels after 1, 4 or 10 weeks by 14%, 18% and 16%,

respectively, at 500 ppm and by 16%, 25% and 19%, respectively, at 1000 ppm (Green, 2003b). This same study showed that feeding 1000 or 3000 ppm thiamethoxam to rats for up to 50 weeks did not lead to cholesterol level reductions at 1, 10, 20, 30, 40 or 50 weeks.

In considering whether this effect on circulating cholesterol concentrations might constitute a key event in hepatocarcinogenesis in mice, it is necessary to recall the effects of thiamethoxam on this parameter in rats in 28-day (Bachmann, 1995) and 90-day (Bachmann, 1996b) studies. There were statistically significant increases in cholesterol concentrations of 22%, 26% and 43%, respectively, in the 28-day study in male rats given diets containing 1000, 2500 or 10 000 ppm thiamethoxam and of 70% in female rats given 10 000 ppm thiamethoxam. In the 90-day study, 5000 ppm thiamethoxam was associated with increases in cholesterol of 22% in males and 24% in females.

There is strong evidence that changes in lipid metabolism in rodents lead to an increase in liver tumours. A large number of drugs have been developed in recent years for the control of lipids (triglycerides and cholesterol) and the prevention of coronary heart disease. These fall into two main classes: the hypolipidaemic drugs, such as the fibrates, and the more recent statins. Both classes have been evaluated in rodent carcinogenicity studies during their development, and the majority of drugs in each class cause liver cancer, particularly in mice, but also in rats (MacDonald et al., 1988; Gerson et al., 1989; Newman & Hulley, 1996; von Keutz & Schluter, 1998). Thiamethoxam is not a peroxisome proliferator, and it has minimal effects on plasma triglycerides in vivo. In specifically lowering plasma cholesterol, its mode of action might resemble that of the statin family of cholesterol-lowering drugs. Changes in cholesterol biosynthesis and/or metabolism are early events, but it is unclear whether they constitute key events in the mode of action of thiamethoxam as a mouse liver carcinogen.

10–50 weeks: Hepatotoxicity

At this stage of continuous dosing in mice (15 examined per group), frank signs of hepatotoxicity were seen (Noakes, 2003a; Weber, 2003), and the statistically significant changes described below were observed.

Plasma cholesterol changes: As described above, reductions in plasma cholesterol were sustained throughout this period.

Plasma clinical chemistry changes: The median AST activity was increased at 2500 ppm in weeks 20 and 40 (but not week 10, 30 or 50) and at 5000 ppm in all weeks from 10 to 50. The median ALT activity was significantly increased at 1250 ppm in weeks 40 and 50 (but not at earlier times) and at 2500 and 5000 ppm in all weeks from 10 to 50. No changes were observed in the activity of alkaline phosphatase (Weber, 2003). On the basis of the dose levels at which the activities of these plasma enzymes are affected, it would appear that this is not a key event in mouse liver carcinogenesis.

Hepatocellular hypertrophy: Hepatocellular hypertrophy incidences and/or mean severity (in parentheses) were statistically significantly increased from the control group value of 0/15 to 7/15 (1.00), 12/15 (1.17) and 12/15 (1.67), respectively, at weeks 30, 40 and 50 for the 2500 ppm group and to 12/15 (1.75), 13/15 (2.15), 14/15 (2.00), 13/15 (2.23) and 14/15 (1.50) at weeks 10, 20, 30, 40 and 50 for the 5000 ppm group. This change was characterized by enlarged centrilobular/midzonal hepatocytes with increased amounts of cytoplasmic glycogen, fat and smooth endoplasmic reticulum (Weber, 2003). The increase in hepatocellular hypertrophy was confirmed in a separate study in which male TifMAGf mice were given diets containing 0 or 2500 ppm thiamethoxam for up to 20 weeks. The incidences (and severity) at week 10 were 0/12 at 0 ppm and 7/12 (1.14) at 2500 ppm and, at week 20, 1/11 (2.00) at 0 ppm and 7/12 (1.29) at 2500 ppm. In this same experiment, neither of the metabolites CGA 322704 (2000 ppm) and CGA 265307 (500 ppm) induced hypertrophy at any time up to 20 weeks (Noakes, 2003a).

Table 59. Intergroup comparison of selected microscopic findings in the liver: total incidence across all time points in mice treated with thiamethoxam

Finding	Dietary concentration (ppm)						
	0	50	200	500	1250	2500	5000
Hepatocellular hypertrophy	0	1	0	2	5	35*	66*
Hepatocellular necrosis	9	11	13	27*	44*	66*	71*
Inflammatory cell infiltration	18	21	18	31*	33*	51*	47*
Hepatocellular apoptosis	0	0	1	6*	28*	51*	63*
Pigmentation	0	1	1	4	8*	38*	57*
Fatty change	30	37	38	52*	61*	57*	12*

From Weber (2003)

* $P < 0.05$ (statistically significant difference from control group mean)

Pigmentation: Pigmentation was statistically significantly increased in incidence and/or mean severity at 500 ppm (week 50), at 2500 ppm (from weeks 20 to 50) and at 5000 ppm (all time points). Yellow/brown pigment granules in the cytoplasm of centrilobular hepatocytes characterized this lesion. Occasionally, pigmented Kupffer cells were observed (Weber, 2003). The pigment was not identified. This increase in pigmentation at 2500 ppm was confirmed in a separate study after 20 weeks of exposure, in which the incidences were 1/11 in the 0 ppm group and 5/12 in the 2500 ppm group (Noakes, 2003a). The pigment could possibly have been haemosiderin or lipofuscin. In the absence of effects on blood cells, the latter is more likely.

Hepatic single-cell necrosis: Hepatic single-cell necrosis was increased significantly in incidence and/or mean severity at 500 ppm in week 40, at 1250 ppm in weeks 20, 30, 40 and 50 and at 2500 and 5000 ppm in weeks 10, 20, 30, 40 and 50. Hepatocellular necroses affected single cells or small groups of cells with mainly centrilobular localization and were often accompanied by inflammatory cells (Weber, 2003).

Inflammatory cell infiltration: Inflammatory cell infiltration was increased significantly in incidence and/or mean severity at 500 ppm in week 50, at 1250 ppm in weeks 40 and 50, at 2500 ppm in weeks 20, 40 and 50 and at 5000 ppm in weeks 30, 40 and 50 (Weber, 2003).

Hepatocellular apoptosis: Hepatocellular apoptosis, as assessed by standard histology, was increased in incidence and/or mean severity at 1250 ppm thiamethoxam in weeks 20 and 30 and at 2500 ppm and 5000 ppm in weeks 10, 20, 30, 40 and 50. Hepatocellular apoptoses affected single cells or small groups of cells with mainly centrilobular localization. Quantitative assessment of the extent of apoptosis using terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick end labelling (TUNEL) (Gavrieli, Sherman & Ben Sasson, 1992) area density showed an increase at 500 ppm in weeks 30 and 40, at 1250 ppm in weeks 30 and 40, at 2500 ppm in weeks 30, 40 and 50 and at 5000 ppm in weeks 10, 20, 30, 40 and 50 (Weber, 2003). Largely consistent with these data, Noakes (2003a) did not show any increase in TUNEL in TifMAGf mice treated for up to 20 weeks with thiamethoxam at 2500 ppm, CGA 322704 at 2000 ppm or CGA 265307 at 500 ppm.

These hepatic manifestations of effects of thiamethoxam, all time points combined, are summarized in Table 59.

In vitro study of cytotoxicity in primary hepatocyte cultures from rats and mice failed to demonstrate any effect of thiamethoxam or the metabolite CGA 256084 at concentrations of up to 5000 µmol/l (Bouis, 1997).

20–50 weeks: Sustained increased cell replication rate

An increased median 5-bromo-2′-deoxyuridine (BrdU) labelling index was observed at 1250 ppm in week 40, at 2500 ppm in weeks 30, 40 and 50 and at 5000 ppm in weeks 10, 30, 40 and 50. These increases were determined across the whole liver lobule (Weber, 2003). These observations were confirmed in a separate study after 20 weeks of exposure to 2500 ppm, but no increase was observed after 10 weeks (Noakes, 2003a). When the increases in cell replication rates were measured specifically in centrilobular hepatocytes, the region of the liver in which single-cell necrosis and apoptosis were concentrated, a statistically significant increase in cell replication rates was also observed in the 500 ppm dose group. The labelling indices (%) increased from 0.15 ± 0.10 in the control group to 0.36 ± 0.31 at 500 ppm and 0.48 ± 0.45 at 1250 ppm. There was no increase (0.10 ± 0.07) at 200 ppm (Soames, 2003). The metabolites CGA 322704 at 2000 ppm and CGA 265307 at 500 ppm did not increase BrdU labelling after 10 or 20 weeks (Noakes, 2003a). An assessment of DNA replicative synthesis in male rats fed thiamethoxam for 28 days failed to demonstrate any significant change in the numbers of proliferating cell nuclear antigen–positive hepatocyte nuclei at dietary concentrations up to 10 000 ppm (Persohn, 1995).

Hepatocellular neoplasia developed to some degree in TifMAGf mice even in the small groups in the comparatively short 50-week dietary experiments with thiamethoxam. At week 40, there were 0, 0, 0, 1, 1, 2 and 1 hepatocellular neoplasms in the 0, 50, 200, 500, 1250, 2500 and 5000 ppm groups, respectively, whereas at 50 weeks, the numbers were 2, 1, 1, 0, 2, 3 and 4, respectively. Most were adenomas, but some carcinomas were also reported.

Carcinogenicity and toxic properties of metabolites: Both the hepatic tumours and the events described above were seen in the Tif:MAGf mouse. The long-term studies with thiamethoxam metabolite CGA 322704 (tested as clothianidin) used another strain, believed to be the CD-1 mouse, in which no carcinogenesis in liver or elsewhere was observed. In order to investigate possible strain effects on the outcome of these studies, both male Tif:MAGf mice and male CD-1 mice were fed on diets containing 0 or 2500 ppm thiamethoxam for 1, 10 or 20 weeks. The end-points monitored in this study were identical to those in the 50-week studies: namely, liver biochemistry, histopathology, apoptosis (TUNEL) and cell replication rates (Noakes, 2003a).

Decreases in plasma cholesterol levels were seen at all time points after exposure to the metabolite CGA 322704. At 10 and 20 weeks, aminotransferase activities were increased, and the histopathological changes noted at these time points in the 50-week mouse study were observed. An increase in cell replication rates was seen at 20 weeks. The magnitude of the changes seen in this study was comparable to those seen in the 50-week mouse study. All of these changes were seen in both Tif:MAGf and CD-1 mice to a similar degree. Thus, the events, at least some of which may be key to a mode of action, are not mouse strain specific.

Two other thiamethoxam metabolites investigated in these studies, CGA 330050 and CGA 265307, were dosed to Tif:MAGf mice at levels selected to mimic systemic exposure to these metabolites following high-dose feeding levels of thiamethoxam. These were 500 and 1000 ppm for CGA 330050 and 500 ppm for CGA 265307. The dose levels of CGA 330050 induced the same changes in the livers of mice as those seen after dietary administration of thiamethoxam. Plasma cholesterol levels were reduced significantly, and the histopathological examination of the livers revealed an increase in hypertrophy, single-cell necrosis, apoptosis and increased cell replication at 10 weeks. Plasma cholesterol was statistically significantly reduced at week 1 by 14% and 16% in the 500 and 1000 ppm groups, respectively, whereas at weeks 4 and 10, there were statistically significant reductions only in the 1000 ppm group of 25% and 31%, respectively. Hepatocellular hypertrophy incidences were 0/12 in all groups at 1 week, but after 10 weeks, they were 0/12, 0/12 and 11/12 (8/12 being of minimal severity) in the 0, 500 and 1000 ppm groups, respectively. Single-cell necrosis/apoptosis did not occur at 1 week, but at 10 weeks, the incidences were 2/12, 2/12 and 6/12 (higher,

but not significant) in the 0, 500 and 1000 ppm CGA 330050 groups. However, BrdU labelling of hepatocyte nuclei was statistically significantly increased after 10 weeks at 1000 ppm. The percentages in the 0, 500 and 1000 ppm groups were 0.20 ± 0.11, 0.36 ± 0.24 and 0.57 ± 0.61, respectively. Therefore, CGA 330050 is seen to play a role in the development of hepatic lesions (Green, 2003d).

CGA 265307 (dosed at 500 ppm in the diet, a dose level selected to mimic systemic exposure to this metabolite following a feeding level of 2500 ppm thiamethoxam) did not induce any of the hepatic lesions seen in thiamethoxam-treated mice when tested in a 20-week study alongside thiamethoxam and CGA 322704 (Noakes, 2003a).

The metabolic relationships of these metabolites (CGA 322704, CGA 330050 and CGA 265307) to each other are shown in Figure 8 above. It is possible for CGA 265307 to be produced from thiamethoxam by two routes, one of which is found in mouse (via CGA 330050), whereas the other pathway can occur in both mice and rats (via CGA 322704).

It is proposed that the key events in a mode of (carcinogenic) action of thiamethoxam in mice are as follows:

1. Exposure to thiamethoxam, its absorption and metabolism in the liver, in which metabolites CGA 322704 and CGA 330050 are produced, both of which are further metabolized to CGA 265307. There are significant quantitative species differences in the metabolism of thiamethoxam.
2. Hepatocellular toxicity induced by CGA 330050 and exacerbated by CGA 265307, seen as increases in hepatocellular single-cell necrosis and apoptosis. Changes in plasma aminotransferase activities are not seen at all critical doses.
3. Sustained cell proliferation in the liver of mice. Adenomas develop from these proliferating cell populations by a non-genotoxic process that has not been identified.

Metabolism to the critical metabolites proceeds in mice to an extent that far exceeds that in rats in vivo. Experiments with mouse, rat and human hepatocytes in vitro indicate close similarities of rat and human hepatocytes and the dissimilarity of mouse hepatocytes. The species differences are not absolute, all metabolites being found in rats as well as in mice, but the quantitative differences are of paramount importance in the induction of toxic and neoplastic effects. As thiamethoxam does not induce hepatocellular neoplasms in rats, it is reasonable to conclude that the compound will not do so in exposed humans either.

(ii) Effects on kidney in rats

Subchronic and chronic studies with thiamethoxam in rats indicated the kidney to be a target organ in males. Histopathologically, renal tubular hyaline change was observed at high doses (≥ 250 ppm) in subchronic studies. In the chronic study, increased incidences of regenerative changes in renal tubules (12-month interim sacrifice) and chronic nephropathy (24-month terminal sacrifice) were the main findings at high doses (≥ 500 ppm) in male animals. The morphological appearance of the predominant finding "renal tubular hyaline change" in subchronic studies as well as its exclusive treatment-related induction in the kidney of male rats suggested this effect to be an α_{2u}-globulin nephropathy. The sponsor of the compound also suggested that the chronic nephropathy that was observed and that seemed to be exacerbated by treatment was also dependent upon α_{2u}-globulin. This assumption is unlikely to be correct, as chronic progressive nephropathy is a separate entity and is not male rat specific, although it has a tendency to be more common in males than in females.

A number of studies were conducted with the objective of providing evidence for α_{2u}-globulin involvement in the renal pathology observed, but there seems to have been little consideration of chronic progressive nephropathy as a component of the renal lesions. Chronic progressive nephropathy develops as a naturally occurring disease of rats that is particularly pronounced in males (Elema

& Arends, 1975). Not only is it a degenerative disease, but it is also highly regenerative, in that cell proliferation is increased in many of the affected tubules. It is important to recognize that this disease is not the result of any chemical treatment, and it is necessary to distinguish its regenerative aspects from preneoplasia (atypical hyperplasia) (Hard & Seely, 2005). Incidence and severity can be influenced by a number of factors, which include strain, sex (Elema & Arends, 1975), diet (Rao, 2002) and hormonal status (Baylis, 1994; Tanaka et al., 1995), in addition to chemical treatment. Chronic progressive nephropathy begins at about 2 months of age, when some rats develop basophilic renal tubules with a thickened basement membrane. Progression involves an increase in the number of tubules affected, tubule degeneration and atrophy and an ongoing renal tubule cell proliferation in which mitotic figures may be frequent (Hard & Seely, 2005). By the time that end-stage (grade 8) chronic progressive nephropathy is reached, there are virtually no normal tubules remaining, and death from renal failure is highly probable. In these advanced stages of this disease and in the same areas of the kidney, there is the development of atypical hyperplasia. Atypical hyperplasia is distinguished from foci of regeneration by the absence of thickening of the tubule basement membranes and the observation of more disorganization and crowding, sometimes with stratification, of tubule epithelial cells. In untreated rats, it does not commonly develop to extreme stages within 2-year studies. Exacerbation of chronic progressive nephropathy currently has no identified biochemical events equivalent to those that occur in α_{2u}-globulin nephropathy, and the process is not easily investigated by experiment. However, this process is becoming more widely recognized as a potential mode of action by which chemicals may influence the incidence of kidney tumours in rats (although this was not an issue with thiamethoxam). Clearly, chronic progressive nephropathy does occur in untreated rats, its severity does increase with advancing age, and it is exacerbated by exposure to some chemicals in comparison with untreated rats. Importantly, however, it has no human counterpart (Hard, Johnson & Cohen, 2009) and therefore should not be taken into consideration in human health risk assessment.

In the 90-day dietary study in rats (Bachmann, 1996b), chronic lesions of the renal tubules were already apparent and were at a higher incidence in the higher dose group rats of both sexes. In the 0, 25, 250, 1250, 2500 and 5000 ppm groups, respectively, the incidences in groups of 10 male rats were 0, 1, 3, 6, 10 and 9 and, in female rats, 4, 5, 7, 7, 9 and 10. Pairwise comparison of the incidences would indicate 250 ppm as a NOAEL in male rats, but, as mentioned above, the lesion has no human significance, and so it may be discounted.

With regard to α_{2u}-globulin nephropathy, there have been extensive investigations and a large number of publications pointing to it being male rat specific and—again—of no human significance. It is emphasized once more that, in the context of thiamethoxam, the issue is only of non-neoplastic renal lesions that are treatment related. There is no requirement to formulate a mode of action for tumour induction. The key events considered key to an α_{2u}-globulin-associated nephropathy are as follows:

- increase in the number and size of hyaline droplets in the renal cortex;
- identification of the protein accumulating in hyaline droplets as α_{2u}-globulin;
- reversible binding of thiamethoxam or a metabolite to α_{2u}-globulin;
- a characteristic sequence of histopathological changes in shorter-term studies (necrosis in the tubule epithelium, exfoliation of cells into proximal tubule lumen, granular cast formation and linear mineralization of the papillary tubules);
- sustained regenerative tubule cell proliferation or foci of tubule hyperplasia.

It may not be necessary to consider all of these events, but at least the early ones should be manifest.

The incidences of hyaline change in groups of 10 rats treated with 0, 25, 250, 1250, 2500 or 5000 ppm thiamethoxam, respectively, in the 90-day study were, in males, 1, 0, 4, 8, 10 and 10 and, in females, 0 in all groups. Furthermore, in males, the severity of the hyaline lesion was scored, on average, in these same groups, respectively, as 1.0, 0, 1.3, 1.5, 1.7 and 2.3. Thus, while there are no specific data on number and size of the hyaline droplets, a dose-related severity increase was recorded by the pathologist. It is significant that there was no report of hyaline droplets in the kidneys of female rats in this experiment (Bachmann, 1996b) and, furthermore, that there was no mention of them in mice, although kidneys were examined (Bachmann, 1996a).

Four retrospective immunohistochemical studies (with a specific anti-α_{2u}-globulin antibody) were performed with the intention of identifying and quantifying the renal accumulation of α_{2u}-globulin in male rats. These studies used material from a 28-day study, a 3-month study, and the 12-month and the 24-month terminations of a carcinogenicity and chronic toxicity study in rats with thiamethoxam. The results showed the treatment-related increase in renal α_{2u}-globulin accumulation in male rats (Weber, 2000a,b,c,d), particularly after 28 days and 3 months of treatment. Another important diagnostic tool for altered α_{2u}-globulin in hyaline droplets is the presence of crystalline material, but this is better seen if Mallory-Heidenhain stain is used, which was not the case in these studies. It was concluded that the renal changes observed in male rats treated with high doses of thiamethoxam represent a mild α_{2u}-globulin nephropathy. As such, it is a male rat–specific lesion that has no human relevance and should not be considered in risk assessment of this compound.

3. Toxicology of metabolites

Clothianidin (the *E*-isomer of CGA 322704) was evaluated at this Meeting of the JMPR.

The acute toxicity of CGA 322704 has been investigated in a rat LD_{50} assay and in a bacterial mutation assay. No other investigations were submitted; however, the carcinogenicity of this compound has been evaluated previously by regulatory authorities (Federal Register, 2003). No indication of carcinogenicity was observed in CD-1 mice.

In an acute oral toxicity study conducted according to OECD guideline 401 (1987), groups of five male and five female fasted Wistar HanIbm:WIST strain rats were each administered CGA 322704 technical (batch No. RV-2793/6; purity 99%) suspended in 0.5% methylcellulose solution in aqueous 0.1% polysorbate 80, at a dose volume of 10 ml/kg bw by gavage, as a single oral dose of 0, 1500 or 2000 mg/kg bw. Mortality, clinical signs and body weight changes were recorded. Autopsies were performed on rats at termination on day 14.

There were no deaths during the study. Tremor, piloerection and hunched posture were observed in all rats in the 2000 mg/kg bw group on the day of treatment, but all rats appeared normal by day 1. No remarkable clinical observations were seen among animals in the 0 and 1500 mg/kg bw groups. Body weights and body weight changes of rats in all groups were not affected by treatment. Autopsy examinations did not reveal any treatment-related abnormalities.

The acute oral LD_{50} of CGA 322704 in male and female rats was greater than 2000 mg/kg bw (Cantoreggi, 1998).

There was no evidence of mutagenic activity in a bacterial reverse mutation assay performed over a dose range of up to 5000 µg/plate. The assay was performed in the presence and absence of an exogenous metabolic activating system (S9 mix) prepared in the standard way from male rats treated with Aroclor 1254 and using *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 and *Escherichia coli* WP2uvrA. At the highest dose, the numbers of revertants per plate were reduced in *S. typhimurium* TA102 and *E. coli* WP2uvrA in the presence of S9 mix (Deparade, 1998).

4. Observations in humans

As a new compound, there is little information on exposure of workers to thiamethoxam, and none on exposure of the general population. There have so far been no reported incidents of adverse reactions during the manufacture or formulation of thiamethoxam. Twelve cases of adverse reactions related to thiamethoxam are recorded in the Syngenta adverse effects database up to July 2009. The symptoms reported were rash, pruritus, erythema and dermal irritation, all of which were transitory.

Comments

Biochemical aspects

In rats given [^{14}C]thiamethoxam labelled in either the thiazole or oxadiazine ring as a single oral dose of 0.5 or 100 mg/kg bw, the radiolabel was rapidly and completely absorbed, based on the recoveries in excreta. The time to reach maximum concentrations in plasma was 1–4 hours. Distribution to the tissues was generally non-selective, but resulted in higher concentrations in liver and blood. Tissue residues in rats amounted to 0.3% of the total applied dose after 7 days.

The depletion from the tissues followed first-order kinetics, with half-lives in all tissues in the range of 2–6 hours, independent of the dose level, the site of the label and the sex of the rats. Seven days after oral administration of 0.5 mg/kg bw, the tissue residues were very low. The absorbed material was rapidly excreted from rats, predominantly in the urine. The routes of elimination and the urinary pattern of the rapidly excreted thiamethoxam and its metabolites were complex and independent of the route of administration, the dose level, pretreatment with non-radiolabelled thiamethoxam, the site of the label and the sex of the animals.

In rats, about 20–30% of the dose was biotransformed, whereas 70–80% was eliminated as unchanged thiamethoxam. Within 24 hours, about 90% of the dose was excreted via kidneys with recovery in urine, and about 4% via the bile with recovery in faeces; total faecal recovery was about 5%. In mice, 30–60% of the dose was biotransformed and eliminated mainly in urine, but faecal elimination accounted for about 19%.

Twenty-two metabolites were isolated from the excreta of rats and identified. The quantitatively most important metabolite was CGA 322704 (clothianidin), which accounted for about 10% of the dose. The individual contributions of all the other metabolites did not exceed 1% of the dose. Plasma concentrations of two of these minor metabolites, CGA 330050 and CGA 265307, were 15- to 140-fold higher in mouse than in rat. The major reaction involved in the biotransformation of thiamethoxam is cleavage of the oxadiazine ring to the corresponding nitroguanidine compound. Minor pathways are reduction of the nitroguanidine group, yielding a hydrazine, followed by either acylation or further reduction to a guanidine derivative, hydrolysis of the guanidine group to the corresponding urea, demethylation of the guanidine group and substitution of the chlorine of the thiazole ring by glutathione. Cleavage between the thiazole and oxadiazine ring occurs to a small extent and is mediated by either glutathione or oxidative dealkylation. The glutathione derivatives are prone to further degradation. Both the thiazole and oxadiazine moieties are susceptible to oxidative attack. These minor pathways proceed to small molecules and ultimately, probably, to carbon dioxide. The small molecules generated may enter the general metabolism. Metabolic degradation of thiamethoxam in mice proceeded via the same pathway as in rats. All major and almost all minor metabolites found in rat excreta were also detected in mouse excreta.

In vitro comparisons of thiamethoxam metabolism in mouse, rat and human liver microsomal preparations clearly support the significantly higher generation of CGA 330050 and CGA 265307 in mice compared with rats and additionally demonstrate that human liver microsomes metabolize thiamethoxam in a manner quantitatively similar to and not exceeding that of rats.

Toxicological data

The acute toxicity of thiamethoxam is low, the oral LD_{50} being 1563 mg/kg bw in rats and 871 mg/kg bw in mice. Signs of toxicity at high doses included tonic or clonic convulsions, ptosis and reduced locomotor activity. The acute dermal LD_{50} of thiamethoxam in rats was greater than 2000 mg/kg bw. The 4-hour acute inhalation LC_{50} of thiamethoxam in rats was greater than 3.72 mg/l (the mean achieved concentration). Thiamethoxam was not irritating to rabbit skin or rabbit eyes. Thiamethoxam was not a skin sensitizer in the Magnusson & Kligman maximization test in guinea-pigs.

The short-term oral toxicity of thiamethoxam administered via the diet was evaluated in mice, rats and dogs. These consisted of 4-week range-finding studies in rats followed by 13-week toxicity studies in rats and dogs and a 52-week toxicity study in dogs. A 13-week range-finding study was also conducted in mice.

The liver was identified as a target organ in mice and rats. Treatment for 13 weeks induced liver hypertrophy, inflammatory cell infiltration and pigmentation of hepatocytes and Kupffer cells in both rodent species. In mice, single-cell necrosis and apoptosis occurred in parallel with these alterations.

The kidney was identified as a target organ in rats, but not in mice or dogs. Both sexes were affected, but there was a difference between the sexes in both morphology and sensitivity. In males, nephrotoxicity was characterized by tubular epithelial hyaline droplet accumulation, acute and chronic tubule lesions, basophilic proliferation and cast formation. The pattern of effects in male rat kidneys resembled α_{2u}-globulin nephropathy, which is generally accepted to be a phenomenon exclusively found in males of some strains of rats. Immunohistochemical studies on kidneys from male rats exposed to thiamethoxam for 28 days or 3, 12 or 24 months with a specific anti-α_{2u}-globulin antibody identified treatment-related increases in renal α_{2u}-globulin accumulation, particularly after 28 days and 3 months of treatment. It was concluded that the renal changes observed in male rats treated with thiamethoxam represent a mild α_{2u}-globulin nephropathy, which is male rat specific and has no human relevance. Renal lesions in females were confined to an increased incidence of chronic tubular lesions and an increase in the severity of nephrocalcinosis. Other observations, particularly basophilic proliferation, tended to be increased in all groups of females at 28 days and may represent the beginnings of chronic progressive nephropathy. However, such observations were not repeated after 3 (or 12) months of exposure. Therefore, the NOAELs of human relevance in rats were derived on the basis of effects in organs other than male kidney in the repeated-dose toxicity studies after 28 days and 3 months of treatment as well as in the 2-year study.

Other target organs and changes in rats were fatty changes in the adrenal cortex, enhanced haemosiderosis or extramedullary haematopoiesis in the spleen and follicular epithelial hypertrophy in the thyroid gland. Thymic and splenic atrophy in dogs and alterations suggestive of delayed maturation of the gonads in dogs and female mice occurred at doses causing substantial growth retardation.

The NOAELs derived from short-term studies in which thiamethoxam was administered orally were as follows:

- The NOAEL in the 90-day dietary study in mice with thiamethoxam was 100 ppm (equal to 14.3 mg/kg bw per day), based on raised platelet counts at 1250 ppm (equal to 176 mg/kg bw per day) in females. Minimal lymphocytic infiltration and hepatocyte hypertrophy were observed in males at 100 and 1250 ppm; in the absence of any other hepatic changes, these were considered an adaptive response or an early sign of mouse-specific hepatotoxicity.
- The NOAEL in the 28-day dietary study in rats was 100 ppm (equal to 8.0 mg/kg bw per day), based on increased plasma cholesterol concentrations at a dose level of 1000 ppm (equal to 81.7 mg/kg bw per day). The male rat–specific kidney effects have no human relevance, and therefore they are not considered for the NOAEL.

- The NOAEL in the 90-day dietary study in rats was 250 ppm (equal to 17.6 mg/kg bw per day in males), based on reduced body weight gain and histological findings in the adrenals at 1250 ppm (equal to 84.9 mg/kg bw per day). Observation of hyaline droplet accumulation in the kidneys of male rats was considered indicative of α_{2u}-globulin involvement, which is male rat specific and has no human significance.
- The NOAEL in the 90-day oral toxicity study in dogs was 250 ppm (equal to 8.23 mg/kg bw per day), based on prolonged thromboplastin times in both sexes at 1000 ppm (equal to 32 mg/kg bw per day).
- The NOAEL in the 52-week oral toxicity study in dogs was 750 ppm (equal to 21 mg/kg bw per day), based on prolonged thromboplastin times and reductions in testis weights at 1500 ppm (equal to 42 mg/kg bw per day).

Long-term toxicity and carcinogenicity studies were performed in mice and rats. The main target organs were the liver in mice and female rats and the kidneys in male rats. In rats, the principal findings were increased incidences of renal tubule regenerative lesions, which were considered to represent the sequelae of the rat-specific nephropathies observed in short-term studies. Minor and morphologically different changes occurred in the spleen of both rats and mice.

The NOAEL in the 78-week dietary study in mice was 20 ppm (equal to 2.63 mg/kg bw per day), based on hepatotoxic effects (i.e. increased liver weights, hepatocellular hypertrophy, pigment deposition, inflammatory cell infiltration and single-cell necrosis) at 500 ppm (equal to 63.8 mg/kg bw per day). Thiamethoxam was tumorigenic in mice and induced hepatocellular adenomas in male and female mice at a dose level of 500 ppm and hepatocellular adenocarcinomas in male mice at 2500 ppm (equal to 354 mg/kg bw per day).

Special studies on thiamethoxam (the *EZ*-isomer mixture, as used in all toxicity studies) were performed to investigate the etiology of adenoma and adenocarcinoma formation in mouse liver during an 18-month oncogenicity study. The hypothesis investigated was that the mouse specificity in this response is due to a very large species difference in metabolism of thiamethoxam, as demonstrated by 15-fold and 140-fold higher plasma concentrations of CGA 330050 and CGA 265307, respectively, in mice than in rats following 10 weeks of dosing with thiamethoxam. This large difference is supported by in vitro comparison of thiamethoxam metabolism by microsomal preparations from mouse, rat and human liver. Although these metabolites also occur with rat microsomes, their concentrations are very much lower than in mice. The data also suggested that humans were likely to be even less susceptible than rats to the hepatic effects of thiamethoxam. The mode of action proposed for development of these tumours is based on the hepatotoxicity of the metabolite CGA 330050 in particular, with CGA 265307 exacerbating its effect, and the subsequent sustained cell proliferation of mouse hepatocytes, leading to the development of a higher incidence of hepatocellular tumours. Not all elements for a mode of action have been identified, but the available data support the contention of a low risk to humans with regard to both hepatotoxicity and carcinogenicity and the absence of any genotoxic involvement. An alternative metabolic pathway to CGA 265307 in both mice and rats is via CGA 322704. The *E*-isomer of CGA 322704 was evaluated at the present Meeting, and it was concluded that it is not carcinogenic in mice or rats.

The NOAEL in the 78-week dietary study in mice for non-hepatic effects is 1250 ppm (equal to 162 mg/kg bw per day), based on reductions in body weight and effects on spleen and stomach at 2500 ppm (equal to 354 mg/kg bw per day).

The NOAEL in the 104-week dietary study in rats was 1000 ppm (equal to 50.3 mg/kg bw per day in females), based on foci of cellular alteration in the liver and increased severity of splenic haemosiderosis at 3000 ppm (equal to 155 mg/kg bw per day). Increased incidences of renal chronic tubular lesions and basophilic proliferation were observed exclusively in male rats at 500 ppm (equal

to 21.0 mg/kg bw per day). These renal lesions were considered to represent the outcome of α_{2u}-globulin-mediated nephropathy, which is widely acknowledged as male rat specific and not relevant in human risk assessment, and therefore they were not used to identify the NOAEL.

Thiamethoxam was tested for genotoxicity and mutagenicity in an adequate range of assays, both in vitro and in vivo. In none of these assays was there any evidence of genotoxic or mutagenic potential.

The Meeting concluded that thiamethoxam is unlikely to be genotoxic.

On the basis of the absence of genotoxicity in vivo, the absence of carcinogenicity in rats and the mode of action by which liver tumours arise in mice, the Meeting concluded that thiamethoxam is unlikely to pose a carcinogenic risk at human dietary exposure levels.

Hyaline change and casts in renal tubules were observed in male rats in the multigeneration studies at 1000 ppm (equal to 45.6 mg/kg bw per day). This observation has no human relevance. Therefore, the relevant NOAEL for parental toxicity is 1000 ppm (equal to 45.6 mg/kg bw per day), based on significantly reduced body weight gain at 2500 ppm (equal to 117.6 mg/kg bw per day in F_0 generation males). The overall NOAEL for reproductive toxicity in the multigeneration studies in rats was 1000 ppm (equal to 74.8 mg/kg bw per day for F_1 males), based on minimal testicular germ cell loss or disorganization, with or without Sertoli cell vacuolation (and unaccompanied by any reduction in epididymal sperm numbers), at 2500 ppm (equal to 191.5 mg/kg bw per day). These effects were not observed in the first study, a difference that could be attributed to a refinement of the methods used for sperm observations between the first and second studies. The overall NOAEL for offspring was 30 ppm (equal to 1.4 mg/kg bw per day for the males), based on marginal reductions in body weight gains of F_{2a} and F_{2b} pups during lactation at 1000 ppm (equal to 45.6 mg/kg bw per day for males) in the first of the two studies.

The NOAEL for maternal toxicity in the developmental toxicity study in rats was 30 mg/kg bw per day, based on slightly decreased body weight gain in dams, providing a LOAEL of 200 mg/kg bw per day. The NOAEL for fetotoxicity was 200 mg/kg bw per day, based on mild reduction in mean fetal body weight at 750 mg/kg bw per day. Further evidence of fetotoxicity at this dose was increased incidences of skeletal anomalies (irregular or absent ossification of the occipital bone) and skeletal variants (poor ossification of sternebra 5, shortened 13th rib and non-ossification of metatarsal 1).

The NOAEL for maternal toxicity in the developmental toxicity study in rabbits was 15 mg/kg bw per day, based on reduction in body weight gain and feed consumption during the treatment period in dams at 50 mg/kg bw per day. The NOAEL for fetotoxicity in rabbits was 50 mg/kg bw per day, based on increased postimplantation loss and reduction in fetal body weights at 150 mg/kg bw per day. Further evidence of fetotoxicity at this dose was increased incidence of delayed and absent ossification as well as an increased incidence of fused sternebrae in fetuses.

The Meeting concluded that thiamethoxam can cause fetotoxicity and skeletal anomalies (malformations and variants), but only at maternally toxic doses.

An acute neurotoxicity study, a 13-week neurotoxicity study and a developmental neurotoxicity study were conducted in rats. A comprehensive set of neurotoxicity end-points was investigated in these studies, including an evaluation of potential to induce neurobehavioural or neuromorphological changes. The studies did not show any specific neurotoxicity after repeated exposure of adult rats or any specific developmental neurotoxicity in the offspring, including at doses causing maternal toxicity. Acute administration of thiamethoxam at dose levels approaching the LD_{50} produces a range of transient neurobehavioural effects, including tonic or clonic convulsions, ptosis and reduced locomotor activity. The NOAEL in the single-dose neurotoxicity study in rats was 100 mg/kg bw, based on transient behavioural changes at 500 mg/kg bw. The NOAEL for systemic toxicity and neurotoxicity in the 13-week neurotoxicity study was 1500 ppm (equal to 95.4 mg/kg bw per day) in males and 3000 ppm (equal to 216.4 mg/kg bw per day) in females, the highest doses tested, based on the

absence of treatment-related effects at these doses. The NOAEL for systemic toxicity in a study of developmental neurotoxicity was 400 ppm (equal to 34.5 mg/kg bw per day), based on decreased body weight gain and feed consumption in dams throughout gestation and postpartum, as well as reduced birth weight, reduced pup body weight gain, some evidence of delayed preputial separation and small changes in brain morphometry, but without any quantitative histological or behavioural changes, at 4000 ppm (equal to 298.7 mg/kg bw per day). The NOAEL for developmental neurotoxicity was 4000 ppm (equal to 298.7 mg/kg bw per day), the highest dose tested.

The Meeting concluded that thiamethoxam is not a neurotoxin in mammals at the tested dose levels, although it is a member of the neonicotinoid chemical class, the biological effects of which in target species are mediated primarily by an interaction with nicotinic acetylcholine receptor sites.

No information on medical surveillance or poisoning incidents was available.

The Meeting concluded that the existing database on thiamethoxam was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.08 mg/kg bw on the basis of a NOAEL of 250 ppm (equal to 8.23 mg/kg bw per day) in a 90-day study of toxicity in dogs, based on prolonged thromboplastin time. A safety factor of 100 was applied. This ADI is protective of the hepatotoxic and hepatocarcinogenic effects observed in mice, which were not observed in rats because of marked species differences in metabolism. It is also protective of the marginally toxic effects observed in a multigeneration study in rats at 46 mg/kg bw per day.

A number of blood chemistry and haematology parameters in dogs were considered as a basis for the ADI, but the only consistently altered parameter was measures of blood coagulation. Other end-points that received consideration included feed consumption reduction in a gavage study of developmental toxicity in rabbits. Renal changes observed in rats arose by processes not relevant for risk assessment at human dietary exposure levels.

An acute reference dose (ARfD) of 1 mg/kg bw was established on the basis of a NOAEL of 100 mg/kg bw in a single-dose study of neurotoxicity in rats. A safety factor of 100 was applied. The transient functional changes in rats appeared to be mild signs of overt toxicity rather than neurotoxicity. The neurotoxicity study was supported by a single-dose study of toxicity in mice, in which clinical signs of toxicity were observed at 500 mg/kg bw, the lowest dose tested.

The metabolite of thiamethoxam, CGA 322704 (clothianidin), was evaluated separately by the present Meeting.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day range-finding study of toxicity	Toxicity	100 ppm, equal to 14.3 mg/kg bw per day	1250 ppm, equal to 176 mg/kg bw per day
	Eighteen-month study of toxicity and carcinogenicity	Toxicity[a]	20 ppm, equal to 2.63 mg/kg bw per day	500 ppm, equal to 63.8 mg/kg bw per day
		Carcinogenicity[b]	20 ppm, equal to 2.63 mg/kg bw per day	500 ppm, equal to 63.8 mg/kg bw per day
Rat	Single-dose study of neurotoxicity	Toxicity	100 mg/kg bw	500 mg/kg bw

Species	Study	Effect	NOAEL	LOAEL
	Ninety-day study of toxicity	Toxicity[c]	250 ppm, equal to 17.6 mg/kg bw per day	1250 ppm, equal to 84.9 mg/kg bw per day
	Twenty-four-month studies of toxicity and carcinogenicity	Toxicity[c]	1000 ppm, equal to 50.3 mg/kg bw per day	3000 ppm, equal to 155 mg/kg bw per day
		Carcinogenicity	3000 ppm, equal to 155 mg/kg bw per day[d]	—
	Two-generation study of reproductive toxicity (1)	Reproductive toxicity	2500 ppm, equal to 117.6 mg/kg bw per day[d]	—
		Parental toxicity[c]	1000 ppm, equal to 45.6 mg/kg bw per day	2500 ppm, equal to 117.6 mg/kg bw per day
		Offspring toxicity	30 ppm, equal to 1.4 mg/kg bw per day	1000 ppm, equal to 45.6 mg/kg bw per day[e]
	Two-generation study of reproductive toxicity (2)	Reproductive toxicity	1000 ppm, equal to 74.8 mg/kg bw per day	2500 ppm, equal to 191.5 mg/kg bw per day
		Parental toxicity[c]	1000 ppm, equal to 61.7 mg/kg bw per day	2500 ppm, equal to 155.6 mg/kg bw per day
		Offspring toxicity	1000 ppm, equal to 74.8 mg/kg bw per day	2500 ppm, equal to 191.5 mg/kg bw per day
	Developmental toxicity study	Maternal toxicity	30 mg/kg bw per day	200 mg/kg bw per day
		Embryo and fetal toxicity	200 mg/kg bw per day	750 mg/kg bw per day
Rabbit	Developmental toxicity study	Maternal toxicity	15 mg/kg bw per day	50 mg/kg bw per day
		Embryo and fetal toxicity	50 mg/kg bw per day	150 mg/kg bw per day
Dog	Ninety-day study of toxicity	Toxicity	250 ppm, equal to 8.23 mg/kg bw per day	1000 ppm, equal to 32 mg/kg bw per day

[a] Mouse particularly susceptible to hepatotoxicity, based on the mode of action of thiamethoxam.

[b] No carcinogenicity relevant to humans based on mode of action considerations of tumorigenic effects in mice.

[c] Toxicity relevant to humans, not including nephrotoxicity specific for male rats.

[d] Highest dose tested.

[e] Marginal LOAEL.

Estimate of acceptable daily intake for humans

0–0.08 mg/kg bw

Estimate of acute reference dose

1 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to thiamethoxam

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and extensive, > 90%
Distribution	Distributed throughout the body; higher concentrations in liver and blood

Potential for accumulation	None
Rate and extent of excretion	High, > 90% within 24 h
Metabolism in animals	22 metabolites identified in rats
Toxicologically significant compounds (animals, plants and environment)	Parent; CGA 330050; CGA 322704 (clothianidin)

Acute toxicity

Rat, LD_{50}, oral	1563 mg/kg bw
Mouse, LD_{50}, oral	871 mg/kg bw
Rat, LC_{50}, inhalation	> 3.7 mg/l (4 h)
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman test)

Short-term studies of toxicity

Target/critical effect	Coagulation in dogs
Lowest relevant oral NOAEL	8.23 mg/kg bw per day (3-month study in dogs)
Lowest relevant dermal NOAEL	60 mg/kg bw per day (liver, 4-week study in rats)
Lowest relevant inhalation NOAEC	No data

Long-term studies of toxicity and carcinogenicity

Target/critical effect	Liver, spleen, stomach (mice)
Lowest relevant NOAEL	2.63 mg/kg bw per day (18-month study in mice)
Carcinogenicity	Carcinogenic in mice, but unlikely to pose a risk at human dietary exposure levels, based on the proposed mode of action

Genotoxicity

	Not genotoxic

Reproductive toxicity

Reproductive target/critical effect	Preweaning weight gain
Lowest relevant reproductive NOAEL	74.8 mg/kg bw per day (rat)
Developmental target/critical effect	Rat: Not teratogenic; reduced live birth weight, delayed ossifications Rabbit: Reduced pup body weight, delayed ossifications and increased incidences of skeletal abnormalities
Lowest relevant developmental NOAEL	50 mg/kg bw per day (rabbit)

Neurotoxicity/delayed neurotoxicity

	No signs of neurotoxicity

Other toxicological studies

	Mechanistic studies relevant to hepatotoxicity and hepatocarcinogenicity in mice and renal toxicity in rats

Medical data

	No reports of toxicity in workers exposed during manufacture or use

Summary

	Value	Study	Safety factor
ADI	0–0.08 mg/kg bw	Ninety-day study of toxicity in dogs	100
ARfD	1 mg/kg bw	Neurotoxicity study in rats supported by a single-dose toxicity study in mice	100

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Appendix 1: Application of the IPCS conceptual framework for cancer risk assessment

(IPCS framework for analysing the relevance of a cancer mode of action for humans)

A1. Hepatocarcinogenesis in mice

A1.1 Introduction

All information relating to a mode of action has been summarized in this document, so reference will be made to the relevant paragraphs rather than redescribing the data in this section. The analytical approach applied to the postulated mode of action is based on the methods developed by the International Programme on Chemical Safety (IPCS) (Sonich-Mullin et al., 2001; Boobis et al., 2006). The incidences of any hepatic tumours in mice after 18 months of exposure to thiamethoxam at 0, 5, 20, 500, 1250 or 2500 ppm were, in males, 11, 7, 10, 19, 22 and 44 in groups of 50 and, in females, 0, 0, 0, 5, 9 and 29 in groups of 50 (Bachmann, 1998b). Most were hepatocellular tumours. The incidences of primary hepatocellular adenomas in these same dose groups of 50 mice of each sex were, respectively, for males, 9, 5, 8, 17, 21 and 39; and, for females, 0, 0, 0, 5, 8 and 28. In addition, hepatocellular carcinomas were significantly increased in males of the highest-dose group, with incidences of 3, 3, 2, 4, 4 and 16, whereas in females, there were a few hepatocellular carcinomas in the two highest dose groups only: 0, 0, 0, 0, 2 and 3. Thus, any explanation for the induction of these tumours should account for their occurrence in both male and female Tif:MAGf(SPF) mice (but particularly in male mice) from a dietary exposure concentration of 500 ppm. Some other neoplasms were also occasionally observed, but they tended to show a random distribution with dose. At the same dose level and higher, there were also a number of non-neoplastic lesions in the liver that were statistically significantly increased. These included pigment deposition (lipofuscin and haemosiderin), foci of cellular alteration, hepatocellular hypertrophy, single-cell necrosis and inflammatory cell infiltration.

There was no evidence for hepatocellular adenoma or carcinoma induction in rats receiving thiamethoxam in their diets at 0, 10, 30, 500 or 1500 ppm (males) or 0, 10, 30, 1000 or 3000 ppm

(females) for 2 years (Bachmann, 1998a). The numbers of hepatocellular adenomas in these groups (of 49 or 50 of each sex) were, for males, 0, 1, 0, 1 and 1; and, for females, 2, 0, 2 and 2. The numbers of hepatocellular adenocarcinomas were also sporadic, being, for males, 0, 0, 1, 1 and 0; and, for females, 0, 0, 1, 0 and 0. Thus, any mode of action should also account for the species specificity of the response. There were, furthermore, no treatment-related tumour findings in any other organ, and, in liver, there was no dose-related increase in non-neoplastic lesions in rats, except for an increase in clear cell foci, which are probably indicative of excess glycogen accumulation, in females, and only at a dose level of 3000 ppm.

The genetic toxicity database for thiamethoxam indicates that it is not a genetic toxin; therefore, modes of action were investigated with, most probably, a dose below which tumours would not be expected to occur (i.e. a "threshold").

A1.2 Postulated mode of action (theory of the case)

Thiamethoxam is metabolized to a metabolite (CGA 330050) in mice that is hepatotoxic, and its action is exacerbated by a subsequent metabolite (CGA 265307) that is a competitive inhibitor in the activity of nitric oxide synthase, which plays a role in ameliorating the effects of hepatotoxic substances (Green, 2003e). This pathway occurs, but is of much less importance in rats, in which CGA 265307 is generated via CGA 322704. This second pathway also occurs in mice. The induced hepatotoxicity is followed by regenerative hepatocellular hypertrophy and proliferation. Neoplasia develops from foci of these altered cells.

A1.3 Key events

1. Thiamethoxam is metabolized via two major pathways, one of which occurs in both mice and rats (→ CGA 322704 → CGA 265307), whereas the other appears less likely to occur at expected exposure levels, on kinetic grounds, except in mice (→ CGA 330050 → CGA 265307). The relative rate of metabolism of thiamethoxam in mice versus rats is about 54:1 for the first pathway and about 87:1 for the second pathway.
2. The actively toxic metabolite, CGA 330050, induces the same changes in mouse liver as thiamethoxam itself. CGA 265307 does not seem to be toxic, but it is a competitive inhibitor with arginine, the natural substrate of nitric oxide synthase. Nitric oxide modulates the adverse effects of TNFα released from endothelial cells during chemical-induced hepatotoxicity. Thus, hepatotoxicity is enhanced by CGA 265307. In the absence of hepatotoxicity, there would be no effect of this metabolite. The expression of hepatotoxicity during weeks 1–10 includes general perturbation of hepatocyte metabolism, such as lowering of plasma cholesterol, induction of cytochrome P450, decreased protein synthesis, and glycogen and lipid accumulation.
3. During weeks 8–50, there is development of hepatocellular hypertrophy, lymphocytic infiltration and pigmentation of hepatocytes and Kupffer cells, single-cell necrosis and apoptosis, and blood chemistry changes.
4. In the latter part of this period (weeks 20–50), there is a sustained increase in cell replication rates among centrilobular hepatocytes.
5. Hepatocellular neoplasms begin to develop (even in controls) in weeks 40–50, but higher incidences in treated groups are observed only at the end of the 18-month experiment.

A1.4 Concordance of dose–response relationships

All of the features considered to contribute to the key events occurred in dose–response relationships; however, some of the studies contributing this information included multiple sampling

times, and therefore it is difficult to separate concentration and time effects. What is critical to the proposed mode of action, however, is that the key events should be observed at 500 ppm or lower.

After 14 days of treatment with a 2500 ppm diet, there were small, non-significant increases in absolute and relative liver weights of male and female mice in one study (Trendelenburg, 1998). In contrast, in another study (Weber, 1998), there were no statistically significant liver weight increases at 2500 ppm after 7, 13, 27 or 59 days in male mice, whereas there were statistically significant increases after 7 and 27 days in female mice at 2500 ppm, but not lower doses. In the 18-month study, relative (to body weight) liver weights were insignificantly increased after 35 weeks only in females at 2500 ppm, but by week 79, the relative liver weights were significantly increased in females from 500 ppm and in males from 1250 ppm. Absolute liver weights were significantly increased in both male and female mice at 79 weeks from 1250 ppm (Bachmann, 1998b).

Trendelenburg (1998) also found that after 14 days of treatment with a 2500 ppm diet, there were small, non-significant increases in hepatic cytochrome P450 in male and female mice at 2500 ppm, but not at 500 ppm. 7-Ethoxyresorufin *O*-deethylase (EROD), PROD and BROD activities were all significantly increased in female mice at 2500 and 500 ppm, and PROD activity was also significantly increased in female mice at 100 ppm. In male mice, however, PROD and BROD activities were significantly increased only at 2500 ppm. Other activities of hepatic enzymes were significantly altered only at 2500 ppm (e.g. testosterone hydroxylations or oxidations, particularly in females, and, in males and females, uridine diphosphate-glucuronosyltransferase, glutathione *S*-transferase and epoxide hydrolase activities) (Trendelenburg, 1998).

In a study of BrdU labelling in the livers of mice, the labelling indices were increased in male mice at 2500 ppm after 3, 7 and 59 days and at 500 ppm at day 13. There was no effect in males at 100 ppm. In females, there were significant increases in labelling index at days 7 and 59 (but not at day 3 or 27) in the 2500 ppm group, but there was no effect at 500 ppm at any time (Weber, 1998). BrdU labelling of liver cells was also measured after treatment of mice with 0, 50, 200, 500, 1250, 2500 or 5000 ppm for up to 50 weeks (Weber, 2003). In this study, the lowest dose at which BrdU labelling was significantly increased was 1250 ppm after 40 weeks, but not at shorter times or at 50 weeks. However, labelling was measured over the entire liver lobules in this study. When the observations were restricted to those regions in which necrosis and apoptosis were concentrated, a significant increase in cell replication rates was also observed at 500 ppm (Soames, 2003).

Apoptotic activity was demonstrated using the TUNEL staining method. TUNEL-positive objects were significantly increased at all doses from 50 ppm at 40 weeks and from 500 ppm at 30 weeks. The increase was dose related from 500 ppm (Weber, 2003).

In the same 59-day study mentioned above (Weber, 1998), there were clearly dose-related increases in the incidence of enlarged centrilobular and midzonal hepatocytes with increased amounts of glycogen, lipids and, to a lesser extent, smooth endoplasmic reticulum. These changes were observed in males across all doses and with an increased severity at day 59, and, in females, there was an increase in incidence and severity at 2500 ppm. Pigment granules were observed in centrilobular hepatocytes and in Kupffer cells in male and female mice at 2500 ppm after 59 days. Dose-related increases in incidence and severity of fatty change were observed in the later multiple sampling time study with doses up to 5000 ppm (Weber, 2003; see above); however, the hepatocytic vacuolation and glycogen deposition were not observed.

Following 7 days of treatment, plasma cholesterol concentrations were significantly reduced in adult male mice at doses of 500 ppm (the lowest tested) and higher (Green, 2003c) (in contrast, they tended to be raised in rats of 1- and 3-month studies and were unaffected in dogs). Serial blood chemistry measurements in male mice over 50 weeks indicated increased AST and ALT activities at 2500 and 5000 ppm at specific sampling times from 10 to 50 weeks, while combining all sampling times resulted in increases of both liver toxicity indicator enzymes from 1250 ppm. Microscopic changes (mainly fatty change) occurred from 500 ppm in this experiment (Weber, 2003).

Combining all sample time points showed a dose-related increase in apoptosis in the liver from 500 ppm, whereas the BrdU labelling index for all time points combined increased from 1250 ppm (Weber, 2003).

In the 18-month study, relative (to body weight) liver weights were insignificantly increased after 35 weeks only in females at 2500 ppm, but by week 79, the relative liver weights were significantly increased in females from 500 ppm and in males from 1250 ppm. Absolute liver weights were significantly increased in both male and female mice at 79 weeks from 1250 ppm. Increased microscopically observable hepatotoxicity was recorded in males and females of the 2500 ppm group at week 35 and from 500 ppm at week 79. Hepatocellular neoplasms were increased in a dose-related manner in male and female mice at week 79 from 500 ppm.

A1.5 Temporal association

In the mouse strain and metabolite comparison study with observations in which Tif:MAGf and CD-1 mice were fed thiamethoxam (2500 ppm), CGA 322704 (2000 ppm) or CGA 265307 (500 ppm) for 1, 10 and 20 weeks (Green, 2003d; Noakes, 2003a), few effects were noted after 1 week, whereas they did occur after 10 and/or 20 weeks. Thiamethoxam-associated hepatic effects observed early in treatment of both Tif:MAGf and CD-1 mice were increases in:

- hepatocellular hypertrophy incidence and/or severity after 10 and 20 weeks;
- hepatocellular necrosis incidence and/or severity after 10 and 20 weeks;
- inflammatory cell infiltration incidence and/or severity after 20 weeks (but not after 10 weeks);
- hepatocellular apoptosis incidence and/or severity after 10 and 20 weeks in CD-1 mice, but only after 10 weeks in TifMAGf mice;
- pigmentation incidence and/or severity after 10 and 20 weeks, but only in CD-1 mice;
- cell proliferation (BrdU immunohistochemistry) in TifMAGf mice at week 20 and in CD-1 mice at weeks 10 and 20.

Apoptosis was not observed in either strain in the Noakes (2003a) study, and none of these effects was observed following parallel treatment with either CGA 322704 or CGA 265307. The use of two strains demonstrated that the effects were not strain specific, but that CD-1 mice seemed to be more susceptible than the TifMAGf mice that were used in the carcinogenicity study with thiamethoxam.

Sustained increases in cell proliferation (240%), measured specifically in centrilobular hepatocytes (the region of the liver in which single-cell necrosis and apoptosis were concentrated), were observed in male mice at 500 ppm at 40 weeks.

A1.6 Strength, consistency and specificity of association of tumour response with key events

The relative rates of thiamethoxam metabolism in mice versus rats in vitro are about 54:1 for thiamethoxam → CGA 322704 → CGA 265307 and about 87:1 for thiamethoxam → CGA 330050 → CGA 265307. CGA 322704 (clothianidin) is not carcinogenic, and, although CGA 330050 has not been tested for carcinogenicity, it is known to be as hepatotoxic as thiamethoxam itself. Thus, the kinetics favours the generation of much larger quantities of the toxic metabolite in mice than in rats, consistent with the species difference in the hepatotoxicity and carcinogenicity of thiamethoxam.

A comparison of the hepatotoxicity of thiamethoxam metabolites CGA 322704, CGA 265307 and CGA 330050 administered in the diet for up to 20 weeks to TifMAGf mice (Green, 2003d) demonstrated that only CGA 330050 induced the same changes as thiamethoxam; no changes were observed following CGA 322704 or CGA 265307 administration at any time. Plasma cholesterol was

reduced after 1 and 10 weeks of CGA 330050 treatment at 500 and 1000 ppm; hepatocellular hypertrophy, single-cell necrosis/apoptosis and cell replication rates were increased after 10 weeks, and plasma AST and ALT activities were increased at weeks 10 and 20. In contrast, feeding Tif:RAIf rats with CGA 330050 at 500 or 1000 ppm for 1 week reduced plasma cholesterol levels and the plasma aminotransferase activities.

A1.7 Biological plausibility and coherence

The mode of action proposed for the induction of liver tumours in mice but not rats is largely supported by the available data, but there appear to be some inconsistencies in the details of the dose–response relationships. Thus, evidence of hepatotoxicity involving all dose levels at which tumour incidences were significantly increased was a relatively late-occurring observation, whereas it was frequently necessary to combine all sample time results in order to demonstrate a significant effect considered to be key to the proposed mode of action.

A1.8 Other modes of action

A number of properties of thiamethoxam have been identified, some of which appear to be time- and dose-related key events that characterize the emergence of liver cancer in thiamethoxam-treated mice in a manner that resembles that of the cholesterol-lowering drugs, statins, which also induce liver tumours in mice. Thus, it might be postulated that thiamethoxam has a statin-like mode of action; however, the studies conducted so far provide no evidence for a statin-like inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, this being the rate-limiting enzyme in cholesterol biosynthesis. Thus, the cholesterol-lowering effect seen in a number of thiamethoxam studies in mice might have no mechanistic significance for neoplasia.

Furthermore, the phenobarbital-like inductions noted in liver phase I and phase II enzymes may be misleading, as the effects on these enzymes are small and probably insufficient to account for the emergence of liver tumours (Trendelenburg, 1998).

Finally, there is no evidence for any genotoxic or mutagenic action of thiamethoxam or its metabolites; therefore, there is no basis for proposing a genotoxic mode of action.

A1.9 Uncertainties, inconsistencies and data gaps

The weight of the evidence suggests that the mode of action for thiamethoxam-induced liver tumours in mice is based on the hepatotoxicity of its metabolite CGA 330050, the effects of which are increased by CGA 265307. The other major metabolic pathway produces the non-toxic CGA 322704, from which CGA 265307 is produced. This reasoning seems persuasive, but there are some weaknesses in the dose–response relationships for hepatotoxicity. They are not as strong as they could be, given that the increase in tumour incidence is observed at 78 weeks from a dose level of 500 ppm. Certain key events occur rather late on the time scale, raising the question as to whether they are truly part of the neoplastic process. It is also questionable whether the procedure of combining data from all sampling times is a legitimate way of demonstrating that there are dose-related responses during preneoplasia consistent with the neoplastic outcome.

A1.10 Assessment of postulated mode of action

A case has been proposed to explain the induction of hepatocellular neoplasms in mice exposed to thiamethoxam. Some weaknesses have been described above, among them being the

rather unusual statistical technique of combining data from all observation times in order to demonstrate a significant effect in properties and the late appearance of others (at all relevant dose levels) considered to be key events in the mice. The temporal and dose-related occurrence of key events in mice is summarized in Figure A1 and Table A1.

Human relevance analysis

1. Is the weight of evidence sufficient to establish a mode of action in animals?

There are many elements in the toxicology of thiamethoxam that are consistent with a mode of action based on large quantitative differences in metabolism between mice and rats and the hepatotoxicity of a particular metabolite produced in mice. If it is accepted that hepatotoxicity is the basis for the neoplasia, then a reasonable case has been made for this mode of action in rodents.

2. Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

Limited studies (Green, 2002) comparing the metabolism of thiamethoxam in vitro by liver microsomal preparations indicate that human microsomes are capable of producing both CGA 330050 and CGA 265307. Therefore, the human relevance of the proposed mode of action cannot be ruled out on qualitative differences in the key events.

3. Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

Limited human in vitro metabolism studies (Green, 2002) suggest that humans are more similar to rats or even less capable than rats, compared with mice, in producing the active metabolite CGA 330050. Thus, the relative rates of thiamethoxam metabolism in mice versus rats in vitro (based on maximum rate $[V_{max}]/K_m$ ratios) are about 54:1 for thiamethoxam → CGA 322704 → CGA 265307 and about 87:1 for thiamethoxam → CGA 330050 → CGA 265307. Similar comparisons of mouse versus human show that the overall relative metabolic rate ratios for these pathways are 371:1 and 238:1, respectively. The relative rate ratios for the critical conversion of thiamethoxam to CGA 330050 are mouse:rat 10.6:1 and mouse:human 25.6:1. Rats do not develop tumours following treatment with thiamethoxam. Thus, mice appear to be particularly sensitive to this mode of action. Because of the threshold nature of the mode of action and the unique sensitivity of mice, it is concluded that humans are unlikely to be at risk for developing tumours following exposures to thiamethoxam.

4. Statement of confidence, analysis and implications

There are, as described above, some weaknesses in the proposed mode of action, in that there may be perceived to be differences in the dose levels at which key events are observed and those at which tumours emerge; however, the differences are not substantial, and it may be that they are no more than manifestations of the differences occurring between experiments conducted at different times and under different conditions. The analysis suggests that there is not any absolute difference between mice on one hand and rats and humans on the other. The crucial difference is quantitative, and the dose levels required to produce liver toxicity and cancer by thiamethoxam exposure would not be reached at human dietary levels of exposure, indeed, in any of these species, but particularly in humans. Furthermore, the absence of any evidence for genotoxicity of thiamethoxam indicates that a non-linear dose–response model for tumours applies to mice.

Figure A1. The dose–response relationship for hepatocellular changes over 10–50 weeks (a–d) compared with the liver tumour incidence over 80 weeks (e)

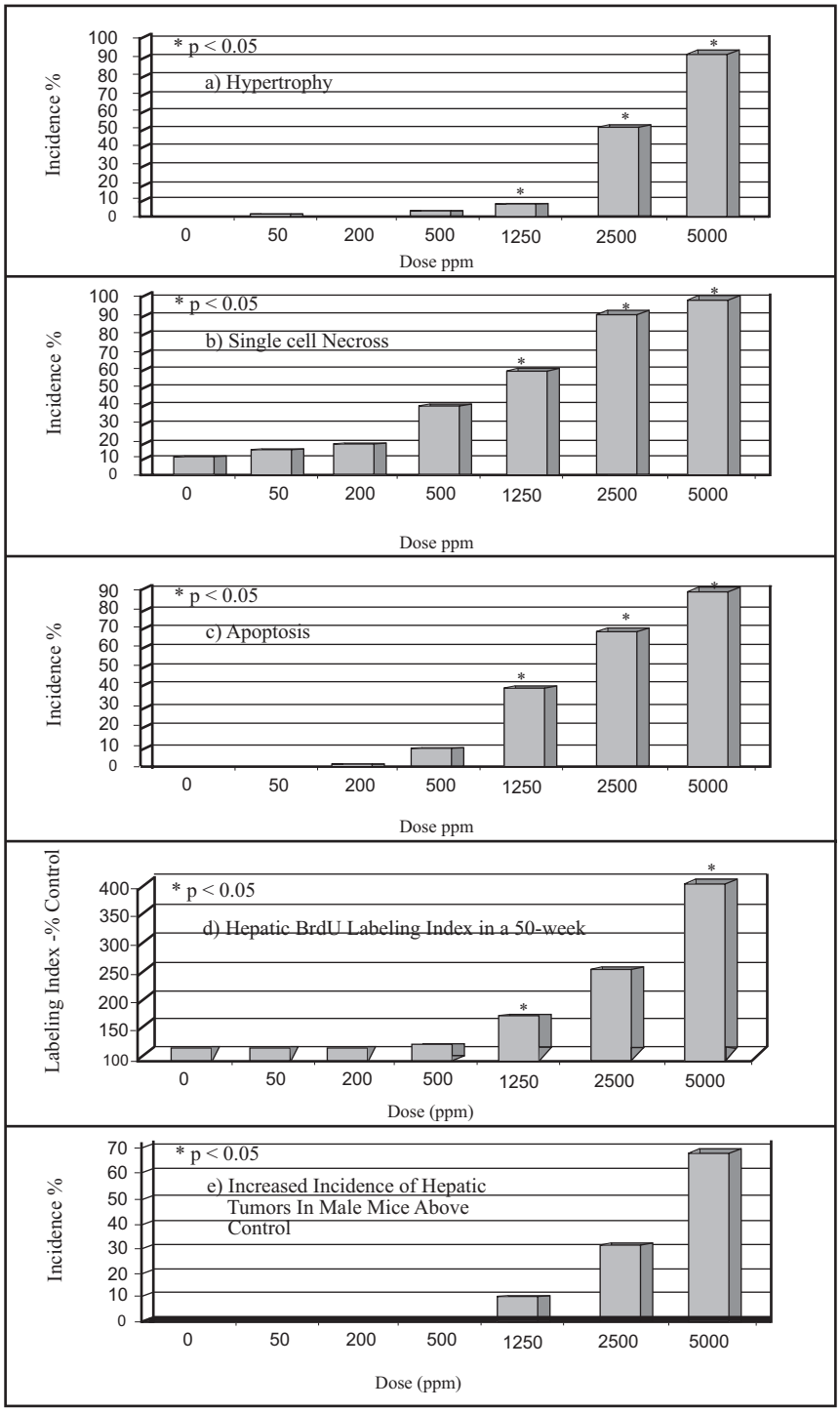

Table A1. Comparison of mode of action data relevant to hepatotoxicity and hepatocarcinogenicity in mice

	Dietary concentration (ppm)						
	0 (control)	5	20	100	500	1250	2500
Males							
Proliferation	—	—	—	None	Weak	—	Significant (3 days)
Enzyme induction	—	—	—	None	Very weak	—	Significant
Tumour incidence (hepatic neoplasia)	11/50 (22%)	7/50 (14%)	10/50 (20%)	—	19/50 (38%)	22/50 (44%)	44/50 (88%)
Historical incidence: mean (range)							
Hepatocellular adenoma		22.14% (10–34%)					
Hepatocellular adenocarcinoma		10.51% (0–16%)					
Females							
Proliferation	—	—	—	Very weak	Weak	—	Significant (7 days)
Enzyme induction	—	—	—	Very weak	Some	—	Significant
Tumour incidence (hepatic neoplasia)	0/50 (0%)	0/50 (0%)	0/50 (0%)	—	5/50 (10%)	9/50 (18%)	29/50 (58%)
Historical incidence: mean (range)							
Hepatocellular adenoma		3.2% (0–16%)					
Hepatocellular adenocarcinoma		0.38% (0–2%)					

ANNEX 1

Reports and other documents resulting from previous Joint Meetings of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and WHO Core Assessment Group on Pesticide Residues

1. Principles governing consumer safety in relation to pesticide residues. Report of a meeting of a WHO Expert Committee on Pesticide Residues held jointly with the FAO Panel of Experts on the Use of Pesticides in Agriculture. FAO Plant Production and Protection Division Report, No. PL/1961/11; WHO Technical Report Series, No. 240, 1962.

2. Evaluation of the toxicity of pesticide residues in food. Report of a Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1963/13; WHO/Food Add./23, 1964.

3. Evaluation of the toxicity of pesticide residues in food. Report of the Second Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1965/10; WHO/Food Add./26.65, 1965.

4. Evaluation of the toxicity of pesticide residues in food. FAO Meeting Report, No. PL/1965/10/1; WHO/Food Add./27.65, 1965.

5. Evaluation of the hazards to consumers resulting from the use of fumigants in the protection of food. FAO Meeting Report, No. PL/1965/10/2; WHO/Food Add./28.65, 1965.

6. Pesticide residues in food. Joint report of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 73; WHO Technical Report Series, No. 370, 1967.

7. Evaluation of some pesticide residues in food. FAO/PL:CP/15; WHO/Food Add./67.32, 1967.

8. Pesticide residues. Report of the 1967 Joint Meeting of the FAO Working Party and the WHO Expert Committee. FAO Meeting Report, No. PL:1967/M/11; WHO Technical Report Series, No. 391, 1968.

9. 1967 Evaluations of some pesticide residues in food. FAO/PL:1967/M/11/1; WHO/Food Add./68.30, 1968.

10. Pesticide residues in food. Report of the 1968 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 78; WHO Technical Report Series, No. 417, 1968.

11. 1968 Evaluations of some pesticide residues in food. FAO/PL:1968/M/9/1; WHO/Food Add./69.35, 1969.

12. Pesticide residues in food. Report of the 1969 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Group on Pesticide Residues. FAO Agricultural Studies, No. 84; WHO Technical Report Series, No. 458, 1970.

13. 1969 Evaluations of some pesticide residues in food. FAO/PL:1969/M/17/1; WHO/Food Add./70.38, 1970.

14. Pesticide residues in food. Report of the 1970 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 87; WHO Technical Report Series, No. 4574, 1971.

15. 1970 Evaluations of some pesticide residues in food. AGP:1970/M/12/1; WHO/Food Add./71.42, 1971.

16. Pesticide residues in food. Report of the 1971 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 88; WHO Technical Report Series, No. 502, 1972.

17. 1971 Evaluations of some pesticide residues in food. AGP:1971/M/9/1; WHO Pesticide Residue Series, No. 1, 1972.

18. Pesticide residues in food. Report of the 1972 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 90; WHO Technical Report Series, No. 525, 1973.

19. 1972 Evaluations of some pesticide residues in food. AGP:1972/M/9/1; WHO Pesticide Residue Series, No. 2, 1973.

20. Pesticide residues in food. Report of the 1973 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 92; WHO Technical Report Series, No. 545, 1974.

21. 1973 Evaluations of some pesticide residues in food. FAO/AGP/1973/M/9/1; WHO Pesticide Residue Series, No. 3, 1974.

22. Pesticide residues in food. Report of the 1974 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 97; WHO Technical Report Series, No. 574, 1975.

23. 1974 Evaluations of some pesticide residues in food. FAO/AGP/1974/M/11; WHO Pesticide Residue Series, No. 4, 1975.

24. Pesticide residues in food. Report of the 1975 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Plant Production and Protection Series, No. 1; WHO Technical Report Series, No. 592, 1976.

25. 1975 Evaluations of some pesticide residues in food. AGP:1975/M/13; WHO Pesticide Residue Series, No. 5, 1976.

26. Pesticide residues in food. Report of the 1976 Joint Meeting of the FAO Panel of Experts on Pesticide Residues and the Environment and the WHO Expert Group on Pesticide Residues. FAO Food and Nutrition Series, No. 9; FAO Plant Production and Protection Series, No. 8; WHO Technical Report Series, No. 612, 1977.

27. 1976 Evaluations of some pesticide residues in food. AGP:1976/M/14, 1977.

28. Pesticide residues in food—1977. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 10 Rev, 1978.

29. Pesticide residues in food: 1977 evaluations. FAO Plant Production and Protection Paper 10 Suppl., 1978.

30. Pesticide residues in food—1978. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 15, 1979.

31. Pesticide residues in food: 1978 evaluations. FAO Plant Production and Protection Paper 15 Suppl., 1979.

32. Pesticide residues in food—1979. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 20, 1980.

33. Pesticide residues in food: 1979 evaluations. FAO Plant Production and Protection Paper 20 Suppl., 1980

34. Pesticide residues in food—1980. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 26, 1981.

35. Pesticide residues in food: 1980 evaluations. FAO Plant Production and Protection Paper 26 Suppl., 1981.

36. Pesticide residues in food—1981. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 37, 1982.

37. Pesticide residues in food: 1981 evaluations. FAO Plant Production and Protection Paper 42, 1982.

38. Pesticide residues in food—1982. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 46, 1982.

39. Pesticide residues in food: 1982 evaluations. FAO Plant Production and Protection Paper 49, 1983.

40. Pesticide residues in food—1983. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 56, 1985.

41. Pesticide residues in food: 1983 evaluations. FAO Plant Production and Protection Paper 61, 1985.

42. Pesticide residues in food—1984. Report of the Joint Meeting on Pesticide Residues. FAO Plant Production and Protection Paper 62, 1985.

43. Pesticide residues in food—1984 evaluations. FAO Plant Production and Protection Paper 67, 1985.

44. Pesticide residues in food—1985. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 68, 1986.

45. Pesticide residues in food—1985 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 72/1, 1986.

46. Pesticide residues in food—1985 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 72/2, 1986.

47. Pesticide residues in food—1986. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 77, 1986.

48. Pesticide residues in food—1986 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 78, 1986.

49. Pesticide residues in food—1986 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 78/2, 1987.

50. Pesticide residues in food—1987. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 84, 1987.

51. Pesticide residues in food—1987 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 86/1, 1988.

52. Pesticide residues in food—1987 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 86/2, 1988.

53. Pesticide residues in food—1988. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 92, 1988.

54. Pesticide residues in food—1988 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 93/1, 1988.

55. Pesticide residues in food—1988 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 93/2, 1989.

56. Pesticide residues in food—1989. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 99, 1989.

57. Pesticide residues in food—1989 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 100, 1990.

58. Pesticide residues in food—1989 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 100/2, 1990.

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This volume contains toxicological monographs that were prepared by the 2010 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Rome on 21–30 September 2010.

The monographs in this volume summarize the safety data on 11 pesticides that could leave residues in food commodities. These pesticides are chlorothalonil metabolite R611965, clothianidin, cyproconazole, dicamba, dithianon, etoxazole, flubendiamide, fluopyram, meptyldinocap, tebuconazole and thiamethoxam. The data summarized in the toxicological monographs served as the basis for the acceptable daily intakes and acute reference doses that were established by the Meeting.

This volume and previous volumes of JMPR toxicological evaluations, many of which were published in the FAO Plant Production and Protection Paper series, contain information that is useful to companies that produce pesticides, government regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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