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Mode of Action Analysis for the Synthetic Pyrethroid Metofluthrin-Induced Rat Liver Tumors: Evidence for Hepatic CYP2B Induction and Hepatocyte Proliferation

Yoshihito Deguchi, Tomoya Yamada,[1] Yukihiro Hirose, Hirohisa Nagahori, Masahiko Kushida, Kayo Sumida, Tokuo Sukata, Yoshitaka Tomigahara, Kazuhiko Nishioka, Satoshi Uwagawa, Satoshi Kawamura, and Yasuyoshi Okuno

Environmental Health Science Laboratory, Sumitomo Chemical Company, Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-8558, Japan

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Two-year treatment with high doses of Metofluthrin produced hepatocellular tumors in both sexes of Wistar rats. To understand the mode of action (MOA) by which the tumors are produced, a series of studies examined the effects of Metofluthrin on hepatic microsomal cytochrome P450 (CYP) content, hepatocellular proliferation, hepatic gap junctional intercellular communication (GJIC), oxidative stress and apoptosis was conducted after one or two weeks of treatment. The global gene expression profile indicated that most genes with upregulated expression with Metofluthrin were metabolic enzymes that were also upregulated with phenobarbital. Metofluthrin induced CYP2B and increased liver weights associated with centrilobular hepatocyte hypertrophy (increased smooth endoplasmic reticulum [SER]), and induction of increased hepatocellular DNA replication. CYP2B1 mRNA induction by Metofluthrin was not observed in CAR knockdown rat hepatocytes using the RNA interference technique, demonstrating that Metofluthrin induces CYP2B1 through CAR activation. Metofluthrin also suppressed hepatic GJIC and induced oxidative stress and increased antioxidant enzymes, but showed no alteration in apoptosis. The above parameters related to the key events in Metofluthrin-induced liver tumors were observed at or below tumorigenic dose levels. All of these effects were reversible upon cessation of treatment. Metofluthrin did not cause cytotoxicity or peroxisome proliferation. Thus, it is highly likely that the MOA for Metofluthrin-induced liver tumors in rats is through CYP induction and increased hepatocyte proliferation, similar to that seen for phenobarbital. Based on analysis with the International Life Sciences Institute/Risk Science Institute MOA framework, it is reasonable to conclude that Metofluthrin will not have any hepatocarcinogenic activity in humans, at least at expected levels of exposure.

Key Words: **hepatocarcinogenesis; constitutive androstane receptor (CAR); phenobarbital; nongenotoxic; RNA interference (RNAi); toxicogenomics.**

In the 1950′s and 1960′s, several chemicals were identified as rodent carcinogens that were also known to be carcinogenic in humans. The 2-year bioassay was developed to provide a standardized screening procedure for evaluating chemicals with the assumption that these were predictive of human carcinogenic risk. In utilizing animals as a bioassay screening model, two fundamental assumptions are made: (1) the results observed in the animal model are relevant to humans (species extrapolation); and (2) the dose administered to the animals is relevant to the exposure levels in humans (dose extrapolation). For many chemicals, particularly DNA reactive carcinogens, these assumptions are reasonable. However, as has been clear for many chemicals, one or both of these assumptions may not be appropriate (Cohen, 2004; Holsapple *et al*., 2006).

In recent years, a mode of action (MOA) framework has been developed through the International Life Sciences Institute/Risk Science Institute (Cohen *et al*., 2004; Meek *et al*., 2003) and the International Programme on Chemical Safety (Boobis *et al*., 2006; Sonich-Mullin *et al*., 2001), including an evaluation of the human relevance of the animal MOA data. This study has been conducted to evaluate the MOA for rat liver tumors induced by Metofluthrin (CAS-No. 240494-70-6; 2,3,5,6-tetrafluoro-4-methoxymethylbenzyl (*EZ*)-(1*RS*,3*RS*;1*RS*,3*SR*)-2,2-dimethyl-3-(prop-1-enyl)cyclopropanecarboxylate) based on this framework.

Metofluthrin is a new Type I pyrethroid insecticide for use in pest control (Lucas *et al*., 2007; Ujihara *et al*., 2004). A basic genotoxicity assessment (including bacterial mutagenicity test, chromosomal aberration test in Chinese hamster lung cells, and micronucleus test in mice) demonstrated that Metofluthrin is not genotoxic (unpublished observations). The carcinogenicity of Metofluthrin has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice. Male and female HanBrl:WIST rats were fed 0 (control), 20, 200, 900, or 1800 ppm Metofluthrin in the diet for 2 years. The incidence of hepatocellular adenomas and/or carcinomas was significantly increased in male rats given 900 or 1800 ppm Metofluthrin and in female rats given 1800 ppm Metofluthrin (see Supplementary Tables 1 and 2). The combined

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[1] To whom correspondence should be addressed. Fax: +81-66466-5354. E-mail: yamadat8@sc.sumitomo-chem.co.jp.

incidences of hepatocellular adenomas and carcinomas of 0, 20, 200, 900, and 1800 ppm groups were respectively 2, 2, 6, 16, and 24% for males and 2, 6, 2, 10, and 24% for females. Historical background incidences for combined liver tumors in the laboratory conducting this bioassay were 0–6% for males and 1–12% for females, respectively. Although the high dose level appeared to exceed the maximum tolerated dose (MTD) in females and, to a lesser extent, in males, it was concluded that Metofluthrin has a weak carcinogenic potential in rats based on the hepatocellular tumors. In contrast, Metofluthrin was not carcinogenic in the liver or any other tissue in male or female CD-1 mice when administered at dietary levels of 100, 1000, and 1750/2500 ppm (unpublished observations).

The existing data from the general toxicity studies (unpublished observations) demonstrated that Metofluthrin showed increased liver weights, enlarged liver, centrilobular hepatocyte hypertrophy, and increased SER, suggesting that the MOA of Metofluthrin-induced liver tumors involves CYP2B isoform induction/constitutive androstane receptor (CAR) activator similar to phenobarbital (Holsapple *et al*., 2006; Whysner *et al*., 1996). The key events for phenobarbital-induced liver tumor formation in mice include induction through CAR of CYP2B isoforms, increased hepatocellular proliferation and eventually liver foci, adenomas and carcinomas. However, there have been no data demonstrating whether Metofluthrin affects CYP induction and/or hepatocellular proliferation in rats. This is the first study investigating the MOA of Metofluthrin for rat liver tumor induction, which was conducted to confirm the postulated MOA. To test each of the postulated key events, several parameters were evaluated in the liver: (1) global gene expression profile using DNA microarrays; (2) CYP2B and CYP3A induction; (3) involvement of CAR in the CYP2B induction; (4) hepatocellular proliferation; (5) gap junctional intercellular communication (GJIC); (6) oxidative stress; and (7) apoptosis. Involvement of CAR in the CYP2B induction was confirmed using the RNA interference (RNAi) technique (a recently developed technique involving sequence-specific gene silencing using short-interfering RNA [siRNA]) (Caplen *et al*., 2001; Elbashir *et al*., 2001; Fire *et al*., 1998).

MATERIALS AND METHODS

Chemicals

Test chemicals were obtained from the following manufacturers: Metofluthrin (Lot No. PK-020301G; purity, 96.6%; storage condition, cold storage) was provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan); phenobarbital-Na (NaPB; Lot No. KLM4036; purity, 98.0%; storage condition, room temperature) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Husbandry

All experiments were performed in accordance with *The Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd.* BrlHan:WIST@Jcl(GALAS) rats aged 8 weeks were purchased from CLEA Japan, Inc., Fuji Breeding Center (Shizuoka, Japan). The BrlHan:WIST@Jcl(GALAS) rat was selected as the test animal for two reasons: its genetic and microbiological properties are known, and HanBrl:WIST is the species used in the 2-year bioassay of Metofluthrin. The animals were acclimated to laboratory conditions for 7 days prior to dosing (the age of animals was 9 weeks old). The animals were housed in a barrier-system animal room. During the course of the study, the environmental conditions in the animal room were targeted within a temperature range of 22–26°C and a relative humidity range of 40–70%, with frequent ventilation (more than 10 times per hour) and a 12-h light (8:00–20:00)/12-h dark (20:00–8:00) illumination cycle. A commercially available pulverized diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water were provided *ad libitum* throughout study. The animals were not fasted overnight prior to sacrifice.

Study 1: Effect of Metofluthrin on Hepatic Microsomal CYP Content and Hepatocellular Proliferation in Rats Treated with Metofluthrin for 1 week

Study design. Male and female rats (9 weeks of age, five to seven rats per dose) were fed diets containing 0 (control), 200, 900, 1800, or 3600 ppm Metofluthrin. In the previous 2-year bioassay, liver tumors were increased at dietary concentrations of 900 and 1800 ppm but not 200 ppm. 200, 900, and 1800 ppm were, therefore, selected for this study. In addition, to better evaluate the effects of Metofluthrin, twice the highest dose level in the 2-year bioassay, 3600 ppm, was used as the highest dietary concentration in this MOA study. Rats were dosed for periods of 1 week (treatment group) or 1 week followed by 1 week of untreated diet (recovery group). In addition, as a positive control group, another group was fed diets containing NaPB 1000 ppm, equivalent to a dose level at which effects on liver have been observed (IARC, 2001). Clinical signs, body weights, and food consumption were monitored throughout the study. Alzet minipumps (Alzet Corporation, Palo Alto, CA) containing 5-bromo-2′-deoxy-uridine (BrdU, Sigma Company, St Louis, MO), with a release rate of 200 mg/h, were implanted in the subcutaneous tissue of rats under anesthesia with ketamine-xylazine (70:8 mg/kg, ip) on the day prior to 7 days of the scheduled sacrifice. After the 7-day treatment period, rats were euthanized by decapitation on the morning of day 8 (without fasting), then livers were excised quickly and weighed. Some liver tissue was stored in RNA stabilization solution (Ambion, Austin, TX) at −20°C until analyzed for gene expression. The rest of the liver tissue was processed for hepatic microsomal CYP isoform expression analysis, histopathology, and cell proliferation measurements.

Gene expression profiling analysis. Gene expression profiling was individually determined for the livers from three male rats in each of the control, Metofluthrin 1800 ppm and NaPB 1000 ppm groups. Rat Genome U34A Arrays were purchased from Affymetrix (Santa Clara, CA). Total RNA samples were extracted from liver using Isogen (Nippon Gene Co., Ltd., Toyama, Japan) and then purified with an RNeasy Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions. Concentrations of total RNA samples were measured by Amersham-Pharmacia spectrophotometer, model Ultraspec 3100pro. For microarray probing, reverse transcription, second-strand synthesis, and probe generation were all accomplished by the standard Affymetrix protocol (Affymetrix) (Lockhart *et al*., 1996). Briefly, from 5 μg of total RNA, first-strand cDNAs were synthesized with SuperScript II reverse transcriptase (Invitrogen, Groningen, the Netherlands) and a T7-(dT)24 primer (Amersham Bioscience, Buckinghamshire, UK), and then double-strand cDNAs were generated with *Escherichia coli* RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase (Invitrogen). From the double-strand cDNA, biotin-labeled cRNAs were prepared using a BioArray High-Yield RNA Transcript Labeling Kit (ENZO Diagnostics, Farmingdale, NY). Twenty micrograms of labeled cRNA was fragmented, and the RGU34A Arrays were hybridized as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix). The arrays were stained with R-Phycoerythrin Streptoavidin (Molecular Probes, Eugene, OR), the fluorescence was intensified by an antibody-amplification method and the arrays were scanned with a GeneArray scanner (Agilent Technologies, Palo Alto, CA).

Microarray data analysis was performed using the Affymetrix data suite system, Microarray Analysis Suite 5.0 (MAS 5.0) (Santa Clara, CA). Tab-delimited files containing data regarding the relative level of expression of a transcript (Signal) and whether a transcript is reliably detected or not (Detection Call; Present(P), Marginal(M), Absent(A)) from the image files have been obtained. The derived signal values were globally normalized and targeted to all probe sets equal to 100 before the following analysis. Average signal values of three biological replicates of each group were calculated. The magnitude and direction of change of a transcript was obtained as Signal Ratio from the average signal values in the treated three replicates and those in the control three replicates to examine gene expression differences between treatment and control. Genes (probe sets) showing greater than or equal to "2" Signal Ratio and "P or M" Detection Call in three treatment animals or showing less than or equal to "0.5" Signal Ratio and "P or M" Detection Call in three control animals were considered as altered.

Hierarchical clustering analysis was performed in GeneSpring 7.2 (Silicon Genetics, San Carlos, CA). A condition tree was prepared using a spearman correlation. Genes whose expression was up- and downregulated with Metofluthrin were analyzed. Total number of genes analyzed is 35; 25 genes upregulated and 10 genes downregulated. Of these 35 genes, Signal Ratio values of three animals treated with Metofluthrin 1800 ppm and three animals treated with NaPB 1000 ppm were used in hierarchical clustering analysis.

Hepatic microsomal CYP isoform expression analysis. (1) Quantitative real-time PCR. Total RNA was extracted from fresh rat liver (approximately 0.5 g) using Trizol reagent (Invitrogen) and RNeasy Mini kit (Qiagen) in accordance with the manufacturer's instructions. cDNA was prepared from total RNA by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR assays for rat CYP2B1/2, CYP3A1, and CYP3A2 mRNA were performed using the primer sets contained in the commercial determination kit (Rat Cytochrome P450 Competitive RT-PCR Set, Takara Bio, Shiga, Japan) and following the instruction manual of the PCR system (GeneAmp 5700 Sequence Detection System, Applied Biosystems, CA). Gene expression level of cyclophilin was measured as an internal control. The individual values were calculated and shown as the relative level to the mean of the values from the control group. (2) Preparation of rat liver microsomes and western blot analysis. Liver microsomes were prepared as described by Lake (1987). A portion of fresh liver (4 - 6 g) was homogenized in 4 volumes of Tris/HCl buffer (pH7.4; 50mM; containing 154mM KCl) with a Potter homogenizer. Liver homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was centrifuged at 105,000 × g for 60 min at 4°C to separate microsomes. Microsomes were washed and resuspended in potassium phosphate buffer (pH7.4; 100mM) to prepare 100% wt/vol microsomal suspension. The protein level in the microsomal suspensions was determined using the Bio-Rad protein assay (Bio-Rad, CA) with bovine serum albumin as the protein standard (Bradford, 1976). Total CYP P450 protein level in liver microsomes was assayed by carbon monoxide difference spectrum with a ultraviolet (UV) spectrophotometer (U-3210, Hitachi, Tokyo, Japan) after reduction with sodium dithionite (Omura and Sato, 1964). Western blot analysis was performed using a goat anti-rat CYP2B1 polyclonal antibody or a rabbit anti-rat CYP3A2 polyclonal antibody (Daiichi Pure Chemicals, Tokyo, Japan). Band intensities were quantified with a luminescent image analyzer (LAS-1000 plus, Fuji Photo Film, Kanagawa, Japan). A goat anti-rat CYP2B1 polyclonal antibody and a rabbit anti-rat CYP3A2 polyclonal antibody were cross reactive with CYP2B and CYP3A subfamily enzymes, respectively. (3) Microsomal enzyme assay. The microsomes of control, Metofluthrin 3600 ppm and NaPB 1000 ppm groups from the Treatment Group were used for measurement of enzyme activities. Hepatic microsomal activity for 7-pentoxyresorufin *O*-depentylation was determined by fluorometric analysis. The fluorescence of the reaction mixture was measured with a fluorescence spectrophotometer (F-4010, Hitachi) with an excitation wavelength of 550 nm and an emission wavelength of 585 nm. The activities were expressed as the rate of resorufin formation, and were calculated based on the fluorescence of a standard curve of resorufin. Hepatic microsomal activity for testosterone 6β-hydroxylation was determined by high-performance liquid chromatography (HPLC) analysis. The 6β-hydroxytestosterone was extracted with addition of 17α-methyltestosterone as an internal standard. The extracts were analyzed by HPLC with UV detection at 245 nm. The activity was expressed as the rate of 6β-hydroxytestosterone formation, and were calculated based on the peak area of a standard curve of 6β-hydroxytestosterone.

Histopathology. Livers were dehydrated, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. In the control, Metofluthrin 3600 ppm and NaPB groups, livers were removed from two rats per sex per group. Small pieces of livers were fixed 10% neutral-buffered formalin over approximately 24 h, and with 2.5% glutaraldehyde solution, postfixed in 2% osmium tetroxide, dehydrated and embedded in epoxy resin. Ultrathin sections were prepared from those pieces, and stained with uranyl ascetate and lead citrate, and examined with the transmission electron microscope.

Hepatocellular proliferation analysis from BrdU-labeling indices. Thin sections of liver prepared from the same paraffin embedding for histopathology were stained immunohistochemically using BrdU monoclonal antibody, then BrdU-labeling indices were determined. At least 2,000 hepatocellular nuclei were counted for each liver using an Image Processor for Analytical Pathology (Sumika Technoservice Corporation, Osaka, Japan). BrdU-labeling indices for the livers were calculated by dividing the number of labeled hepatocellular nuclei by the total number of hepatocellular nuclei counted.

Study 2: Confirmation Study for Involvement of CAR in the MOA for CYP2B Induction Using the RNAi Technique

Rat hepatocytes. On day 0, primary cultured hepatocytes were obtained from a single male BrlHan:WIST@Jcl(GALAS) rat at age of 10 weeks by a modified two-step collagenase digestion method as previously described (Seglen, 1976). Rat liver was perfused and hepatocytes were dispersed from digested liver and washed with William's E medium (GIBCO) three times by centrifugation. The hepatocytes were cultured in supplemented William's E medium (5% fetal bovine serum [Gibco], 100 U/ml penicillin (Nakaraitesque, Japan), 100 μg/ml streptomycin (Nakaraitesque, Japan), 2mM L-glutamine (Nakaraitesque, Japan), 0.1μM insulin (Sigma-Aldrich), 1μM dexamethasone (Sigma-Aldrich), 0.2μM ascorbic acid (Sigma-Aldrich), and 10mM nicotinamide; Sigma-Aldrich) in a six-well plate coated with collagen I (AsahiTechnoGlass, Japan), at a density of approximately 7 to 8 × 10^5 cells/well, and allowed to attach for 3 h at 37°C in a humidified chamber. After 3 h, the culture dishes were gently swirled and fresh medium was added after removing the unattached hepatocytes.

Transfection. On day 1, cells were rinsed and supplemented with serum/antibiotics free medium (2 ml). siRNA (100nM) for CAR (Sthealth RNAi, Invitrogen) or negative control (Stealth RNAi Negative Control with Medium GC, Code No.; 12935-300, Invitrogen), and 4 μl of Lipofectamine RNAiMAX (Invitrogen) were each diluted with 200 μl of serum/antibiotics free medium according to the manufacturer's instructions, and the two solutions were gently mixed. After 20 min, the transfection mixtures (400 μl) were added to the cells. The sequences of the siRNA are listed in Supplementary Table 3. After 4 h, the media were changed to the supplemented Williams E medium containing serum and antibiotics.

Chemical treatment. Following the transfection (on day 1), hepatocytes were treated with 50μM NaPB or 50μM Metofluthrin in medium for 2 days. A concentration of 50μM NaPB was selected as this concentration has been previously shown to induce CYP2B-dependent enzyme activity in cultured rat hepatocytes (Madan *et al*., 1999). A medium concentration of 50μM was selected for Metofluthrin. Metofluthrin concentration in liver at 900–1800 ppm dose is estimated to be about 1–10μM (unpublished data), and 50μM was expected to be sufficient to detect the CYP2B induction potential of Metofluthrin by this *in vitro* system. This concentration was lower than the toxic concentration to CHL cells used in the study of in vitro chromosomal aberration test on Metofluthrin (unpublished data), and no cytotoxicity (e.g., cell death) was observed in the primary cultured hepatocytes.

Medium was changed on a daily basis thereafter. The control group was conducted in the same manner without test chemical. The experiment was repeated three times; one experiment was examined using two wells, and the other two experiments were examined using four wells for each group. Because similar findings were observed for these three experiments with different rats, representative data are presented in this paper.

Analysis of isolated RNA using quantitative real-time PCR. Total RNA was isolated, reverse-transcribed into complementary DNA and performed quantitative real-time PCR as previously described (Kobayashi *et al*., 2005). Briefly, on day 3, hepatocytes were washed with phosphate-buffered saline (PBS) and the total RNA was extracted using Isogen (Nippon Gene, Japan). Total RNA was purified using RNeasy Mini kit (Qiagen) with on-column DNase treatment to avoid genomic DNA contamination. cDNA was prepared from 400 μg of total RNA by reverse transcription using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR assays for rat CAR, CYP2B1, and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA were performed using TaqMan Universal Master Mix (for CAR and GAPDH mRNA) (Applied Biosystems) and Power SYBR Green PCR Master Mix (for CYP2B1 mRNA) (Applied Biosystems) following the instruction manual of the PCR system (GeneAmp 7500 Sequence Detection System, Applied Biosystems). The primer and probe sets are listed in Supplementary Table 4. The CAR and CYP2B1 mRNA level were normalized to those of GAPDH mRNA level.

Study 3: Confirmation Study for Transient Enhancement of Hepatocellular Proliferation Induced by Metofluthrin

Study design. Male rats (9 weeks of age, five rats per dose) were fed diets containing 0(control), 2700 ppm Metofluthrin or NaPB 1000 ppm for 1 and 2 weeks. Though the dose level of 3600 ppm was evaluated in Study 1, it is a much higher dose level than those of the 2-year bioassay and it proved to be toxic. Thus, 2700 ppm was used in Study 3. Using the same method as Study 1, liver weight and hepatocellular proliferation based on BrdU-labeling indices were evaluated.

Study 4: Effect of Metofluthrin on Hepatic GJIC, Oxidative Stress and Apoptosis in Rats Treated with Metofluthrin for 1 Week

Study design. Male and female rats (9 weeks of age, eight rats per dose) were fed diets containing 0 (control), 200, 900, 1800, or 3600 ppm Metofluthrin or 1000 ppm NaPB. Study design was the same as that of Study 1. The liver samples were provided for assessment of GJIC capacity *ex vivo*, oxidative stress and apoptosis.

***Assessment of GJIC capacity* ex vivo.** The effect of Metofluthrin on GJIC in rat liver was evaluated by fluorescent dye cut-loading with minor modifications (Rice, 2004). Six rats were assigned randomly for the assessment. Soon after the liver was removed, 5-mm-thick slices of liver were cut and two to three incisions (1-mm depth) were made with a blade, followed by dripping onto the slices a mixture composed of 0.05% Lucifer Yellow CH and 0.05% Rhodamine B isothiocyanate-dextran in PBS. After 3 min, the slices were washed in PBS three times, then embedded and frozen in OCT compound. Six-micrometers-thick frozen sections were obtained from each animal and photographed by fluorescence microscopy in a blinded fashion. Dye transfer was measured in each section and the mean and SD presented.

Parameters associated with oxidative stress (lipid peroxidation/total and reduced glutathione). The effect of Metofluthrin on the lipid peroxidation level in rat liver was determined by colorimetric assay using LPO-586 (Bioxytech, OR). The method is based on a reaction of N-methyl-2-phenylindole with malondialdehyde (MDA) and 4-hydroxyalkenal at 45°C. One molecule of MDA or 4-hydroxyalkenal reacts with two molecules of N-methyl-2-phenylindole to yield a stable chromophore with maximal absorbance at 586 nm. The effect of Metofluthrin on total glutathione (GSH) level in rat liver was determined by the dithionitrobenzoic acid-glutathione disulfide reductase method using a GSH assay kit (Cayman Chemical Company, MI). Liver homogenates were deproteinized with metaphosphoric acid, and the total GSH was measured according to the manufacturer's instructions. The effect of Metofluthrin on reduced GSH level in rat liver was determined by the thioether method using GSH-400 (Bioxytech, OR) according to the manufacturer's instructions.

Apoptosis (cytoplasmic histone-associated DNA fragments level). The effect of Metofluthrin on apoptosis in rat liver was assessed by quantitation of DNA fragments using Cell Death Detection ELISA (enzyme-linked immunosorbent assay) plus (Roche Diagnostics, Penzberg, Germany). This test is based on the detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), which are generated during apoptosis. ELISA was performed according to the manufacturer's instructions.

Statistical Analyses

Statistical analysis was performed with the statistical analysis system software (SAS, version 6.1) (SAS Institute, Cary, NC). For experiments using multiple dose levels of test chemical, the data were initially tested for homogeneity using Bartlett's test. For the data found to be homogeneous ($p > 0.05$), Dunnett's multiple analysis (Dunnett, 1955) was performed. If the data were not homogeneous ($p \leq 0.05$), Steel's multiple analysis was performed. For experiments using a single dose level of test chemical, the data were initially tested for homogeneity using the *F*-test for homogeneity of variance. If the variance was homogeneous, the data were analyzed by the Student's *t*-test, and if heterogeneous, by the Aspin-Welch test (Aspin, 1949; Welch, 1938). The Mann-Whitney's *U* test was used for analysis of the gradable histopathological findings, comparing test substance and control groups. The Fisher's exact probability test was similarly employed for analysis of clinical signs and gross pathological findings. Each evaluation was by 2-tailed tests with 0.05 and 0.01 as the levels of significance.

RESULTS

Study 1: Effect of Metofluthrin on Hepatic Microsomal CYP Content and Hepatocellular Proliferation in Rats Treated with Metofluthrin for 1 Week

Data from the Treated Group in Study 1 are summarized in Supplementary Tables 5 and 6. The treatment of rats with Metofluthrin at 3600 ppm revealed tremor, which is a typical clinical sign caused by exposure to high doses of pyrethroids (Soderlund *et al*., 2002; U.S. EPA, 2003). Two females of the Treatment Group and one male of the Recovery Group were found dead at that dose level, suggesting that 3600 ppm exceeded the MTD. The treatment of males at 1800 ppm and females at 1800 and 3600 ppm Metofluthrin and both sexes at 1000 ppm NaPB resulted in increased liver weights, which were associated with enlarged/dark liver and centrilobular hepatocellular hypertrophy, but no cytotoxicity. Electron microscopy revealed dilatation and/or proliferation of SER but no proliferation of peroxisomes in rats treated with 3600 ppm Metofluthrin or 1000 ppm NaPB.

For the global gene expression profile analysis, Supplementary Figure 1 illustrates a Venn diagram of significant alterations in the gene expression observed. Numbers of probe sets with twofold alteration of Metofluthrin and NaPB groups were, respectively, 25 and 85 for upregulation and 10 and 14 for downregulation. Of probes upregulated in the Metofluthrin group, 84% (21 out of 25) were also upregulated in the NaPB group. Of probes downregulated in the Metofluthrin group,

40% (4 out of 10) were also downregulated in the NaPB group. Clustering analysis was evaluated in genes altered by Metofluthrin treatment. It demonstrated that the profile of these genes is generally similar between Metofluthrin- and NaPB-treated animals (Supplementary Fig. 2). Table 1 lists the genes (probe sets) whose expression was significantly altered by Metofluthrin. Most genes whose expression was upregulated with Metofluthrin were metabolic enzymes including glutathione-*S*-transferase (GSTs), CYPs, UDP-glycosyltransferase (UGTs), etc. These genes (probe sets) were also upregulated by NaPB with greater potency.

Although treatment with Metofluthrin did not show marked induction of microsomal proteins or total CYP levels (data not shown), Metofluthrin treatment increased CYP2B1/2 and 3A1 mRNA levels dose dependently (Fig. 1). Statistically significant increases of CYP2B mRNA levels were observed at 1800 and 3600 ppm Metofluthrin in males, and at 900, 1800, and 3600 ppm Metofluthrin in females. Whereas the mRNA for CYP3A1 was increased statistically significantly at 3600 ppm in males and 1800 and 3600 ppm Metofluthrin in females (Fig. 1), CYP3A2 mRNA was not increased at any of these dose levels (Supplementary Tables 5 and 6). Similarly, there was an increase

TABLE 1
Genes with Altered Expression after 1-Week Treatment with Metofluthrin

Probe set name	Gene symbol	Gene title	Metofluthrin	NaPB
Upregulation				
S72506_s_at	**Yc2**	**Glutathione *S*-transferase Yc2 subunit**	**13.3**	**10.3**
S82820mRNA_s_at	**Yc2**	**Glutathione *S*-transferase Yc2 subunit**	**9.9**	**8.6**
rc_AA945082_at	**—**	**Transcribed locus**	**6.9**	**6.3**
L00320cds_f_at	**Cyp2b15**	**Cytochrome P450, family 2, subfamily b, polypeptide 15**	**5.2**	**59.4**
M33550cds_s_at	**Cyp2c40**	**Cytochrome P450, family 2, subfamily c, polypeptide 40**	**4.4**	**12.3**
K00996mRNA_s_at	**Cyp2b15 /// Cyp2b2**	**Cytochrome P450, family 2, subfamily b, polypeptide 15 /// cytochrome P450, family 2, subfamily b, polypeptide 2**	**4.4**	**23.1**
D38061exon_s_at	**Ugt1a6**	**UDP glycosyltransferase 1 family, polypeptide A6**	**4.2**	**4.6**
S56936_s_at	**Ugt1a6**	**UDP glycosyltransferase 1 family, polypeptide A6**	**4.0**	**3.8**
K01721mRNA_s_at	**Cyp2b2**	**Cytochrome P450, family 2, subfamily b, polypeptide 2**	**3.9**	**13.1**
M11251cds_f_at	**Cyp2b15**	**Cytochrome P450, family 2, subfamily b, polypeptide 15**	**3.9**	**50.1**
AF045464_s_at	**Akr7a3**	**Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)**	**3.8**	**2.9**
rc_AI171506_g_at	**Me1**	**Malic enzyme 1**	**3.8**	**3.6**
M13234cds_f_at	**Cyp2b2**	**Cytochrome P450, family 2, subfamily b, polypeptide 2**	**3.7**	**17.8**
M64986_g_at	**Hmgb1**	**High mobility group box 1**	**2.9**	**2.9**
rc_AI171506_at	**RGD1307103**	**Similar to RIKEN cDNA 1190003A07 (predicted)**	**2.8**	**2.7**
M26594_at	**Me1**	**Malic enzyme 1**	**2.8**	**3.3**
M13506_at	**Udpgtr2**	**Liver UDP-glucuronosyltransferase, phenobarbital-inducible form**	**2.7**	**4.9**
AF001898_at	**Aldh1a1**	**Aldehyde dehydrogenase family 1, member A1**	**2.6**	**6.4**
rc_AI029805_at	LOC499337	Similar to dedicator of cytokinesis 8	2.4	2.625[a]
rc_AI008020_at	**Me1**	**Malic enzyme 1**	**2.3**	**2.5**
X90710_at	Adh4	Alcohol dehydrogenase 4 (class II), pi polypeptide	2.2	1.5
rc_AA891737_at	—	Transcribed locus	2.2	1.7
J00728cds_f_at	**Cyp2b2**	**Cytochrome P450, family 2, subfamily b, polypeptide 2**	**2.1**	**10.0**
rc_AI639470_g_at	LOC361776	LOC361776	2.1	1.5
M26125_at	**Ephx1**	**Epoxide hydrolase 1, microsomal**	**2.1**	**2.9**
Downregulation				
U02553cds_s_at	**Dusp1**	**Dual specificity phosphatase 1**	**0.22**	**0.30**
S74351_s_at	**Dusp1**	**Dual specificity phosphatase 1**	**0.29**	**0.46**
V01225mRNA_s_at	**Amy2_predicted**	**Amylase 2, pancreatic (predicted)**	**0.29**	**0.30**
rc_AI176456_at	LOC682651	Similar to metallothionein-2 (MT-2) (metallothionein-II) (MT-II)	0.39	1.21
S81478_s_at	**Dusp1**	**Dual specificity phosphatase 1**	**0.44**	**0.45**
L37333_s_at	G6pc	Glucose-6-phosphatase, catalytic	0.45	1.04
rc_AI102562_at	Mt1a	Metallothionein 1a	0.48	1.30
X13044_at	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	0.49	1.24
rc_AA859980_at	Acat2	Acetyl-coenzyme A acetyltransferase 2	0.49	1.75
rc_AA998683_g_at	Hspb1	Heat shock 27-kDa protein 1	0.50	1.20

Note. Data represent the expression ratio between control and chemical treatment. Significant alteration is change more than twofold and evaluated as obvious by ''Difference Call.'' Genes with alteration were listed in order of larger alteration. Bold text means significant alteration that is common to Metofluthrin and NaPB.

[a]Although this probe set shows greater than ''2'' Signal Ratio, Detection Call in three treatment animals was P and P & A, respectively. For this reason, this probe set was not considered as altered in NaPB group.

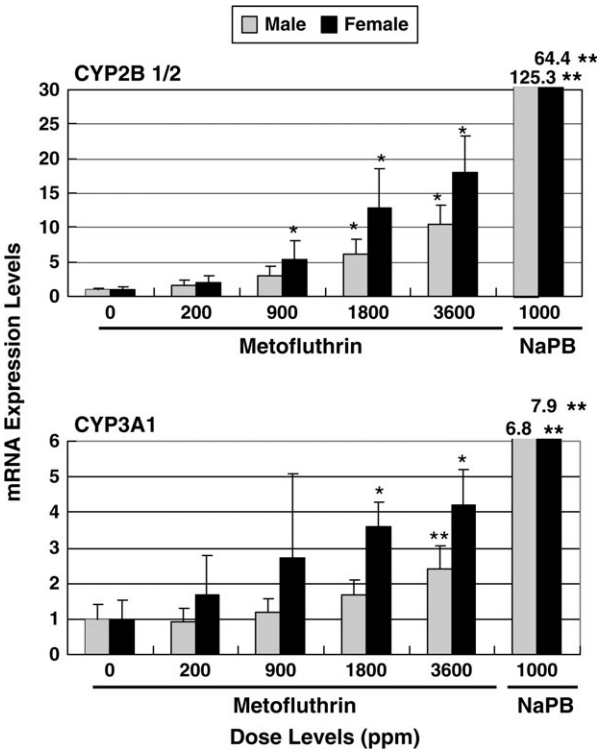

FIG. 1. Relative CYP2B1/2 and CYP3A1 mRNA expression levels in the liver of rats treated with Metofluthrin or phenobarbital for 1 week. Values represent group mean ± SD, $N = 5–7$. Group mean values ± SD in phenobarbital 1000 ppm group was: for CYP2B1/2, 125.3 ± 36.8 in males, 64.4 ± 25.1 in females; for CYP3A1, 6.8 ± 2.6 in males, 7.9 ± 3.1 in females. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

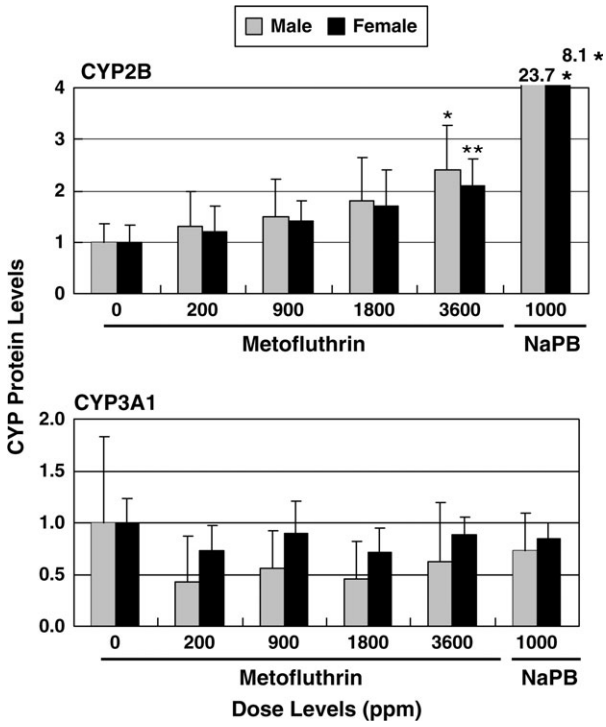

FIG. 2. Relative CYP2B and CYP3A protein levels in the liver of rats treated with Metofluthrin or phenobarbital for 1 week. Values represent group mean ± SD, $N = 5–7$. Group mean values ± SD in phenobarbital 1000 ppm group was for CYP2B, 23.7 ± 14.6 in males, 8.1 ± 4.9 in females; for CYP3A, 0.73 ± 0.37 in males, 0.85 ± 0.15 for CYP3A in females. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

in CYP2B protein levels dose dependently, whereas there was no increase in CYP3A protein levels at any of these doses (Fig. 2). Statistical significance of CYP 2B protein levels was observed at 3600 ppm in both sexes. Hepatic activities of 7-pentoxyresorufin *O*-depentylase (CYP2B marker) were increased in both sexes treated with Metofluthrin 3600 ppm, and hepatic activities of testosterone 6β-hydroxylase (CYP3A marker) were increased in females treated with Metofluthrin 3600 ppm (Fig. 3).

NaPB also increased all parameters described above excluding CYP3A protein levels, but the extent of changes was much greater than those of Metofluthrin (Figs. 1–3, Supplementary Tables 5 and 6).

Hepatocyte cell replicative DNA synthesis (determined as BrdU-labeling indices) was also significantly increased by treatment with 900 and 1800 ppm Metofluthrin in males and 1000 ppm NaPB in both sexes (Fig. 4). In females, labeling indices, although not statistically significant at the small sample sizes used in this study, showed tendencies for increases at 1800 and 3600 ppm Metofluthrin. Owing to high values in the control group, significant increases in the BrdU-labeling index were not observed in female rats. However, compared with the BrdU-labeling index value of the female 200 ppm Metofluthrin group (i.e., value expressed as 1.0), the labeling index values of the 900, 1800, and 3600 ppm were 1.2-, 2.1-, and 1.9-fold, respectively.

All of these effects in the Metofluthrin- or NaPB-treated rats were reversible back to control levels upon cessation of treatment (data not shown).

Study 2: Confirmation Study for Involvement of CAR in the MOA for CYP2B Induction Using the RNAi Technique

RNAi is a sequence-specific gene silencing technique using siRNA, and we introduced this technique to knockdown CAR mRNA *in vitro*. CAR mRNA expression levels were significantly suppressed to 37% of the control by CAR-siRNA (Fig. 5A). CYP2B1 mRNA in control hepatocytes was clearly induced by NaPB, whereas hepatocytes treated with CAR-siRNA had marginal induction by NaPB (Fig. 5B). Elevated expression levels of CYP2B1 mRNA by NaPB in the CAR-siRNA–treated group were only 21% of that in the control group (Cont-siRNA–treated group). This value of 21% is derived by subtracting 0.050 (the value of Untreated) from both 0.206 (NaPB + Cont-siRNA) and 0.083 (NaPB + CAR-siRNA), respectively, and then, calculating the percentage of (NaPB + CAR-siRNA) compared with (NaPB + Cont-siRNA) form the alternatives. Reproducibility of these findings was confirmed in two other experiments.

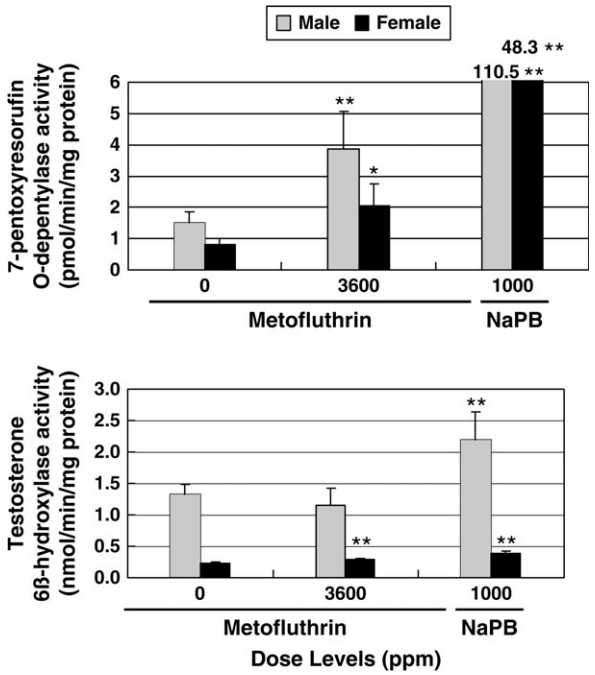

FIG. 3. Hepatic activities of 7-pentoxyresorufin *O*-dependylase (CYP2B marker) and testosterone 6β-hydroxylase (CYP3A marker) levels in rats treated with Metofluthrin or phenobarbital for 1 week. Values represent group mean ± SD, $N = 5–7$. Group mean values ± SD in phenobarbital 1000 ppm group was: for CYP2B marker, 110.5 ± 13.4 in males, 48.3 ± 14.2 in females; for CYP3A marker, 2.2 ± 0.43 in males, 0.39 ± 0.04 in females. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

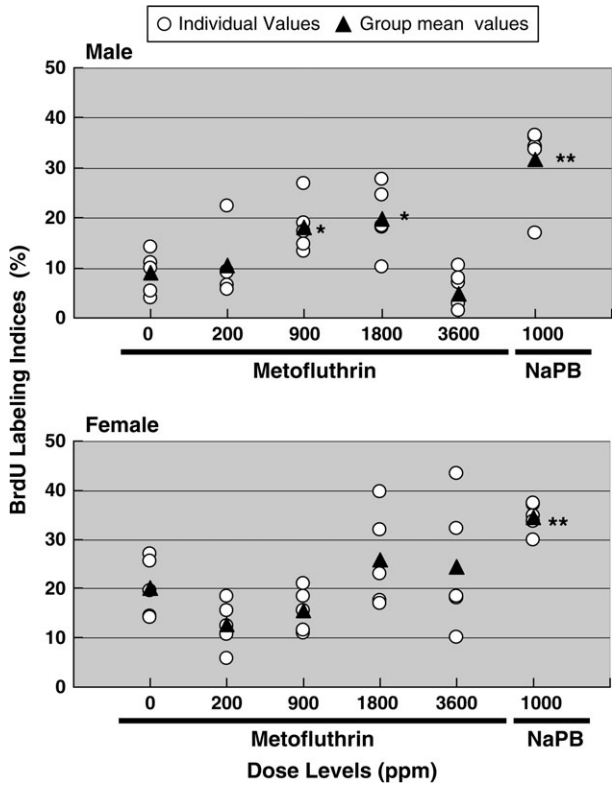

FIG. 4. Hepatocyte cell replicative DNA synthesis determined as BrdU-labeling indices in the liver of rats treated with Metofluthrin or phenobarbital for 1 week. Open circles represent data from individual rats; closed triangles represent group mean ± SD, $N = 5–7$. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

In the experiment for Metofluthrin, CAR mRNA expression levels were significantly suppressed to 34% of control by CAR-siRNA (Fig. 5C). CYP2B1 mRNA in control hepatocytes was significantly induced by Metofluthrin, whereas hepatocytes treated with CAR-siRNA had marginal induction by Metofluthrin (Fig. 5D). Elevated expression level of CYP2B1 mRNA by Metofluthrin in the CAR-siRNA–treated group was only 32% of that in the control group (Cont-siRNA–treated group). Reproducibility of these findings was also confirmed in two other experiments.

Study 3: Confirmation Study for Transient Enhancement of Hepatocellular Proliferation Induced by Metofluthrin

The treatment of male rats with Metofluthrin at 2700 ppm revealed no death or abnormality and did not affect body weight or food consumption during the 2-week treatment, demonstrating that treatment with Metofluthrin at 2700 ppm did not show excess toxicity (data not shown) as was seen at 3600 ppm. One-week treatment with Metofluthrin at 2700 ppm to rats affected liver morphology and enzyme induction, evidenced by increases of liver weights, hepatocyte cell replicative DNA synthesis, and CYP2B expression (Fig. 6). Most findings, except BrdU-labeling indices, were also observed after 2 weeks of treatment. However, the BrdU-labeling index values returned to control levels when Metofluthrin treatment was continued for 2 weeks (Fig. 6).

Study 4: Effect of Metofluthrin on Hepatic GJIC, Oxidative Stress, and Apoptosis in Rats Treated with Metofluthrin for 1 Week

Data from the Treated Group in Study 2 are summarized in Supplementary Tables 7 and 8. Treatment of rats with Metofluthrin at 3600 ppm revealed tremor and death (three-female of the Recovery Group). Again, the dose level of 3600 ppm exceeded the MTD consistent with study 1. The treatment of males at 1800 and 3600 ppm Metofluthrin, females at 3600 ppm Metofluthrin and both sexes at 1000 ppm NaPB resulted in increased liver weights with statistical significance, associated with enlarged and/or dark liver and centrilobular hepatocellular hypertrophy.

Regarding the distances of fluorescent dye transfer, statistically significant decreases were noted in both sexes of the 1800 ppm Metofluthrin, females of the 3600 ppm Metofluthrin and both sexes of the NaPB 1000 ppm groups. Furthermore, the tendency for decreases was observed in both sexes at 900 ppm Metofluthrin (Fig. 7). After the recovery period of 1 week,

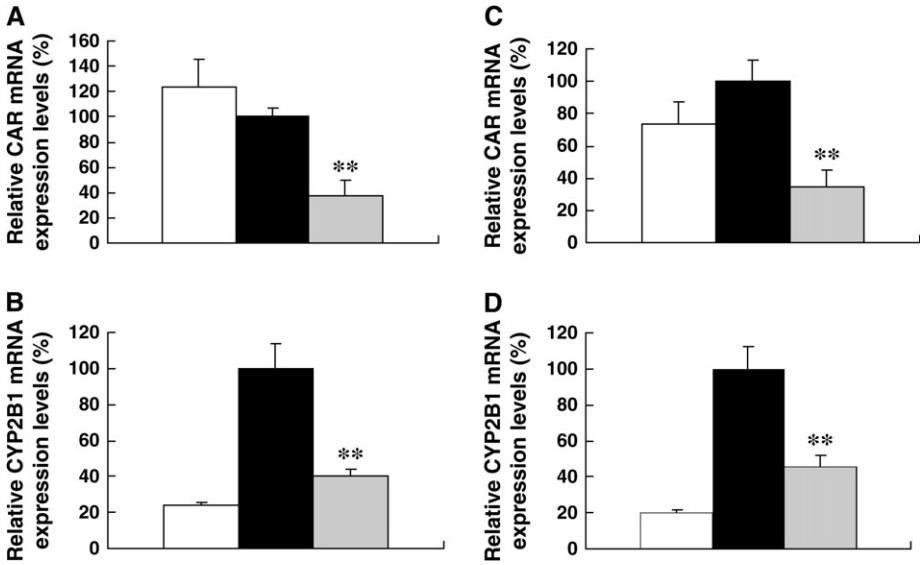

FIG. 5. CAR mRNA knockdown (A and C), and NaPB or Metofluthrin induced CYP2B1 mRNA expression levels (B and D). White columns indicate untreated groups (i.e., without siRNA, NaPB nor Metofluthrin). Black columns indicate Cont-siRNA–treated groups with NaPB (A and B) or Metofluthrin (C and D) treatment, respectively. Gray columns indicate CAR-siRNA–treated groups with NaPB (A and B) or Metofluthrin (C and D) treatment, respectively. CAR and CYP2B1 mRNA levels of groups with Cont-siRNA transfection are shown by 100%. Mean ± SD, $N = 4$. **Indicate statistically significant differences from the Cont-siRNA treated group ($p < 0.01$).

the distances in all treatment groups were comparable to the control group (data not shown).

Lipid peroxidation levels in liver were not markedly changed in any groups other than females receiving 1000 ppm NaPB. These results suggested that NaPB and Metofluthrin did not remarkably affect the MDA level under the conditions of this study. In both sexes, statistically significant increases or slight increases in total GSH and reduced GSH levels were observed in liver from the Treatment Group receiving 900, 1800, or 3600 ppm Metofluthrin or 1000 ppm NaPB (Fig. 8). These results suggested induction of antioxidant capacity by NaPB and Metofluthrin. After the recovery period of 1 week, the changes were generally comparable to the control group, excepting that significant decreases were observed in the total and reduced GSH levels in males that received 1000 ppm NaPB in the Recovery Group (data not shown).

In both sexes, cytoplasmic histone-associated DNA fragments as an apoptosis marker were not changed in any groups other than males receiving NaPB 1000 ppm from the Treatment Group (Supplementary Tables 7 and 8, Supplementary Fig. 3).

DISCUSSION

Different carcinogens may have different MOAs. Some MOAs lead to cancers in both experimental rodents and humans, but others that lead to cancers in rodents do not do so in humans, at least under realistic circumstances of human exposure. To improve the process of carcinogen hazard identification, and to avoid misidentification of harmless substances as possible human carcinogens, it has become imperative that a MOA analysis be undertaken and that data to support such analyses be collected in a thorough and scientifically rigorous manner (Cohen, 2004; Cohen *et al.*, 2003, 2004; Meek *et al.*, 2003; Rice, 2004). Based on the findings in the present studies, the MOA of Metofluthrin-induced rat liver tumor involves the effects of liver CYP induction and stimulation of increased hepatocellular proliferation.

The liver is by far the most common target tissue affected in the rodent bioassay (Gold *et al.*, 2001; McClain, 1994). For liver cancer, numerous DNA reactive and non-DNA reactive MOAs have been identified, including direct DNA damage, peroxisome proliferation, CYP induction, metal overload and oxidative damage, estrogenic activity or cytotoxicity and regeneration, some of which appear to be relevant to humans and some do not. These MOAs can readily be identified in short-term evaluations ranging from a few days to a few months (Cohen, 2004).

Short-term tests for genotoxic potential have demonstrated that Metofluthrin is not a genotoxic agent (unpublished observations), indicating that the MOA by which Metofluthrin induced liver tumor is nongenotoxic. The observations in general toxicity studies suggested that Metofluthrin may be a CYP inducer. Furthermore, Metofluthrin did not cause cytotoxicity or peroxisome proliferation, does not produce iron overload (evaluated by histopathology), and did not show evidence of estrogens activity (evaluated by histopathology of estogen-sensitive tissues). These findings support the hypothesis that the MOA by which Metofluthrin induces liver tumors appears to be similar to that of phenobarbital.

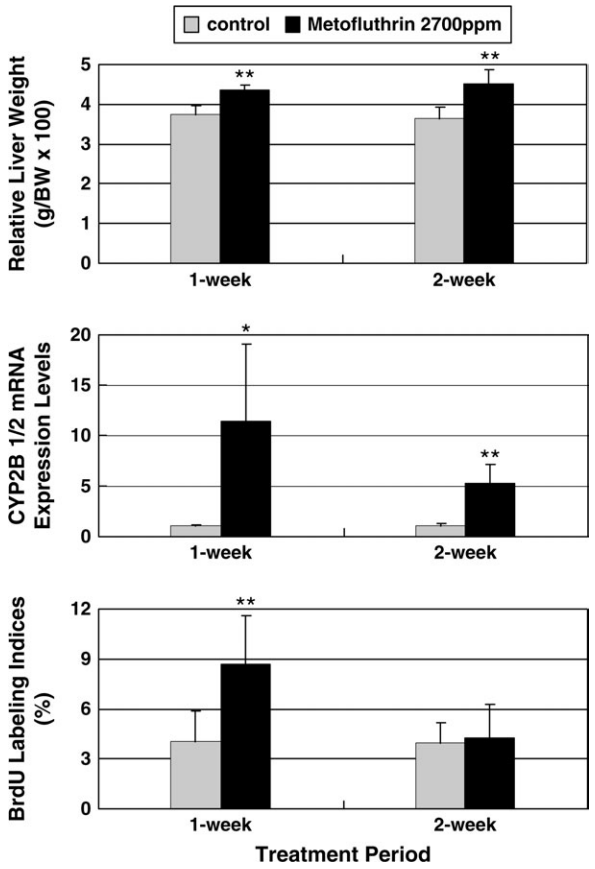

FIG. 6. Time-course alteration of liver weight, CYP2B1/2 mRNA expression levels and hepatocyte cell replicative DNA synthesis in the liver of rats treated with Metofluthrin for 1 or 2 weeks. Values represent group mean ± SD, $N = 5$. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

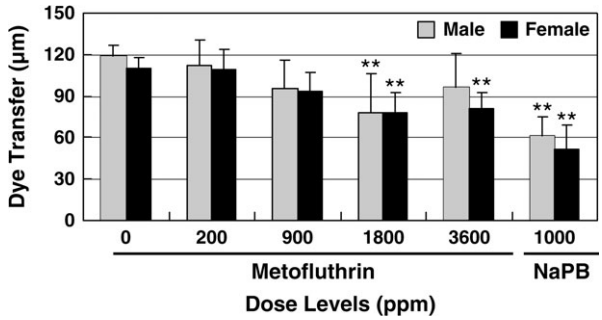

FIG. 7. Hepatic GJIC capacity by fluorescent dye transfer in the liver of rats treated with Metofluthrin or phenobarbital for 1 week. Values represent group mean ± SD, $N = 8$. **Indicates statistically significant differences from the control ($p < 0.01$).

The present studies were conducted to test this hypothesis. Central to this study is a comparison of the effects of Metofluthrin to those of phenobarbital. Phenobarbital is known to induce hepatic xenobiotic metabolism and to promote liver and thyroid tumors in rodents (IARC, 2001; McClain, 1994; Whysner *et al.*, 1996). The study with phenobarbital is important, because some of the biochemical changes seen with phenobarbital are rodent specific, exhibit a threshold, and have no relevance to human risk (McClain, 1994; Whysner *et al.*, 1996). Phenobarbital has been used therapeutically in humans as a sedative, hypnotic and antiepileptic for almost a century at exposure levels in humans similar to those producing tumors in rodents. Many patients receive high doses of phenobarbital daily over extended periods. Extensive epidemiological investigations of these patients receiving pharmacologically active doses have not shown any significant elevation of liver or thyroid tumors (IARC, 2001; Olsen *et al.*, 1989, 1995).

Based on global gene expression profile analysis in the present study, most genes with upregulated expression following Metofluthrin treatment were metabolic enzymes, including GSTs, CYPs, UGTs, etc., well known metabolic enzymes also induced by phenobarbital (Ganem and Jefcoate, 1998; Kodama and Negishi, 2006). However, CYP2B induction activity by Metofluthrin was much less potent than phenobarbital, consistent with the tumorigenic effects of Metofluthrin in rats appearing to be weaker than phenobarbital at achievable doses in rodent bioassays.

CYP2B induction has been shown to be associated with liver tumor formation in rodents for phenobarbital and related compounds (Holsapple *et al.*, 2006; Rice *et al.*, 1994; Whysner *et al.*, 1996) and involves activation of nuclear receptors, particularly CAR (Ueda *et al.*, 2002; Wei *et al.*, 2000; Yamamoto *et al.*, 2004; Yoshinari *et al.*, 2001). In the study reported here, phenobarbital treatment at 1000 ppm to both sexes resulted in significant inductions of mRNA levels of CYP2B, CYP3A1 and CYP3A2, and CYP2B protein levels as shown in previous studies (IARC, 2001). The treatment with Metofluthrin also produced significant increases in hepatic CYP2B mRNA (1800 ppm and higher in males, 900 ppm and higher in females), CYP3A1 mRNA (3600 ppm in males, 1800 ppm and higher in females), and CYP2B protein levels (3600 ppm in both sexes). Increased hepatic activity of 7-pentoxyresorufin *O*-dependentylase (CYP2B marker) was also confirmed in Metofluthrin 3600 ppm–treated rats.

In mice lacking CAR (CAR knockout mice), phenobarbital does not induce hepatic CYP2B isoforms, does not increase liver weight or stimulate replicative DNA synthesis and is not a tumor promoter, suggesting that CAR plays an essential role in the mouse liver carcinogenicity of phenobarbital (Wei *et al.*, 2000; Yamamoto *et al.*, 2004). Although there is no direct information for the role of CAR in rat liver carcinogenicity, the data from a number of MOA studies of phenobarbital, including CYP2B induction, increased cell proliferation, hepatocellular hypertrophy, etc., suggest that liver tumor promotion of phenobarbital in rats also appears to be mediated through CAR similar to mice. Because there are no rats lacking CAR (CAR knockout rats), the induction of CYP2B isoforms has been considered as an acceptable surrogate for CAR

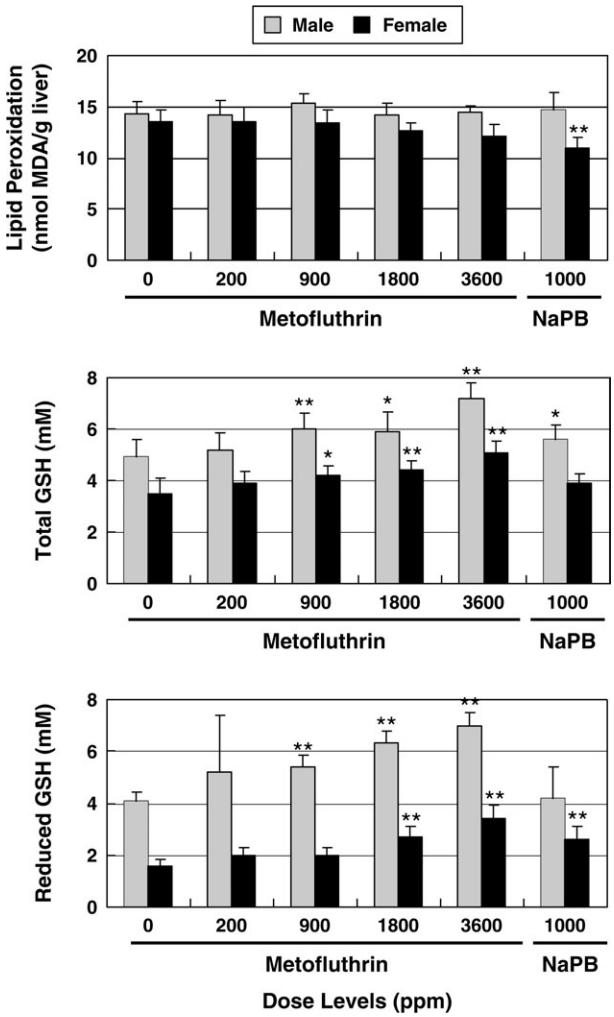

FIG. 8. Lipid peroxidation, total GSH and reduced GSH levels in the liver of rats treated with Metofluthrin or phenobarbital for 1 week. Values represent group mean ± SD, $N = 8$. * and ** indicate statistically significant differences from the control (*$p < 0.05$, **$p < 0.01$).

activation (Price *et al.*, 2007; Pustylnyak *et al.*, 2005; Yoshinari *et al.*, 2001). Recently, the RNAi technique was developed for sequence-specific gene silencing (Caplen *et al.*, 2001; Elbashir *et al.*, 2001; Fire *et al.*, 1998). Therefore, in this study, RNAi was applied to knockdown CAR mRNA in rat hepatocytes. Although phenobarbital and Metofluthrin did not lead to significant alteration in the level of CAR mRNA expression, both chemicals induced CYP2B1 mRNA in normal rat hepatocytes. However, both chemicals did not significantly induce CYP2B1 mRNA in CAR knockdown rat hepatocytes. This is the first direct evidence demonstrating that induction of CYP2B by phenobarbital or Metofluthrin is mediated through CAR in rat hepatocytes as well as mouse hepatocytes (Wei *et al.*, 2000; Yamamoto *et al.*, 2004).

Phenobarbital treatment resulted in significant increases of hepatocyte replicative DNA synthesis (evidenced by hepatocyte BrdU-labeling indices). This was also increased by the 1-week Metofluthrin treatment with 900 and 1800 ppm in males, and tended to increase at 1800 and 3600 ppm in females. No effects were observed at this endpoint in either sex given 200 ppm Metofluthrin. The observation of no increase of BrdU labeling in 3600 ppm males was consistent with no significant increase of liver weight. The reason for these findings is unknown, however, it was considered that severe toxic effects at 3600 ppm in male rats might induce non-specific alterations and cell proliferation might be suppressed. Moreover, treatment with Metofluthrin at 2700 ppm showed a significant increase of the BrdU-labeling index after a 1-week treatment period but not after 2 weeks of treatment, demonstrating that the Metofluthrin-induced DNA synthesis based on BrdU-labeling indices in rat liver is transient, consistent with observations by phenobarbital (Furukawa *et al.*, 2000; IARC, 2001; Jones and Clarke, 1993; Kolaja *et al.*, 1996; Whysner *et al.*, 1996).

Apart from the above, it was demonstrated that there is an inhibition of GJIC in male and female rats at a Metofluthrin dose level of 1800 ppm, but not at 900 ppm. This is a characteristic finding for non-DNA-reactive chemicals that produce hepatocellular tumors in rodents, including CYP inducers (Neveau *et al.*, 1990; Ruch and Klaunig, 1988). Though oxidative stress was not identified as a key event for liver tumor formation by phenobarbital and other CAR activators (Holsapple *et al.*, 2006; Whysner *et al.*, 1996), it has been implicated as an important factor in the carcinogenesis process for both genotoxic and nongenotoxic mechanisms (Klaunig and Kamendulis, 2004). Oxidative stress in the liver after administration of phenobarbital or Metofluthrin was assessed by MDA as an indicator of lipid peroxidation, as well as total glutathione (GSH + GSSG) and reduced GSH as a measure of antioxidant capacity. However, no evidence was obtained for the involvement of oxidative stress in phenobarbital or Metofluthrin-induced rat liver tumor formation. Unlike oxidative stress, inhibition of apoptosis is considered a key event in the MOA for phenobarbital-induced rat liver tumors (Holsapple *et al.*, 2006; Whysner *et al.*, 1996). An apoptosis marker (cytoplasmic histone associated DNA-fragments level) was not markedly changed with Metofluthrin or phenobarbital treatment in our studies. Indeed, in this study, inhibition of apoptosis was only observed in male and not in female rats given phenobarbital. The method employed for determining apoptosis in this study may lack the sensitivity of morphological procedures.

Previous studies have demonstrated that the effects of chronic administration of phenobarbital on rat hepatic xenobiotic metabolizing enzymes are reversible on cessation of treatment (Crampton *et al.*, 1977; Lake *et al.*, 1978). The current study demonstrated that most of the parameters induced by 1-week treatment with Metofluthrin or phenobarbital were similar to control levels within 1 week after cessation of chemical treatment. These findings suggest that non-neoplastic hepatic alterations induced by Metofluthrin are reversible, similar to the effects with phenobarbital.

The present study demonstrated that all hepatic effects of Metofluthrin are dose dependent and exhibit a threshold. At dose levels of 900 and 1800 ppm, Metofluthrin significantly induces hepatic xenobiotic metabolizing enzymes and replicative DNA synthesis in rats, with the pattern of induction being similar, but less marked, to that observed after treatment with phenobarbital 1000 ppm. In contrast to 900 ppm and higher dose levels of Metofluthrin, treatment with Metofluthrin at 200 ppm, a concentration not associated with carcinogenicity for both sexes in the Metofluthrin bioassay, had no effects on any of the hepatic parameters measured in this study.

In conclusion, the findings in the present study demonstrated that there is both direct and indirect evidence for CYP2B induction by Metofluthrin administration with biologically important consequences, demonstrating similarity between Metofluthrin and phenobarbital. Based on the evidence, including a comparison with the results with phenobarbital, acting by a similar MOA, it is reasonable to conclude that Metofluthrin will not have any hepatocarcinogenic activity in humans. In addition, the present studies suggest that functional genomics may provide reliable evidence for accurate categorization of test chemicals, and also that RNAi technique is a great tool for toxicological research.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Sumitomo Chemical Co., Ltd. (owns the patent on Metofluthrin).

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