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Developmental Toxicity and Genotoxicity Studies of 1,1,1,3,3,3-Hexachloropropane (HCC-230fa) in Rats

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ABSTRACT

The potential developmental toxicity and the *in vitro* and *in vivo* genotoxicity of HCC-230fa were assessed. In the developmental toxicity study, groups of 25 mated Crl:CD®(SD)BR rats were exposed (whole body) by inhalation to HCC-230fa over days 7-21 of gestation; the day of confirmed mating was designated as gestation day 1 (GD1). Exposures were 6 hours per day at concentrations of 0, 0.5, 2.5, or 25 ppm. Body weight, food consumption, and clinical observation data were collected during the study. On day 22 of gestation, the dams were euthanized and examined grossly. The fetuses were removed and subsequently weighed, sexed, and examined for external, visceral, head, and skeletal alterations. Evidence of maternal and developmental toxicity was observed at 25 ppm, and was noted as significant, compound-related reductions in mean maternal body weight, weight change, and food consumption. Significant fetal effects also were observed at 25 ppm as compound-related reductions in mean fetal weight and increased fetal malformations (filamentous tail, situs inversus, absent vertebrae) and variations (rudimentary cervical ribs, delayed sternebral ossification). There was no evidence of either maternal or developmental toxicity at 0.5 or 2.5 ppm. The genotoxicity of HCC-230fa was examined in a bacterial reversion assay and in erythrocyte micronucleus studies in two species by different routes of administration. No increases in the number of revertants were observed in the bacterial reversion assay. In one micronucleus study, HCC-230fa was administered by inhalation to rats as part of a 90-day study at doses indicated above. For the second study, ICR mice were given a single ip dose at 0, 166, 330 or 660 mg/kg. In both micronucleus studies, a significant increase in micronucleated erythrocytes was observed. The results of these studies suggest that HCC-230fa affects rapidly dividing cells and may have long-term consequences for occupational exposures.

INTRODUCTION

The chlorinated hydrocarbons have been the subject of exhaustive research and testing for many decades. The interest in this class of compounds stemmed from their use as solvents, blowing agents for plastics and as chemical intermediates. Over this long history of use and toxicological investigations, the liver has been the primary target organ for toxicity although effects in the kidney and central nervous system also have been observed. In contrast to the large body of data for the chloroethanes and chloromethanes, limited data have been published on the chlorinated propanes. Recently, we investigated the subchronic toxicity of 1,1,1,3,3,3-hexachloropropane (HCC-230fa) in rats (Bamberger et al., 2001). In that study, effects in the liver and kidney were observed at inhalation concentrations of 25 ppm (the highest level tested), with minimal effects observed in the testes. The liver effects observed with HCC-230fa in the subchronic toxicity study were generally consistent with those observed with other chlorinated propanes (Johannsen et al., 1988a, 1988b; Kolesar et al., 1995) tested at similar inhalation concentrations.

Prior to conducting the subchronic 90-day study, a 14-day range finding inhalation study was conducted with HCC-230fa (DuPont unpublished data, 1996). In that study groups of male rats were exposed 6 hr/day, 5 days a week to 10, 50, or 150 ppm of HCC-230fa vapors. Rats from all test groups had oligospermia with germ cell debris in the epididymides. Rats exposed to 150 ppm had severely decreased leukocyte counts at the end of the exposure period. Compound-related microscopic changes were also observed in the liver, lungs, spleen, thymus, mesenteric lymph node, sternal bone marrow (atrophy), seminal vesicles, testes, and epididymides in rats sacrificed after the final exposure. In addition, rats exposed to 50 and 150 ppm exhibited testicular degeneration characterized by loss of spermatogonia, scattered individual germ cell necrosis, and occasional germ cell debris within lumina. No other microscopic effects were seen in the 10 ppm exposure group. After a 14-day recovery period, the same microscopic changes that were present previously continued to be observed. Lesions in the testes and epididymides were more severe in the 150 ppm exposure animals at 14 days of recovery than after the final exposure, indicating continued progression of the testicular lesion. The testes after 14 days of recovery were essentially devoid of germ cell epithelium. The subchronic data suggested that HCC-230fa might be attacking rapidly dividing cells. Furthermore, because of the effects on sperm cells and the structural relationship to 1,2-dibromo-3-chloropropane (DBCP) there was concern for possible reproductive and developmental toxicity for HCC-230fa.

The purpose of the toxicology program on HCC-230fa was to develop occupational exposure guidelines for this compound. Given the large toxicology database on chlorinated hydrocarbons, it seemed reasonable to develop occupational exposure guidance levels for HCC-230fa based largely on those data. Indeed, a structure-activity relationship analysis using the database of the other chloropropanes suggested that the target organs for toxicity, and hence the occupational guidance, would be similar for this compound. Given the results of the 14-day study, however, it was clear that additional data were needed to assess other endpoints of toxicity for HCC-230fa. Although other studies were being considered at the time, e.g., multi-generation reproduction study, a developmental toxicity study was undertaken initially because of the structural similarity of HCC-230fa to other chlorinated hydrocarbons that produced developmental effects. In addition, because of the observed genotoxicity with some chlorinated hydrocarbons, a series of genotoxicity studies was conducted. A second purpose of this

toxicology program, therefore, was to compare the results from testing of other halogenated propane, ethane and methane analogs to the results of the HCC-230fa program to better understand the relationship between structure and toxicity.

MATERIALS AND METHODS

Test Material

HCC-230fa (CAS # 3607-78-1), also known as 2,2-dihydroperchloropropane, is a colorless liquid with a strong halocarbon odor. The chemical has a boiling point of 260°C at 760 mm Hg and a low vapor pressure (3.3 mm Hg at 57.9°C). The compound was supplied as a liquid by DuPont Fluoroproducts (Deepwater, NJ). The purity of the test substance, determined prior to study start and at the conclusion of the study, was >99.9%.

Animal Husbandry

For the developmental toxicity study, a total of 140 female (nulliparous and non-pregnant) Crl:CD®(SD)IGS BR rats were received from Charles River Breeding Laboratories (Raleigh, NC). The rats were approximately 63 days old on the day of arrival and were quarantined for 6 days and housed an additional three days prior to testing. Stock male rats of the same strain and from the same supplier were used for breeding. Rats were weighed and observed for clinical signs of disease during the quarantine and pretest periods. Rats were housed singly in suspended, stainless steel, wire-mesh cages in a Bioclean® room (laminar flow air circulation pattern).

For the inhalation micronucleus study, male and female rats were received from Charles River Breeding Laboratories (Raleigh, NC), and were approximately five weeks of age on the day of arrival. Rats used for this study were a subset of those used in the subchronic study reported by Bamberger et al. (2001). For studies using mice (ip micronucleus study), male (28-35 g) and female (23-31 g) ICR mice were obtained from Harlan Sprague Dawley (Frederick, MD). The mice were housed up to 5/cage (sexes separate) in polycarbonate cages maintained on stainless steel racks equipped with automatic watering manifolds. Heat-treated hardwood chips were used for bedding.

Animal rooms were maintained on a timer-controlled, 12-hour light/12-hour dark cycle. Environmental conditions of the rooms were targeted to be within a temperature range of 23 ± 2°C and a relative humidity range of 50 ± 10%. Animals were fed Purina Certified Rodent Chow®#5002 (rats) or Harlan TEKLAD Certified Rodent 7012C chow (mice) and tap water were available *ad libitum*. Feed and water were removed during inhalation exposures. Animals were humanely cared for and sacrificed according to principles provided by the Society of Toxicology as described in the "Guiding Principles in the Use of Animals in Toxicology".

Atmosphere Generation and Analysis

Vapor atmospheres of HCC-230fa were generated by metering the liquid test substance into a heated, Instatherm flask (146-183°C) with a Harvard Apparatus Model 22 Syringe Infusion Pump. Nitrogen, introduced into the flask, carried the HCC-230fa vapor through heated, non-reactive stainless steel lines (91-140°C) into an air stream which was directed into the top of the exposure chamber. The chamber concentration of HCC-230fa was controlled by varying the amount of HCC-230fa evaporated in the chamber air stream. Nitrogen and air streams were passed through the control chamber at approximately the same flow rates as those used in the exposure chambers. In addition, the nitrogen was passed through a heated Instatherm flask (107-147°C). Homogeneous distribution of the compound within the chamber was determined prior to initiating the study. The exposure chambers (New York University style) used on this study were constructed of stainless steel and glass and had a nominal internal volume of 1.4 m^3. The chambers were operated in a one-pass, flow-through mode with air flow rates adequate to provide sufficient oxygen for rats and enable adequate distribution of HCC-230fa in the chambers.

Chamber airflow was set to achieve at least 12 air changes per hour within the exposure chamber. Chamber temperature was targeted to 23 ± 1°C. Chamber relative humidity was targeted at 50 ± 10%. Chamber oxygen concentration was targeted to at least 19%. Airflow, temperature, and relative humidity were monitored continually with a Lander Control Systems Toxicology Monitoring System and were recorded at 15-minute intervals during each exposure. Percent oxygen was measured with a Biosystems Model 3100R Oxygen Monitor and recorded 2 times during each exposure.

The atmospheric concentration of HCC-230fa was determined by gas chromatography at approximately 45-minute intervals during each 6-hour exposure. Vapor samples were drawn by vacuum pump from two representative areas of the chamber where animals were exposed. Chamber atmosphere samples were directly injected into a Hewlett Packard Model 5880 Gas Chromatograph equipped with a flame ionization detector for determination of HCC-230fa concentration. All samples were chromatographed isothermally at 135°C on a 30 meter Alltech AT™-35 column. The atmospheric concentration of HCC-230fa was determined from a standard curve derived from vapor standards. The vapor standards were prepared prior to each exposure by injecting known volumes of liquid HCC-230fa into Tedlar® bags that contained known volumes of air.

Dose Level Selection

A pilot developmental toxicity study was conducted to assist in setting dose levels for the main developmental study (DuPont unpublished data, 1997). Groups of eight mated female rats were exposed by inhalation to concentrations of 0, 10, 50 or 100 ppm HCC-230fa over days 7-21 of gestation; the day of confirmed mating was designated as gestation day 1 (GD1). Except for reduced food consumption, no adverse effects were observed at 10 ppm. At 100 ppm, there was excessive maternal toxicity such that all of the animals were sacrificed *in extremis* on days 14 or 15, prior to the conclusion of the inhalation exposures. At necropsy, all of the animals were pregnant, but all litters were in the process of resorption. Significant maternal toxicity (reduced weight gains) was observed at 50 ppm, and an increased number of

resorptions and reduced live fetuses were observed. Among the surviving fetuses, increased variations and malformations were noted, and included gastroschisis, domed heads, and absent eyes. Although no effects were observed at 10 ppm in the pilot study, exposure concentrations of 0, 0.5, 2.5 and 25 ppm were selected for the inhalation developmental toxicity study. The low and intermediate concentrations were selected because of continued uncertainty associated with observing toxicological effects below 10 ppm, particularly since only eight animals had been used in the pilot study. Furthermore, since the data generated from this developmental toxicity study would be used in setting an occupational exposure limit, we wanted to ensure a no-observable-adverse-effect level (NOAEL) would be definitively established.

Two micronucleus studies were conducted. For one study, rats were exposed to HCC-230fa for 90 days. Dose levels for this study were 0, 0.5, 2.5 and 25 ppm as described in Bamberger et al. (2001). The positive control material, cyclophosphamide, was administered by oral intubation at a dose of 40 mg/kg (10 mL/kg) approximately 24 hr prior to the scheduled sacrifice.

The second micronucleus study was conducted in mice by intraperitoneal (ip) injection. Dose levels for the study were based on a pilot toxicity study conducted at dose levels of 400, 800, 1000 and 1200 mg/kg. Corn oil was the vehicle. At these dose levels, mortality and clinical signs of toxicity were observed at 800 mg/kg and above. An ip LD_{50} of 827 mg/kg was calculated by probit analysis. Based on these data, dose levels of 0, 166, 330 and 660 mg/kg, in corn oil (20 mL/kg), were used for the micronucleus study. Cyclophosphamide (60 mg/kg, 20 mL/kg) was administered ip as the positive control.

Experimental Procedures

Developmental Toxicity

Before exposures commenced on day 7 of gestation, females selected for the study were ranked by their gestation day (GD) 1 body weights and randomly assigned to control or experimental groups. Twenty-five mated females were assigned to each dosage group; the day of confirmed mating was designated as GD1. Body weights were recorded on days 1, and 7 through 22 of gestation. Food consumption was measured on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 22 of gestation. Individual clinical observations were recorded each morning and each afternoon of the exposure period (days 7-21 of gestation) and once on day 22 of gestation.

In addition to random assignment to dose groups, bias was controlled by coding the dams to group designation prior to scheduled euthanasia. The dams and litters remained coded for the collection of all postmortem and fetal data. On day 22 of gestation, females were euthanized by carbon dioxide asphyxiation and examined for gross pathologic changes. The uterus was opened and the types of implantations (live and dead fetuses and resorptions) and their positions were recorded. The uterus of each apparently nonpregnant rat was opened and stained with ammonium sulfide (Salewski, 1964) to detect early resorptions. Corpora lutea were counted and the number recorded for each ovary.

Live fetuses were weighed, sexed, and examined for external alterations. The first live fetus and thereafter every other fetus in each litter were decapitated and examined for visceral

alterations (Staples, 1974) and the sex was verified. Stages of renal papillary development were determined as described previously (Woo and Hoar, 1972). The heads were fixed in Bouin's fluid and examined (Barrow and Taylor, 1969). All externally malformed fetuses were examined for visceral alterations.

The remaining fetuses were sacrificed by an intraperitoneal injection of sodium pentobarbital. All fetuses were fixed in ethanol, eviscerated (if not done earlier during the visceral examination), macerated in 1% aqueous potassium hydroxide solution, stained with alizarin red S and examined for skeletal alterations (Staples and Schnell, 1964).

In Vitro Genotoxicity Studies

Four strains of *S. typhimurium* (TA1535, TA97a, TA98 and TA100) and one *E. coli* strain [WP2uvrA (pkm101)] were used. Bacterial growth was initiated in a 10-hour pre-culture, and sterility of the cultures was determined. The plates were exposed to HCC-230fa at concentrations of 0, 10, 50, 75, 100, 250 or 500 μg/plate closed vessels at 37° C for 48 hours. Toxicity was evident by the absence of colonies or the appearance of microcolonies at concentrations of 250 μg/plate and greater. Positive indicators used in the *Salmonella* studies included sodium azide (2 μg/plate), 2-aminoanthracene (1, 2 or 25 μg/plate), ICR-191 acridine (2 μg/plate), and 2-nitrofluorene (25 μg/plate). Methyl methanesulfonate (1000 μg/plate) was used in the *E. coli* study. The number of revertants was determined after the 48-hour incubation period.

Erythrocyte Micronucleus Studies

Rats used for the inhalation micronucleus study were a satellite group of rats used in the 90-day subchronic study (Bamberger, et al., 2001). Rats were exposed for 6 hours/day for 90 days to HCC-230fa concentrations of 0, 0.5, 2.5 or 25 ppm. Positive control animals, exposed to air only, were treated orally with 40 mg/kg of cyclophosphamide approximately 24 hr prior to sacrifice. All rats were killed by CO_2 asphyxiation. One femur was taken from each of ten randomly selected rats/sex/group, and bone marrow was collected by flushing with 0.2 mL fetal calf serum into a test tube containing fetal bovine serum; the total volume was about 1.5-2.0 mL. The collected cells were centrifuged at 200 x g for 5 min, and the excess serum aspirated from the tube. The cells were re-suspended in the remaining fluid. The cells were placed on microscopic slides, three slides per animal, and fixed in methanol for 8 min.

The slides were stained for 3 min with acridine orange (0.0125 mg/mL) in phosphate buffer (pH 7.2). At least 2000 polychromatic erythrocytes (PCE) per animal were examined for micronuclei. The frequency of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells was calculated, and the ratio of polychromatic to mature cells determined. Cellular inclusions that were irregularly shaped or stained, or out of the focal plane of the cell were considered artifacts and were not included in the cell counts. The unit of scoring was the micronucleated cell. PCEs with more than one micronucleus were scored as a single MNPCE. The frequency of micronuclei in polychromatic cells provides an index of induced genetic damage.

For the second micronucleus study conducted by ip injection, mice were allocated to groups of 15 per sex. For the high dose group (660 mg/kg) an additional five mice/sex were included as replacements in the event of mortality prior to the scheduled sacrifice. HCC-230fa (166, 330 and 660 mg/kg), the negative control (corn oil) and the positive control material (cyclophosphamide, 60 mg/kg) were administered as a single ip injection. The dose volume was 20 mL/kg. At 24, 48 or 72 hr following administration, five mice/sex/dose were killed by CO_2 asphyxiation. One femur was removed and bone marrow was collected by flushing with fetal calf serum into a test tube containing fetal bovine serum. The total volume was about 1 mL. The cells were centrifuged at 100 x g for 5 min, and excess serum aspirated from the tubes. The cells were then re-suspended in the remaining fluid. The cell suspension was placed on microscopic slides, 2-4 slides per animal, and fixed in methanol and stained with May-Gruenwald-Giemsa stain.

At least 1000 polychromatic erythrocytes per animal were examined for micronuclei. The frequency of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells was calculated, and the ratio of polychromatic to mature cells determined. The criteria applied for determination of micronuclei was the same as described above.

Statistical Analyses

Developmental Toxicity Study:

The litter (the proportion of affected fetuses per litter or the litter mean) was considered the experimental unit for statistical evaluation (Haseman and Hogan, 1975; Piegorsch and Haseman, 1991; Staples and Haseman, 1974). The level of significance selected was $p \leq 0.05$. Trend tests were sequentially applied to the data such that if a statistically significant dose response was detected, data from the top dosage group was excluded and the test was repeated until no statistically significant trend was detected (Selwyn, 1995). Maternal weight, weight change, and food consumption data were analyzed using a linear contrast of means from analysis of variance (Snedecor and Cochran, 1967). Incidence data (pregnancy, clinical observations) were analyzed for trend using the Cochran-Armitage test (Snedecor and Cochran, 1967). Litter data, corpora lutea counts, and incidences of fetal alteration data were analyzed by Jonckheere's test (Jonckheere, 1954). When more than 75% ties occurred in reproductive or fetal parameters, the asymptotic p-values generated by the standard large sample version of Jonckheere's test were not considered reliable. In these cases, an exact α value was calculated using permutation methodology (Patefield, 1982). For the analysis of mean fetal weight, an analysis of covariance was applied to test for any effect of dosage level on fetal weight while accounting for the variance contribution of overall litter size and sex ratio (Dempster, et al., 1984). Trends were detected by a linear contrast of least square means generated by the ANCOVA.

Genotoxicity Studies:

No statistical analyses were conducted for the bacterial reverse mutation assay. However, for this study, trials were evaluated independently and the average number and standard deviation of revertants were calculated. The test material was classified as positive when the average number of revertants in any strain was at least two-fold greater than the

average number of revertants in the negative control, and there was a positive dose-response relationship.

For the inhalation micronucleus study, data for percent micronucleated PCEs (MNPCEs) and proportion of PCEs among 2000 erythrocytes were transformed prior to analysis using the arcsine square-root function. This transformation is appropriate for percentages and proportions since the transformed data more closely approximate a normal distribution than do the nontransformed proportions (Sokal and Rohlf, 1969). Transformed data for each variable (MNPCE or PCE frequency) were analyzed separately for normality of distribution and equal variances using the Shapiro-Wilk's and Bartlett's tests, respectively (Shapiro and Wilk, 1965; Snedecor and Cochran, 1967). If the results of this test indicated that the transformed values for each variable were normally distributed in both sexes, parametric methods were used. If there was evidence of non-normality in either sex, nonparametric methods were employed for that variable using nontransformed proportions. Positive indicator data were not included in the evaluation of normality of distribution.

For the second micronucleus study conducted in mice, statistical significance was determined using the Kastenbaum-Bowman tables that are based on the binomial distribution (Kastenbaum and Bowman, 1970). The test material was considered positive if a dose-response was observed for MNPCEs and if one or more of the doses was significantly elevated above the control group.

For all of these studies, significance was judged at a level of $p \leq 0.05$.

RESULTS

Developmental Toxicity Study:

There was no evidence of a compound-related toxicity at either 0.5 or 2.5 ppm. There was evidence of significant, compound-related maternal and developmental toxicity at 25 ppm. At 25 ppm, mean maternal body weight was significantly reduced starting on day 9 and the reduction in mean body weight persisted until the end of the study (data not shown). The mean final body weight (day 22 of gestation) was about 10% lower than control body weight using the absolute or adjusted (final body weight adjusted for the products of conception) final weight. The decrease in body weight was also reflected in a reduced body weight gain of maternal animals at 25 ppm (Table 1). For the entire exposure period, days 7-22 of gestation, overall weight gain was 25% or 48% lower than the control, using either the absolute or adjusted final weight, respectively, to calculate weight gain. There was a consistent compound-related reduction in mean maternal food consumption at 25 ppm. When averaged over the entire dosing period, days 7-22 of gestation, mean food consumed was 11% lower than the control value. There were no compound-related increases in either maternal clinical observations or gross postmortem findings at 25 ppm.

At the lower exposure levels, 0.5 and 2.5 ppm, there were no compound-related effects on maternal weight, weight change, food consumption, clinical observations, or postmortem findings. However, at 2.5 ppm, mean maternal weight change was significantly reduced over

days 15-17. This reduction contributed to a slight reduction (about 6% lower than control) over the entire exposure period, days 7-22 of gestation. While this reduced maternal weight gain may be spurious, it is most likely reflective of the lower number of live fetuses per litter at this level rather than an actual compound-related effect. This conclusion is supported by the lack of any appreciable reduction (102% of control) in maternal weight gain when the adjusted final weight was used to calculate overall gain.

There were no early deaths of maternal rats (Table 2). A statistically significant reduction by trend analysis was observed for the total number of live fetuses at 2.5 and 25 ppm HCC-230fa. Although not statistically different from controls, there was a concomitant increase in total number of resorptions, accounted for by early resorptions, in rats at 2.5 and 25 ppm. However, no dead fetuses were observed. A 12% reduction in mean fetal weight was observed in fetuses at 25 ppm.

A statistically significant increase in fetal malformations at 25 ppm was observed (Table 3) and consisted of filamentous tail, situs inversus, and absent vertebrae. All fetuses in the 25 ppm group that exhibited any malformation had more than one malformation. Also at 25 ppm, a statistically significant increase in fetal variations consisting of rudimentary cervical ribs and delayed sternebral ossification was observed (Table 4). These variations were consistent with the decreased fetal weight seen at this exposure level. There were no compound-related effects on either fetal weight or on fetal malformations and variations at 0.5 or 2.5 ppm. There were no compound-related effects on total resorption incidence, early delivery incidence, implantation counts, live and dead fetus counts, resorption counts, or fetal sex ratio at any exposure level tested.

Genetic Toxicity Studies:

Two micronucleus studies were conducted with HCC-230fa, and in both studies HCC-230fa induced an increase in micronuclei (Tables 5 and 6). At the end of the 90-day inhalation study, a statistically significant increase in MNPCEs was observed in female rats exposed to 25 ppm HCC-230fa. Slight bone marrow cytotoxicity was noted in male rats at the high concentration suggesting that the compound was reaching the target tissue.

In the second study, reductions in PCEs were observed in some of the treated mice, primarily in the high dose group, indicting bone marrow cytotoxicity. Because of the significant reductions in PCEs at the high dose group, a full counting (1000 cells) could not be done for two female mice at 72 hr. A significant increase in MNPCEs was observed at all dose levels for male or female mice at 24, 48 and 72 hrs (Table 6). Indeed, at 72 hrs there was a dose-dependent increase in MNPCEs for female mice.

HCC-230fa concentrations of 10-5000 µg/plate were tested for mutagenicity and concentrations of 250 µg/plate and greater resulted in toxicity. However, no increase in revertants was observed at any of the concentrations tested (Table 7).

DISCUSSION

Exposure concentrations for the developmental toxicity study were the same as 90-day inhalation study (Bamberger, et al., 2001). During the conduct of the study, temperature, humidity, and oxygen levels were maintained within acceptable ranges (data not shown). Daily mean exposure concentrations were well within the target exposure concentrations, and varied only by approximately 1% throughout the study (data not shown).

Maternal toxicity was observed in the developmental toxicity study at 25 ppm, and was observed as a significant decline in body weight gain and food consumption. No other maternal effects were observed at 25 ppm HCC-230fa, and no maternal toxicity was evident at the lower concentrations. Developmental toxicity studies with other chlorinated propanes are limited. In an unpublished study, Schroeder (1985) administered by gavage 1,1,2,2,3-pentachloropropane to timed-pregnant rats on days 6-15 of gestation. No effects were observed on reproductive outcomes in this study. Similarly, no maternal effects were observed in timed pregnant rats or rabbits treated by gavage with 1,2-dichloropropane (Kirk et al., 1995). Also, for the shorter chain chlorinated methanes and ethanes, no maternal effects were observed. Several investigators have reported no maternal effects with 1,1,1-trichlorethane or 1,2-dichloroethane when administered in drinking water to rats or mice, and no maternal effects were noted in rats in an inhalation developmental toxicity study with 1,2-dichloromethane (George et al., 1989; Lane et al., 1982; York et al., 1982; Hardin and Manson, 1980). In an inhalation study in rats with 1,2-dichloroethane, no maternal effects were observed (Payan et al., 1995).

Incorporation of a double bond into the alkane backbone does not seem to change the maternal toxicity of this class of compounds. In inhalation rat studies with 1,2-dichlorothylene or trichloroethylene, no maternal effects were observed (Hurtt et al., 1993; Dorfmueller et al., 1979). Furthermore, increasing the length of the carbon backbone does not appear to change the maternal toxicity. No maternal effects were observed in an inhalation study with 1,2-dichlorobutene or in an oral gavage study with 1-hexene (Kennedy et al., 1982; Gingell et al., 2000). Maternal toxicity was, however, observed in rats and rabbits in an inhalation study with 1,2-dichloropropene at concentrations of 60 and 120 ppm administered on days 6-15 (rats) or days 6-18 (rabbits) of gestation (Hanley et al., 1987).

Overall, with this class of compounds, one could reasonably predict that no maternal toxicity would be observed with short chain chlorinated alkanes or even alkenes. Introduction of fluorine into these simple short chain molecules also does not result in maternal effects (Rusch et al., 1999, 1995; Brock et al., 1995, 1996). With the introduction of bromine into the molecule, however, an increase in the toxicity seems apparent. In an inhalation study in rats and mice with 1,2-dibromoethane (Short et al., 1978), significant maternal toxicity, reduced body weight gain and food consumption were observed along with a significant increase in resorptions at high inhalation doses (80 ppm). In this study, the experimental animals (rat) were exposed to the compound 23 hr/day which could lead to increased stress on the animal and thereby contribute to the toxicity. Complete developmental toxicity studies for the brominated propanes have not been published although 1- and 2-bromopropane are receiving attention because of concerns over the

reproductive toxicity of these compounds in Korean workers and effects on ovulation (Sekiguchi et al., 2002; Takeuchi et al., 1997).

In the current developmental toxicity study, there was a significant reduction in the number of live fetuses (13.7 and 14.3, respectively) at 2.5 and 25 ppm HCC-230fa. However, this finding is not considered to be compound related for several reasons. First, there was no clear dose response relationship. Secondly, while the value for the 2.5 ppm group was below the lower end of the historical control range, the value observed in the high dose group was at the low end of the historical control values (14.3-16.4) for our laboratory. Finally, there was no evidence of embryolethality (dead fetuses or resorptions), a finding that would contribute to a compound-related reduction in live fetuses. Rather the reduction in live fetuses is considered related to a combination of the slight reduction in the number of implantations and the very slight increase in resorptions. The historical values for the laboratory for the number of implantations range from 14.5-17.4, and the historical control values for resorptions per litter range from 0.2-1.3. Clearly, the values observed in the current study are well within these historical control values.

Significant increases in the number of malformations and fetal variations were observed at 25 ppm and were considered to be compound related. No effects were observed at the lower concentrations. Although some of these malformations and variations are normally observed in control populations, a malformation of situs inversus is a rare occurrence. Because the incidence of this finding was increased, this finding along with other variations and malformations, e.g., filamentous tail, reduced sternebrae ossification, absent vertebrae, are considered related to exposure.

No fetal effects were observed in either rats or rabbits administered 1,2-dichloropropane by gavage (Kirk et al., 1995). Hanley, et al. (1987) also found no fetal effects, in the absence of maternal toxicity (see above) when 1,2-dichloropropene was administered to either rats or rabbits. No developmental toxicity studies have been conducted with other chlorinated propanes. Also, Schroeder (1985) reported no fetal malformations or fetal toxicity in rats administered pentachloropropane. No other developmental toxicity studies with chlorinated propanes have been reported.

No biologically meaningful developmental effects were reported for 1,2-dichloroethane or dichloroethylene when administered to rats or rabbits by inhalation or via drinking water (Kirk et al., 1995; George et al., 1989; Hurtt et al., 1993). Ruddick and Newsome (1979) investigated the developmental toxicity of DBCP. In that study, DBCP, which has well established reproductive effects, did not produce fetal malformation although fetal toxicity (reduced body weights) was observed. When fluorine is added to the propane backbone (1,1,1,3,3-pentafluoropropane or 1,1,1,3,3,3-hexafluoropropane), no developmental effects are produced (Rusch et al., 1999; Brock et al., 2000). In contrast, 1-bromopropane caused a reduction in implantation rates and numbers of pups in rats (Takeuchi, et al., 2001). Fetal malformations or alterations have not been reported for 1- or 2-bromopropane.

Overall, it appears that most chlorinated propanes and ethanes are not developmentally toxic, or are the fluorine substituted propanes. HCC-230fa seems to be unique in its biological

effects on the developing fetus. From a structure-activity perspective, the introduction of bromine and chlorine, compared with fluorine, into an alkane backbone increases the general toxicity of these compounds. This is not overly surprising since it has been well established that brominated compounds tend to be more toxic than their chlorine counterparts and, more so, than their fluorine congener. Indeed, this concept of reduced toxicity with the addition of fluorine has been well recognized for many years and served as a basis for the manufacture and use of chlorofluorocarbons (CFC) and the CFC alternatives in a variety of commercial applications. From a mechanistic point of view, the results of this and previously reported studies (Bamberger et al., 2001) suggests that HCC-230fa affects rapidly dividing cells. Precisely how this toxicity might occur remains unknown but may be related to the metabolic conversion to a chlorinated ketone. Some chlorinated ketones are known to be quite toxic causing effects on testicular function as well as inducing developmental toxicity (Becci et al., 1982; Britelli et al., 1979; John et al., 1982). The toxicity may be due to metabolism around the central carbon of the propane or propene backbone, and this may further explain the slightly greater toxicity of the chlorinated propenes. A concern for HCC-230fa was the potential for this compound to be metabolized to heaxchloroacetone, a compound suspected of being a reproductive toxicant, and possibly a developmental toxicant (Kaiser et al., 1998), and its structural similarity to DBCP. Initial metabolism studies with HCC-230fa indicated that the compound is not metabolized to a ketone (Sumner et al., 2000). This study, however, was rather limited so that additional metabolism studies with HCC-230fa are needed to further examine a possible metabolic role in a mechanism of toxicity.

In the genotoxicity studies, HCC-230fa did not induce mutagenic effects in the bacterial assay. However, in the micronucleus evaluations, an increase in micronuclei was observed in both studies. It is noteworthy that a positive response to HCC-230fa was observed via two routes of administration and in two species. Several investigators have examined the induction of micronuclei when a compound was administered by the oral or ip route, and found no significant differences. Urethane, N, N-dimethylurethane, benzene (Ashby et al., 1990; Tice et al., 1989) and several other chemicals have been examined in mice in micronuclei assays by both the ip and oral route. Although lower doses of the compound were needed to induce micronuclei by the ip route, compared to the oral route, positive responses were observed. Species and strain differences also have been examined for the induction of micronuclei. Luke et al. (1988) showed that the induction of micronuclei by benzene was dependent on the strain of mice following a 13-week inhalation exposure. The induction of micronuclei in DBA/2 mice was generally greater than in B6C3F1 or C57B1/6 mice, although a positive response was observed in all three strains. Also, other investigators examined the differential response to the induction of micronuclei in rats and mice. Simula and Priestly (1992), Madle et al. (1986) and others (Shimada et al., 1992; Holden et al., 11997) showed no differences in response to chemicals (mitomycin C, benzo(a)pyrene, cyclophosphamide) for the induction of a positive micronuclei response. In contrast, however, Wakata et al. (1998) demonstrated for some chemicals (benzene, 5-fluorouracil, etc.) that the mouse was, at times, 3-fold more sensitive to the induction of micronuclei in bone marrow cells than the rat when the compound was administered by the same route.

Another interesting observation was the apparent delayed effect observed with micronuclei induction by HCC-230fa (see Table 6). Although this occurred only in females, the

maximum increase in MNPCEs at 660 mg/kg occurred at the 72 hr harvest. A delay in maximum micronuclei formation has been observed with other compounds that are known anti-metabolites, e.g. 5-fluorouracil, 6-mercaptopurine (Hayashi et al., 1994; CSGMT, 1990). This limited observation with HCC-230fa further supports our supposition that this compound may be acting as an anti-metabolite. This supposition was raised in our previous publication (Bamberger et al., 2001).

In the current study, our results appear to be consistent with those of others showing an effect via two routes of exposure, although the response by inhalation was much less than that for ip injection. An obvious difference in response between the two species is related to dose. At the high concentration in the subchronic inhalation study (25 ppm), toxicity of the liver, kidney, testes and other organs was quite evident (Bamberger et al., 2001), but no clear evidence of bone marrow toxicity or effects on hematological parameters were observed. At the higher concentrations used in the 14-day pilot study, bone marrow atrophy was observed in rats exposed to 150 ppm clearly indicating that the compound is reaching the target tissue for induction of micronuclei. Thus, the difference in response between the rat and mouse is most likely related to the dose of HCC-230fa, and the concentrations used in the 90-day study were not sufficiently high to detect clear genotoxic effect. Alternatively, the species differences observed in the current study with HCC-230fa might be due to toxicokinetic differences between the rat and mouse. Sumner et al., (2000) have examined the metabolism of HCC-230fa in the rat but not the mouse. Clearly, additional work would be needed to further examine this relationship.

Genotoxicity studies with chlorinated propanes have been limited. 1,2,3-Trichloropropane was positive in a bacterial reversion assay (Ratpan and Plaumann, 1988), but no other studies have been reported for the other chlorinated propanes. However, brominated and mixed chlorinated and brominated propanes have been examined in a number of assays. Positive results in sister chromatid exchange and bacterial reversion assays have been observed with di- and tribromopropanes and DBCP (Belitsky et al., 1994; Ratpan and Plaumann, 1988; Lag et al., 1994). However, the addition of fluorine to the propane molecule tends to stabilize and reduce the genotoxic potential of these compounds. Negative genotoxicity studies have been reported for hexafluoropropane and pentafluoropropane (Rusch et al., 1999; Brock et al., 2000). Like the findings with the developmental toxicity studies, addition of bromine to the three carbon chain results in greater mutagenic activity than the addition of fluorine (Lag, et al., 1994).

In summary, the inhalation administration of HCC-230fa produced evidence of maternal and developmental toxicity in rats at 25 ppm. Developmental toxicity was evidenced by a significant, compound-related reduction in mean fetal weight and a significant increase in fetal malformations (filamentous tail, situs inversus, absent vertebrae) and variations (rudimentary cervical ribs, delayed sternebral ossification). There was no evidence of either maternal or developmental toxicity at 0.5 or 2.5 ppm. HCC-230fa produced no increase in the number of revertants in the bacterial assay. However, in both micronucleus studies, a significant increase in micronucleated erythrocytes was observed. The results of these studies further suggest that HCC-230fa may affect rapidly dividing cells and may have long-term consequences for occupational exposures.

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Table 1
Maternal Body Weight Changes of Rats Exposed by Inhalation to HCC-230fa

Days of Gestation	Control (0 ppm)	0.5 ppm	2.5 ppm	25 ppm
1-7	22.4 (6.85)[a]	19.0 (7.14)	18.3 (6.43)	19.3 (7.40)
7-9	4.4 (4.76)	4.4 (5.54)	2.7 (4.60)	-0.4 (5.70)
9-11	8.3 (4.01)	10.2 (3.75)	9.0 (4.26)	6.4 (5.05)
11-13	9.7 (3.74)	10.4 (5.69)	9.5 (4.87)	5.7 (5.70)*
13-15	10.1 (2.52)	9.3 (6.38)	11.3 (5.46)	8.2 (5.68)*
15-17	17.6 (4.90)	16.5 (4.59)	14.3 (6.79)*	12.6 (12.5)*
17-19	29.3 (4.97)	27.8 (4.92)	27.7 (5.55)	27.2 (12.76)
19-21	30.1 (4.42)	32.1 (6.29)	28.7 (6.34)	21.7 (6.70)*
21-22	32.4 (6.51)	33.6 (5.97)	30.4 (11.48)	24.5 (5.29)*
7-22	141.9 (12.72)	144.3 (14.51)	133.5 (21.09)	105.9 (13.32*
7-22[b]	44.8 (9.31)	49.3 (9.18)	45.7 (14.28)	23.2 (7.82)*

* Significant trend, $p \leq 0.05$
[a] Mean ($\pm$ standard deviation)
[b] Weight changes calculated using final body weight minus products of conception.

Table 2

Reproductive Outcome of Rats Exposed by Inhalation to HCC-230fa[a]

Concentration (ppm):		0	0.5	2.5	25
No. Mated		25	25	25	25
No. Pregnant		25	25	22	24
No. Early Deliveries		0	0	0	0
No. Deaths		0	0	0	0
No. With Total Resorptions		0	0	0	0
No. Litters		25	25	22	24
Means Per Litter					
Mean Corpora Lutea		17.7 (3.2)[c]	16.6 (2.6)	16.0 (2.4)	16.9 (2.5)
Implantations		15.4 (2.0)	15.1 (1.8)	14.5 (2.3)	15.0 (2.2)
Live Fetuses:	Total	15.1 (1.8)	14.6 (1.8)	13.7 (2.6)*	14.3 (2.4)*
	Males	8.2 (1.9)	6.7 (2.1)	6.9 (2.1)	7.1 (2.7)
	Females	6.9 (2.1)	8.0 (2.0)	6.8 (1.7)	7.2 (1.9)
Resorptions:	Total	0.3 (0.6)	0.4 (0.6)	0.9 (1.0)	0.7 (1.1)
	Early	0.3 (0.6)	0.4 (0.6)	0.9 (1.0)	0.7 (1.1)
	Late	0.0	0.0	0.0	0.0
Dead Fetuses		0.0	0.0	0.0	0.0
Mean Fetal Weight:	Total	5.04 (0.32)	5.09 (0.25)	5.10 (0.30)	4.43 (0.25)*
Sex Ratio[b]		0.54	0.45	0.50	0.49

[a] timed pregnant rats were exposed to 6 hours per day on gestation days 7-22.
[b] number male fetuses/total number fetuses per litter.
[c] Mean (± standard deviation)
* Significant trend

Table 3

Incidence of Fetal Malformations

Concentration (ppm):	0	0.5	2.5	25
No. Examined[a]	377(25)	366(25)	301(22)	344(24)
No. Affected	0 (0)	0 (0)	1 (1)	6 (5)
Abdomen – Gastroschisis	...[b]	…	...	1(1)
Tail				
Filamentous	…	...	...	2(2)*
Kinked	…	...	...	1(1)
Heart &/or Greater Vessels				
Septal Defect	…	…	1(1)	…
Dextrocardia	…	...	...	1(1)
Thorax - Situs Inversus	...	...	…	2(2)*
Brain – Distended Ventricles	…	…	…	1(1)
Anophthalmia	…	…	…	1(1)
Rib – Fused	…	...	...	1(1)
Vertebra				
Absent	…	...	...	2(2)*
Fused	…	…	…	1(1)

[a]Number examined and affected, including the number affected with the listed malformations, are expressed as Fetuses (Litters).
[b]For ease of reading, zeros have been replaced with dashes for the listed malformations.
*Significant trend

Table 4

Incidence of Fetal Variations

Concentration (ppm):	0	0.5	2.5	25
No. Examined[a]	377(25)	366(25)	301(22)	344(24)
No. Affected	195 (25)	163 (25)	124 (25)	174 (22)
Rib - Rudimentary Cervical	5(4)	...[b]	3 (2)	27(12)*
Sternebra- Misaligned	...	…	2(2)	1(1)
Heart and/or Greater Vessels - Patent Ductus Arteriosus	2(2)	1(1)	3(3)	4(4)
Kidney, Papilla	84(24)	60(22)	43(18)	34(19)
- Small Papilla - Size 1	7(6)	6(4)	2(2)	2(2)
- Small Papilla - Size 2	37(16)	25(17)	18(14)	15(12)
- Papilla - Size 3	41(21)	29(16)	24(14)	17(13)
Skull - Retarded Ossification	14(7)	6(4)	10(5)	10(7)
Sternebra - Retarded Ossification	16(5)	5(4)	7(4)	33(11)*
Vertebra - Retarded Ossification	111(25)	106(21)	72(21)	115(24)

[a] Numbers examined and affected, including the numbers affected with the listed variations, are expressed as Fetuses (Litters).

[b] For ease of reading, zeros have been replaced with dashes for the listed variations.

* Significant trend

Table 5

Frequency of Micronuclei in Bone Marrow Of Rats Exposed by Inhalation to HCC-230fa[a]

Concentration (ppm)	Sex	Mean Percent PCE	Mean Percent MNPCE
0 [b]	M F	48.5 (9.2)[c] 45.7 (11.5)	0.29 (0.18) 0.30 (0.23)
0.5	M F	47.5 (9.3) 48.0 (7.2)	0.36 (0.23) 0.31 (0.21)
2.5	M F	44.7 (6.15) 43.3 (5.25)	0.18 (0.12) 0.24 (0.11)
25	M F	37.2 (7.84)* 49.3 (8.19)	0.29 (0.17) 0.50 (0.19)*
CP (40mg/kg)	M F	40.0 (10.5) 34.9 (10.5)	2.65 (3.45)* 1.16 (0.56)*

[a] Two thousand polychromatic erythrocytes (PCE) per animal were scored for micronuclei; MNPCE – micronucleated PCE. For each animal the proportion of PCEs among 1000 erythrocytes were counted.

[b] Rats (10/sex/group) were exposed 6 hours per day, 5 days/week for 90 days. Cyclophosphamide, as the positive control, was administered 24 hours prior to the scheduled sacrifice.

[c] Mean values were determined based on 10/sex/dose group. Values in parenthesis are standard deviations.

* Statistically significant difference from control, $p \leq 0.05$.

Table 6

Frequency of Micronuclei in Bone Marrow of Mice Treated with HCC-230fa by ip Injection[a]

Treatment (mg/kg)	Sex	Time (hr)	Mean Percent PCE	Mean Percent MNPCE
Corn Oil[b]	M F	24 24	58 (9.0) 65 (11.0)	0.08 (0.11) 0.08 (0.11)
166	M F	24 24	55 (4) 55 (4)	0.10 (0.10) 0.28 (0.30)
330	M F	24 24	57 (7) 69 (7)	0.5 (0.28)* 0.48 (0.53)*
660	M F	24 24	62 (6) 52 (9)	0.14 (0.06) 0.20 (0.20)
CP, 60	M F	24 24	50 (8) 52 (4)	3.22 (1.85)* 2.78 (0.97)*
Corn Oil	M F	48 48	54 (9) 53 (11)	0.04 (0.09) 0 (0)
166	M F	48 48	53 (2) 51 (9)	0.12 (0.16) 0.34 (0.43)*
330	M F	48 48	55 (2) 56 (2)	0.46 (0.38)* 0.12 (0.08)
660	M F	48 48	55 (2) 51 (14)	0.2 (0.16) 0.84 (0.79)*
Corn Oil	M F	72 72	58 (2) 65 (5)	0.12 (0.04 0.06 (0.06)
166	M F	72 72	53 (10) 61 (4)	0.06(0.09) 0.04 (0.06)
330	M F	72 72	52 (8) 58 (5)	0.26 (0.24) 0.48 (0.24)*
660	M F	72 72	41 (18) 19 (21)	0.14 (0.11) 1.48 (2.11)*[c]

[a] One thousand polychromatic erythrocytes (PCE) per animal were scored for micronuclei; MNPCE – micronucleated PCE. For each animal the proportion of PCEs among 1000 erythrocytes were counted. Standard deviations are in parenthesis.

[b] Mice (5/sex/group) were treated once with HCC-230fa by ip injection, and bone marrow was collected 24, 48 and 72 hrs after dosing.

[c] Only 3850 cells were evaluated due to bone marrow toxicity.

* $p \leq 0.05$ (Kastenbaum-Bowman Tables)

Table 7
Mean Number of Revertants in *S. typhimurium* and *E. coli* Following Exposure to HCC-230fa[a]

	TA 98		TA 97a		TA 100		TA 1535		WP2uvrA(pKM101)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Concentration (µg/plate)	Without S-9									
0	18	12	81	86	129	91	13	14	119	116
10	16	14	74	93	114	77	13	12	137	115
50	11	11	75	95	92	75	8	9	121	103
75	14	174	71	84	102	76	12	10	160	140
100	13	12	83	79	98	78	11	9	136	147
250	--[b]	7	8	33	--[b]	11	--[b]	5	75	73
500	0	--[b]	--[b]	3	0	2	--[b]	0	--[b]	18
NAAZ (2 µg/plate)[c]	-	-	-	-	711	580	346	369	--	-
ICR191 (2 µg/plate)	-	-	1339	1263	-	-	-	-	-	-
2NF (25 µg/plate)	1471	1274	-	-	-	-	-	-	-	-
MMS (1000 µg/plate)	-	-	-	-	-	-	-	-	1703	1051
Concentration (µg/plate)	With S-9									
0	19	13	124	95	136	104	12	12	124	113
10	17	18	118	97	124	124	9	10	141	135
50	18	13	126	84	128	115	10	9	152	136
75	14	16	110	88	110	104	10	11	157	141
100	15	21	110	110	112	115	10	12	142	148
250	4	6	33	43	20	43	--[b]	6	93	70
500	--[b]	--[b]	0	8	0	8	--[b]	4	--[b]	48
2-AA (1 µg/plate)	-	-	-	-	998	1200	269	447	-	-
2-AA (2 µg/plate)	1592	1451	1299	883	-	-	-	-	-	-
2-AA (25 µg/plate)									1321	789

[a] Mean of triplicate cultures
[b] Colony formation was reduced or absent
[c] NAAZ – sodium azide; ICR191 – ICR-191 acridine; 2-AA – 2-aminoantracene; MMS – methylmethanesulfonate; 2-NF – 2-aminofluorene