Evaluation of the human relevance of the constitutive androstane receptor-mediated mode of action for rat hepatocellular tumor formation by the synthetic pyrethroid momfluorothrin

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ABSTRACT — High dietary levels of the non-genotoxic synthetic pyrethroid momfluorothrin increased the incidence of hepatocellular tumors in male and female Wistar rats. Mechanistic studies have demonstrated that the mode of action (MOA) for momfluorothrin-induced hepatocellular tumors is constitutive androstane receptor (CAR)-mediated. In the present study, to evaluate the potential human carcinogenic risk of momfluorothrin, the effects of momfluorothrin (1-1,000 μM) and a major metabolite Z-CMCA (5-1,000 μM) on hepatocyte replicative DNA synthesis and CYP2B mRNA expression were examined in cultured rat and human hepatocyte preparations. The effect of sodium phenobarbital (NaPB), a prototypic rodent hepatocarcinogen with a CAR-mediated MOA, was also investigated. Human hepatocyte growth factor (hHGF) produced a concentration-dependent increase in replicative DNA synthesis in rat and human hepatocytes. However, while NaPB and momfluorothrin increased replicative DNA synthesis in rat hepatocytes, NaPB, momfluorothrin and Z-CMCA did not increase replicative DNA synthesis in human hepatocytes. NaPB, momfluorothrin and Z-CMCA increased CYP2B1/2 mRNA levels in rat hepatocytes. NaPB and momfluorothrin also increased CYP2B6 mRNA levels in human hepatocytes. Overall, while NaPB and momfluorothrin increased replicative DNA synthesis in rat hepatocytes, NaPB, momfluorothrin and Z-CMCA did not increase replicative DNA synthesis in human hepatocytes. NaPB, momfluorothrin and Z-CMCA increased CYP2B1/2 mRNA levels in rat hepatocytes. NaPB and momfluorothrin also increased CYP2B6 mRNA levels in human hepatocytes. Overall, while momfluorothrin and NaPB activated CAR in cultured human hepatocytes, neither chemical increased replicative DNA synthesis. Furthermore, to confirm whether the findings observed in vitro were also observed in vivo, a humanized chimeric mouse study was conducted. Replicative DNA synthesis was not increased in human hepatocytes of chimeric mice treated with momfluorothrin or its close structural analogue metofluthrin. As human hepatocytes are refractory to the mitogenic effects of momfluorothrin, in contrast to rat hepatocytes, the data support the hypothesis that the MOA for momfluorothrin-induced rat liver tumor formation is not relevant for humans.

Key words: Carcinogenesis, Liver tumor, Nongenotoxic, MOA, Cell proliferation, Humanized mice

INTRODUCTION

epsilon-Momfluorothrin (CAS-No. 1065124-65-3, 2,3,5,6-Tetrafluoro-4-(methoxymethyl)benzyl (Z)-(1R,3R)-3-(2-cyanopropl-1-enyl)-2,2-dimethylcyclopropanecarboxylate, referred to as momfluorothrin in this article) is a non-genotoxic type I pyrethroid (ECHA, 2014). Two-year treatment with momfluorothrin produced hepatocellular tumors in RccHan:WIST rats at 1,500 and 3,000 ppm for males and 3,000 ppm for females.
In our previous paper (Okuda et al., 2017), we evaluated the mode of action (MOA) for rat hepatocellular tumor formation by momfluorothrin based on the frameworks which have been developed through the International Life Sciences Institute (ILSI) (Cohen et al., 2004; Meek et al., 2003; Seed et al., 2005) and the International Programme on Chemical Safety (IPCS) (Boobis et al., 2006, 2008; Sonich-Mullin et al., 2001). This MOA study demonstrated that momfluorothrin produces liver hypertrophy, induces hepatic microsomal cytochrome P450 (CYP) 2B enzymes and increases hepatocellular proliferation in wild-type rats but not in constitutive androstane receptor (CAR) knockout rats (Okuda et al., 2017). Moreover, alternative MOAs for momfluorothrin-induced rat hepatocellular tumor formation including cytotoxicity, activation of the peroxisome proliferator-activated receptor alpha (PPARα), accumulation of iron, statin-like effects and hormonal perturbation have been excluded (Okuda et al., 2017). These findings demonstrate that the MOA for hepatocellular tumor formation by momfluorothrin is the same as that of some other nongenotoxic mitogenic CAR activators such as metofofluthrin a close structural analogue to momfluorothrin (Yamada et al., 2009) and phenobarbital (PB) (Elcombe et al., 2014).

Since increased hepatocellular replicative DNA synthesis is considered to be the critical key event for CAR-mediated hepatocellular tumorigenesis (Elcombe et al., 2014), it is important to determine whether the test compound has a mitogenic effect on human hepatocytes or not. We have provided some experimental data showing a key species difference; those were that while PB stimulates replicative DNA synthesis in rodent hepatocytes, such proliferative effects are not observed in cultured human hepatocytes (Yamada et al., 2015; Hirose et al., 2009) nor in human hepatocytes of chimeric mice (Yamada et al., 2014). The lack of this key event in humans strongly supports the previous conclusion that the rodent carcinogenicity by CAR activators such as PB is not relevant to humans based on experimental and epidemiology data (Elcombe et al., 2014).

Based on the MOA for momfluorothrin-produced rat hepatocellular tumors being the same as that for PB and metofluthrin (Okuda et al., 2017), the MOA for momfluorothrin-induced rat hepatocellular tumors is also postulated as not relevant in humans. To confirm this hypothesis, in the present study we have examined species differences in mitogenic effects of momfluorothrin and its major metabolite Z-CMCA ((Z)-(1R,3R)-3-(2-cyanoprop-1-ethyl)-2,2-dimethylcyclopropane carboxylic acid) in both cultured rat and human hepatocytes.

Recently, mice with human hepatocyte chimeric livers have been produced by transplanting human hepatocytes into albumin enhancer/promoter-driven urokinase-type plasminogen activator-transgenic severe combined immunodeficient (uPA/SCID) mice (Tateno et al., 2015a, 2015b, 2011, 2004, 2013). The host mouse hepatocytes are replaced with human hepatocytes in the livers of the chimeric mice. Importantly, human hepatocytes in the livers of chimeric mice are susceptible to growth enhancing activities. The treatment of chimeric mice with human growth hormone (hGH) increases the repopulation speed and the replacement index of transplanted human hepatocytes, as determined by increased replicative DNA synthesis and the up-regulation of hGH-related signalling molecules (Masumoto et al., 2007). Furthermore, treatment with epidermal growth factor (Yamada et al., 2014) and partial hepatectomy (personal communication, Dr. Chise Tateno) also enhances hepatocellular proliferation in this chimeric mouse model. These findings demonstrated that transplanted human hepatocytes in the chimeric mice are responsive to hepatocyte mitogens. Therefore, in addition to the cultured human hepatocytes, the effects of momfluorothrin and metofluthrin on replicative DNA synthesis in human hepatocytes of chimeric mice were examined in the present studies.

**MATERIALS AND METHODS**

All animal experiments were performed in accordance with The Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd.; and all experiment using human hepatocyte preparations were performed in accordance with The Guide for Biosafety of Sumitomo Chemical Co., Ltd. Chimeric mouse studies were performed in accordance with the ethical approval of the PhoenixBio Ethics Board.

**Test chemicals**

The chemicals were obtained from the following manufacturers: Momfluorothrin (Lot No. 9CM0109G;
purity 95.7%), Z-CMCA (Lot No. SK0712171; Purity 99.8%), and epsilon-Metofluthrin (Lot No. 100702; purity 98.8%; referred to as metofluthrin in this article), Sumitomo Chemical Co., Ltd. (Tokyo, Japan); Sodium phenobarbital (NaPB, Lot No. KLM4036, purity 98.0%), Wako Pure Chemical Industries, Ltd. (Osaka, Japan); human recombinant hepatocyte growth factor (hHGF), Sigma-Aldrich (St. Louis, MO, USA); human epidermal growth factor (hEGF; AF-100-15), Peprotech (Rocky Hill, NJ, USA) for the cultured hepatocyte study and Wako Pure Chemical Industries, Ltd. for the chimeric mouse study.

**Cultured hepatocyte study**

**Hepatocytes**

Rat hepatocytes were isolated by a two-step perfusion procedure from Harlan RccHanTM:WIST male rats aged 10 weeks (purchased from Japan Laboratory Animals, Inc., Hanno Breeding Center, Saitama, Japan) (Hirose et al., 2009; Yamada et al., 2015). Viability (range 80-92%) was determined by trypan blue exclusion. Rat hepatocytes were cultured in William's medium E (GIBCO, Waltham, MA, USA) containing 2 mM L-glutamine (Nakaraitesque, Kyoto, Japan), 0.1 μM bovine insulin (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), 10 mM nicotinamide (Wako Pure Chemical Industries), 0.2 mM L-ascorbic acid (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 % (v/v) fetal bovine serum (GIBCO).

Cryopreserved human hepatocytes for the cell culture study were obtained from Celsis IVT (Baltimore, MD, USA). A total of ten different human hepatocyte preparations were used in this study. Information on the donors of the hepatocyte preparations used is presented in Table 1. Human hepatocytes were thawed and plated according to the supplier's instructions. Briefly, cryopreserved hepatocytes were thawed at 37°C for 1 min, transferred into 20 mL in William’s medium E containing 2 mM L-glutamine, 0.1 μM bovine insulin, 1 μM dexamethasone, 10 mM nicotinamide, 0.2 mM L-ascorbic acid, 0.5 ng/mL hEGF (Peprotech), and 10 % (v/v) fetal bovine serum. The supernatant was discarded and the hepatocytes were resuspended in Williams’ medium E containing the additions described above (Yamada et al., 2015).

For assays of CYP2B mRNA induction, rat hepatocytes were plated at a density of 4.0 x 10⁵ cells/well per 2 mL of medium containing the above additions in 6-well plates (two rats) and 6.0 x 10⁵ cells/well per 500 μL of medium containing the above additions in 24-well plates (3 rats); human hepatocytes were plated at a density of 3.0 x 10⁵ cells/well per 500 μL of medium containing the above additions in 24-well plates. For assays of replicative DNA synthesis, rat and human hepatocytes were plated at a density of 1.0 x 10⁴ and 3.5 x 10⁴ cells/well per 100 μL of medium containing the above additions, respectively, in 96-well plates. All tissue culture plates were coated with collagen I (AsahiTechnoGlass, Shizuoka, Japan) and the hepatocytes were cultured at 37ºC in a humidified incubator under an atmosphere of 95% air/5% carbon dioxide (Yamada et al., 2015; Hirose et al., 2009).

**Chemical treatment**

For assays of CYP2B induction, rat hepatocytes were incubated for 48 hr in medium containing the

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Human hepatocytes were obtained from Celsis (Bioreclamation IVT) (# 1-10) or BD Biosciences (# 11-13).

Endpoints determined: A: cell viability; B: BrdU incorporation; C: CYP2B mRNA expression; and D: liver weight.

CVA: Cerebrovascular attack; ICH: Intracerebral hemorrhage; MVA: Motor vehicle accident; RD: Respiratory Disease.
above additions and in William’s medium E containing 2 mM L-glutamine, 0.7 μM bovine insulin, and 50 μM hydrocortisone hemisuccinate (Sigma-Aldrich), respectively. Rat and human hepatocytes were treated with momfluorothrin (1, 5, 10, 50, 100, 500, and 1,000 μM), Z-CMCA (5, 10, 50, 100, 500, and 1,000 μM) and NaPB (500 (rat hepatocytes only) and/or 1,000 μM). As a vehicle control, all media were supplemented with dimethyl sulfoxide (DMSO) at a final concentration of 0.1% (v/v). The medium was changed after 4 hr (only rat) from plating the hepatocytes and then at 24-hr intervals. For each concentration of the test chemicals the assays were run in three wells of 6-well plates or in three wells of 24-well plates. Studies were performed with hepatocyte preparations from five rats and seven human donors, and each assay was performed on a different isolation and donor (not pooled).

For assays of replicative DNA synthesis, rat and human hepatocytes were incubated for 48 hr in medium same as CYP2B induction assay. Rat and human hepatocytes were treated with momfluorothrin (1, 5, 10, 50, 100, 500, and 1,000 μM), Z-CMCA (5, 10, 50, 100, 500, and 1,000 μM for rat hepatocytes; 5, 100, 500, and 1,000 μM for human hepatocytes), NaPB (500 and 1,000 μM) and recombinant hHGF (10 and 100 ng/mL). In the media containing momfluorothrin, Z-CMCA, NaPB and hHGF for rat and human hepatocytes, 0.5 ng/mL EGF was added to activate DNA synthesis (Runge et al., 1999). As a vehicle control, all media were supplemented with DMSO at a final concentration of 0.1% (v/v). The medium was changed after 4 hr (only rat) from plating the hepatocytes and then at 24-hr intervals. Assays were run in either eight or twelve wells of 96-well plates at each concentration of the test chemicals and hHGF. Studies were performed with hepatocyte preparations from eight rat and seven human donors.

The concentration of momfluorothrin or Z-CMCA in the liver at the momfluorothrin 1,500-3,000 ppm dose (liver tumor occurrence dose levels in 2-year bioassay) was estimated to be less than 110 μM, based on concentration of 14C-label in the liver of rat metabolism study. In addition, test chemicals at the concentrations employed were soluble in the medium and the effects of test chemicals should be evaluated without producing significant cytotoxicity during the culture period. Thus, momfluorothrin (1-1,000 μM) and Z-CMCA (5-1,000 μM) were used in this experiment. For NaPB and hHGF, concentrations indicating expected response were used based on previous studies (Hirose et al., 2009; Yamada et al., 2015).

**Determination of DNA synthesis**

The extent of DNA synthesis was determined by measuring 5-Bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) incorporation into DNA over a 24-hr period in humidified incubator at 37°C, using a Cell Proliferation ELISA BrdU (chemiluminescent) kit (Roche, Mannheim, Germany) as previously described (Yamada et al., 2015; Hirose et al., 2009). BrdU (100 μM) was added to the medium at the point of the medium change after 24 hr of chemical treatment. After 24 hr of treatment with the test chemicals and hHGF in medium containing BrdU, hepatocytes were dried and fixed according to the kit supplier’s instructions. The luminescences of the samples were measured with a luminometer (EnVision, PerkinElmer, Waltham, MA, USA), with the measurements being conducted by Sumika Technoservice Corporation (Hyogo, Japan). The proliferation rate was calculated from the luminescent intensity compared to untreated controls.

**CYP2B mRNA expression analysis by quantitative real-time PCR**

At the end of the treatment period the medium was removed and the hepatocytes (approximately 2-4 x 10^5 cells) were washed with phosphate buffered saline (PBS, pH 7.4). Total RNA from hepatocytes was extracted using Isogen solution (Nippon Gene, Tokyo, Japan) and RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment to avoid genomic DNA contamination. Total RNA was quantified by UV analysis at 260 nm and 280 nm using a UV spectrometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). The total RNA solution was stored at -80°C until required for complementary DNA (cDNA) generation. cDNA was prepared from total RNA by reverse transcription using the High Capacity cDNA Reverse Transcription Kit for reverse transcription polymerase chain reaction (RT-PCR) (Applied Biosystems, Waltham, MA, USA) according to the kit supplier’s instructions. The reaction mixture (20 μL) containing 10x RT Buffer containing total RNA (10-100 ng) (2 μL), 25x dNTP mix (0.8 μL), 10x RT Random Primers (2 μL), 20U/μL RNase Inhibitor (1 μL) and 50U/μL MultiScribe Reverse Transcriptase (1 μL) in diethyl pyrocarbonate-treated water was incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA solution was stored at -80°C until required for real-time PCR assays. Quantitative real-time PCR assays for rat CYP2B1/2 and human CYP2B6 were performed following the instruction manual of the PCR system (7500 Fast Real-Time PCR System, Applied Biosystems) as previously described (Yamada et al., 2015). In addition, levels of rat and human Glyceraldehyde-3-phosphate dehy-
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drogenase (GAPDH) mRNA were determined as internal controls. The primer and probe sets and reaction condition used have been previously described (Yamada et al., 2015).

Cell viability analysis

Under the same conditions as for the experiments for replicative DNA synthesis, cell viability was analyzed employing a Cell counting kit (Dojindo Laboratories, Kumamoto, Japan) as previously described (Yamada et al., 2015). Briefly, rat and human hepatocytes were incubated in 96-well plates at a density of 1.0 x 10^4 and 3.5 x 10^4 cells/well, respectively, for 48 hr with medium containing momfluorothrin (10, 50, 100, and 1,000 μM), Z-CMCA (50, 100 and 1,000 μM), NaPB (1,000 μM), or hHGF (10 and 100 ng/mL). As a vehicle control, all media were supplemented with DMSO at a final concentration of 0.1% (v/v). The medium was changed after 4 hr (only rat) from plating the hepatocytes and then at 24-hr intervals. After the test chemical treatment, medium containing the Cell counting kit reagent was added to each well and the cells incubated for 4 hr at 37°C. The plates were read using a microplate reader (SH-1000 Lab, Corona Electric, Ibaraki, Japan) at a wavelength of 450 nm. The measurements were conducted by Sumika Technoservice Corporation. Cell viability was determined with two preparations each of cultured rat and human hepatocytes. Since an initial screening conducted in two preparations each of cultured rat and human hepatocytes (Lot numbers of human hepatocytes were BHL and ETA) showed that momfluorothrin and Z-CMCA had no marked cytotoxicity, cell viability was not examined in other preparations.

Chimeric mouse study

Animals

The in-life phase of the experiments using chimeric mice was performed at PhoenixBio Co., Ltd. (Hiroshima, Japan). Chimeric mice with human hepatocytes (PXB mouse®, PhoenixBio Co., Ltd.) were produced as previously described (Tateno et al., 2013, 2004). Briefly, cryopreserved human hepatocytes from donors BD85, BD87 and BD195 were purchased from BD Biosciences, Woburn, MA, USA. Human hepatocytes from donors BD85 and BD87 were transferred to homozygotic cDNA-uPA+/+/SCID mice and hepatocytes from donor BD195 were transferred to hemizygotic cDNA-uPA wild+/-SCID mice (which is an improved type from homozygotic SCID mice) (Tateno et al., 2015a) aged 20-30 days as donor cells for the chimeric mice. Information on the donors of the hepatocyte preparations used is presented in Table 1. Hepatocytes from these three donors were selected for transplantation because cells from young subjects are more responsive to the stimulation of hepatocellular proliferation (Masumoto et al., 2007). For the purposes of transplantation, vials of cryopreserved human hepatocytes (5-10 x 10^6 cells/vial) were thawed and transplanted into 20-60 cDNA-uPA/SCID mice (2.5 x 10^4 viable cells/mouse). Since the human albumin (hAlb) concentration in mouse blood correlates well with the replacement index (Tateno et al., 2004), the hAlb concentration in the blood samples was measured to predict the replacement index of human hepatocytes in mouse livers. The range of replacement indices in chimeric mice used in Experiments I, II and III was estimated as 75-89%, 90-100%, and 81-98%, respectively. Chimeric mice have previously been shown to have almost confluent human hepatocytes at 64 days to 81 days after transplantation (Tateno et al., 2004). To reduce possible variation of background levels of replicative DNA synthesis, treatment with test chemicals was commenced more than 70 days after transplantation (animal ages 15-17 weeks). Human hepatocytes in chimeric mice are considered to be deficient in growth hormone (GH) because the human GH receptor is unresponsive to mouse GH (Souza et al., 1995). Due to a lack of human GH in the chimeric mice, the chimeric mouse liver spontaneously becomes fatty in the human hepatocyte regions about 70 days after transplantation (Tateno et al., 2011). Therefore, to mimic the normal in vivo condition and to decrease steatosis, Alzet mini-pumps (Model 1002, Alzet Corporation, Palo Alto, CA, USA) containing recombinant human GH (Wako Pure Chemical Industries Ltd.), with a release rate of 8.2 μg/hr, were implanted in the subcutaneous tissue of mice under isoflurane anesthesia on the day prior to 7 days before the commencement of treatment with test chemicals. As shown below, since human EGF increased replicative DNA synthesis in chimeric mice under the same study conditions, human GH administration is unlikely to be a confounding factor in this model.

The animals were housed in a clean room with HEPA filtered air. During the course of the study, the environmental conditions in the animal room were set to maintain a temperature range of 18-28°C and a relative humidity range of 30-80%, with frequent ventilation and a 12-hr light (8:00-20:00)/12-hr dark (20:00-8:00) illumination cycle. A commercially available pelleted diet and pulverized diet containing 300 mg/100 g of vitamin C (not able to produce in these mice) (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) was provided ad libitum before treatment and during treatment, respectively. Filtered tap water was provided ad libitum through-
out the study. The animals were not fasted overnight prior to sacrifice. Human-albumin concentration of peripheral blood was determined to confirm replacement indices at 1 week prior to assignment. On the day of group assignment, all animals were weighted. Mice were randomly assigned to each group (5 or 8 animals/dose) based upon body weight and replacement indices, so that there was no significant difference in mean body weight and replacement indices among the groups.

**Study design**

The highest dose level in the 2-year rat study in which hepatocellular tumors were increased by treatment with momfluorothrin and metofluthrin was used in this study; namely 3,000 ppm for momfluorothrin, 1,800 ppm for metofluthrin. The dose level of momfluorothrin was originally set at 3,000 ppm in Experiment I, but it was decreased to 1,100 ppm in Experiments II and III due to animal deaths in Experiment I. Since 1,500 ppm momfluorothrin, which was the 2nd higher dose level in the 2-year rat study, also showed a palatability problem with similar degree as 3,000 ppm in the preliminary dose setting study (data not shown), 1,100 ppm was used in Experiments II and III. Chemical intake (170 and 146 mg/kg/day in Experiments II and III, respectively; Table 2) was higher than or equivalent to those at 146 mg/kg/day in Experiments II and III. The 2-year rat study, also showed a palatability problem with similar degree as 3,000 ppm. The oral route was selected because it is one of the potential exposure routes for humans and to be consistent with the exposure route utilized in the momfluorothrin and metofluthrin rat carcinogenicity and MOA studies. Diet containing the test compounds was provided to animals *ad libitum* for 7 days. Due to a clear enhancement of hepatocellular proliferation having been observed in rats in previous studies after 7-day treatment with metofluthrin (Yamada *et al.*, 2009) and momfluorothrin (Okuda *et al.*, 2017), a 7-day treatment period was selected for the present study. In addition, the dose level of hEGF was selected based on a preliminary dose-range finding study.

Mortality, body weight and food consumption were monitored throughout the study. After the 7-day treatment period, blood was collected from all surviving animals from the heart under isoflurane anesthesia without prior fasting. All organs and tissues from all animals were subjected to gross pathological examination. The liver of all animals was weighed under wet condition. Relative organ weight (organ weight to body weight ratio) was calculated on the basis of the body weight on the day of euthanasia. The available number of animals per group for evaluation is shown in Table 2.

**Liver histopathology-light microscopy**

Segments of livers were cut into pieces of less than 5 mm in thickness, showing the largest area, from the left lateral lobe, right lateral lobe, left medial lobe and the right medial lobe from all surviving animals, and were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

**Cell proliferation analysis**

The cell proliferation rate was evaluated as replicative DNA synthesis determined as the BrdU labeling index as previously described (Yamada *et al.*, 2014). Four hundred milligrams of BrdU was dissolved in 1.0 mL DMSO and diluted with distilled water to 10.0 mL total for injection. Alzet mini-pumps (Model 2001, Alzet Corporation) were filled with 200 μL of this solution (40 mg BrdU/mL), and were then implanted into the subcutaneous tissue of mice on the day prior to 7 days before the scheduled euthanasia. According to the manufacturer, the pump supplied 40 μg/hr of BrdU for 7 days. This corresponds to a total release of: 40 μg/hr × 24 hr/day × 7 days = 6,720 μg BrdU. To minimize the delay between implantation and the onset of release, the filled pumps were incubated in saline at 37°C for about 4 hr before implantation. The pumps were implanted subcutaneously in the back region under isoflurane anesthesia and remained there until necropsy.

After necropsy, segments of liver and duodenum from all animals were removed. Hepatocyte replicative DNA synthesis was determined in the livers from all surviving animals by an immunohistochemical method using BrdU monoclonal antibody (Yamada *et al.*, 2014). An image analysis system was used to evaluate the replicative DNA synthesis of human hepatocytes in chimeric mice. Glass slides were scanned at 20x magnification using Olympus VS120 virtual slide scanning system (Olympus, Tokyo, Japan) and Definiens Tissue Studio software (Definiens, Munich, Germany). Areas consisting of human hepatocytes (without inflammation or necrosis) were selected manually with at least one area from each of 4 lobes (lateral left lobe, lateral right lobe, medial right lobe and medial left lobe) and custom-made image analysis algorithms were applied to the digital slides to automatically detect and quantify the number of positively and negatively stained hepatocytes. The total number of evaluated human hepatocytes was more than 8,000 per chimeric mouse. Sections of duodenum were also prepared and stained immunohistochemically as a positive control on
the same glass slide as the liver using BrdU monoclonal antibody to confirm appropriate administration of BrdU.

**CYP2B mRNA expression analysis by quantitative real-time polymerase chain reaction (PCR)**

After necropsy, a piece of liver from all surviving animals was removed and stored in RNA stabilization solution (Life Technologies Co., Carlsbad, CA, USA) at 4°C overnight. After that, the RNA stabilization solution was removed and these samples were moved to a deep freezer at -80°C for analysis for gene expression. After the above sampling, the remaining liver tissue was frozen in liquid nitrogen and stored at -80°C for possible future analysis. Expression levels of human CYP2B6 mRNA and mouse Cyp2b10 mRNA were determined in livers of all surviving animals. Levels of human and mouse GAPDH mRNA were determined as internal controls. Quantitative real-time PCR assays for human CYP2B6 and human and mouse GAPDH mRNA were performed as described previously (Yamada et al., 2015). Primer and probe sequences for mouse Cyp2b10 mRNA were GAAAGTAGAGATGTCACAT for Forward primer, GTAAGTGGGACCTTGGGCTAT for Reverse primer, TCATGTTGAGTCACTTCCCTTTCCTCCC for Probe. To avoid possible contamination with the mouse genome in chimeric mouse liver, each primer set for human mRNA was shown not to work with mouse mRNA and vice versa.

**Statistical analysis**

For comparison among multiple groups, if the variables exhibited a normal distribution by the Bartlett-test, the Dunnett-test was applied for a comparison of the treated groups with the control group. The Steel-test was applied instead of the Dunnett-test when the data did not exhibit a normal distribution. For comparison between two groups, the F-test was applied to compare treated groups with the control group. If the variance was homogeneous, Student’s t-test was used. If the variance was heterogeneous, the Aspin-Welch-test was used. Each evaluation except for the replicative DNA synthesis in chimeric mouse study was by 2-tailed tests with 0.05 and 0.01 as the levels of significance.

**RESULTS**

**Cultured hepatocyte study**

To compare the responses between rat and human hepatocytes to treatment with momfluorothrin, Z-CMCA, NaPB and hHGF, the data obtained for cell viability, CYP2B mRNA expression, and replicative DNA synthesis for both species are plotted in Figs. 1-3.

![Fig. 1](image)  
**Fig. 1.** Effect of hHGF, NaPB, momfluorothrin and Z-CMCA on cell viability in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with hHGF (10 and 100 ng/mL), NaPB (1,000 μM), momfluorothrin (10-1,000 μM) and Z-CMCA (50-1,000 μM) for 48 hr and cell viability was determined by a cell counting kit. Results are presented as individual values expressed as percentage of control at each concentration of hHGF, NaPB, momfluorothrin and Z-CMCA in each of two rat and human hepatocyte preparations.
Fig. 2. Effect of NaPB, momfluorothrin and Z-CMCA on CYP2B mRNA expression in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with NaPB (500 and 1,000 μM), momfluorothrin (1-1,000 μM) or Z-CMCA (5-1,000 μM) for 48 hr and rat CYP2B1/2 and human CYP2B6 mRNA levels determined by quantitative real-time PCR. Results are presented as mean ± S.D. (n = 2-5 rat and n = 1-7 human hepatocyte preparations) expressed as percentage of control at each concentration of NaPB, momfluorothrin or Z-CMCA. Values significantly different from control (DMSO only treated) are: * p < 0.05 and ** p < 0.01 in rat hepatocytes; and ## p < 0.01 in human hepatocytes.

Fig. 3. Effect of hHGF, NaPB, momfluorothrin and Z-CMCA on replicative DNA synthesis in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with hHGF (10 and 100 ng/mL), NaPB (500 and 1,000 μM), momfluorothrin (1-1,000 μM) or Z-CMCA (5-1,000 μM) for 48 hr and replicative DNA synthesis determined by BrdU incorporation over the last 24 hr of culture. Results are presented as mean ± S.D. (n = 3-8 rat and n = 5-10 human hepatocyte preparations) expressed as percentage of control at each concentration of hHGF, NaPB, momfluorothrin or Z-CMCA. Values significantly different from control (DMSO only treated) are: * p < 0.05 and ** p < 0.01 in rat hepatocytes; and # p < 0.05 and ## p < 0.01 in human hepatocytes.
With respect to cell viability (Fig. 1), none of the treatments produced a marked reduction in formazan production by dehydrogenase enzymes. Some small decreases in formazan production were observed in rat hepatocytes treated with 1,000 μM momfluorothrin and human hepatocytes treated with 1,000 μM Z-CMCA. The increases in formazan production at some concentrations of momfluorothrin, NaPB and hHGF observed in rat hepatocytes may be due to increased metabolic activity as a result of either CYP2B enzyme induction and/or increased replicative DNA synthesis.

Treatment with 1,000 μM NaPB produced a less marked effect on CYP2B mRNA levels in human than in rat hepatocytes (Fig. 2). The effects of momfluorothrin and Z-CMCA on CYP2B mRNA levels were also less marked in human than in rat hepatocytes, with Z-CMCA only producing an induction of CYP2B mRNA levels in rat hepatocytes.

The treatment of rat hepatocytes with 100 ng/mL hHGF and human hepatocytes with 10 and 100 ng/mL hHGF resulted in significant increases in replicative DNA synthesis, thus confirming the functional viability of the rat and human hepatocyte preparations used in these studies to treatment with a mitogenic agent (Fig. 3). In rat hepatocytes, significant increases in replicative DNA synthesis were observed after treatment with 500 and 1,000 μM NaPB and 5 and 10 μM momfluorothrin, whereas replicative DNA synthesis was reduced by treatment with 100-1,000 μM momfluorothrin. In contrast to the effects observed in rat hepatocytes, the treatment of human hepatocytes with 500 and 1,000 μM NaPB and 1-1,000 μM momfluorothrin had no significant increase in replicative DNA synthesis. Treatment with 5-1,000 μM Z-CMCA had no significant effects on replicative DNA synthesis in either rat or human hepatocytes.

Chimeric mouse study

A summary of the data obtained is presented in Table 2. In three separate studies, chimeric mice transplanted with human hepatocytes from different donors were treated for 7 days with diets containing 1,800 ppm metoﬂuthrin (average chemical intake, 239-285 mg/kg/day) and with 1,100 or 3,000 ppm momfluorothrin (average chemical intake, 146-170 mg/kg/day for 1,100 ppm; 410 mg/kg/day for 3,000 ppm). As described in the Methods section, the dose level of momfluorothrin was changed in Experiments II and III due to animal interim deaths (Days 2 and 3, commencement of treatment is counted as Day 0) in Experiment I. Regarding metoﬂuthrin, while no mortality was observed in Experiments I and III, two of five animals treated with 1,800 ppm metoﬂuthrin were found dead during the early phase of treatment (Days 2 and 3) in Experiment II. The average chemical intakes in these 7-day studies were higher than those observed at tumorigenic dose levels administered to male rats in the 2-year bioassays, which were 38 and 78 mg/kg/day for 900 and 1,800 ppm mg/kg/day metoﬂuthrin, respectively, and 73 and 154 mg/kg/day for 1,500 and 3,000 ppm momfluorothrin, respectively. With the exception of interim deaths due to suppressed food consumption possibly resulting from neurotoxicity during the early phase of treatment (data not shown), no severe toxic effects were observed in surviving animals during the study.

Although serum or liver concentrations of the test chemicals were not examined in the present study, significant increases in mouse Cyp2b10 mRNA expression in the liver (Table 2) demonstrated that treatment with metoﬂuthrin or momfluorothrin significantly stimulated CAR in the mouse hepatocytes of the chimeric mice. Increased levels of human CYP2B6 mRNA were also observed in liver of the chimeric mice, but the effects were relatively weak, being 1.1-1.4 fold of control in animals given 1,800 ppm metoﬂuthrin and 1.4 fold of control in animals given 3,000 ppm momfluorothrin.

The chimeric mouse livers were characterized histologically with respect to the extent of chimerism. The areas consisting of human hepatocytes were easily distinguishable from the areas of mouse hepatocytes by H&E staining (Fig. 4A). Some positive staining cells to BrdU were detected in the areas of human hepatocytes in the control animals (Fig. 4B). The BrdU labelling index was only determined in human hepatocytes and not in mouse hepatocytes, in which the rate of replicative DNA synthesis was relatively high even in controls (Fig. 4B), and thus was difficult to compare between control and treatment animals in mouse hepatocytes of the chimeric mice. The cause of the high spontaneous DNA synthesis in the mouse hepatocytes is unclear but may be related to induced synthesis of DNA and/or hepatocyte damage by expression of uPA with consequent regeneration.

Under these conditions, treatment with metoﬂuthrin or momfluorothrin did not result in any increases in replicative DNA synthesis of human hepatocytes (Table 2, Fig. 5A). However, as a positive control, hEGF treatment increased replicative DNA synthesis in human hepatocytes though statistical significance was not observed due to the small number of animals examined (Table 2, Fig. 5A). When the data from all three experiments were combined, statistically significant increased replicative DNA synthesis was observed in the hEGF group (Fig. 5B). Representative photos of liver sections in control and hEGF-treated animals are shown.

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Table 2. Summary of findings in chimeric mouse study.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Donor ID; BD87</th>
<th>Groups</th>
<th>Control</th>
<th>Metofluthrin 1800 ppm</th>
<th>Momfluorothrin 3000 ppm</th>
<th>hEGF 600 μg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of animals examined</td>
<td>5</td>
<td>5</td>
<td>3*</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average Chemical intake (mg/kg/day)</td>
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<td>272</td>
<td>410</td>
<td>0.6</td>
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<td></td>
<td>Liver weight Absolute (g)</td>
<td>2.78 ± 0.36</td>
<td>2.89 ± 0.32</td>
<td>2.36 ± 0.51</td>
<td>2.75 ± 0.25</td>
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</tr>
<tr>
<td></td>
<td>Liver weight Relative (g/body weight x 100)</td>
<td>13.47 ± 1.80</td>
<td>14.43 ± 2.13</td>
<td>11.83 ± 1.43</td>
<td>13.78 ± 1.13</td>
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</tr>
<tr>
<td></td>
<td>Replicative DNA synthesis in human hepatocytes (%)</td>
<td>11.59 ± 6.77</td>
<td>4.63 ± 2.03</td>
<td>6.71 ± 6.76</td>
<td>21.18 ± 9.03</td>
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</tr>
<tr>
<td></td>
<td>Human CYP2B6 mRNA (% of control average)</td>
<td>100 ± 18.93</td>
<td>111 ± 11.90</td>
<td>136 ± 17.70*</td>
<td>124 ± 18.24</td>
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<tr>
<td></td>
<td>Mouse Cyp2b10 mRNA (% of control average)</td>
<td>100 ± 33.49</td>
<td>424 ± 118.90**</td>
<td>1692 ± 600.04*</td>
<td>154 ± 26.17*</td>
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<th>Metofluthrin 1800 ppm</th>
<th>Momfluorothrin 3000 ppm</th>
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<td>Number of animals examined</td>
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<td>5</td>
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<td></td>
<td>Average Chemical intake (mg/kg/day)</td>
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<td>239</td>
<td>170</td>
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<td></td>
<td>Liver weight Absolute (g)</td>
<td>2.30 ± 0.20</td>
<td>2.14 ± 0.08</td>
<td>2.17 ± 0.21</td>
<td>2.53 ± 0.23</td>
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<tr>
<td></td>
<td>Liver weight Relative (g/body weight x 100)</td>
<td>11.71 ± 0.79</td>
<td>11.72 ± 0.68</td>
<td>11.19 ± 0.67</td>
<td>12.94 ± 0.87*</td>
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<td></td>
<td>Replicative DNA synthesis in human hepatocytes (%)</td>
<td>7.44 ± 4.00</td>
<td>5.23 ± 3.08</td>
<td>4.59 ± 1.61</td>
<td>12.22 ± 5.51</td>
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<td>Human CYP2B6 mRNA (% of control average)</td>
<td>100 ± 12.90</td>
<td>137 ± 14.75*</td>
<td>93 ± 12.77</td>
<td>135 ± 41.88</td>
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<td>Mouse Cyp2b10 mRNA (% of control average)</td>
<td>100 ± 55.9</td>
<td>281 ± 136.5*</td>
<td>266 ± 105.9**</td>
<td>112 ± 27.2</td>
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<th>Experiment</th>
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<th>Groups</th>
<th>Control</th>
<th>Metofluthrin 1800 ppm</th>
<th>Momfluorothrin 3000 ppm</th>
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<td>Number of animals examined</td>
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<tr>
<td></td>
<td>Average Chemical intake (mg/kg/day)</td>
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<td>285</td>
<td>146</td>
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<td>Liver weight Absolute (g)</td>
<td>2.63 ± 0.40</td>
<td>2.61 ± 0.34</td>
<td>2.61 ± 0.40</td>
<td>3.21 ± 0.39*</td>
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<tr>
<td></td>
<td>Liver weight Relative (g/body weight x 100)</td>
<td>11.98 ± 1.59</td>
<td>12.44 ± 1.68</td>
<td>11.94 ± 1.21</td>
<td>14.94 ± 2.00*</td>
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<tr>
<td></td>
<td>Replicative DNA synthesis in human hepatocytes (%)</td>
<td>8.37 ± 4.53</td>
<td>5.01 ± 1.76</td>
<td>4.55 ± 1.32</td>
<td>15.46 ± 5.61</td>
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<tr>
<td></td>
<td>Human CYP2B6 mRNA (% of control average)</td>
<td>100 ± 14.79</td>
<td>121 ± 19.78*</td>
<td>86 ± 16.29</td>
<td>120 ± 29.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse Cyp2b10 mRNA (% of control average)</td>
<td>100 ± 26.9</td>
<td>305 ± 96.6**</td>
<td>191 ± 14.1**</td>
<td>122 ± 55.5</td>
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</table>

Results are presented as mean ± S.D., and values in parenthesis are fold change of control. Values significantly different from control are: * p < 0.05 and ** p < 0.01. For the momfluorothrin group of the experiment I, since five of eight animals found dead during treatment, group mean was evaluated in survived three animals. For the metofluthrin group of the experiment II, since two of five animals were found dead during treatment, group mean was evaluated in survived three animals.
in Figs. 4C and 4D, respectively.

DISCUSSION

The stimulation of replicative DNA synthesis via CAR activation is the critical key event in the proposed MOA for momfluorothrin-induced rat liver tumor formation, as demonstrated by the CAR knockout rat study, CAR knockdown hepatocyte study using RNAi and hepatic global gene expression analysis (Okuda et al., 2017). Therefore, effects of momfluorothrin and its major metabolite Z-CMCA on CYP2B mRNA induction and replicative DNA synthesis were examined in cultured rat and human hepatocyte preparations and in human hepatocytes of chimeric mice in the present study. Cyp2B is well-known as an associative event but not a key event for hepatocellular tumorigenesis related to CAR activation (Elcombe et al., 2014). Induction of total cytochrome P450 content and individual subfamily enzymes generally correlate poorly with carcinogenicity (Elcombe et al., 2002). Thus, Cyp2B is a biomarker for CAR activation, but the increases in Cyp2B levels do not correlate with the extent of cell proliferation. The treatment of rat and human hepatocytes with 1,000 μM NaPB significantly increased CYP2B mRNA levels, demonstrating that under the experimental conditions employed in this study the cultured hepatocytes maintain enough CAR signaling functions to be able to respond to treatment with CYP2B enzyme inducers.

hHGF is a well-known growth factor which is known to stimulate replicative DNA synthesis in hepatocytes (Runge et al., 1999; Yamada et al., 2015; Hirose et al., 2009). In the present study, hHGF increased replicative DNA synthesis in rat and human hepatocytes in a concentration-dependent manner. These results confirmed the functional viability of the rat and human hepatocyte preparations used in this study to the effect of a known hepatocyte mitogen.

The toxicokinetic characteristics of momfluorothrin have been determined in vivo and in vitro (ECHA, 2014). In the single dose study, absorption of both isomers was extensive (> 80%) with no potential for bioaccumulation.
in tissues or organs, underwent ester cleavage to generate Z/E-CMCA and subsequent downstream metabolites and conjugates, and was excreted rapidly within 24-48 hr. Z/E-CMCA, were also the major metabolites observed in an in vitro study using rat and human liver microsomes (Data not published but technical report is referred in ECHA 2014). Thus, the major metabolites of the momfluorothrin-RTZ and momfluorothrin-RTE isomers in humans in vivo are likely to be Z/E-CMCA, making the in vivo rat studies a valid model for estimating human metabolism of momfluorothrin. Though momfluorothrin concentrations in rat liver have not been determined, plasma concentrations of momfluorothrin and Z-CMCA were determined in rats treated with momfluorothrin at 3,000 ppm in the diet for 3 and 7 days. The plasma concentration of momfluorothrin was around 0.3 μM at both time points, and the concentration of Z-CMCA was approximately 10-fold higher than that of momfluorothrin (unpublished data).

Since test chemical concentrations in the liver are expected to be more than 2~10-fold higher than plasma concentrations based on findings of the momfluorothrin metabolism study (ECHA, 2014), the concentration range used in the present study (1~1000 μM for momfluorothrin, 5~1,000 μM for Z-CMCA) would cover these expected concentrations in the liver. The treatment of rat hepatocytes with 1,000 μM momfluorothrin and human hepatocytes with 1,000 μM Z-CMCA resulted in slight decreases in formazan production, suggesting that high concentrations of momfluorothrin or Z-CMCA could be toxic to rat or human hepatocytes. However, the toxic effects observed at high concentrations in vitro does not appear to occur in vivo, as no hepatotoxic effects, such as necrosis, were observed in in vivo rat studies at doses up to the maximum tolerated dose (MTD) (ECHA, 2014; Okuda et al., 2017). Thus, these findings suggest that the plasma concentrations of these chemicals in the carcinogenicity study with momfluorothrin were less than 1,000 μM.

The treatment of rat hepatocytes with 500 and 1,000 μM NaPB significantly increased replicative DNA synthesis consistent with previous findings (Hirose et al., 2009). Replicative DNA synthesis in rat hepatocytes was significantly increased by treatment with 5 and 10 μM momfluorothrin to 1.6- and 1.8-fold control, respectively. However, the treatment of rat hepatocytes with 5-1,000 μM Z-CMCA had no statistically significant effect on replicative DNA synthesis, suggesting that momfluorothrin is likely the causative agent for rat liver tumor production rather than its major metabolite Z-CMCA.

In evaluating the relevance of rodent liver tumours induced by a CAR activation MOA for humans, the key species difference is that such compounds increase replicative DNA synthesis in rodent hepatocytes but not in human hepatocytes (Cohen, 2010; Elcombe et al., 2014; Lake et al., 2015; Wood et al., 2015; Kushida et al., 2016). For human hepatocytes, in contrast to hHGF, replicative DNA synthesis was not increased by treatment with 500 and 1,000 μM NaPB, 1-1,000 μM momfluorothrin or 5-1,000 μM Z-CMCA in the present study. The lack of effect of NaPB on replicative DNA synthesis in cultured human hepatocytes is consistent with previous findings (Hirose et al., 2009; Yamada et al., 2015; Parzefall et al., 1991). In keeping with the properties of the prototypic CAR activator PB, two closely
structurally related pyrethroid insecticides metofluthrin (Yamada et al., 2015; Hirose et al., 2009) and momfluorothrin (present study) were demonstrated not to stimulate replicative DNA synthesis in cultured human hepatocytes. In addition, no stimulation of replicative DNA synthesis by NaPB in human hepatocytes has already been demonstrated in vivo in the study utilizing chimeric mice with human hepatocytes (Yamada et al., 2014). The present study demonstrated that momfluorothrin and metofluthrin did not increase in vivo human hepatocyte replicative DNA synthesis in chimeric mice employing hepatocytes from three different donors. Thus, correlation can be made between the findings in the human cells in the chimeric mice and the effects utilizing human cells in vitro compared to rodent cells.

The conclusion that the MOA for rodent liver tumour formation by a nongenotoxic CAR activator is not relevant for humans is supported by available epidemiological data (Elcombe et al., 2014). For example, a recent evaluation of the literature for PB concluded that there was no evidence of a specific role of PB in human liver cancer risk (La Vecchia and Negri, 2014). Critically, in such studies showing no evidence of increased cancer risk, the subjects received PB for many years at doses which produced similar plasma levels to those which are carcinogenic in mice (Monro, 1993).

**Human applicability of the proposed mode of action**

In terms of the human relevance of an animal carcinogenic MOA there are three questions to consider (Boobis et al., 2006) before reaching a conclusion. These are: 1. Is the weight of evidence sufficient to establish a MOA in animals? 2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans? 3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?

As described by Okuda et al. (2017), the postulated MOA is similar to that of certain other non-genotoxic agents which are CAR activators including that of a close structural analogue metofluthrin (Yamada et al., 2009). Alternative MOAs for momfluorothrin-induced rat liver tumour formation have been excluded. Thus, a plausible MOA for momfluorothrin-induced rat liver tumour formation has been established, and therefore, the answer to question 1 is yes. In assessing the relevance of animal MOA data to humans, a concordance table has been suggested as being of considerable value (Boobis et al., 2006). Such a table is presented in Table 3. This includes not only the data for the effects of momfluorothrin in the rat, but also the available data for humans. In contrast to PB, which produced liver tumors in both rat and mouse, momfluorothrin showed rat specific tumorigenicity of the liver. As discussed previously (Okuda et al., 2017), this is not specific to momfluorothrin but other CAR activators also show rat specificity. Since momfluorothrin did not produce hepatocellular tumors in mice, our current study focused on species differences between rat and human and thus mouse was not included in this comparative discussion.

A number of studies have shown that CAR is present in human liver and that this receptor can be activated by drugs and other compounds (Moore et al., 2003). Hence, it is probable that at high doses momfluorothrin could activate CAR in human liver. Treatment with momfluorothrin increased CYP2B6 mRNA levels in human hepatocytes.

### Table 3. Comparison of key and associative events on MOA for liver tumorigenesis of momfluorothrin in rats and humans.

<table>
<thead>
<tr>
<th>Key (K) and Associative (A) Event</th>
<th>Evidence in Rats</th>
<th>Evidence in Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of CAR (K)</td>
<td>Inferred from CAR-siRNA studies and from induction of CYP2B enzymes</td>
<td>Probable at high doses (inferred from induction of CYP2B mRNA in cultured hepatocytes and in vivo in chimeric mice with human hepatocytes)</td>
</tr>
<tr>
<td>Induction of CYP2B (A) [as a marker for CAR activation]</td>
<td>Direct experimental evidence in vivo and in vitro in cultured hepatocytes</td>
<td>Probable at high doses (Experimental evidence in vitro in cultured hepatocytes and in vivo in chimeric mice with human hepatocytes)</td>
</tr>
<tr>
<td>Hepatocellular hypertrophy (A)</td>
<td>Direct experimental evidence in vivo</td>
<td>Possible at very high doses # (Experimental evidence in vivo in chimeric mice with human hepatocytes treated with NaPB)</td>
</tr>
<tr>
<td>Increased hepatocellular proliferation (K)</td>
<td>Direct experimental evidence in vivo and in vitro in cultured hepatocytes</td>
<td>Not predicted to occur (Not observed in cultured hepatocytes and in vivo in chimeric mice with human hepatocytes)</td>
</tr>
<tr>
<td>Altered hepatic foci (K)</td>
<td>Direct experimental evidence in vivo</td>
<td>Not predicted to occur</td>
</tr>
<tr>
<td>Liver tumours</td>
<td>Yes</td>
<td>Not predicted to occur</td>
</tr>
</tbody>
</table>

# based on studies in human subjects given anticonvulsant drugs
cytes (Fig. 2). Thus, at high doses momfluorothrin has the potential to activate CAR and induce CYP2B enzymes in human liver.

Studies in human subjects given anticonvulsant drugs (which induce hepatic CYP enzymes) have shown that prolonged treatment with high doses can increase liver size in humans, which is associated with liver hypertrophy and increased smooth endoplasmic reticulum (Aiges et al., 1980; Pirttiaho et al., 1978). Thus, by comparison with the effects of such anticonvulsant drugs, at high doses momfluorothrin has the potential to produce hypertrophy in human liver.

As the stimulation of replicative DNA synthesis is the critical key event in the proposed MOA for momfluorothrin-induced rat hepatocellular tumour formation (Okuda et al., 2017), the effect of momfluorothrin on replicative DNA synthesis has been studied in cultured rat and human hepatocytes (Fig. 3). Using hepatocyte cultures, increases in replicative DNA synthesis following treatment with hHGF were observed in both rat and human hepatocytes. However, increased replicative DNA synthesis following momfluorothrin treatment was only observed in rat hepatocytes, not in human hepatocytes. These results demonstrate that momfluorothrin only induced replicative DNA synthesis in rat and not in human hepatocytes. This conclusion is also strongly supported by the chimeric mouse study, where no increase in replicative DNA synthesis in human hepatocytes was also observed (Table 2, Fig. 4).

The data obtained in these studies with the CAR activator momfluorothrin is in agreement with literature data on other CAR activators that have shown increased replicative DNA synthesis in cultured rodent hepatocytes but not in cultured human hepatocytes (Hirose et al., 2009; Lake et al., 2015; Elcombe et al., 2014; Parzefall et al., 1991; Yamada et al., 2015).

Table 3 summarizes the available rat and human data for the key and associative events in the proposed MOA for momfluorothrin-induced rat liver tumour formation. Overall, while some of the key (activation of CAR) and associative (CYP2B enzyme induction and hepatocellular hypertrophy) events in the MOA for momfluorothrin-induced rat hepatocellular tumour formation could occur in human liver, the available experimental data demonstrate that human hepatocytes appear to be refractory to the mitogenic effects of momfluorothrin.

Some studies of PB treatment indicated that PB induces cell cycle transcriptional responses in humanized CAR (Huang et al., 2005) and humanized CAR/PXR (Luisier et al., 2014) mouse liver, and, furthermore, that PB-treatment produced liver tumors in humanized CAR/PXR mice similar to wild type mice but to a significantly lesser extent than the wild type mice (Braeuning et al., 2014). Therefore, some researchers have suggested that the human relevance of the tumorigenicity of PB through CAR activation remains the subject of an ongoing debate (Braeuning, 2014; Braeuning and Schwarz, 2016; Braeuning et al., 2015; Marx-Stoelting et al., 2017; Groll et al., 2016). We recognize that the human receptors of hCAR/hPXR mouse model are operational in a mouse hepatocyte environment. While PB can induce replicative DNA synthesis in hCAR/hPXR mice (Luisier et al., 2014), such effects are not observed in cultured human hepatocytes (Hirose et al., 2009; Yamada et al., 2015; Parzefall et al., 1991) or in human hepatocytes of chimeric mice with humanized livers (Yamada et al., 2014), where human CAR and other receptors are acting in a human environment. This is not the case with the hCAR and hPXR receptors in hepatocytes of hCAR/hPXR mice, where the human receptors function in the context of mouse target gene regulatory elements and chromatin structure and hence gene regulation and protein interactions may differ from human hepatocytes (Braeuning et al., 2014; Luisier et al., 2014). Namely, although the human CAR/PXR has been inserted, the downstream genes are still mouse and may be the basis for those studies still showing increased cell proliferation. Therefore, we believe the positive results from the hCAR/hPXR mouse model require careful consideration with respect to implications for human relevance. Although we have no data for momfluorothrin in the hCAR/hPXR mouse model, our present study strongly supports the previous conclusion that the CAR-mediated MOA is qualitatively not relevant to humans.

As examination of the available data demonstrates that the MOA for momfluorothrin-induced rat liver tumour formation is qualitatively not plausible for humans, there is no need to consider quantitative differences in either kinetic or dynamic factors between rats and humans. In addition to a quantitative difference in response, a quantitative difference in exposure, which is not generally the basis for quantitative differences under the WHO/IPCS framework, is also discussed here. It should be noted that likely human chronic exposure to momfluorothrin would be orders of magnitude lower than momfluorothrin dose levels required to produce liver tumours in the rat. Thus, not only is there a qualitative difference between the rat and human in the response of the liver cells to the pyrethroid CAR activators regarding induction of liver tumours, but also a marked quantitative difference in the level of exposure. Thus, based on quantitative considerations, the confidence in a lack of effect in humans at
expected exposures is even stronger than that based only on qualitative considerations. Consequently, momfluorothrin is of no carcinogenic hazard or risk for humans.

Since pyrethrins, including natural pyrethrins, have been used for many years as insecticides for household, agricultural, and other applications, the Agency for Toxic Substances and Disease Registry (ATSDR) provided an excellent review entitled “Toxicological Profile for Pyrethrins and Pyrethroids” (ATSDR, 2003). According to this review, no reports were located regarding cancer in humans or animals following inhalation or dermal exposure to pyrethrins or natural pyrethroids. However, in the case of oral exposure to these chemicals, while pyrethrins and some pyrethroids have been shown to cause tumors in rodent models (Tsujii et al., 2012), no reports were located regarding cancer in humans. Although there are no epidemiological data for momfluorothrin or metofluthrin so far, the conclusion in this study may also be supported by epidemiological data for pyrethroids/natural pyrethrins (ATSDR, 2003).

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The authors thank Ms. Miwa Kondo, Keiko Tanaka, Keiko Maeda, and Yoko Obara, for their expert technical assistance. We also thank the other contributors to this research project from Sumitomo Chemical Company Ltd., Sumitomo Chemical (UK) PLC, and Sumika Technoservice Corporation. Professors Samuel M. Cohen and Brian G. Lake consult for Sumitomo and Sumika Technoservice Corporation. Professors Hirose, Y., Nagahori, H., Yamada, T., Deguchi, Y., Tomigahara, Y., Nishioka, K., Uwagawa, S., Kawamura, S., Isobe, N., Lake, B.G. and Okuno, Y. (2009): Comparison of the effects of the synthetic pyrethroid Metofluthrin and phenobarbital on CYP2B form induction and replicative DNA synthesis in cultured rat and human hepatocytes. Toxicology, 258, 64-69.

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Human relevance of momfluorothrin-induced liver tumors in rats

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Since pyrethrins, including natural pyrethrins, have been used for many years as insecticides for household, agricultural, and other applications, the Agency for Toxic Substances and Disease Registry (ATSDR) provided an excellent review entitled “Toxicological Profile for Pyrethrins and Pyrethroids” (ATSDR, 2003). According to this review, no reports were located regarding cancer in humans or animals following inhalation or dermal exposure to pyrethrins or natural pyrethroids. However, in the case of oral exposure to these chemicals, while pyrethrins and some pyrethroids have been shown to cause tumors in rodent models (Tsujii et al., 2012), no reports were located regarding cancer in humans. Although there are no epidemiological data for momfluorothrin or metofluthrin so far, the conclusion in this study may also be supported by epidemiological data for pyrethroids/natural pyrethrins (ATSDR, 2003).

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