Human Risk Assessment for Rat Liver Tumors Induced by a Constitutive Androstane Receptor Activator Momfluorothrin

March, 2018

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Executive Summary

Many chemicals have been shown to produce tumors in rats and mice, especially liver is the most common target organ affected. Momfluorothrin, a new pesticide developed by Sumitomo Chemical Co. Ltd., induced hepatocellular tumors in the rat two-year bioassay and which is a close structural analogue of pyrethroid insecticide metofluthrin. The metofluthrin also induced rat liver tumors and results from the mode of action (MOA) analysis showed constitutive androstane receptor (CAR)-mediated MOA. Therefore, the MOA for momfluorothrin-induced rat hepatocellular tumors is also predicted to be the CAR-mediated MOA as it is for metofluthrin. A series of MOA analysis was conducted based on the International Programme on Chemical Safety (IPCS) framework to evaluate the human cancer risks of momfluorothrin in the present research.

The IPCS framework composed of the following three questions. Question 1 is to establish an animal MOA for momfluorothrin-induced rat liver tumors, question 2 is to demonstrate the qualitative differences in the key events between rats and humans, and question 3 is to demonstrate the quantitatively differences in either kinetic or dynamic factors between rats and humans.

In chapter 1, to establish the animal MOA for rat hepatocellular tumors induced by momfluorothrin, a series of *in vivo* and *in vitro* MOA analysis were conducted using wild type (WT) and CAR knockout (KO) rats or RNA interference (RNAi) technique. As a result of MOA analyses, defined key events (i.e. CAR activation, hepatocellular proliferations) and associative events (hepatic cytochrome P450 (CYP) 2B induction, increased liver weights, hepatocellular hypertrophy) in the CAR-mediated MOA were

identified in WT rats with strong dose-dependency and temporal consistency. In further studies using CAR KO rats and RNAi technique, it was elucidated that hepatocellular proliferation and CYP2B activity induced by momfluorothrin were depend on CAR activation. Thus, a plausible MOA for momfluorothrin-induced rat liver tumor formation have been established as CAR activated MOA and the answer to question 1 in the IPCS framework is yes.

In chapter 2, to evaluate of human relevancy for rat hepatocellular tumors induced by momfluorothrin, some *in vitro* and *in vivo* assays were conducted using human culture hepatocytes and chimeric mice with human hepatocytes. In rat and human hepatocyte studies with momfluorothrin, CYP2B gene expression was significantly increased in both hepatocytes but replicative DNA synthesis was only increased in rat and not in human hepatocytes. This conclusion is strongly supported by the chimeric mouse study, where no increase in replicative DNA synthesis in human hepatocytes was also observed. As examination of the available data demonstrates that the MOA for momfluorothrin-induced rat liver tumor formation is qualitatively not plausible for humans and the answer to question 2 is yes. In addition, no stimulation of replicative DNA synthesis by NaPB and metofluthrin was demonstrated in chimeric mice with human hepatocytes.

In conclusion, these data suggested that CAR activators including momfluorothrin have no carcinogenic risk for humans.

Introduction

I. Cancer bioassay

The current standard for evaluation of possible carcinogenic activity of a chemical in humans is the two-year bioassay in rodents, usually rats and mice. These animal cancer bioassays have been used for more than a half century to determine whether pesticides, pharmaceuticals, consumer products, industrial chemicals, food additives and other products might cause cancer or other health problems in humans. Inherent in rodent-based assessments was the assumption that the observation of tumors in laboratory animals could be meaningfully extrapolated to identify potential human carcinogens (Cohen, 2010; Boobis *et al*., 2006). However, some of them have been identified rodents-specific tumors throughout mechanism studies have brought together a fuller biological understanding of how chemicals induce neoplasia in animal studies (Whysner, Ross, and Williams 1996; Holsapple *et al*., 2006; Meek *et al*., 2014; Elcombe *et al*., 2014; Corton *et al*., 2014). Therefore, it is necessary to evaluate and extrapolate appropriately the human cancer risks from positive results of rodent carcinogenicity study.

II. IPCS Framework for analyzing the relevance of a cancer MOA for human

In the early 2000s, as an analytical tool to provide a means of evaluating systematically the data available on specific carcinogenic response to a chemical in a transparent manner, frameworks for analyzing the MOAs by which chemicals produce tumors in laboratory animals and the relevance of such tumor data for human risk assessment have been developed by the IPCS and by the International Life Sciences Institute (ILSI) (Boobis *et al.*, 2006, 2008; Cohen *et al.*, 2004; Meek *et al.*, 2003;

Sonich-Mullin *et al.*, 2001). An MOA has been defined as a "biologically plausible sequence of key and associative events leading to an observed effect supported by robust experimental observations and mechanistic data" (Boobis *et al.*, 2006). 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 (Fig. 1).

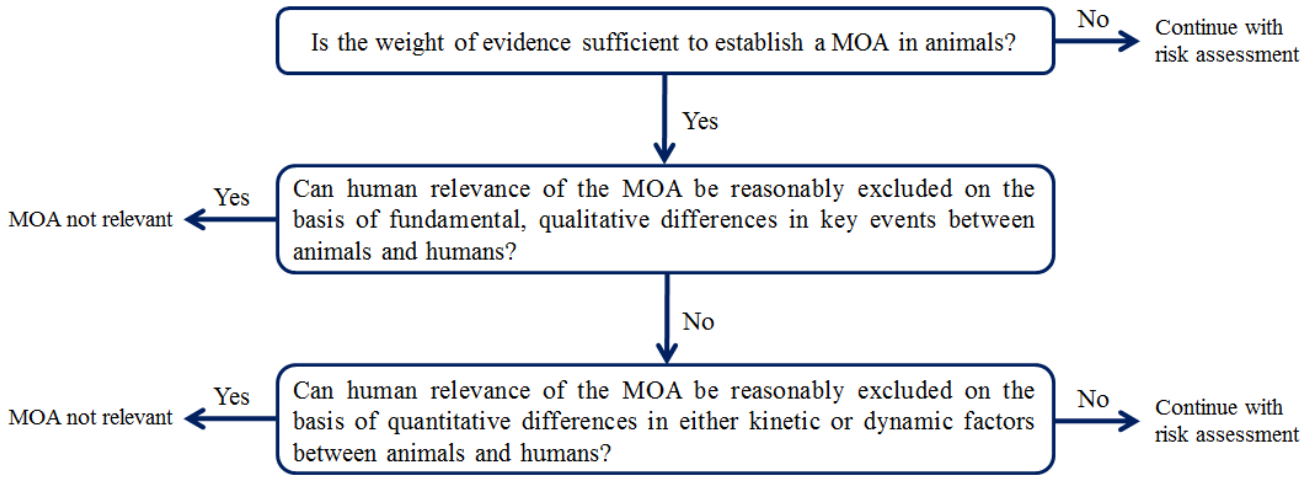

Figure 1. IPCS general scheme illustrating the main steps in evaluating the human relevance of an animal MOA for tumor formation (Figure is modified from Boobis *et al.*, 2006).

The questions have been designed to enable an unequivocal answer *yes* or *no*, but recognizing the need for judgment regarding sufficiency of weight of evidence (WoE). Answers leading to the left side of the diagram indicate that the WoE is such that the MOA is not considered relevant to humans. In contrast, answers leading to the right side of the diagram indicate either that the WoE is such that the MOA is likely to be relevant to humans.

III. Rats cancer bioassay in Momfluorothrin

Many chemicals have been shown to produce tumors in rats and mice. Analysis of rodent bioassay data demonstrates that for both the rat and the mouse liver is the most common target organ affected (Huff *et al*., 1991; Gold *et al*., 2001). In our recent case, *Epsilon*-Momfluorothrin (CAS# 1065124-65-3; 2,3,5,6-Tetrafluoro-4-(methoxymethyl)benzyl(Z)-(1R,3R)-3-(2-cyanoprop-1-enyl)-2,2-dimethylcyclopropane carboxylate, referred to as momfluorothrin in this report, Figure 2A) was also induced hepatocellular tumors in the rat carcinogenicity study.

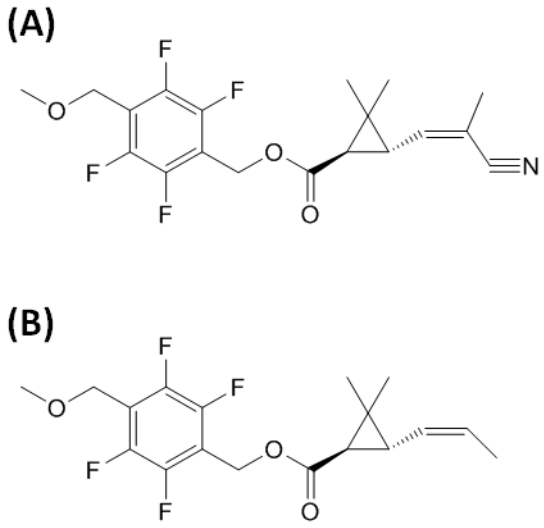

Figure 2. Chemical structures of momfluorothrin (A) and metofluthrin (B).

Momfluorothrin was developed as a type I synthetic pyrethroid insecticide consisting of two main isomers (RTZ: RTE ratio is 9:1) for use to treat crawling insects in both indoor residential settings and outdoor commercial/residential/barn settings. A summary of the results of 2-year bioassay using male and female Wistar rats fed diet containing momfluorothrin at 0, 200, 500, 1500, and 3000 ppm are shown in Table 1. The

incidences of the total number of animals with hepatocellular adenomas and/or carcinomas were 2, 0, 4, 12, and 33% for males, and 0, 0, 2, 2, and 10% for females, respectively. The combined incidence of hepatocellular adenoma and carcinoma was significantly increased in male and female rats given 3000 ppm momfluorothrin, with a non statistically significant increase being observed in male rats given 1500 ppm. The incidences of hepatocellular adenoma, carcinoma, and combined in males given 1500 and 3000 ppm were equivalent to or higher than the maximum incidence of the historical control data, and the combined incidence of female rats given 3000 ppm was within the historical control data, but incidence of carcinoma was equivalent to the maximum incidence of the historical control data. Overall, treatment with momfluorothrin in rats for 2 years produced hepatocellular tumours in males at 1500 and 3000 ppm (73 and 154 mg/kg/day) and in females at 3000 ppm (182 mg/kg/day) (ECHA, 2014).

This research report was summarized based on the published data from Okuda *et al*., 2017a, 2017b, and Yamada *et al.*, 2014.

Table 1. Summary of liver alterations in rats treated with momfluorothrin in the 2-year tumorigenicity study.

Sex		Males					Females				
Dose levels of momfluorothrin (ppm)		**0**	**200**	**500**	**1500**	**3000**	**0**	**200**	**500**	**1500**	**3000**
Organ weight											
104 weeks	Relative liver weights	1.00	1.04	1.08	1.22**	1.66**	1.00	0.99	1.05	1.20**	1.46**
Light microscopy (incidence)											
104 weeks	Hepatocellular hypertrophy	1/51	1/51	0/51	5/51	14/51**	0/51	0/51	0/51	3/51	10/51**
Preneoplastic or neoplastic findings (incidence and %)											
104 weeks	Eosinophilic cell foci	0/51	2/51	3/51	3/51	20/51**	2/51	0/51	2/51	5/51	9/51*
		(0%)	(4%)	(6%)	(6%)	(39%)	(4%)	(0%)	(4%)	(10%)	(18%)
	Hepatocellular adenomas	1/51	0/51	2/51	4/51	8/51*	0/51	0/51	1/51	1/51	4/51
		(2%)	(0%)	(4%)	(8%)	(16%)	(0%)	(0%)	(2%)	(2%)	(8%)
	Hepatocellular carcinomas	0/51	0/51	0/51	4/51	9/51**	0/51	0/51	0/51	0/51	1/51
		(0%)	(0%)	(0%)	(8%)	(18%)	(0%)	(0%)	(0%)	(0%)	(2%)
	Combined hepatocellular	1/51	0/51	2/51	6/51	17/51**	0/51	0/51	1/51	1/51	5/51*
	adenomas/carcinomas	(2%)	(0%)	(4%)	(12%)	(33%)	(0%)	(0%)	(2%)	(2%)	(10%)
Historical control data [a]		Average		Range (min - max)			Average		Range (min - max)		
	Eosinophilic cell foci	6.55%		0.0 - 44.0%			7.42%		0.0 - 56.0%		
	Hepatocellular adenomas	2.54%		0.0 - 8.0%			2.80%		0.0 - 10.2%		
	Hepatocellular carcinomas	0.47%		0.0 - 2.8%			0.32%		0.0 - 2.0%		
	Combined hepatocellular adenomas/carcinomas	3.01%		0.0 - 10.0%			3.12%		0.0 - 12.0%		

Note. Data are unpublished but refereed to ECHA (2014). For histopathology, data represent the number of animals with the lesion/total number of animals examined and incidence is shown in parentheses.

Values of relative liver weight are presented as fold of the control at each dose level.

Values significantly different from control (0 ppm) are: * $p < 0.05$, ** $p < 0.01$. Values within shaded areas indicate toxicologically significant change.

a: Historical control data on liver tumors on 104-weeks studies in RccHanTM:WIST, Wistar Hannover rats compiled from 104 weeks bioassays performed at Harlan Laboratories Ltd. Itingen/Switzerland.

Table is adapted from Okuda *et al*., (2017a).

Chapter 1

MOA analysis for rat hepatocellular tumors produced by the synthetic pyrethroid momfluorothrin: evidence for CAR activation and mitogenicity in male and female rats

Introduction

Postulated MOA for rodent liver tumors formation by momfluorothrin

According to the Cohen (2010), there are two ways that a chemical can alter the incidence of cancer: (1) by damaging DNA directly or (2) indirectly by increasing the number of DNA replications resulting in an increase in the spontaneous errors in DNA. Several MOAs which have been identified for liver carcinogenesis both in humans and in rodent models are shown in Table 2. MOAs that have evidence of human relevance are highlighted in bold letters (e.g. DNA-reactive carcinogens (genotoxic compound), estrogen receptor activation, increased cytotoxicity, infections, and metal overload). While, there are a number of MOAs with no relevance to humans, including peroxisome proliferation, enzyme induction, statine-mediated alterations in liver metabolism, and increased apoptosis (Cohen, 2010; Cohen and Arnold, 2011).

Table 2. Modes of action for hepatocellular carcinogenesis (refer to Cohen, 2010).

I. **DNA reactivity**
II. Increased cell proliferation
    A. *Receptor mediated*
        1. PPARα (peroxisome proliferation)
        2. Enzyme induction (CAR, PXR, AHR)
        3. **Estrogen**
        4. Statins
        5. **Cytotoxicity**
        6. Other

    B. *Non-receptor mediated*
        1. **Cytotoxicity**
        2. **Infections**
        3. **Iron (copper) overload**
        4. Increased apoptosis
        5. Other

Note. MOA appearing in bold letters are likely to be relevant to humans.

Momfluorothrin is clearly not genotoxic, being negative in a variety of *in vivo* and *in vitro* genotoxicity assays (Ames test, *in vitro* chromosomal aberration test, *in vitro* gene mutation assay, unscheduled DNA synthesis (UDS) assay and mouse micronucleus test) (ECHA, 2014). Moreover, momfluorothrin is a close structural analogue of the type I pyrethroid insecticide metofluthrin (Fig. 2B) and high doses of metofluthrin have also been shown to produce hepatocellular tumors in rats (Deguchi *et al.*, 2009). Results in *in vivo* and *in vitro* MOA studies, metofluthrin induced hepatic CYP2B subfamily enzymes as a surrogate marker of CAR and hepatocellular replicative DNA synthesis in rats (Deguchi *et al.*, 2009; Hirose *et al.*, 2009; Kushida *et al.*, 2016; Yamada *et al.*, 2009, 2015). From a read-across approach, the MOA for rat hepatocellular tumors induced by momfluorothrin was postulated the same as that of metofluthrin, that is CAR-mediated MOA. This MOA is similar to that of certain other non-genotoxic agents which are CAR activators, such as phenobarbital (PB) (Carmichael *et al.*, 2011; Holsapple *et al.*, 2006; Osimitz and Lake, 2009) which can produce liver tumors in rats and mice (IARC, 2001; Whysner *et al.*, 1996).

Key and associative events in the CAR-activated MOA

In CAR-activated MOA, many key events (i.e. an empirically observable causally precursor step to the adverse outcome that is itself a necessary element of the MOA) and associative events (i.e. biological processes that are themselves not causal necessary key events for the MOA, but are reliable indicators or markers for key events) have been identified (Elcombe *et al.*, 2014). Key events are required events for the MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events. Associative events can often be used as surrogate markers for a key event in a MOA

evaluation or as indicators of exposure to a xenobiotic that has stimulated the molecular initiating event or a key event.

In a recent evaluation of the MOA for PB-induced rodent liver tumor formation, key events in the MOA were considered to be CAR activation, altered gene expression specific to CAR activation, increased cell proliferation, and the development of altered hepatic foci leading to liver tumor formation; whereas associative events included the induction of CYP enzymes (in particular the CYP2B subfamily enzymes), liver hypertrophy (increased liver weight and hepatocellular hypertrophy) and inhibition of apoptosis (Elcombe *et al*., 2014). If a key event (or events) is an essential element for carcinogenesis, it must precede the appearance of the tumors (Cohen and Arnold, 2016). A scheme of key and associative events in the postulated MOA for momfluorothrin-induced rat hepatocellular tumor formation is shown in Figure 3. To determine whether this postulated MOA (CAR activated MOA) is correct, *in vivo* and *in vitro* experiments were conducted following the IPCS framework against the modified Bradford Hill considerations (Meek *et al.*, 2014; Sonich-Mullin *et al.*, 2001) which are:

1. Postulated MOA.
2. Key events; associated critical parameters.
3. Dose-response relationships.
4. Temporal association.
5. Strength, consistency, and specificity of association of key events and tumor response.
6. Biological plausibility and coherence.
7. Possible alteration MOAs.
8. Uncertainties, Inconsistencies, and data gaps.

9. Conclusion about the MOA.

These obtained experimental and analytical data have already been published from Toxicological Sciences (Okuda *et al*., 2017a).

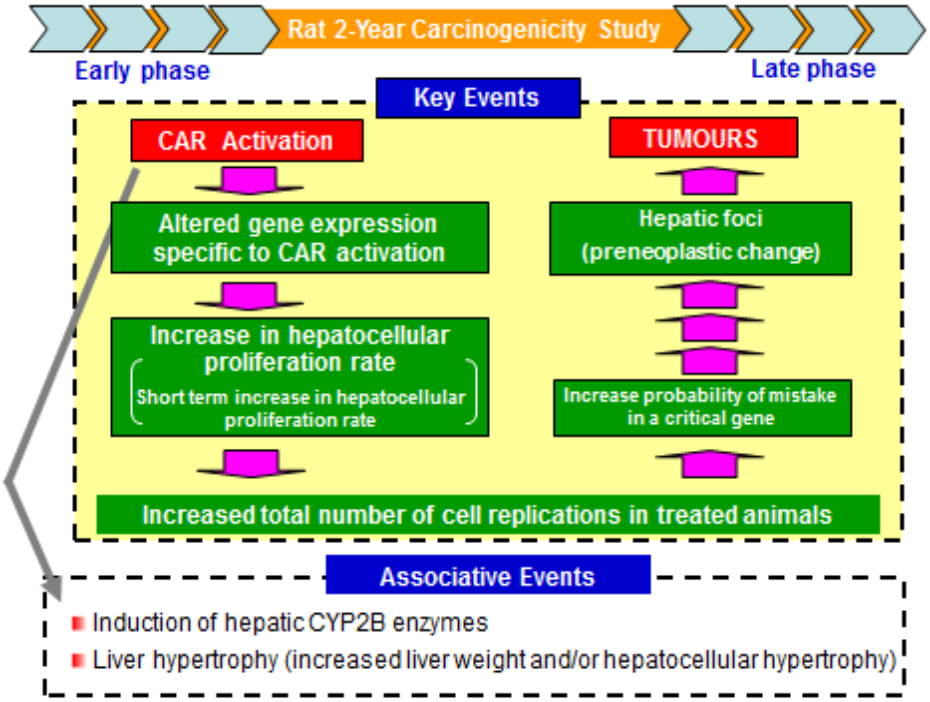

Figure 3. Schematic representation of key and associative events in the proposed MOA for momfluorothrin-induced rat hepatocellular tumor formation. The MOA for momfluorothrin-induced rat liver tumor formation is postulated to involve activation of the CAR, which results in a pleiotropic response including the stimulation of CYP2B subfamily enzymes, hepatocellular hypertrophy and increased hepatocellular proliferation. Although hepatocyte labeling index values, determined as 5-bromo-2´-deoxy-uridine (BrdU) labeling index, may return toward control levels with continued momfluorothrin treatment, the number of cell replications in treated animals will be enhanced due to the increased total number of hepatocytes per animal. The continued stimulation of cell proliferation may lead to tumor formation as a result of critical errors being produced during cell replication and/or to the enhanced proliferation of spontaneously initiated pre-neoplastic hepatocytes (Cohen and Arnold, 2011; Schulte-Hermann *et al.*, 1983). Prolonged treatment results in the formation of altered hepatic foci and liver tumors. Figure is adapted from Okuda *et al*., (2017a).

Materials and Methods

Chemicals

Test chemicals were obtained from the following manufacturers: momfluorothrin (Lot no. 9CM0109G; purity 95.7%; storage condition, cold storage) and *epsilon*-metofluthrin (Lot no.100702; purity 98.8%; storage condition, cold storage; referred to as metofluthrin in this article) were provided by Sumitomo Chemical Co., Ltd. (Tokyo, Japan); phenobarbital-Na (NaPB; Lot no.AWJ4960; purity 98.0%; storage condition, room temperature) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and husbandry

All experiments were performed in accordance with *The Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd.*

HarlanRccHanTM:WIST (Wistar strain) rats aged 9 weeks were purchased from Japan Laboratory Animals, Inc., Hanno Breeding Center (Saitama, Japan) as this was the strain of rats used in the 2-year cancer bioassay. In addition, CAR KO rats with a Crl:CD (SD) genetic background aged 10 weeks and wild-type Crl:CD (SD) rats (WT) aged 11 weeks were purchased from SAGE labs, Inc. (Boyertown, Pennsylvania, 19512, USA) and Charles River Japan, Inc., Hino Breeding Center (Shiga, Japan), respectively. SD genetic background CAR KO rats were used as Wistar background KO rats were not available. Similar responses to momfluorothrin in the liver of wild type rats of both strains were observed in the present study (described later). Prior to the MOA analysis, BrlHan:WIST@Jcl(GALAS) (Wistar strain) rats aged 4 weeks were purchased from

CLEA Japan, Inc., Fuji Breeding Center (Shizuoka, Japan) and used in the toxicity screening study at the early stage of development of this chemical; liver samples from the BrlHan:WIST@Jcl(GALAS) rats were subjected to the global gene expression profile analysis and the data are presented in this paper.

Animals were acclimatized to laboratory conditions for 7 days prior to treatment in the *in vivo* assay. During the course of the study, the environmental conditions in the animal room were set to maintain a temperature range of 22-26°C and a relative humidity range of 40 - 70%, with frequent ventilation (more than 10 times per hour) and a 12 hour light (8:00 - 20:00)/ 12 hour dark (20:00 - 8:00) illumination cycle. A commercially available pulverized diet (CRF-1; Oriental Yeast Co., Ltd, Tokyo) and filtered tap water were provided *ad libitum* throughout the study. The animals were not fasted overnight prior to sacrifice in the present MOA studies, but they were fasted in the toxicity screening study.

Design for in vivo studies using Wistar rats

In the present MOA study, three different experiments were conducted for evaluating time-course, dose-dependency and reversibility. Male and female rats (ten animals/dose/sex) fed diets containing momfluorothrin at 0 (control) and 3000 ppm (the highest dose in the 2-year bioassay, a tumor inducing dose level) for 7 and 14 days to evaluate the time-course of changes. For evaluation of the dose-dependency, momfluorothrin was administered at 0, 200, 500, 1500, and 3000 ppm to both sexes (ten animals/dose/sex) for 7 days; these levels were consistent with those of the 2-year bioassay. Furthermore, the reversibility of hepatic effects was evaluated in male rats (ten animals/dose) by cessation of treatment for 7 days after 7-days treatment with

momfluorothrin at 0 and 3000 ppm.

Design for in vivo studies using CAR KO rats

Male Crl:CD (SD) (WT) and CAR KO rats (five animals/group, respectively) were fed diets containing momfluorothrin at 0 or 3000 ppm for 7 days to clarify whether the hepatocellular proliferation involved CAR activation. As shown below, since momfluorothrin treatment at 3000 ppm for 7 days significantly increased CYP2B activity, liver weight and replicative DNA synthesis in male Wistar rats, the same treatment time was selected for this experiment. This model has been quite recently developed and only limited data have been published (Chamberlain *et al.*, 2014). Thus, two additional CAR-mediated MOA liver tumor inducers NaPB (1000 ppm) (Rossi *et al.*, 1977) and metofluthrin (1800 ppm) (Yamada *et al.*, 2009) were examined in the present study to confirm reliability of the CAR KO rat model. Administration of PB in drinking water (500 ppm, the corresponding daily intake was of 39.5 mg/kg/day in males and of 46.7 mg/kg/day in females) caused liver adenomas in male and female Wistar rats (Rossi *et al.*, 1977). In this study, treatment of 1000 ppm NaPB in diet revealed a similar range of daily intake (approximately 50 mg/kg/day, see Table 5).

Although we consider that eight to ten rats per group is preferred for reliable evaluation of BrdU labeling under the study conditions we used, due to limitation of the number of the CAR KO rat availability, five animals per group were used in the present study. Since small numbers of animals were used (5 rats/group), to observe any expected CAR-mediated alterations (even a tendency) in the WT animals, two sets of experiments (5 rats/group in each experiment) were conducted in the WT rats.

Observations and tissue sampling

Mortality, body weights, and food consumption were monitored throughout the studies. For evaluation of replicative DNA synthesis, Alzet minipumps (Model 2ML1; Alzet Corporation, Palo Alto, CA, USA) containing BrdU (a structural analog of thymidine that incorporates into nuclear DNA and is used as a surrogate marker of cell proliferation (Wood *et al.*, 2015), Sigma Company, St Louis, MO, USA) with a release rate of 200 μg/hour, were implanted in the subcutaneous tissue of rats under isoflurane anesthesia on the day prior to 4 days of the scheduled euthanization to avoid the effects of a palatability problem at the early phase of the study. After 7- or 14-day treatment period, rats were sacrificed under deep anesthesia by isoflurane without prior fasting on the morning of day 8 or 15, and then livers were excised quickly and weighed. Some liver tissue was stored in RNA stabilization solution (Ambion, Austin, TX, USA) at -80°C until analyzed for gene expression. The remaining liver tissue was processed for hepatic CYP enzyme activity analysis, histopathology, and cell proliferation measurement.

Liver histopathology

Segments of livers from all surviving animals were fixed in buffered 4% paraformaldehyde or 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. In addition, the left lateral lobe in the control and treatment groups was prefixed by perfusing 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) using a syringe for 2 animals/group in Wistar rats. The sample blocks were post-fixed in 2% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultra-thin sections were prepared, stained with uranyl

acetate and lead citrate, and examined by JEM-1400 transmission electron microscopy (JEOL, Tokyo, Japan).

Hepatocyte replicative DNA synthesis from BrdU-labeling indices

Hepatocyte replicative DNA synthesis was determined in the livers from all surviving animals by an immunohistochemical method using BrdU monoclonal antibody (Deguchi *et al.*, 2009; Yamada *et al.*, 2014). BrdU labeling was analyzed microscopically in a blinded manner with more than 2000 hepatocytes per rat being evaluated. A small section of duodenum from each animal was also processed to serve as a control for confirming systemic availability of BrdU and immunohistochemical staining.

Quantitative real-time polymerase chain reaction of the selected genes

Using liver samples from male Wistar rats treated with momfluorothrin at 3000 ppm for 7 and 14 days, hepatic CYP1A2, CYP2B1/2, CYP3A1, CYP3A2, and CYP4A1 mRNA expression levels were analyzed by quantitative real-time PCR. Hepatic CYP2B1/2 and CAR mRNA levels were also analyzed in samples from the *in vitro* RNAi experiment using rat hepatocytes and in *in vivo* study of CAR KO rats, respectively. Total RNA from hepatocytes was extracted using Isogen solution (Nippon Gene) and RNeasy Mini Kit (Qiagen) 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). The total RNA solution was stored at -80 ºC 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) 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 ºC for 10 min, 37 ºC for 120 min and 85 ºC for 5 min. The cDNA solution was stored at -80 ºC until required for real-time PCR assays. The primer and probe sets are shown in Table 3.

Table 3. Primer and prove set.

Species	Target mRNA	Forward Primer	Reverse Primer	Probe	Product size
Rat	CYP1A2	GAAGCCCAGAACCTGTGAACA	CCGATGTCTCGGCCATCTT	CAGGCCTGGCCACGCTTCTCC	70bp
	CYP2B1/2	GCTCAAGTACCCCCATGTCG	ATCAGTGTATGGCATTTTACTGCGG	Not used	109bp
	CYP3A1	AGTCGTCCTGGTGCTCCTCTAC	CCCAGGAATCCCCTGTTTCT	ATTTGGGACCCGCACACATGGACT	73bp
	CYP3A2	AAACCACCAGCAGCACACTCT	CAGGGCCCCATCGATCTC	TCTTGTATTTCCTGGCCACTCACCCTGA	95bp
	CYP4A1	TCCAGGTTTGCACCAGACTCT	TCCTCGCTCCTCCTGAGAAG	CCCGACACAGCCACTCATTCCTGC	67bp
	GAPDH	GCTGCCTTCTCTTGTGACAAAGT	CTCAGCCTTGACTGTGCCATT	TGTTCCAGTATGATTCTACCCACGGCAAG	129bp
Mouse	Cyp2b10	CAGGTGATCGGCTCACACC	TGACTGCATCTGAGTATGGCATT	Not used	70bp
	GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	CCGCCTGGAGAAACCTGCCAAGTATG	77bp
Human	CYP2B6	TTGTTCTACCAGACTTTTTCACTCATC	GGAAAGTATTTCAAGAAGCCAGAGA	TCTGTATTCGGCCAGCTGTTTGAGCTC	83bp
	GAPDH	GACACCCACTCCTCCACCTTT	CATACCAGGAAATGAGCTTGACAA	CTGGCATTGCCCTCAACGACCA	79bp

Hepatic microsomal CYP enzyme activity

Hepatic microsomal CYP enzyme activity was determined in selected liver samples such as from the dose-response study. A portion of liver (approximately 0.5 g) was homogenized in 4 volumes of 154 mM KCl containing 50 mM Tris/HCl buffer pH7.4 using a Potter-type Teflon-glass homogenizer. Whole liver homogenates were centrifuged at 9,000 × g for 20 min at 4 °C to separate S9 fractions. The protein content of the S9 fractions was determined using the DC protein assay kit (Bio-Rad, CA) employing bovine serum albumin as standard (Bradford, 1976). CYP2B activity was determined as 7-pentoxyresorufin O-depentylase (PROD) activity by fluorometric analysis using the specific substrate for CYP2B enzyme. The reaction mixture (200 µL) consisted of 3 µM 7-pentoxyresorufin, 10 µM dicoumarol, 1 mM NADPH, 1 µL S9 fraction in 100 mM Tris/HCl buffer pH7.4 in 96-well microplates. After incubation for 10 min at 37 ºC, the reaction was stopped by addition of 100 µL acetonitrile. The fluorescence of the sample was measured with a microplate reader (Saffire II, Tecan) with an excitation wavelength of 550 nm and an emission wavelength of 589 nm. Enzyme activity was calculated from the fluorescence of a standard curve of the resorufin product.

Evaluation for CAR involvement on the MOA for CYP2B1/2 mRNA induction in cultured rat hepatocytes using the RNAi technique

The assay was basically conducted as previously described (Deguchi *et al.*, 2009). On day 0, primary cultured hepatocytes were obtained from a single male rat per experiment (HarlanRccHanTM:WIST rats, at the age of 9 weeks) by a modified two-step collagenase digestion method. Rat liver was perfused and hepatocytes were

dispersed from digested liver and washed with William's E medium (GIBCO) three times by centrifugation. The hepatocytes were cultured in supplemented 2 mL William's E medium (5% fetal bovine serum [GIBCO], 100 U/ml penicillin [Nakaraitesque], 100 μg/ml streptomycin [Nakaraitesque], 2 mM L-glutamine [Nakaraitesque], 0.1 μM insulin [Sigma-Aldrich], 1 μM dexamethasone [Sigma-Aldrich], 0.2 mM ascorbic acid [Sigma-Aldrich], and 10 mM nicotinamide [Sigma-Aldrich]) in a six-well plate coated with collagen I (AsahiTechnoGlass), at a density of approximately 4 x 10^5 cells/well, and allowed to attach for 3 hours at 37 ^oC in a humidified chamber. After 3 hours, the culture dishes were gently swirled and fresh medium was added after removing unattached hepatocytes.

On day 1, cells were rinsed and supplemented with serum/antibiotics free medium (2 mL). siRNA (1 μg) for CAR (sense strand: 5'-GCUCACACACUUUGCAGAUAUCAAU-3', antisense strand: 5'-AUUGAUAUCUGCAAAGUGUGUGAGC-3', Hayashi-Kasei Co., Ltd.) or negative control (NC; Stealth RNAi Negative Control with Medium GC, Code No.; 12935-300, Invitrogen), and 1 μL of MATra-si Reagent (IBA) were each diluted with 200 μL of serum/antibiotics free medium according to the manufacturer's instructions, and the two solutions were gently mixed. After 20 min, the transfection mixtures (200 μL) were added to the cells, and the culture plates were placed on a magnet plate (IBA) for 15 min. After 4 hours, the medium was changed to the supplemented Williams E medium containing serum and antibiotics.

Following the transfection (on day 1), hepatocytes were treated with 50 μM of NaPB and 100 μM of momfluorothrin in medium for 2 days. A concentration of 50 μM of NaPB was selected as this concentration has previously been shown to induce

CYP2B-dependent enzyme activity in cultured rat hepatocytes (Deguchi *et al.*, 2009; Hirose *et al.*, 2009). Medium was changed on a daily basis thereafter. The control group was treated in the same manner without test chemical. The experiment was examined using three wells for each group. On day 3, hepatocytes were washed with phosphate-buffered saline (PBS) and the total RNA was extracted using Isogen (Nippon Gene, Japan). During the experiment, no cytotoxicity was observed visually and there was no change in expression levels of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH).

Global gene expression analysis

Since detailed MOA for momfluorothrin-induced liver tumor production was evaluated for the key and associate evens in the current MOA studies, the additional data of global gene expression analysis in the general toxicity study of momfluorothrin may not be essential for prediction of the MOA for momfluorothrin-induced liver tumor production. However, in the general toxicity studies, animals are usually fasted prior to euthanization to avoid confounding by possible variation of food consumption. In contrast, since it is well known that fasting alters the cytochrome P450 profile affecting drug/chemical metabolism (Maronpot *et al.*, 2010; Sohn and Fiala, 1995), the data of global gene expression analysis in the general toxicity study with fasting may provide valuable information for considering newly postulated MOA of other compounds at the point of departure of the MOA study. The analysis was determined in livers from male BrlHan:WIST@Jcl(GALAS) rats (4 animals per groups) treated with momfluorothrin at 0 and 3000 ppm for 2 weeks; the animals were treated under the same conditions as in the MOA studies described above, excepting for fasting prior to euthanization. Details

of the analytical methods are shown in Supplementary materials. The gene expression data can be downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (Accession No. GSE94738; http://www.ncbi.nlm.nih.gov/geo/info/linking.html.). The obtained gene expression data were subjected to hierarchical clustering analysis with GeneSpring GX 13.1 software (Agilent Technologies). Clustering was conducted with the probe sets, whose expression was known to be changed in our reference data; carbon tetrachloride (CCl_4) and thioacetamide as cytotoxic compounds (Abe *et al.*, 2014; Manibusan *et al.*, 2007), clofibrate as a peroxisome proliferator-activated receptor alpha (PPARα) activator (Corton *et al.*, 2014), and NaPB as a CAR activator (Elcombe *et al.*, 2014) for 2 weeks.

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. Two-tailed tests were employed for evaluation except for BrdU labeling index and BrdU labeling index was evaluated by one-tail test with $p \leq 0.05$ and 0.01 as the levels of significance.

Results

Analysis for selected hepatic CYP mRNA levels

In the MOA studies, the effect of momfluorothrin on some selected CYP mRNA levels were determined in the livers of male Wistar rats treated with momfluorothrin at 3000 ppm for 7- and 14 days (Fig. 4). While hepatic CYP2B1/2 mRNA levels were significantly increased after 7 and 14-day treatment (18- and 16-fold, respectively), CYP3A1 and CYP4A1 mRNA levels were only marginally increased after 7- or 14-day treatment (less than 1.5-fold). No significant changes were observed in CYP1A2 and CYP3A2 mRNA levels. These findings suggested that momfluorothrin activates CAR but not either Aryl hydrocarbon receptor (AhR) or PPARα.

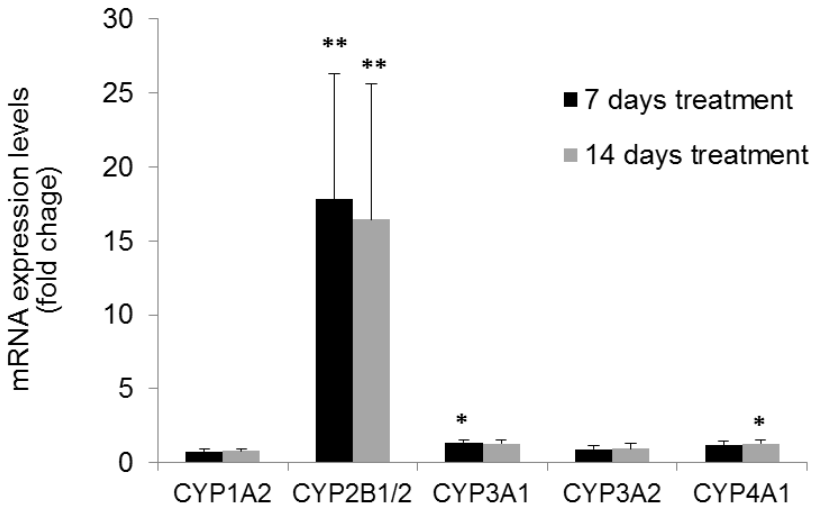

Figure 4. Selected hepatic CYP mRNA expression levels in Wistar male rats treated with momfluorothrin. Male Wistar rats were treated with momfluorothrin at 0 (control) and 3000 ppm for 7 and 14 days and selected hepatic CYP mRNA expression levels were examined by RT-PCR. Data are presented as fold increase to control as mean ± SD (N=6). Values statistically different from control are: *p<0.05; ** p<0.01. Figure is adapted from Okuda *et al.*, (2017a).

Evaluation of CAR involvement in CYP2B induction in cultured rat hepatocytes using the RNAi technique

To confirm whether hepatic CYP2B induction (i.e. CYP2B1/2 mRNA) by momfluorothrin involves CAR activation, the effect of CAR knockdown by the RNAi technique on CYP2B induction by momfluorothrin was investigated in a rat cultured hepatocyte system. A concentration range finding experiment with 5 - 1000 μM momfluorothrin demonstrated that CYP2B1/2 mRNA was significantly increased at 100 μM and higher concentrations with the peak effect being observed at 100 μM (2.6-fold of control) (Fig. 5A). Subsequent experiments were performed with 100 μM momfluorothrin.

When rat hepatocytes were treated with CAR-siRNA together with either 50 μM NaPB or 100 μM momfluorothrin, CAR mRNA levels were significantly suppressed by 80% and 81% of each negative CAR-siRNA control (NC) in NaPB and momfluorothrin treated groups, respectively (Fig. 5B and 5C). Under the CAR-suppressed condition, CYP2B1/2 mRNA levels in NaPB and momfluorothrin-treated groups were also reduced, respectively, by 67% (close to statistical significance, p=0.070) and 68% (close to statistical significance, p=0.067) compared with each NC (Fig. 5D and 5E). In addition, the data from CAR KO mice (Yamamoto *et al.,* 2004) and rat cultured hepatocytes with CAR siRNA (Deguchi *et al.,* 2009) demonstrated that CAR activation is necessary to induce CYP2B mRNA by NaPB. Therefore, these findings demonstrate that momfluorothrin is also a CAR activator bacause CYP2B1/2 mRNA was induced CAR dependently as well as NaPB.

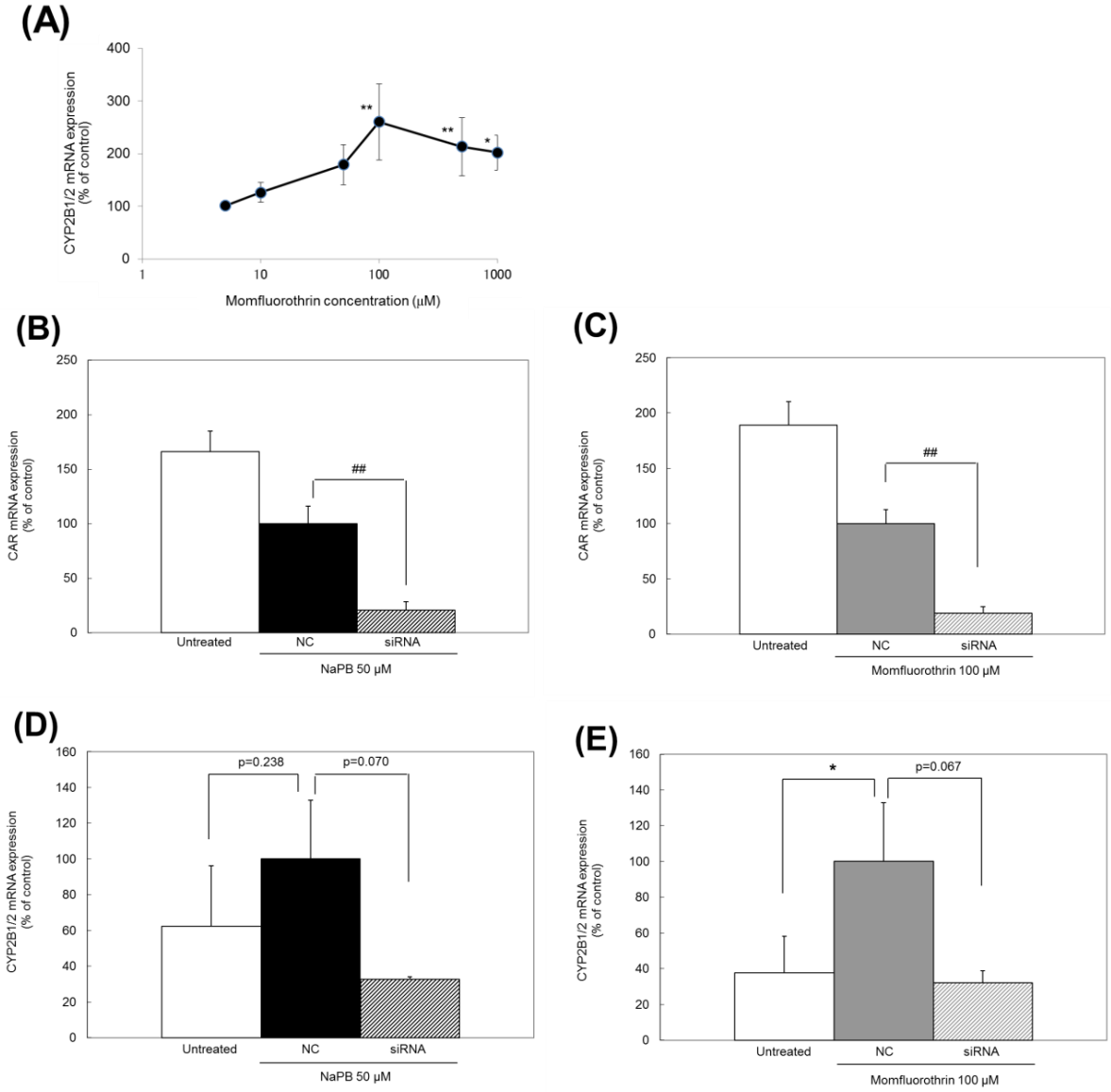

Figure 5. Relative CAR (B, C) and CYP2B1/2 (D, E) mRNA expression levels in cultured hepatocytes treated with 50 μM NaPB (B, D) and 100 μM momfluorothrin (C, E). The concentration of momfluorothrin was determined based on the range finding study (A) and 100 μM momfluorothrin selected for the siRNA studies (B to E). NC means control siRNA as a negative control. NaPB + NC (B, D) and momfluorothrin + NC (C, E) are shown as percentage of control siRNA (NC) values. Data are presented as mean values±SD (N=3). For part A, values significantly different from control are: **p*<0.05; ***p*<0.01. For parts B-E, values significantly different between untreated and NC treated hepatocytes are **p*<0.05 and between NC and either 50 μM NaPB or 100 μM momfluorothrin are $^{\#\#}p$ <0.01. Figure is adapted from Okuda *et al*., (2017a).

In vivo momfluorothrin time-course study in Wistar rats

To investigate the time course of momfluorothrin-induced hepatic effects, male and female rats were treated with 0 (control) and 3000 ppm momfluorothrin for 7 and 14 days. The data from this *in vivo* study are summarized in Table 4. There were no severe toxicities such as death or marked suppression of body weight and food consumption up to 14 days. As shown in Fig. 6A, relative liver weights were significantly increased to a similar extent in both sexes after 7 and 14 days treatment. While replicative DNA synthesis was also significantly increased after 7- and 14-days treatment in both sexes, the increase in replicative DNA synthesis was less marked after 14 days than after 7 days in both sexes (Fig. 6B).

Table 4. Summary of liver alterations in rats treated with momfluorothrin in the MOA studies.

Sex			Males					Females				
Momfluorothrin dose (ppm)			0	200	500	1500	3000	0	200	500	1500	3000
Time-cause study	7 days treatment	Absolute liver weights	1.00	ND	ND	ND	1.12**	1.00	1.08	1.05	1.07	1.11*
		Relative liver weights	1.00	ND	ND	ND	1.17**	1.00	1.09*	1.04	1.10**	1.16**
		Replicative DNA synthesis	1.00	ND	ND	ND	5.30**	1.00	1.15	1.17	1.69*	2.79**
		CYP1A2 mRNA	1.00	ND	ND	ND	0.72	ND	ND	ND	ND	ND
		CYP2B1/2 mRNA	1.00	ND	ND	ND	17.78**	ND	ND	ND	ND	ND
		CYP3A1 mRNA	1.00	ND	ND	ND	1.37*	ND	ND	ND	ND	ND
		CYP3A2 mRNA	1.00	ND	ND	ND	0.93	ND	ND	ND	ND	ND
		CYP4A1 mRNA	1.00	ND	ND	ND	1.17	ND	ND	ND	ND	ND
		PROD activity	ND	ND	ND	ND	ND	1.00	0.88	1.00	1.88*	6.18*
		Hepatocellular hypertrophy	0/10	ND	ND	ND	4/10*	0/10	0/10	0/10	0/10	3/10
		Proliferation of SER	0/2	ND	ND	ND	0/2	ND	ND	ND	ND	ND
	14 days treatment	Absolute liver weights	1.00	ND	ND	ND	1.24**	1.00	ND	ND	ND	1.05
		Relative liver weights	1.00	ND	ND	ND	1.26**	1.00	ND	ND	ND	1.12*
		Replicative DNA synthesis	1.00	ND	ND	ND	1.90*	1.00	ND	ND	ND	1.65*
		CYP1A2 mRNA	1.00	ND	ND	ND	0.79	ND	ND	ND	ND	ND
		CYP2B1/2 mRNA	1.00	ND	ND	ND	16.44**	ND	ND	ND	ND	ND
		CYP3A1 mRNA	1.00	ND	ND	ND	1.28	ND	ND	ND	ND	ND
		CYP3A2 mRNA	1.00	ND	ND	ND	0.97	ND	ND	ND	ND	ND
		CYP4A1 mRNA	1.00	ND	ND	ND	1.30*	ND	ND	ND	ND	ND
		PROD activity	ND	ND	ND	ND	ND	1.00	ND	ND	ND	6.88*
		Hepatocellular hypertrophy	0/10	ND	ND	ND	8/10**	0/10	ND	ND	ND	0/10
		Proliferation of SER	0/2	ND	ND	ND	0/2	ND	ND	ND	ND	ND
Dose-dependency study	7 days treatment	Absolute liver weights	1.00	1.01	1.05	1.08	1.09*	1.00	1.08	1.05	1.07	1.11*
		Relative liver weights	1.00	1.01	1.05	1.10**	1.14**	1.00	1.09*	1.04	1.10**	1.16**
		Replicative DNA synthesis	1.00	1.13	1.17	1.77*	2.47**	1.00	1.15	1.17	1.69*	2.79**
		PROD activity	1.00	0.60	0.80	1.20	2.80**	1.00	0.88	1.00	1.88*	6.18*
		Hepatocellular hypertrophy	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10	0/10	3/10
Recovery study	7 days treatment +7 days recovery	Absolute liver weights	1.00	ND	ND	ND	1.04	ND	ND	ND	ND	ND
		Relative liver weights	1.00	ND	ND	ND	1.05	ND	ND	ND	ND	ND
		PROD activity	1.00	ND	ND	ND	1.28	ND	ND	ND	ND	ND
		Hepatocellular hypertrophy	0/10	ND	ND	ND	0/10	ND	ND	ND	ND	ND

Note. Values excluding histopathological findings are presented as fold of the control at each dose level. For hepatocellular hypertrophy, data represent the number of animals with the lesion/total number of animals examined. Values significantly different from control (0 ppm) are: * $p < 0.05$, ** $p < 0.01$. Values within shaded areas indicate toxicologically significant change. For female, combined time-cause and dose-dependency studies was conducted. Thus, the female data of the 7-day treatment in the time-course study are adopted from those of the dose-dependency study. So, data of the control and 3000 ppm groups were repeatedly presented. PROD: 7-pentoxyresorufin O-depentylase, SER: smooth endoplasmic reticulum, ND; not determined. Table is adapted from Okuda *et al*., (2017a).

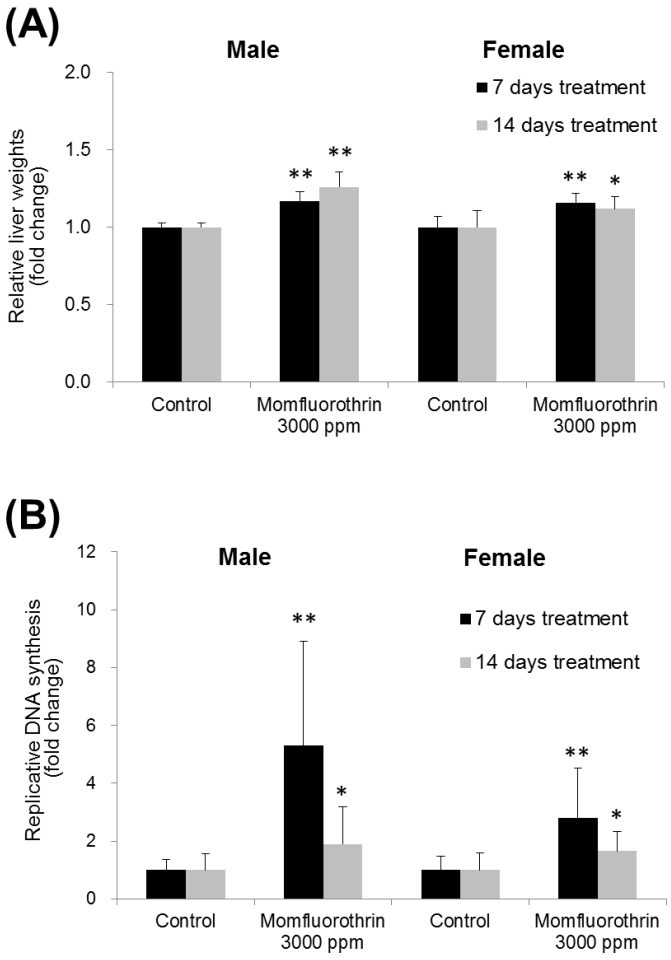

Figure 6. Time-course effects on liver of Wistar rats treated with momfluorothrin. Relative liver weights (A) and hepatocyte replicative DNA synthesis (B) were determined in livers of male and female Wistar rats (N=10/group) treated with 0 (control) and 3000 ppm momfluorothrin for 7 or 14 days. Data are presented as mean values±SD. Values statistically significant from control are: *p < 0.05; **p < 0.01. Figure is adapted from Okuda *et al.*, (2017a).

In vivo momfluorothrin dose-dependency and reversibility study in Wistar rats

Male and female Wistar rats were treated with 0 (control), 200, 500, 1500 and 3000 ppm momfluorothrin for 7 days. The selected data are presented in Table 4. Significant decreased body weight gains were observed at 1500 and 3000 ppm on day 3 due to palatability problems, however, these recovered to control levels by the end of the treatment period.

At the two highest momfluorothrin dose levels (1500 and 3000 ppm), relative liver weight (Fig. 7A) and replicative DNA synthesis (Fig. 7B) were significantly increased in both male and female rats. A small non dose-dependent increase in relative liver weight was also observed in female rats given 200 ppm momfluorothrin (Fig. 7A). PROD activity was also statistically significantly increased in both sexes at 1500 and 3000 ppm (except for males at 1500 ppm) (Fig. 7C). Increased PROD activity in males at 1500 ppm was a marginal change without statistical significance (1.2-fold of control). At 3000 ppm in both sexes, hepatocellular hypertrophy was observed in 2 and 3 of 10 animals in males and females, respectively (Table 4). In contrast, male and female rats administered 200 and 500 ppm revealed no treatment related changes in hepatocyte replicative DNA synthesis, hepatocellular hypertrophy and PROD activity.

All of the effects on liver weight, hepatocellular hypertrophy and PROD activity observed after 7 days of treatment with momfluorothrin returned to control levels upon cessation of treatment for 7 days (Table 4). The reversibility of the effect of momfluorothrin on hepatocyte replicative the DNA synthesis was not examined.

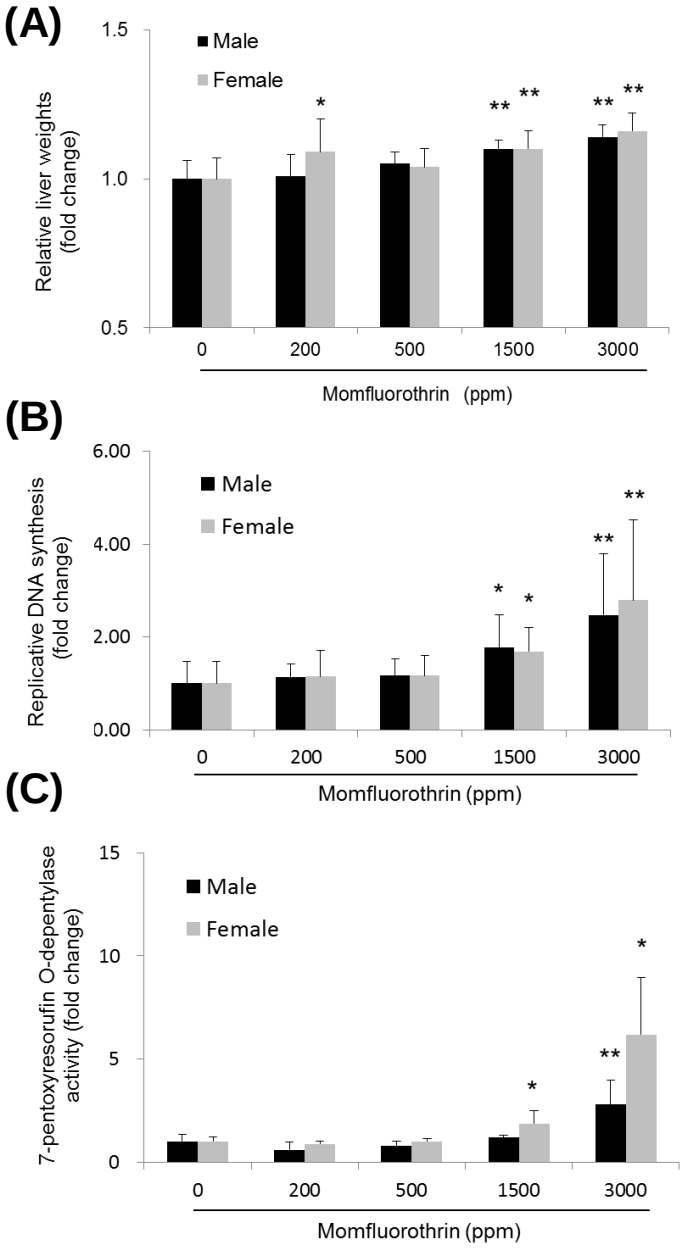

Figure 7. Dose-dependency of alterations in the Wistar rat liver treated with momfluorothrin. Relative liver weights (A), hepatocyte replicative DNA synthesis determined as BrdU labeling index (B), and hepatic PROD activity (C) were evaluated using male and female Wistar rat livers treated with momfluorothrin at 0, 200, 500, 1500, and 3000 ppm for 7 days. Data are presented as the mean values ± SD; 10 animals/dose/sex for A and B, 6 animals/dose/sex for C. Values statistically significant from control are: *$p < 0.05$; **$p < 0.01$.

Figure is adapted from Okuda *et al*., (2017a).

In vivo momfluorothrin study in WT and CAR KO rats

The hepatic effects of momfluorothrin were investigated in an *in vivo* study using a CAR KO rat model, which has only recently become commercially available. Male WT and CAR KO rats were given 0 (control) and 3000 ppm momfluorothrin for 7 days. In addition, the effects of 1800 ppm metofluthrin and 1000 ppm NaPB at, two known CAR-mediated MOA liver tumor inducers, were also investigated. The data are presented in Table 5. In the first experiment with WT rats, two of five animals treated with momfluorothrin showed severe toxicity due to a palatability problem as evidenced by marked decrease of body weights accompanied by considerable suppression of food consumption. However, there was individual variation of sensitivity to this palatability problem; the other 3 rats showed no such severe toxicities. These toxicities of momfluorothrin were also observed in the second experiment with WT rats and CAR KO rats, but the toxicity was less than those in the first experiment with WT rats.

Under such conditions, NaPB, metofluthrin (except in the first experiment, where a 1.6-fold increase was observed) and momfluorothrin significantly increased CYP2B1/2 mRNA levels in WT rats suggesting CAR functionally responded in WT rats; but not in CAR KO rats, indicating that this CAR KO rat model is reliable (Fig. 8A and 8B, Table 5). Replicative DNA synthesis was evaluated with groups of 5 rats in two separate experiments in WT rats. NaPB significantly increased (8.1 fold control) or increased (8.4 fold control) replicative DNA synthesis in the first and second experiments with WT rats, respectively; whereas metofluthrin increased (3.9 fold control) or significantly increased (4.1 fold control) and momfluorothrin significantly increased (3.1 fold control after excluding 2 animals with bad health condition in the first experiment) or increased (8.7 fold control) in the first and second experiments with WT rats, respectively.

Table 5. Summary of findings of the 7-day treatment study in WT and CAR KO Sprague Dawley rats

	WT rats								CAR KO rats			
	1st experiment (Animal No.1-5)				2nd experiment (Animal No.6-10)							
	Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm	Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm	Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm
Number of animals tested	5	5	5	5	5	5	5	5	5	5	5	5
Test Item Intake (mg/kg/day)	-	50.1	88.3	84.4	-	52.5	84.8	110.9	-	50.2	77.1	123.2
Death of animals	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Final Body Weight (g)	431.3±19.0	447.7±23.3	448.8±18.6	396.8±20.9*	451.0±18.3	442.7±10.5	432.8±18.8	418.8±24.8*	454.9±30.4	472.1±20.9	447.7±32.2	456.7±16.6
Total Body Weight Gain (g)	21.4±14.9	25.3±10.9	25.7±8.2	-15.7±24.1*	27.9±7.8	32.8±10.3	23.6±9.1	0.3±13.4**	23.1±7.3	40.6±15.6	18.2±8.9	21.8±5.8
Food Consumption at termination of treatment (g/animal/day)	129.1±14.4 a	153.8±5.3*	149.0±3.0	82.3±31.1	147.2±19.7	159.3±3.9	140.1±7.8	107.0±11.8**	148.5±6.6	160.7±13.9	139.8±20.3	135.2±11.8
Liver Weight Absolute (g)	13.78±0.88	18.03±0.89**	17.10±1.00**	13.61±1.92	15.57±0.47	18.08±1.26**	15.76±0.84	14.39±1.72	16.12±0.82	17.36±1.51	16.86±1.80	17.24±1.19
(fold control)	1.00	1.31	1.24	0.99	1.00	1.16	1.01	0.92	1.00	1.08	1.05	1.07
Relative (g/body weight×100)	3.20±0.20	4.03±0.23**	3.82±0.31**	3.42±0.35	3.46±0.17	4.09±0.30**	3.64±0.19	3.43±0.28	3.55±0.14	3.68±0.24	3.76±0.16	3.77±0.17
(fold control)	1.00	1.26	1.19	1.07	1.00	1.18	1.05	0.99	1.00	1.04	1.06	1.06
CYP2B1/2 mRNA level (% of control average)	100±47	31341±15125**	156±36	5416±2616*	100±16	24273±6593**	192±62*	2483±1636*	100±35	112±14	115±12	99±10
(fold control)	1.00	313.41	1.56	54.16	1.00	242.73	1.92	24.83	1.00	1.12	1.15	0.99
Replicative DNA synthesis (%)	0.90±0.53	7.28±5.53*	3.50±3.57	1.74±1.56 b	0.50±0.33	4.20±5.82	2.06±1.47*	4.36±6.35	3.56±1.38	3.70±2.24	1.78±0.93*	5.34±3.71
(fold control)	1.00	8.09	3.89	1.93 b	1.00	8.40	4.12	8.72	1.00	1.04	0.50	1.50

Due to limitation in the availability of the CAR KO rats, only five animals per dose could be used. For evaluation of replicative DNA synthesis, we consider that the numbers of animals per dose is sufficient although more optimal numbers would be eight to ten rats per dose. Since small number of animals examined (N=5) may result in an increased coefficient of variation (CV) and decreases the statistical power of the assay a second, experiment using wild type rats was conducted.

a: For the control group of the 1st experiment, food consumption was calculated by excluding two of the five animals due to severe diet spillage.

b: For the momfluorothrin group of the 1st experiment, two of five animals had severe toxicity as evidenced by marked suppression of body weight with decreased food consumption. When data of these two toxic animals are excluded, replicative DNA synthesis is 2.77 ± 0.96 (N=3). This value is 3.1-fold of control and statistically significant (p<0.01) compared to control, and is presented in Figure 5C as the data of the 1st experiment of wild-type rats.

Significantly different from control (F-test/Student t, or Welch test) : * p<0.05, ** p<0.01. Table is adapted from Okuda *et al*., (2017a).

Table 5. Summary of findings of the 7-day treatment study in WT and CAR KO Sprague Dawley rats (continued)

		WT rats								CAR KO rats			
		1st experiment (Animal No.1-5)				2nd experiment (Animal No.6-10)							
		Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm	Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm	Control	NaPB 1000 ppm	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm
Number of animals tested		5	5	5	5	5	5	5	5	5	5	5	5
Histopathology: Liver													
Hypertrophy, hepatocyte, centrilobular	±	0/5	1/5 $$	5/5 $$	0/5	0/5	1/5 $$	4/5 $	0/5	0/5	0/5	0/5	0/5
	+	0/5	4/5	0/5	0/5	0/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5
Hypertrophy, hepatocyte, diffuse	±	0/5	0/5	0/5	4/5$	0/5	0/5	0/5	3/5	0/5	0/5	0/5	0/5
Increased vacuole, glycogen-like, hepatocyte, diffuse	±	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Infiltration, mononuclear, focal	±	3/5	3/5	4/5	3/5	4/5	4/5	2/5	3/5	4/5	3/5	4/5	2/5
Vacuolation, hepatocyte, focal	±	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Vacuolation, hepatocyte, centrilobular	±	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Necrosis, hepatocyte, focal	±	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5

For histopathology, data represent the number of animals with the lesion/total number of animals examined. Grade of histopathology: ±, Slight; +, Mild; 2+, Moderate; 3+, Severe.

Significantly different from control (Wilcoxon rank sum test): $ p<0.05, $$ p<0.01. Table is adapted from Okuda *et al*., (2017a).

In contrast to the WT rats, these chemicals did not produce any significant increases in replicative DNA synthesis in the CAR KO rats (Fig. 8C, Table 5). Increased liver weights and/or hepatocellular hypertrophy were also observed in WT rats treated with NaPB, metofluthrin or momfluorothrin, while no such effects were observed in the CAR KO rats treated with these chemicals even at similar intakes of test agents between WT and CAR KO rats (Table 5). Taken together, these data demonstrate that the induction replicative DNA synthesis in rats treated with NaPB, metofluthrin and momfluorothrin is mediated through CAR.

When responses to momfluorothrin between Wistar rats (Table 4) and SD rats (Table 5, WT rats in the CAR KO rat study) were compared, both strains responded qualitatively similarly to momfluorothrin, as demonstrated by increases in CYP2B1/2 mRNA levels and replicative DNA synthesis. Quantitatively, the increases in CYP2B1/2 mRNA levels was greater in SD rats, with the increases being 40-fold (mean of 2 experiments with 54-fold and 25-fold increases) and 18-fold in SD and Wistar rats, respectively. However, for replicative DNA synthesis the response was equivalent in both SD and Wistar rats, the increases being 5.3-fold (mean of 2 experiments with 1.93-fold and 8.72-fold increases) and 5.3-fold, respectively. Overall, these findings demonstrate that SD rats are responsive to momfluorothrin and hence that SD CAR KO rats may be employed to evaluate the CAR-dependency of the hepatic effects of momfluorothrin.

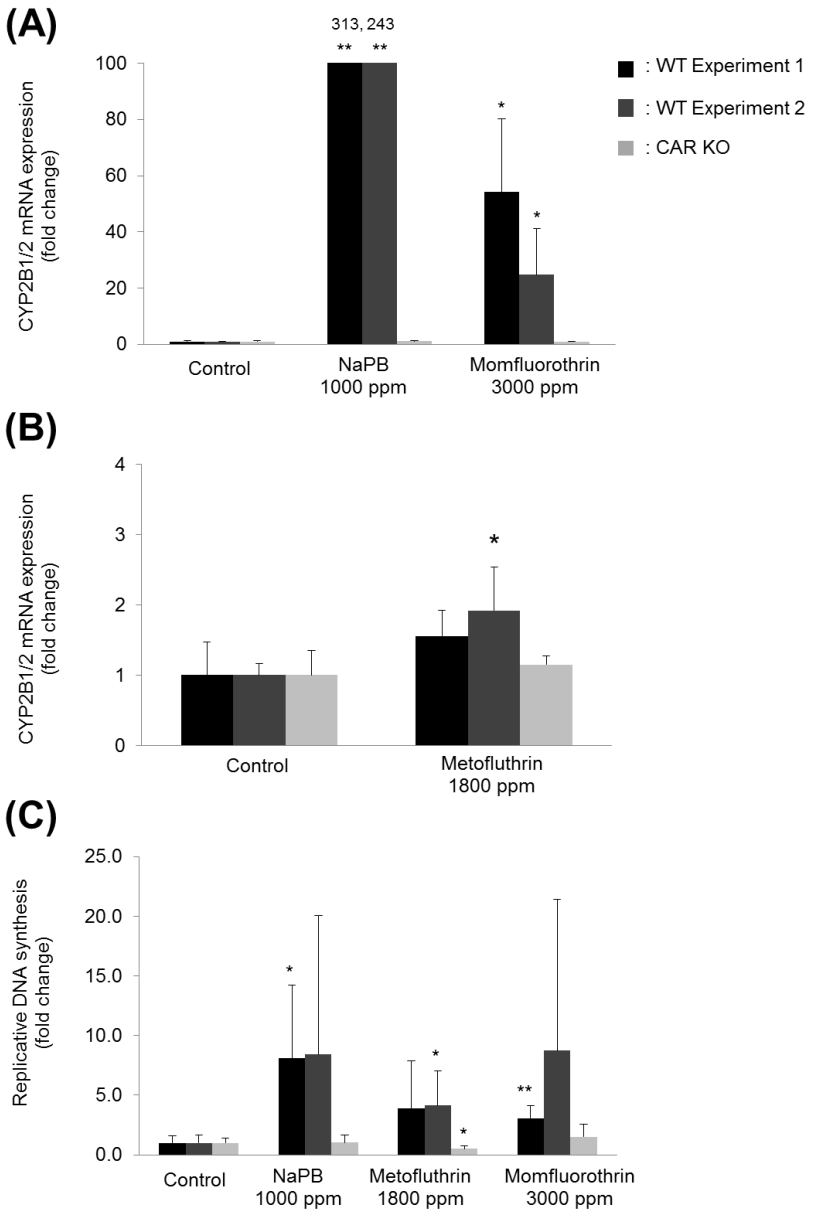

Figure 8. Effect of 1000 ppm NaPB, 1800 ppm metofluthrin and 3000 ppm momfluorothrin on CYP2B1/2 mRNA expression levels (A and B) and replicative DNA synthesis (C) in WT and CAR KO rats. Data are presented as mean values±SD (5 animals/group) with two experiments being performed in WT rats. For the momfluorothrin group in the first experiment, two of five animals revealed severe toxicity as evidenced by severe suppression of body weight with decreased food consumption. When data of the two toxic animals are excluded, replicative DNA synthesis is 2.77 ± 0.96 (N=3). This value is 3.1-fold of control and statistically significant ($p < 0.01$) compared to control, and thus that value is plotted in this figure. Values statistically significant from control are: *$p < 0.05$; **$p < 0.01$. Figure is adapted from Okuda *et al.*, (2017a).

Hepatic global gene expression and CYP mRNA levels

Since this analysis was conducted prior to the present MOA studies, as described in the Methods section, the study conditions were not completely the same as the present MOA studies. However, the data obtained is useful in helping establish the MOA for momfluorothrin-induced liver tumor formation. The global gene expression profile analysis were conducted in the liver of male Wistar rats treated with momfluorothrin at 3000 ppm for 14 days. Numbers of probe sets with 1.5-fold alterations compared to control group were 107 as up-regulation and 268 as down-regulation. Of these, some overlapped with genes altered after 14-day treatment with a CAR activator NaPB (1000 ppm): up-regulated gene were Akr7a3, Aldh1a1, Aldh1a4, App, Atpif1, Ces2, Cd14, Cyp2b2/Cyp2b15, Ephx1, Ddc, Gsta2, Gstm1, RGD1562732_predicted, Snx10, Udpgtr2 and Zdhhc2; down-regulated genes were Atf5, Eif4g2, C4bpb, Ciapin1, LOC678772, Nrd1, Oat, Spetex-2G, Srebf1 and Zdhhc23. The result of hierarchical clustering analysis showed that the gene expression pattern of the liver from rats treated with momfluorothrin at 3000 ppm was clustered most closely to that of NaPB (Fig. 9).

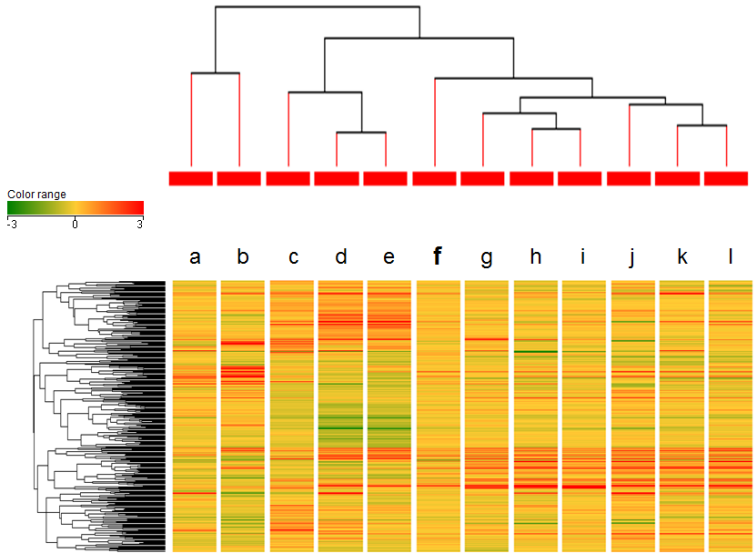

Figure 9. Hierarchical clustering analysis in Wistar male rats treated with momfluorothrin. Data are shown for clofibrate at 2500 ppm (a) and at 300 mg/kg bw/day (b), carbon tetrachloride at 100 mg/kg bw/day (c), thioacetamide at 200 ppm (d) and 15 mg/kg bw/day (e), momfluorothrin at 3000 ppm (f), and NaPB at 1000 ppm (g - k) and at 100 mg/kg bw/day (l). Wistar (a, d, f, g, i, and j), Crj:CD (SD) (b, c, e, and l), or F344 (h and k) male rats (3 or 4 animals/dose) were treated with test substances for 14 days.

Discussions

In two-year carcinogenicity study, the incidence of hepatocellular tumors was increased in both male and female rats treated with higher dose of momfluorothrin. To assess the human relevance of these rat liver tumors, a series of mechanism study was conducted by using WT and KO rats or RNAi technique to elucidate the MOA for rat hepatocellular tumors induced by momfluorothrin and discussed with nine of the modified Bradford Hill considerations as below.

1. Postulated MOA for momfluorothrin-induced rat liver tumor formation

Metofluthrin, a close structural analogue to momfluorothrin, also increased hepatocellular tumors in rats. The MOA for tumor formation by metofluthrin in rats was shown to be similar to that of the prototypic CAR activator phenobarbital (Kushida *et al.*, 2016; Yamada *et al.*, 2009, 2015). This MOA involves activation of CAR, which results in a pleiotropic response including the stimulation of altered gene expression specific to CAR activation, hepatocellular hypertrophy and increased hepatocellular proliferation. Prolonged treatment results in the formation of altered hepatic foci and liver tumors. Activation of CAR, increased cell proliferation and the development of altered hepatic foci are considered to be key events in the MOA for tumor formation by such compounds as they constitute necessary steps in the MOA (Elcombe *et al.*, 2014). The induction of CYP2B enzymes and liver hypertrophy (both morphological changes and increases in liver weight) are considered as associative events and as such represent reliable markers of CAR activation (Elcombe *et al.*, 2014). Based on a close structural analogue, metofluthrin, the MOA for momfluorothrin-induced rat liver tumor formation

was postulated to also involve activation of CAR. The MOA data was summarized in Table 4 and 5 and the validity of this hypothesis was discussed below.

2. MOA data

Activation of the nuclear CAR (Key event #1)

CYP2B enzyme induction in rodent liver involves activation of CAR (Deguchi *et al.*, 2009; Wei *et al.*, 2000; Yamamoto *et al.*, 2004). To probe the role of CAR in the MOA for momfluorothrin-induced rat liver tumor formation, the RNAi technique was employed. CAR-siRNA was used to reduce CAR mRNA levels in order to examine CAR involvement on the effect of momfluorothrin on CYP2B1/2 mRNA induction in Wistar rat hepatocytes, employing a similar experimental design to that previously used in the metofluthrin study (Deguchi *et al.*, 2009). NaPB was employed as a positive control for these studies. The treatment of Wistar rat hepatocytes with CAR-siRNA significantly reduced CAR mRNA in the presence of either NaPB and momfluorothrin, resulting in a reduction in the magnitude of induction of CYP2B1/2 mRNA levels by both compounds (close to significant, p=0.07) (Fig. 5D and 5E). These findings demonstrate that momfluorothrin induces CYP2B1/2 mRNA through CAR in Wistar rat hepatocytes and hence momfluorothrin is a CAR activator. This was also strongly supported by the *in vivo* study in CAR KO rats; where 3000 ppm momfluorothrin increased CYP2B1/2 mRNA in WT rats but not in CAR KO rats (Fig. 8A, Table 5).

Hepatic CYP2B induction (Associative event)

In Wistar male rats, hepatic CYP2B activity was induced 2.8-fold after 7 days treatment with 3000 ppm of momfluorothrin (Fig. 7C, Table 4). A marginal increase in

CYP2B activity to 1.2-fold of control was observed in male rats given 1500 ppm momfluorothrin for 7 days (Table 4). In addition, hepatic CYP2B1/2 mRNA levels in male rats given 3000 ppm momfluorothrin for 7 or 14 days were robustly increased by 16 or 18 fold, respectively (Fig. 4, Table 4), suggesting that CYP2B mRNA levels would also have been significantly increased at lower doses of momfluorothrin. In females, increases in hepatic CYP2B activity were observed at momfluorothrin dose levels of 1500 and 3000 ppm after 7 days (Fig. 7C, Table 4) and at a dose level of 3000 ppm after 14 days (Table 4).

Liver hypertrophy (Associative event)

Treatment with momfluorothrin resulted in significant increases in liver weights associated with hepatocellular hypertrophy throughout the liver lobule (Table 4). In addition, increased smooth endoplasmic reticulum, characteristic of enzyme inducers (Ghadially, 1997), was observed in liver sections from male and female rats given a high 6000 ppm dose level of momfluorothrin for 13 weeks (ECHA, 2014).

By transmission electron microscopy, the treatment of male rats with 3000 ppm momfluorothrin for 7 and 14 days did not result in any apparent increase in numbers and size of peroxisomes. This confirms the results of the selected CYP mRNA analysis and the global gene expression studies that momfluorothrin is not PPARα agonist in rat liver (Fig. 4, Fig. 9, and Table 4).

Altered gene expression specific to CAR activation (Key event #2)

Activation of CAR in both the mouse and rat results in changes in the expression of a large number of genes involved in phase I and II xenobiotic metabolism, cell

proliferation, apoptosis and energy metabolism (Elcombe *et al.*, 2014). Although studies in WT mice and CAR-KO mice have demonstrated that not all genes affected by PB are CAR-dependent, some of genes were only induced in WT mice, suggesting CAR specific genes. The altered genes by momfluorothrin treatment at 3000 ppm for 14 days overlapped with genes altered after 14-day treatment with NaPB as shown in this study (Okuda *et al*., 2017a). In addition, those by momfluorothrin also overlapped with genes altered after 7-day treatment with a CAR activator metofluthrin (1800 ppm) or NaPB (1000 ppm); i.e., CYP family 2, subfamily b; polypeptide 2 aldehyde dehydrogenase; aldo-keto reductase; UDP-glucuronosyltransferase; glutathione-S-transferase and epoxide hydrolase (Deguchi *et al.*, 2009). Interestingly, CAR specific genes demonstrated by Ueda et al. (2002), those are also included CYP family 2, subfamily b; aldehyde dehydrogenase; and glutathione-S-transferase, also overlapped. These findings suggest that momfluorothrin alters gene expression specific to CAR.

Increased cell proliferation (Key event #3)

The treatment of male and female Wistar rats with 1500 and 3000 ppm momfluorothrin for 7 days resulted in significant increases in replicative DNA synthesis (Fig. 7B, Table 4). Significant increases in replicative DNA synthesis were also observed in male and female Wistar rats given 3000 ppm momfluorothrin for 14 days (Table 4). However, the magnitude of the increase in replicative DNA synthesis after 14 days was less marked than that observed after 7 days in both sexes (Fig. 6B, Table 4). This finding is consistent with previous studies with other mitogenic rodent liver CAR activators where short-term treatment results in a peak stimulation of hepatocyte labeling index values (Cohen and Arnold, 2011; Elcombe *et al.*, 2014; Lake, 2009; Lake

et al., 2015; Yamada *et al.*, 2009). Although the hepatocyte labeling index returns to control levels with continued treatment with the compound, the overall number of cell replications per animal is increased. This is because treatment with such chemicals causes a sustained increase in liver weight which results in an overall increase in the number and size of hepatocytes (Cohen and Arnold, 2011; Elcombe *et al.*, 2014; Lake, 2009). For example, in a study employing a stereological technique, an increase in the total number of hepatocytes per animal was observed in rats treated with PB for 12 weeks (Carthew *et al.*, 1998). Thus, although hepatocyte labeling index values may return to control levels after continued momfluorothrin treatment, the number of cell replications in treated animals continues to be enhanced due to the increase in the total number of hepatocytes per animal. The continued stimulation of cell proliferation may lead to tumor formation as a result of critical errors being produced during cell replication and/or enhanced proliferation of spontaneously initiated pre-neoplastic hepatocytes (Cohen and Arnold, 2008; Schulte-Hermann *et al.*, 1983).

Furthermore, momfluorothrin increased replicative DNA synthesis in WT rats (Tables 4 and 5), but not in CAR KO rats (Table 5), demonstrating that CAR activation is required for momfluorothrin-increased hepatocellular proliferation.

Clonal expansion leading to altered hepatic foci (Key event #4)

The treatment of male and female Wistar rats with momfluorothrin for 2 years resulted in liver tumor formation. In addition to the formation of liver tumors, the chronic treatment of male and female rats with 3000 ppm momfluorothrin also resulted in significant increases in eosinophilic hepatocellular foci (Table 1).

Liver adenoma/carcinoma (Key event #5)

The incidences of the total number of animals with hepatocellular adenomas and/or carcinomas of the 0, 200, 500, 1500 and 3000 ppm groups were 2, 0, 4, 12 and 33% for males, and 0, 0, 2, 2 and 10% for females, respectively (Table 1). Treatment with 3000 ppm momfluorothrin significantly increased the incidence of hepatocellular adenoma in both sexes and of hepatocellular carcinoma in male rats. The combined incidence of hepatocellular adenoma and carcinoma was significantly increased in male and female rats given 3000 ppm momfluorothrin, with a non statistically significant increase being observed in male rats given 1500 ppm momfluorothrin. Therefore, the incidences of hepatocellular adenoma, carcinoma, and combined in males given 1500 and 3000 ppm momfluorothrin were equivalent to or higher than the maximum incidence of the historical background; and the combined incidence of female rats given 3000 ppm momfluorothrin was within the historical background incidence, while incidence of carcinoma was equivalent to the maximum incidence of the historical background. Overall, treatment with momfluorothrin in rats for 2 years produced hepatocellular tumors in males at 1500 and 3000 ppm and in females at 3000 ppm. The no tumorigenic dose levels (no observed effect levels for tumors) in male and female rats were established at 500 ppm and 1500 ppm, respectively.

3. Concordance of dose-response relationships

The present MOA study was performed with momfluorothrin doses used in the 2-year bioassay. As shown in Fig. 7, the effects of momfluorothrin on CYP2B enzyme induction, hypertrophy (liver weight), and cell proliferation (replicative DNA synthesis) showed similar dose-dependency. In particular, in males and females at 3000 ppm, those

effects were significantly increased and corresponded with the tumor inducing dose (Table 6). In addition to the 7-day study, increases in hepatocyte hypertrophy and relative liver weights were also observed at 1500 ppm and above in both male and female Wistar rats after treatment for 52 weeks or longer (ECHA, 2014).

In males at 1500 ppm, the increased incidence of liver tumor was equivocal; not statistically significant, but equivalent to or higher than the maximum incidence of the historical background in male Wistar rats. Thus 1500 ppm in males appeared to be borderline for tumor production. The increased relative liver weights and hepatocellular proliferation observed in rats given 1500 ppm momfluorothrin correlated with the observed tumor formation (Table 6). The treatment of male Wistar rats with 1500 ppm momfluorothrin did not result in a significant increase in CYP2B enzyme activity (Fig. 7C), hence the dose response between tumor formation and CYP2B induction was not completely matched (Table 6). However, there were 16-18 fold increases in CYP2B1/2 mRNA levels in male Wistar rats given 3000 ppm momfluorothrin for 7 and 14 days (Table 4), which suggest that some increase in CYP2B1/2 mRNA levels would have been observed in male rats treated with 1500 ppm momfluorothrin. Moreover, hepatocellular tumor incidence at this dose level was only marginally increased (not statistically significant) and increased incidences of eosinophilic hepatocellular foci were only observed at 3000 ppm in both sexes (Table 1). The tumor incidence data suggests that the potency for CAR activation by momfluorothrin would be less at a dose level of 1500 ppm than at 3000 ppm.

Table 6. Temporality and dose-response for MOA key events related to male and female Wistar rat liver tumors.

Temporal →

Dose level ↓

	Dose (ppm)	***Key Event #1*** CAR activation: Biomarker CYP2B mRNA and activity	***Key Event #1*** CAR activation: *Associative Event*: Increased liver weight and hypertrophy	***Key Event #2*** A altered gene expression specific to CAR activation	***Key Event #3*** Increased cell proliferation [a]	***Key Event after recovery***	***Key Event #4*** Clonal expansion leading to altered hepatic foci	***Key Event #5*** Liver adenomas/ carcinomas
Time points		7, 14 days	7, 14 days 13, 52, 104 weeks	14 days	7, 14 days	7 days after 7 days treatment	104 weeks	104 weeks
Males								
WT	200	-	-	ND	-	ND	-	-
	500	-	-	ND	-	ND	-	-
	1500	- [c]	+ (7D -104W)	ND	+ (7D) [d]	ND	-	+ (104W) [e]
	3000	+ (7 - 14D)	+ (7D- 104W)	+ (14D)	+ (7 - 14D)	-	+ (104W)	+ (104W)
CAR KO	3000 [b]	-	-	ND	-	ND	ND	ND
Females								
WT	200	-	-	ND	-	ND	-	-
	500	-	+ (52W)	ND	-	ND	-	-
	1500	+ (7D)	+ (7D - 104W)	ND	+ (7D) [d]	ND	-	-
	3000	+ (7 - 14D)	+ (7D - 104W)	ND	+ (7 - 14D)	ND	+ (104W)	+ (104W)

WT: wild type Wistar rats. CAR KO: CAR Knock out rats. + with shadow indicates effect present at indicated time points of the treatment. - indicates effect absent at indicated time points of the treatment. ND indicates No data.

a: Although hepatocyte labeling index values, determined as BrdU labeling index, may return toward control levels with continued momfluorothrin treatment for more than 14 days, the number of cell replications in treated animals appeared to be enhanced due to the increased total number of hepatocytes per animal. In this Table, “Increased cell proliferation” means “Increased total cell proliferation”.

b: The key/associative events in the CAR KO rat study were determined after 7 days treatment.

c: Increased PROD activity in males at 1500 ppm was a marginal change without statistical significance (1.2-fold of control).

d: Cell proliferation in both sexes at 1500 ppm were determined only after 7 days treatment.

e: The combined incidence of hepatocellular adenoma and carcinoma was increased in male rats given 1500 ppm momfluorothrin, with a non-statistically significant. As the incidences of hepatocellular adenoma, carcinoma, and combined in males given 1500 ppm momfluorothrin were equivalent to or higher than the maximum incidence of the historical background, 1500 ppm was concluded as tumor inducing dose level. Table is adapted from Okuda *et al*., (2017a).

Indeed, the present data demonstrate that the effects on hepatocellular proliferation, CYP2B enzyme induction and liver hypertrophy were less marked in male Wistar rats given 1500 compared to 3000 ppm momfluorothrin (Table 4).

In females given 1500 ppm momfluorothrin, while the early events such as the increased hepatic CYP2B activity, relative liver weight and hepatocellular proliferation were observed after 7 days of treatment, liver tumor formation was not increased (Table 6).

4. Temporal Association

If a key event (or events) is an essential element for carcinogenesis, it must precede the appearance of the tumors. Data are available for the effect of treatment of male and female Wistar rats with momfluorothrin at various time points ranging from 7 days to 2 years (Table 6). The treatment of male and female Wistar rats with momfluorothrin for 7 days resulted in an induction of CYP2B enzymes. Cell proliferation, assessed as the hepatocyte labeling index, was increased in male and female Wistar rats given tumorigenic dose levels of momfluorothrin for 7 and 14 days. Momfluorothrin produced increases in liver hypertrophy (assessed both by morphology and as increases in liver weight) at a number of early time points and also at later time points. Examination of liver sections from the momfluorothrin two year bioassay revealed increases in altered cell foci at the tumorigenic dose level of 3000 ppm in male and female Wistar rats. Three of 51 male Wistar rats given 1500 ppm momfluorothrin also had altered cell foci (Table 1). As altered foci are the precursor lesions for subsequent tumor formation in rodent liver (Elcombe *et al.*, 2014), it is considered that the liver foci developed before the appearance of liver tumors. Overall, there is a logical temporal sequence for all key

and associative events in momfluorothrin-induced liver tumor formation, in which all key and associative events precede tumor formation (Table 6).

In females at 1500 ppm, while the early events such as the increased hepatic CYP2B activity, relative liver weights and hepatocellular proliferation were observed after 7 days of treatment, liver tumor was not increased (Table 6). Incidence of the eosinophilic hepatocellular foci was observed at 1500 ppm with a non-statistically significant increase and higher than average but less than highest incidence of historical control (Table 1). These finding suggest that 1500 ppm momfluorothrin induced the early events but not later events promoting from foci to tumor in female Wistar rats.

5. Strength, consistency and specificity of association of key events and tumor response

As mentioned above, the effects of momfluorothrin on key and associative events at early phase of treatment correlated with the dose-relationship for liver tumor formation. Furthermore there is a logical temporal sequence for all key and associative events in momfluorothrin-induced liver tumor formation, in which all key and associative events precede tumor formation.

The effects of momfluorothrin on liver weight, hepatocellular hypertrophy and CYP2B enzyme induction after 7 days of treatment were shown to be reversible after 7 days of cessation of treatment. Therefore, effects of short term treatment with momfluorothrin on the liver are reversible, which is consistent with the known hepatic effects of other mitogenic rodent liver CAR activators (LeBaron *et al.*, 2013, 2014; Osimitz and Lake, 2009; Tinwell *et al.*, 2014; Yamada *et al.*, 2014).

6. Biological plausibility and coherence

The proposed MOA for liver tumor formation by momfluorothrin involves activation of CAR which results in the key event of increased cell proliferation. Associative events include induction of CYP2B enzymes and liver hypertrophy. Prolonged treatment results in the formation of altered hepatic foci and liver tumors (Table 6). This MOA is similar to that of several other non-genotoxic rodent liver carcinogenic agents which are CAR activators (Currie *et al.*, 2014; Lake *et al.*, 2015; LeBaron *et al.*, 2013, 2014; Osimitz and Lake, 2009; Tinwell *et al.*, 2014; Yamada *et al.*, 2009, 2014).

As described earlier, the key events for the MOA for momfluorothrin-induced rat liver tumor formation comprise CAR activation, increased cell proliferation and the development of altered hepatic foci as these constitute necessary steps in the MOA. The induction of CYP2B enzymes and liver hypertrophy comprise associative events and represent reliable markers of CAR activation. The role of CAR in CYP2B enzyme induction and increased hepatocellular proliferation by momfluorothrin was also demonstrated in cultured rat hepatocytes using the RNAi technique and in an *in vivo* study in CAR KO rats. These findings are consistent with those of metofluthrin (Deguchi *et al.*, 2009; Yamada *et al.*, 2009).

7. Other modes of action

Liver tumors can be produced in rodents by both genotoxic and non-genotoxic agents (Cohen and Arnold, 2011; Williams, 1997). Momfluorothrin is clearly not genotoxic, being negative in a variety of *in vivo* and *in vitro* genotoxicity assays (Ames test, *in vitro* chromosomal aberration test, *in vitro* gene mutation assay, UDS assay and

mouse micronucleus test)(ECHA, 2014). Liver tumors can be produced in rodents by various non-genotoxic MOAs including cytotoxicity, activation of CAR, activation of PPARα, porphyria, statins and hormonal perturbation (Cohen, 2010; Cohen and Arnold, 2016; Holsapple *et al.*, 2006; Klaunig *et al.*, 2003; Meek *et al.*, 2003). In the general toxicity studies, utilizing both histopathology and electron microscopy techniques, there was no evidence of hepatocellular toxicity (e.g. necrosis, fatty liver), peroxisome proliferation, porphyria, statin-like alterations, increased iron deposition or any evidence of hormonal perturbation. In addition, as shown in Fig. 4, unlike effects on CAR, gene expression profiling analysis studies demonstrated no marked alterations in either PPARα, AhR or PXR signaling. Overall, it is considered that the hepatic effects of momfluorothrin in the rat and subsequent liver tumor formation are mediated by activation of CAR.

Although some oxidative stress related genes were increased in the global gene expression analysis (Okuda *et al*., 2017a), the direct endpoints related to oxidative stress have not examined. Oxidative stress in the liver after administration of PB or metofluthrin was assessed by MDA as an indicator of lipid peroxidation, as well as total and reduced glutathione as a measure of antioxidant capacity. However, no evidence was obtained for the involvement of oxidative stress in PB or metofluthrin-induced rat liver tumor formation (Deguchi *et al.*, 2009). In the liver of rats treated with momfluorothrin, there was no histopathological evidence indicating increased oxidative stress, such as degenerative findings like necrosis and fibrosis. Though oxidative stress has been implicated as an important factor in the carcinogenesis process for both genotoxic and nongenotoxic mechanisms (Klaunig and Kamendulis, 2004), it was not identified as a key event for liver tumor formation by PB and other CAR activators

(Elcombe *et al.*, 2014).

8. Uncertainties, inconsistencies, and data gaps

No data have been obtained for effects of momfluorothrin treatment on apoptosis and inhibition of gap junctional intercellular communication, which were identified as an associative event and an associative event/modulating factor, respectively, in the MOA for PB-induced rodent liver tumor formation proposed by Elcombe *et al.* (2014). I consider that, since decreased apoptosis or inhibition of gap junctional intercellular communication are not key events (Elcombe *et al.*, 2014), these data gaps do not alter the overall conclusion regarding the postulated MOA for momfluorothrin-induced rat liver tumors.

9. Conclusions

These data are considered adequate with a high degree of confidence to explain the development of liver tumors in rats following chronic administration of momfluorothrin. As described above, a plausible MOA for momfluorothrin-induced rat liver tumor formation have been established as CAR activated MOA, with a strong dose-dependency and temporal consistency, that is similar to certain other non-genotoxic mitogenic rodent liver carcinogenic agents which are CAR activators including PB and metofluthrin (Currie *et al.*, 2014; Kushida *et al.*, 2016; Lake *et al.*, 2015; LeBaron *et al.*, 2013, 2014; Osimitz and Lake, 2009; Tinwell *et al.*, 2014; Yamada *et al.*, 2014, 2015). Alternative MOAs for momfluorothrin-induced rat liver tumor formation have been excluded. Therefore, the answer to question 1 “Is the WoE sufficient to establish a MOA in animals?” in the IPCS framework is **Yes**.

Chapter 2

Evaluation of human relevancy for rat hepatocellular tumors induced by momfluorothrin

2-1

Utility analysis of novel humanized chimeric mice with human hepatocytes for human risk assessment treated with CAR activator, NaPB

Introduction

In the previous chapter, it was identified the MOA for momfluorothrin-induced rat liver tumors mediated by CAR activation pathway. CAR is the molecular target of CAR activators (i.e. PB, metofluthrin, and momfluorothrin) and activation of this receptor is an essential for the rodent liver tumor development (Okuda *et al*., 2017a; Yamada *et al*., 2015; Elcombe *et al*., 2014; Huang *et al*., 2005; Wei *et al*., 2000; Yamamoto *et al*., 2004). A number of studies using PB have shown that the CAR is also present in the human liver and that the receptor can be activated by various drugs and chemicals (Elcombe *et al*., 2014; Molnar *et al*., 2013; Moore *et al*., 2003), but CAR-mediated liver non-genotoxic carcinogenesis is not generally considered to be human-relevant (Cohen, 2010; Elcombe *et al*., 2014; Holsapple *et al*., 2006; Lake, 2009) because the key species difference is that while PB stimulates replicative DNA synthesis in rodent hepatocytes, such effects are not observed in cultured human hepatocytes (Elcombe *et al*., 2014; Hirose *et al*., 2009; Lake, 2009; Parzefall *et al*., 1991). In addition, there is no clear evidence for PB-associated liver cancer risk in humans based on epidemiological data from a large number of clinical studies including long-term therapeutic treatment of epileptics (Friedman *et al*., 2009; La Vecchia & Negri, 2014). Based on these data, evaluation utilizing the ILSI/IPCS framework (Boobis *et al*., 2006) demonstrates that the MOA for PB-induced liver carcinogenicity is not relevant for humans (Cohen, 2010; Elcombe *et al*., 2014; Holsapple *et al*., 2006; Lake, 2009).

In addition to the use of hepatocyte culture systems, if the key species difference in PB-stimulated hepatocyte replicative DNA synthesis could be evaluated in an *in vivo* experiment, the results obtained would provide important data for the human risk

assessment of CAR activators like PB. One possible *in vivo* system for such a study is the use of humanized mice (Foster *et al*., 2014; Kitamura and Sugihara, 2014). 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 with liver disease (Tateno *et al*., 2004; 2013). The host mouse hepatocytes are replaced with human hepatocytes in the livers of the chimeric mice to the degree indicated by the replacement index, which is the occupancy ratio of the human hepatocyte area to the total (human and mouse) area on histological sections. In some cases, the replacement index in these mice can be as high as 96%. The transplanted human hepatocytes express mRNA for a variety of human drug-metabolizing enzymes and transporters, in a manner similar to that of the donor liver (Tateno *et al*., 2004; 2013). Chimeric mouse livers constructed with human hepatocytes contain a small percentage of mouse hepatocytes and mouse hepatic sinusoidal cells (mainly Kupffer cells, endothelial cells, and stellate cells). Human hepatocytes have been shown to cooperate with mouse hepatic sinusoidal cells in liver functions such as enzyme induction (Tateno *et al*., 2004) and viral infection (Mercer *et al*., 2001). Interestingly, human hepatocytes in the livers of chimeric mice are also 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 GH-related signalling molecules (Masumoto *et al*., 2007). Furthermore, partial hepatectomy also enhances hepatocellular proliferation in chimeric mice (personal communication, Dr. Chise Tateno). These

findings thus suggest that transplanted human hepatocytes in chimeric mice are responsive to hepatocyte mitogens.

The objective of the present study was to examine whether studies with chimeric mice with humanized liver could provide further support for previous evaluations of the MOA for PB-induced rodent liver tumor formation which have concluded that this mitogenic MOA is not relevant for humans (Cohen, 2010; Elcombe *et al*., 2014; Holsapple *et al*., 2006; Lake, 2009). The effects of NaPB-treatment on key and associative events observed at the early phase of treatment were compared in CD-1 mice, Wistar Hannover rats, and in chimeric mice with humanized liver.

These obtained experimental and analytical data have already been published from Toxicological Sciences (Yamada *et al*., 2014).

Materials and Methods

Test chemical.

Momfluorothrin and metofluthrin were provided by Sumitomo Chemical Co., Ltd. (See chapter 1). NaPB (Lot No. TLQ4248; purity, 98.0%) and human epidermal growth factor (hEGF; AF-100-15), Peprotech (EC, London, United Kingdom) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and husbandry.

All experiments were performed in accordance with *The Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd.; The Guide for Biosafety of Sumitomo Chemical Co., Ltd.; or with the ethical approval of the PhoenixBio Ethics Board.*

CD-1 mouse and Wistar Hannover rat.

Male Crlj:CD1(ICR) mice aged 9 weeks and male HarlanRCCHanTM:WIST rats (WH rats) aged 9 weeks were purchased from Charles River Japan, Inc., Hino Breeding Center (Shiga, Japan) and Japan Laboratory Animals, Inc., Hanno Breeding Center (Saitama, Japan), respectively. The animal acclimatization and environmental conditions were same as chapter 1.

Chimeric mouse.

The in-life phase of the experiment using chimeric mice and SCID mice was performed at PhoenixBio Co., Ltd. (Higashihiroshima, Japan). Chimeric mice with human hepatocytes (PXB mouse®, PhoenixBio Co., Ltd) were produced as previously described (Tateno *et al.*, 2004; 2013). Briefly, cryopreserved human hepatocytes (donor

ID: BD195) were purchased from BD Biosciences, Woburn, MA, USA. Human hepatocytes were transferred to homozygotic cDNA-uPA$^{+/+}$/SCID mice aged 20-30 days as donor cells for the chimeric mice because cells from young subjects are more responsive to stimulation of hepatocellular proliferation (Masumoto *et al.*, 2007). For transplantation, vials of cryopreserved human hepatocytes (5-15 x 10^6 cells/vial) were thawed and transplanted into 20-60 uPA/SCID mice (2.5 x 10^5 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. Chimeric mice have previously been shown to have almost confluent human hepatocytes at 64 days and 81 days after transplantation (Tateno *et al.*, 2004). To reduce possible variation of background levels of replicative DNA synthesis, treatment with NaPB was commenced more than 70 days after transplantation (animal age 14-17 weeks). Human hepatocytes in chimeric mice are considered to be deficient in GH because the human GH receptor is unresponsive to mouse GH (Souza *et al.*, 1995). Due to a lack of human GH in 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 minipumps (Model 1002, Alzet Corporation, Palo Alto, CA, U.S.A.) containing recombinant human GH (Wako Pure Chemical Industries Ltd.), with a release rate of 2.05 μg/hr, were implanted in the subcutaneous tissue of mice under isoflurane anesthesia on the day prior to 7 days before the commencement of chemical treatment. 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 hour light (8:00 - 20:00)/ 12 hour dark (20:00 - 8:00) illumination cycle. A commercially available pulverized diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo) containing vitamin C (300 mg/100g diet) and filtered tap water were provided *ad libitum* throughout the study. The animals were not fasted overnight prior to sacrifice. Mice were randomly assigned to each group based upon body weight and replacement indices, so that there was no significant difference in mean body weight and replacement indices among the groups.

Design for study.

In the hEGF study, the effects of hEGF treatment (150 μg/kg, 4 times per day, i.p., for 2 days) on human hepatocyte replicative DNA synthesis were determined in chimeric mice (5 animals/dose).

The design of the NaPB study is summarised in Table 7. Male CD-1 mice and WH rats (8 animals/dose) were fed diets containing 0 (control), 500, 1000, 1500, or 2500 ppm NaPB for 7 days. In previous bioassays, liver tumors were increased in a number of mouse strains at a NaPB dietary level of 500 ppm (IARC, 2001; Whysmer *et al.*, 1996) and at a dietary level of 1000 ppm in male mice of the low spontaneous tumor incidence C57BL/10J mouse strain (Jones *et al.,* 2009). Therefore, dietary levels of 500 and 1000 ppm were selected for this study. In addition, in order to evaluate the effects of higher doses of NaPB, dietary levels of 1500 and 2500 ppm were also investigated. In the dose setting study where chimeric mice (3 mice/dose) with less than a 70% replacement index were given NaPB at dose levels of 0, 1500, 2000, and 2500 ppm, one of three animals died at each of 2000 and 2500 ppm doses. Diets containing 0 (control)

or 1500 ppm were fed to chimeric mice (5 mice/dose) with more than a 70% replacement index for 7 days in the main study. However, 3 of 5 chimeric mice given 1500 ppm NaPB also died. Replicative DNA synthesis and other data was thus only obtained from two animals at 1500 ppm. Therefore, additional chimeric mice fed diets containing 0 (control), 500, and 1000 ppm were investigated and the combined data from these two experiments were evaluated to obtain dose-response effects of NaPB in chimeric mice. SCID mice were treated with 0 (control) and 1500 ppm for comparison with the highest dose group in the chimeric mouse study. As significant increases in hepatocyte replicative DNA synthesis, determined as the hepatocyte labeling index, are observed transiently at the early phase of treatment with NaPB in rodents (Cohen and Arnold, 2011; Elcombe *et al*., 2014; Lake, 2009), a 7-day treatment period was selected for the present study.

Mortality, body weight, and food consumption were monitored throughout the study. Alzet minipumps (Model 2001 in CD-1 and chimeric mice, Model 2ML1 in rats; Alzet Corporation) containing BrdU (Sigma Company, St Louis, MO, U.S.A.), with a release rate of 40 and 200 μg/hr, respectively, were implanted in the subcutaneous tissue of mice and rats, under anesthesia with diethyl ether or isoflurane on the day prior to 7 days (2 days for the hEGF study) before the scheduled euthanasia. After the 7-day treatment period, blood was collected at euthanasia from all surviving animals from the abdominal aorta under diethyl ether anesthesia (CD-1 mice and WH rats) and from the heart under isoflurane anesthesia (chimeric mice and SCID mice) without prior fasting.

Table 7. Summary of study design of NaPB study.

Animals	CD-1 mice	Wistar Hannover rats	Chimeric mice [a]	SCID mice
Sex	Male			
Age (weeks)	10	10	14	10-14
Number of animals per dose	8	8	5 [b]	5
NaPB dose levels (ppm)	0, 500, 1000, 1500, 2500	0, 500, 1000, 1500, 2500	0, 500, 1000, 1500 [c]	0, 1500
Human GH treatment [d]	No	No	Yes	No
Administration route	In diet			
Administration period (days)	7			
Measurements [e]	Body weight, food consumption, plasma concentration of PB, hepatic CYP2B activity (PROD activity), hepatocyte replicative DNA synthesis (BrdU labeling index), liver histopathology (light and electron microscopy), functional transcriptomic and metabolomic analyses, and selected gene expression determined by quantitative real-time polymerase chain reaction			

a: The range of replacement index with human hepatocytes in chimeric mice used in the present study was 73-90%.

b: Control (0 ppm) group of chimeric mice consisted of 9 animals because data from two experiments were combined.

c: Chimeric mice were not examined at 2500 ppm due to death in preliminary dose setting study. Data at 1500 ppm in chimeric mice is from two surviving animals.

d: Due to a lack of human GH in 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 minipumps containing recombinant human GH were implanted in the subcutaneous tissue of mice on the day prior to 7 days before the commencement of NaPB treatment.

e: Electron microscopy was only examined in chimeric mice, and functional transcriptomic and metabolomic analyses were not examined in SCID mice.

Table is adapted from Yamada *et al.*, (2014).

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. After necropsy, a piece of liver from all surviving animals was removed and stored in RNA stabilization solution (Life Technologies Co., Carlsbad, CA) 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, whereas the rest of the liver tissue was processed for hepatic microsomal CYP enzyme activity, histopathology, and replicative DNA synthesis measurements. The blood samples were immediately centrifuged at 2150 × g for 15 min at 4 °C and the plasma samples obtained were immediately extracted with a 3-fold volume of acetonitrile. The supernatants were stored at -80 °C until examined by liquid chromatography/mass spectrometry (LC/MS) analysis.

Plasma concentration of PB.

Chromatographic separation was performed by a CapcellPak C18 MG II column (35 x 3 mm, 3 µm, Shiseido). The mobile phase was A; 0.1% formic acid in water and B; acetonitrile delivered at a flow rate of 0.5 mL/min, and the gradient condition was %B = 5% (0 min) - 100% (5 min). MS data acquisition was accomplished in selected reaction monitoring mode (SRM) for PB (negative, m/z=231/188) with an atmospheric pressure chemical ionization interface using a TSQ Vantage (Thermo Fisher Scientific Inc.). Standard curves were obtained using NaPB.

Quantitative real-time PCR.

CYP2B mRNA expression levels were determined in livers of all surviving animals from each model. Quantitative real-time PCR assays were performed as described chapter 1. The primer and probe sequences are listed in Table 3.

Hepatic CYP enzyme activity.

Liver postmitochondrial supernatant (S9) fractions were prepared and CYP2B activity was determined as PROD by fluorometric analysis using the specific substrate for CYP2B enzyme as shown chapter 1.

Liver histopathology.

Livers from all surviving animals were examined by light microscopy and transmission electron microscopy in described chapter 1. In addition, for chimeric mice, the right lateral lobe in the control and NaPB 1500 ppm groups was prefixed by perfusing 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) using a syringe for 2 animals/group. Human hepatocyte-originated areas of these liver samples were cut into piece of less than 5 mm in thickness with a razor blade in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4).

Hepatocyte replicative DNA synthesis.

The cell proliferation rate was evaluated as replicative DNA synthesis determined as the BrdU labeling index. BrdU labeling was analyzed microscopically in a blinded manner with more than 2000 hepatocytes per animal being evaluated in CD-1 mice and WH rats. An image analysis system was used to evaluate the BrdU labeling indices of

human hepatocytes in chimeric mice and mouse hepatocytes in SCID 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 or SCID mouse.

Statistical analysis.

See chapter 1.

Results

Summary of the effect of NaPB treatment on some selected endpoints are shown in Table 8. In this study data are expressed on a plasma PB µg/mL basis, rather than on a ppm dietary dose level basis, in order to permit a clearer evaluation of species differences in the effects of NaPB treatment.

Mortality and Body weight

One CD-1 mouse given 2500 ppm NaPB, three chimeric mice given 1500 ppm NaPB and one control chimeric mouse died during treatment (data not shown), suggesting that the maximum tolerated dose of NaPB in chimeric mice is lower than that in both CD-1 mice and WH rats. A statistically significant suppression of final body weight with concomitant lower food consumption was observed in WH rats treated with NaPB 2500 ppm (data not shown). The body weights of the two surviving chimeric mice given 1500 ppm NaPB were similar to those of control chimeric mice.

NaPB Intake and Plasma Levels

NaPB intakes, calculated from food consumption and NaPB concentration in diet data, and plasma PB levels were increased in a dose-dependent manner in CD-1 mice, WH rats, and chimeric mice (Table 8). While mean plasma PB levels were similar in CD-1 mice and WH rats, chimeric mice had the highest plasma PB levels at each NaPB dietary level. The ranges of PB plasma levels in chimeric mice given 500, 1000 and 1500 ppm NaPB were 17.3-35.9 µg/mL, 40.1-149.4 µg/mL, 86.3-146.1 µg/mL, respectively.

Table 8. Summary for responses to NaPB treatment in CD-1 mice, Wistar Hannover rat, chimeric mice and SCID mice

	CD-1 mice				WH rats				Chimeric mice			SCID mice
(ppm)	500	1000	1500	2500	500	1000	1500	2500	500	1000	1500 [c]	1500
Overall NaPB intake (mg/kg/day)	59	120	180	250	30	60	85	120	69	150	230	220
Plasma NaPB levels (µg/mL)	12	30	43	70	8.0	20	35	61	27	75	120	43
Absolute liver weight [a]	**1.2**	**1.5**	**1.5**	**1.6**	**1.1**	**1.2**	**1.2**	**1.2**	**1.2**	**1.2**	1.2	**1.4**
Relative liver weight [a]	**1.2**	**1.4**	**1.5**	**1.7**	**1.1**	**1.2**	**1.2**	**1.3**	1.1	1.1	1.1	**1.5**
PROD activity [a]	**4.4**	**5.4**	**6.4**	**6.4**	**22**	**27**	**24**	**20**	**6.8**	**14**	14	**13**
CYP2B mRNA levels [a]	**43**	**59**	**61**	**62**	**170**	**220**	**230**	**250**	**6.9**	**7.4**	11	**160**
Hepatocyte hypertrophy [b]	**8/8**	**8/8**	**7/7**	**7/7**	**8/8**	**6/8**	**8/8**	**8/8**	1/5	1/5	2/2	**5/5**
BrdU labeling index [a]	**5.7**	**13**	**18**	**17**	**3.9**	**4.6**	**4.7**	**4.4**	1.9	1.3	1.4	**4.6**

a: Vales are presented as fold control at each dose level. Values statistically significantly different from control ($p<0.05$) are presented with bold red.

b: Results are presented as number of animals with findings per number of animals examined.

c: Data at 1500 ppm in chimeric mice which is from two surviving animals was not analyzed statistically.

Table is adapted from Yamada *et al.*, (2014).

In this paper most data are expressed on a plasma PB µg/mL basis, rather than on a ppm dietary dose level basis, in order to permit a clearer evaluation of species differences in the effects of NaPB treatment.

Liver weight and morphology

Statistically significant, dose-dependent increases in absolute liver weight were observed in CD-1 mice, WH rats and chimeric mice (Table 8). In the highest NaPB dose group of chimeric mice, the increase was not statistically significant owing to the small number of animals that survived. Statistically significant, dose-dependent increases in relative liver weight were observed in CD-1 mice and WH rats, whereas NaPB only produced small increases in relative liver weight in chimeric mice (Table 8). The chimeric mouse livers were characterized morphologically (Fig. 10A) and histologically (Fig. 10B) with respect to the extent of chimerism, in that they contained both white and red areas (Fig. 10A). The white areas consisted of human hepatocytes and were easily distinguishable from the areas of mouse hepatocytes. The red nodules that were distributed sporadically in the livers of chimeric mice represent colonies of transgene-deleted host hepatocytes, as reported previously (Sandgren *et al.*, 1991). In the human hepatocytes of chimeric mice treated with 1500 ppm NaPB, cytosolic glycogen areas were decreased and the size of the cells was slightly increased in the centrilobular area (Figs. 10C and 10D). These hepatic changes suggest that human-originated hepatocytes exhibited slight hypertrophic changes after NaPB treatment, the effect being less marked than that observed in WH rats and CD-1 mice (data not shown).

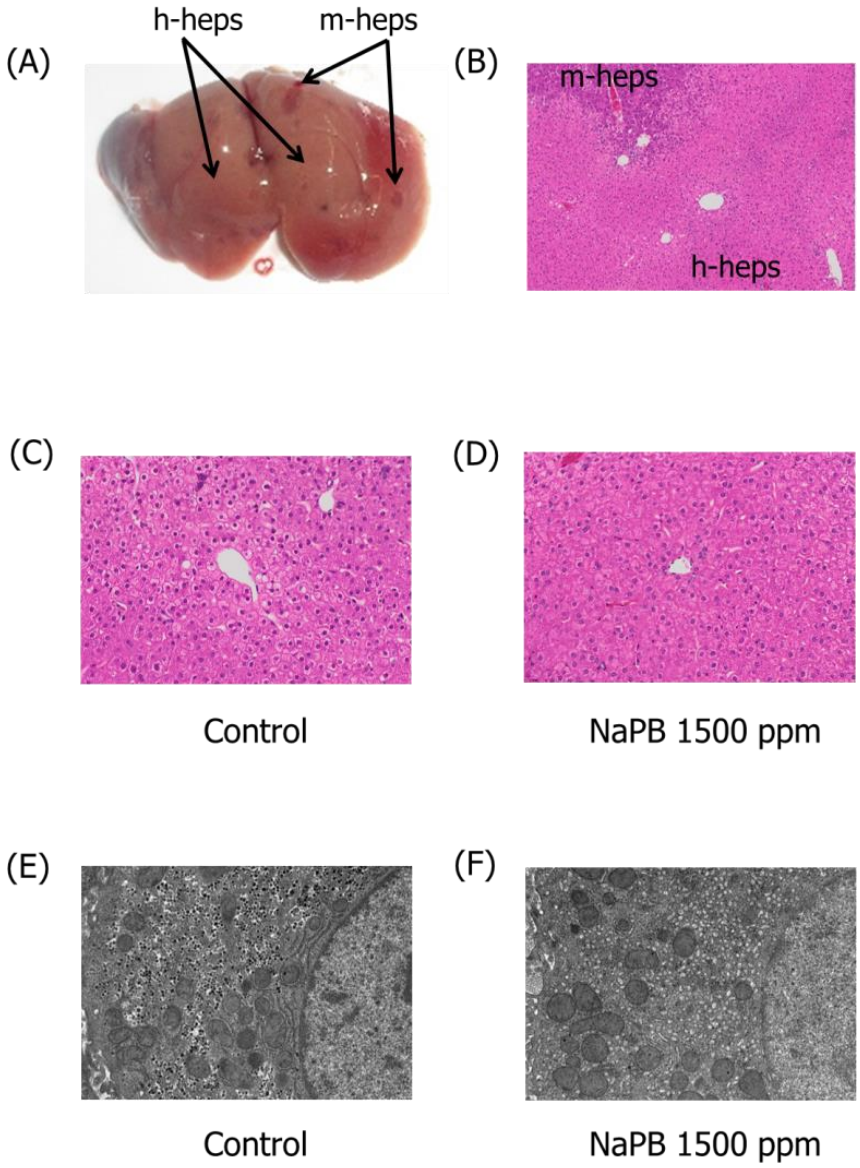

Figure 10. Liver gross pathology and histology in chimeric mice. Photographs present gross (A) and histological (B) appearance of livers of control chimeric mice, with h-heps and m-heps representing human hepatocytes and mouse hepatocytes, respectively. Histopathology (C, D) and ultrastructure (E, F) of human hepatocyte-originated areas of chimeric mice given 0 (C, E) and 1500 ppm (D, F) NaPB are also presented. Centrilobular hepatocellular hypertrophy (D) and proliferation of SER (F) was observed in NaPB treated chimeric mice. Figure is adapted from Yamada *et al.*, (2014).

Electron microscopic evaluation was only conducted in chimeric mice treated with 1500 ppm NaPB, which supported the light microscopic changes, since an increase of SER was observed in the human-originated hepatocytes (Figs. 10E and 10F).

Hepatic CYP2B gene expressions and enzyme activity

CYP2B mRNA levels by NaPB were significantly increased in three animal models, but the increase was much less in chimeric mice than in CD-1 mice and WH rats (6.9, 7.4, and 11.3-fold at 500, 1000, and 1500 ppm in chimeric mice, respectively) (Fig. 11A). While PROD activity was increased in a dose-dependent manner up to 1000 ppm NaPB (mean plasma PB level 75.2 μg/mL) in chimeric mice. The maximum fold change of the PROD activity in chimeric mice (approximately 14-fold control) was between WH rats (approximately 20~27-fold control) and CD-1 mice (approximately 5~6-fold control) (Fig. 11B). In the highest NaPB dose group of chimeric mice the increase was not statistically significant owing to the small number of animals that survived.

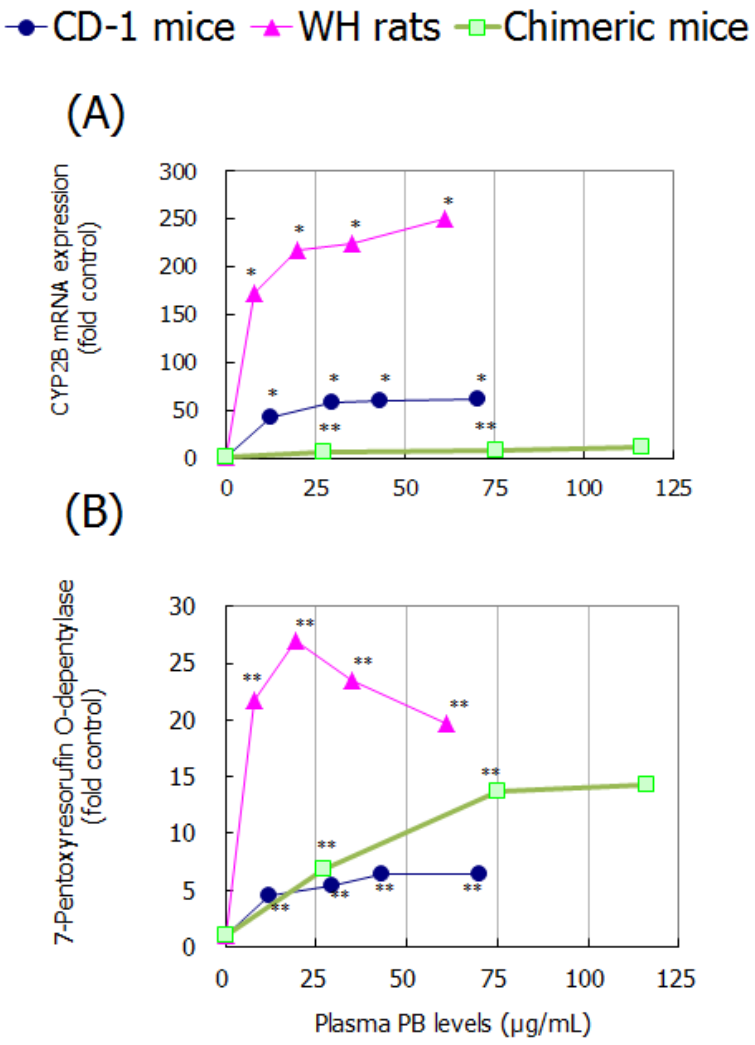

Figure 11. Effect of NaPB treatment on CYP2B mRNA expression (A) and hepatic 7-pentoxyresorufin O-dependylase (CYP2B marker) activity (B) in CD-1 mice, WH rats and chimeric mice. The mRNAs were determined by quantitative real-time PCR and presented as fold control (0 ppm) levels. Mean values from 5-9 animals except for data at 1500 ppm in chimeric mice which is from two surviving animals are presented as fold control at each dose level. Values significantly different from control (0 ppm) are: *p<0.05 and **p<0.01. Figure is adapted from Yamada *et al*., (2014).

Replicative DNA Synthesis

In CD-1 mice and WH rats, replicative DNA synthesis was significantly increased by treatment with NaPB at all dose levels (Figs. 12A). While human hepatocytes in chimeric mice did not show any significant increase in replicative DNA synthesis at NaPB dose levels of 1000 and 1500 ppm, even though plasma PB levels (75±46.9 and 116 (86 and 146) μg/mL for 1000 and 1500 ppm) were higher than those of the highest 2500 ppm CD-1 mouse group (70.2±48.7 μg/mL) (Figs. 12A). Interestingly, treatment with hEGF significantly increased replicative DNA synthesis in human hepatocytes of chimeric mice (Fig. 12B). Thus, these data clearly demonstrate that transplanted human hepatocytes in chimeric mice maintain the mitogen signaling. All individual labeling index values in NaPB-treated chimeric mice were within the control range (2.4~10.7 %) except for one outlier given 500 ppm NaPB (16.6 %), suggesting that the marginally higher values in NaPB-treated chimeric mice are not biologically significant (Fig. 12C).

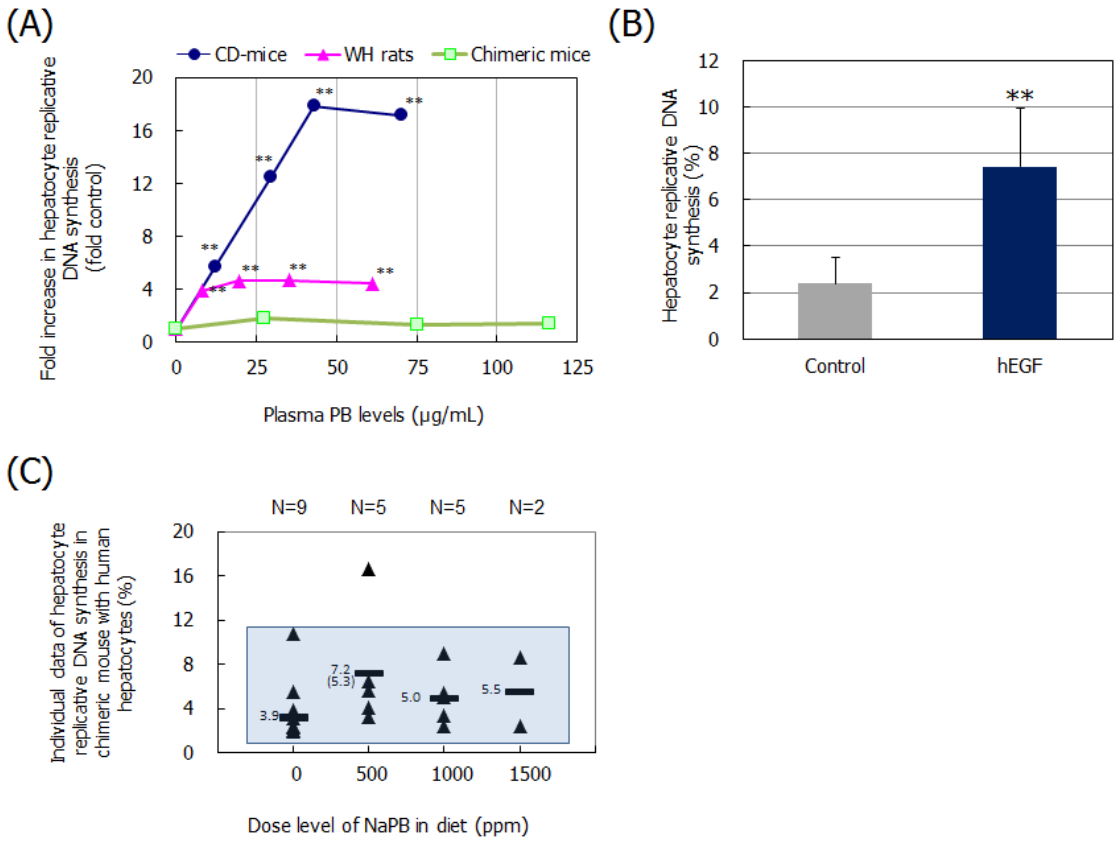

Figure 12. Effect of NaPB treatment on replicative DNA synthesis in CD-1 mice, WH rats, and chimeric mice. (A) Mean values are presented as fold control at each dose level, (B) the effect of hEGF treatment (150 μg/kg x 4 times /day, i.p., 2 days) on hepatocyte replicative DNA synthesis in chimeric mice, (C) Individual values (triangles) with mean value of the dose groups (bars) in chimeric mice. The shaded area represents the control range. While at 500 ppm mean value of all animals examined is 7.2%, it is 5.3% when one outlier (16.6%) is excluded. Values significantly different from control (0 ppm) are: **p<0.01. In chimeric mice, no values were significantly different (p>0.05) from control (0 ppm) irrespective of inclusion of one outlier. Figure is adapted from Yamada *et al*., (2014).

Discussions

The treatment of rats and mice with NaPB results in activation of CAR leading to a pleiotropic response, which includes liver hypertrophy, induction of CYP2B and other xenobiotic metabolising enzymes, increased hepatocyte proliferation and ultimately in the development of altered hepatic foci and liver tumors. A number of evaluations have identified the key and associative events in the MOA for NaPB-induced rodent liver tumor formation (Cohen, 2010; Elcombe *et al.*, 2014; Holsapple *et al.*, 2006; Lake, 2009). The pivotal key event in this MOA is the stimulation of hepatocyte replicative DNA synthesis. Although NaPB produces cell proliferation in rat and mouse hepatocytes, other models are refractory to the mitogenic effects of this compound (Elcombe *et al.*, 2014; Lake, 2009). While NaPB induces replicative DNA synthesis in rodent hepatocytes both *in vivo* and *in vitro*, the absence of an increase in cultured human hepatocytes suggests that the MOA for NaPB-induced rodent liver tumor formation is qualitatively not plausible for humans (Elcombe *et al.*, 2014).

Apart from *in vitro* studies with cultured human hepatocytes, another possible model for investigating the effects of NaPB and related compounds on human hepatocytes is to utilise immunocompromised mice with humanized livers. The uPA/SCID model used in this investigation (Tateno *et al.*, 2004; 2013) has been used in a number of toxicity and metabolism studies to predict human response to drugs and other compounds (Foster *et al.*, 2014; Kitamura and Sugihara, 2014). The data obtained in this study clearly demonstrate that transplanted human hepatocytes in chimeric mice are responsive to hEGF, a hepatocyte mitogen.

CAR is present in human liver and can be activated by drugs and other compounds,

including NaPB (Elcombe *et al.,* 2014; Molnar *et al.,* 2013; Moore *et al.,* 2003). In the present study the treatment of chimeric mice with human hepatocytes with NaPB resulted in significant dose-dependent increases in mRNA levels of known CAR-target genes including CYP2B6. There was also a good correlation between plasma PB levels and CYP2B6 mRNA levels (Fig. 11A), confirming the functional viability of CAR signalling in the human hepatocytes of the chimeric mice. The higher induction of PROD activity in chimeric mice compared to CD-1 mice may be due in part to contamination by mouse hepatocytes, because unlike the mRNA studies, the method for examination of PROD activity used in this study does not distinguish between human and mouse hepatocytes and PROD is not generally used as a marker of CYP2B6 (Fig. 11B). Although the lower responsiveness of chimeric mice to NaPB compared to CD-1 mice and WH rats may be partially attributable to lower expression of CAR mRNA, species differences in the signalling of CAR target genes are likely contribute to differences in potency for each response, especially for hepatocellular proliferation.

In keeping with previous studies, the treatment of CD-1 mice and WH rats with NaPB resulted in significant increases in hepatocyte replicative DNA synthesis. Treatment with NaPB also increased replicative DNA synthesis in hepatocytes of SCID mice (Table 8). In contrast to CD-1 mice, WH rats and SCID mice, the treatment of chimeric mice with NaPB did not result in any increase in hepatocyte replicative DNA synthesis (Table 8).

The plasma levels of PB observed in chimeric mice with human hepatocytes after treatment with NaPB were higher than those obtained in both CD-1 mice and WH rats. In addition, the dose setting study established that that NaPB dietary levels of >1500 ppm could not be administered to chimeric mice with human hepatocytes. It is therefore

considered that the lack of effect of NaPB on replicative DNA synthesis in chimeric mice with human hepatocytes is an accurate finding, as higher dose levels of NaPB could not be examined in this study. Moreover, the plasma levels of PB observed in chimeric mice with human hepatocytes in this study following treatment with 1000 and 1500 ppm NaPB were around 3-5 fold higher than those reported in human subjects given therapeutic doses of 3-6 mg/kg where plasma levels ranged from 10-25µg/mL (Monro, 1993).

In conclusion, some of the key and associative events in the MOA for NaPB-induced rodent liver tumor formation are observable in human liver. However, the key species difference is that although NaPB is a mitogenic agent in rat and mouse liver, no such effect is observed in human liver. To date, the major evidence that NaPB is not mitogenic in human liver comes from studies with cultured human hepatocytes (Elcombe *et al.,* 2014; Hirose *et al*., 2009; Lake, 2009). The data obtained in this study provides additional *in vivo* evidence for a marked species difference in the mitogenic effects of NaPB.

Chapter 2

Evaluation of human relevancy for rat hepatocellular tumors induced by momfluorothrin

2-2

Analysis for the human relevancy for rat hepatocellular tumors induced by momfluorothrin using cultured rat and human hepatocytes and humanized chimeric mice

Introduction

In chapter 1, the MOA for rat hepatocellular tumor formation by momfluorothrin was evaluated based on the ILSI/IPCS framework (Boobis *et al*., 2006, 2008; Sonich-Mullin *et al.,* 2001). This MOA study demonstrated that momfluorothrin produces liver hypertrophy, induces hepatic CYP2B enzymes and increases hepatocellular proliferation in WT rats but not in CAR KO rats (Okuda *et al.*, 2017a). Moreover, alternative MOAs for momfluorothrin-induced rat hepatocellular tumor formation including cytotoxicity, activation of PPARα, accumulation of iron, statin-like effects and hormonal perturbation have been excluded (Okuda *et al.,* 2017a). 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 metofluthrin, a close structural analogue to momfluorothrin (Yamada *et al.*, 2009), and 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.

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

These obtained experimental and analytical data were published from the *Journal of Toxicological Sciences* (Okuda *et al*., 2017b).

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.*

Test chemicals

The chemicals were obtained from the following manufacturers: momfluorothrin (See chapter 1), metofluthrin (See chapter 1), and Z-CMCA (Lot No. SK0712171; Purity 99.8 %), Sumitomo Chemical Co., Ltd. (Tokyo, Japan); NaPB (Lot No. KLM4036, purity 98.0%), Wako Pure Chemical Industries, Ltd. (Osaka, Japan); human recombinant hepatocyte growth factor (hHGF), Sigma-Aldrich (St. Louis, USA); hEGF (See chapter 2-1), Peprotech (EC, London, United Kingdom).

Cultured hepatocytes study

Hepatocytes

Rat hepatocytes were isolated by a two-step perfusion procedure from HarlanRccHanTM: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) (See chapter 2 in materials and methods)

Cryopreserved human hepatocytes for the cell culture study were obtained from Celsis IVT (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 9. Human hepatocytes were thawed and plated according to the supplier's instructions. Briefly, cryopreserved hepatocytes were thawed at 37 ºC 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, 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 4.0×10^5 cells/well per 2 mL of medium containing the above additions in 6-well plates (two rats) and 6.0×10^4 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 3.0×10^5 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 1.0×10^4 and 3.5×10^4 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, Japan) and the hepatocytes were cultured at 37 ºC in a humidified incubator under an atmosphere of 95 % air/5 % carbon dioxide (Hirose *et al.*, 2009; Yamada *et al.*, 2015).

Table 9. Details of human hepatocyte preparations studied

#	Lot No. of donor	Gender	Ethnicity	Age	Smoking	Alcohol	Drug use	Cause of death	Experiments	Endpoints determined
1	**BHL**	Male	Caucasian	28	Yes	Yes	Yes	ICH-Stroke	Cultured cell studies	A, B
2	**ETA**	Female	Caucasian	80	Yes	No reported	No reported	CVA		A, B
3	**CDP**	Male	Caucasian	58	No	No	No	CVA		B
4	**DOO**	Male	Caucasian	57	No	No	No	Anoxia; 2nd to CVA		B, C
5	**FCL**	Female	Hispanic	10 months	No	No	No reported	Anoxia/ drowning		B, C
6	**IPH**	Female	Caucasian	52	No	No	No	Anoxia; 2nd to CVA		
7	**LLA**	Male	Caucasian	26	Yes	Yes	No reported	Head trauma		
8	**LMP**	Female	Caucasian	38	Yes	Yes	No reported	CVA		
9	**MMM**	Female	Caucasian	3	No	No	No	Drowning		
10	**QOQ**	Male	Caucasian	66	Yes	Yes	No	CVA		
11	**BD87**	Male	Caucasian	2	No	No reported	No	MVA	Chimeric mouse studies	B, C, D
12	**BD85**	Male	African American	5	No	No reported	Yes (RD)	Anoxia		
13	**BD195**	Female	Hispanic	2	No	No reported	No	MVA		

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.

Table is adapted from Okuda *et al*., (2017b).

Chemical treatment

For assays of CYP2B induction, rat and human hepatocytes were incubated for 48 hours in medium containing the 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 1000 µM), Z-CMCA (5, 10, 50, 100, 500, and 1000 µM) and NaPB (500 (rat hepatocytes only) and/or 1000 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 hours (only rat) from plating the hepatocytes and then at 24 hour 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 hours in medium same as CYP2B induction assay. Rat and human hepatocytes were treated with momfluorothrin (1, 5, 10, 50, 100, 500, and 1000 µM), Z-CMCA (5, 10, 50, 100, 500, and 1000 µM for rat hepatocytes; 5, 100, 500, and 1000 µM for human hepatocytes), NaPB (500 and 1000 µM) and recombinant hHGF (10 and 100 ng/mL), which is a well-known growth factor stimulating replicative DNA synthesis in hepatocytes (Hirose *et al.*, 2009; Runge *et al.*, 1999; Yamada *et al.*, 2015). 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 hours (only rat) from plating the hepatocytes and then at 24 hour 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 rats and ten human donors.

Determination of DNA synthesis

The extent of DNA synthesis was determined by measuring BrdU (Sigma-Aldrich) incorporation into DNA over a 24 hour period in humidified incubator at 37 ºC, using a Cell Proliferation ELISA BrdU (chemiluminescent) kit (Roche, Germany) as previously described (Hirose *et al.*, 2009; Yamada *et al.*, 2015). BrdU (100 µM) was added to the medium at the point of the medium change after 24 hours of chemical treatment. After 24 hours 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), 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 PBS (pH 7.4). Total RNA preparation and quantitative real-time PCR assays for rat CYP2B1/2 and human CYP2B6 were performed as described chapter 1. In addition, levels of rat and human GAPDH mRNA were determined as internal controls. The primer and probe sets are listed in Table 3.

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 hours with medium containing momfluorothrin (10, 50, 100, and 1000 µM), Z-CMCA (50, 100 and 1000 µM), NaPB (1000 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 hours (only rat) from plating the hepatocytes and then at 24 hour intervals. After the test chemical treatment, medium containing the Cell counting kit reagent was added to each well and the cells incubated for 4 hours 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 hepatocyte (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.

Humanized chimeric mice study

The in-life phase of the experiments using chimeric mice was performed at PhoenixBio Co., Ltd. (Hiroshima, Japan). Three experiments focused on the cell proliferation effects on human hepatocytes were conducted with chimeric mice

produced by individual three donors (donor ID; BD87, BD85, and BD195, see Table 9). 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.*, 2015) aged 20-30 days as donor cells for the chimeric mice because cells from young subjects are more responsive to stimulation of hepatocellular proliferation (Masumoto *et al.*, 2007). The range of replacement indices in chimeric mice used in Experiments I (BD87), II (BD85) and III (BD195) was estimated as 75-89%, 90-100%, and 81-98%, respectively. 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 3000 ppm for momfluorothrin, 1800 ppm for metofluthrin. The dose level of momfluorothrin was originally set at 3000 ppm in Experiment I, but it was decreased to 1100 ppm in Experiments II and III due to animal deaths (Days 2 and 3, commencement of treatment is counted as Day 0) in Experiment I. Since 1500 ppm momfluorothrin, which was the 2nd higher dose level in the 2-year rat study, also showed a palatability problem with similar degree as 3000 ppm in the preliminary dose setting study (date not shown), 1100 ppm was used in the Experiments II and III. Chemical intake (170 and 146 mg/kg/day in Experiment II and III, respectively; Table 10) was higher than or equivalent to those at tumorigenic dose levels in males in the rat 2-year bioassay; 73 mg/kg/day at 1500 ppm and 154 mg/kg/day at 3000 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-days treatment with momfluorothrin (see chapter 1) and metofluthrin (Yamada *et al.*, 2009).

Mortality, body weight, and food consumption were monitored throughout the study. Alzet minipumps (Model 2001; Alzet Corporation) containing BrdU (Sigma Company, St Louis, MO, U.S.A.), with a release rate of 40 μg/hr were implanted in the subcutaneous tissue of mice, under anesthesia with isoflurane on the day prior to 7 days before the scheduled euthanasia. After the 7-day treatment period, blood collection, gross pathological examination, liver weight, and storage of liver sample were conducted with same methods as chapter 2-1.

Quantitative real-time PCR.

CYP2B mRNA expression levels were determined in livers of all surviving animals from each model. Quantitative real-time PCR assays were performed as described chapter 1. The primer and probe sequences are listed in Table 3.

Hepatic CYP enzyme activity.

Liver postmitochondrial supernatant (S9) fractions were prepared and CYP2B activity was determined as PROD by fluorometric analysis using the specific substrate for CYP2B enzyme as shown chapter 1.

Liver histopathology.

Livers from all surviving animals were examined by light microscopy and transmission electron microscopy in described chapter 1. Human hepatocyte-originated areas of these liver samples were cut into piece of less than 5 mm in thickness with were fixed in 10 % neutral buffered formalin.

Hepatocyte replicative DNA synthesis.

The cell proliferation rate was evaluated as replicative DNA synthesis described in chapter 2-1. The total number of evaluated human hepatocytes was more than 8,000 per chimeric mouse.

Statistical analysis.

See chapter 1.

Results

Cultured hepatocytes 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 presented in Figs. 13-15.

Cell viability

With respect to cell viability (Fig. 13), 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 1000 μM momfluorothrin and human hepatocytes treated with 1000 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.

CYP2B gene expression

Treatment with 1000 μM NaPB produced a less marked effect on CYP2B mRNA levels in human than in rat hepatocytes (Fig. 14). 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.

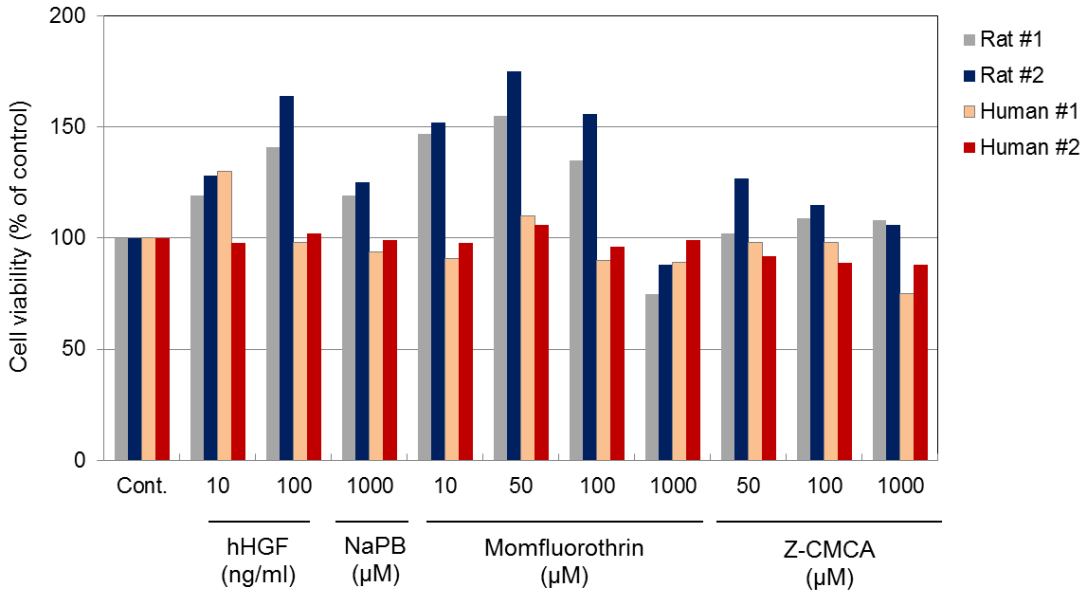

Figure 13 Effect of hHGF, NaPB, momfluorothrin and Z-CMCA on cell viability in cultured rat and human hepatocyte. Rat and human hepatocytes were treated with hHGF (10 and 100 ng/ml), NaPB (1000 μM), momfluorothrin (10-1000 μM) and Z-CMCA (50-1000 μM) for 48 hours and cell viability was determined by 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. Figure is adapted from Okuda *et al*., (2017b).

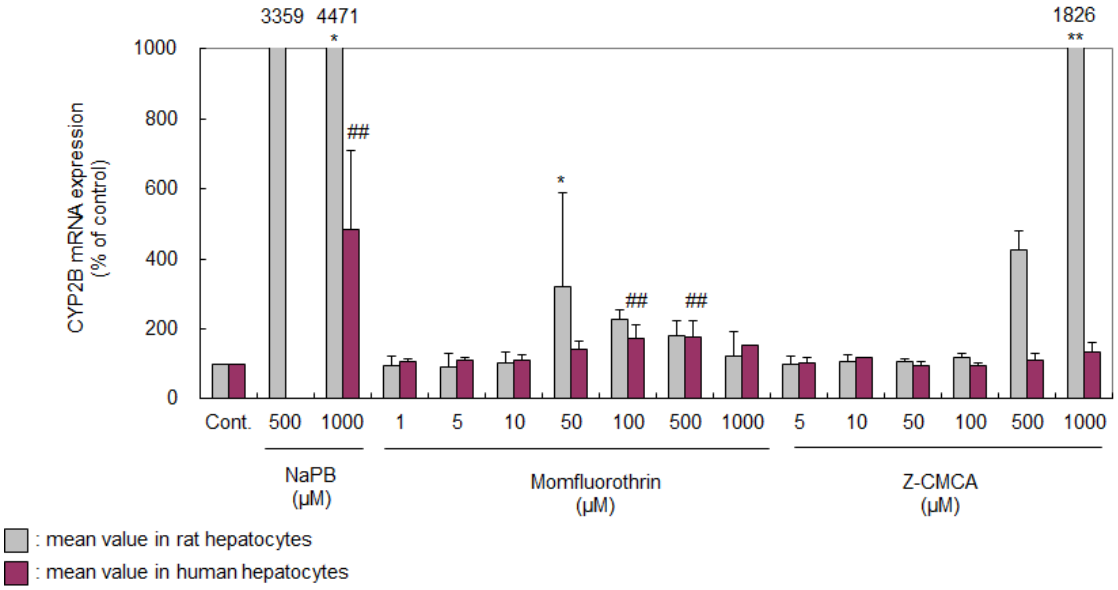

Figure 14. Effect of NaPB, momfluorothrin and Z-CMCA on CYP2B mRNA expression in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with NaPB (500 and 1000 μM), momfluorothrin (1-1000 μM) or Z-CMCA (5-1000 μM) for 48 hours and rat CYP2B1/2 and human CYP2B6 mRNA levels determined by quantitative real-time PCR. Results are presented as mean ± SD (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. Figure is adapted from Okuda *et al.*, (2017b).

Replicative DNA synthesis

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. 15). In rat hepatocytes, significant increases in replicative DNA synthesis were observed after treatment with 500 and 1000 μM NaPB and 5 and 10 μM momfluorothrin, whereas replicative DNA synthesis was reduced by treatment with 100-1000 μM momfluorothrin. In contrast to the effects observed in rat hepatocytes, the treatment of human hepatocytes with 500 and 1000 μM NaPB and 1-1000 μM momfluorothrin had no significant increase in replicative DNA synthesis. Treatment with 5-1000 μM Z-CMCA had no significant effects on replicative DNA synthesis in either rat or human hepatocytes.

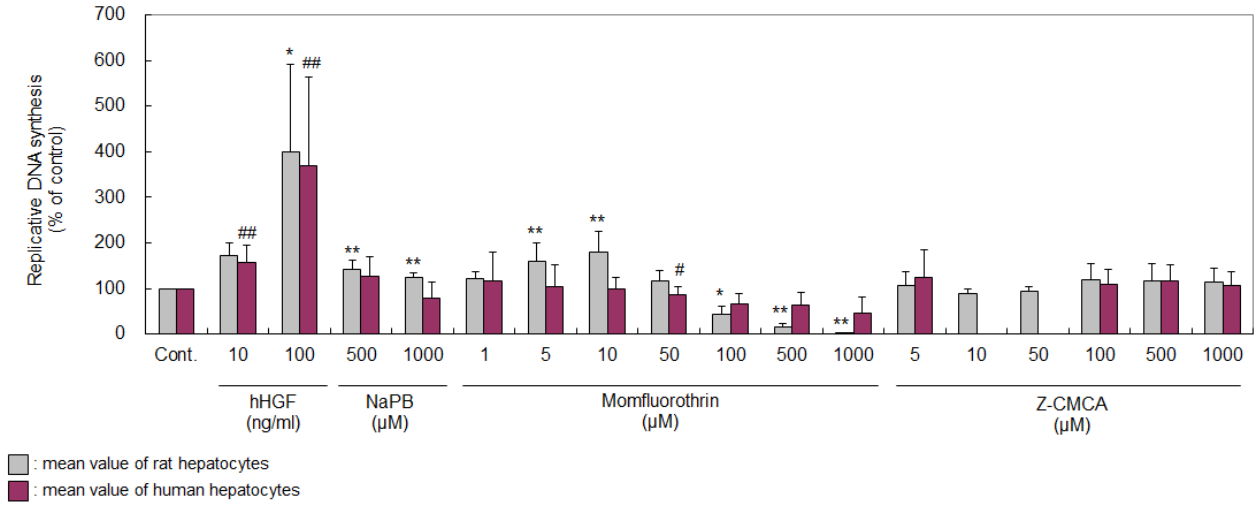

Figure 15. Effect of hHGF, NaPB, momfluorothrin and Z-CMCA on replicative DNA synthesis in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with hHGF (10 and 100 ng/ml), NaPB (500 and 1000 μM), momfluorothrin (1-1000 μM) or Z-CMCA (5-1000 μM) for 48 hours and replicative DNA synthesis determined by BrdU incorporation over the last 24 hours of culture. Results are presented as mean ± SD (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. Figure is adapted from Okuda *et al*., (2017b).

Chimeric mice study

A summary of the data obtained is presented in Table 10. In three separate experiments, chimeric mice transplanted with human hepatocytes from different donors were treated for 7 days with diets containing 1800 ppm metofluthrin (average chemical intake, 239-285 mg/kg/day) and with 1100 or 3000 ppm momfluorothrin (average chemical intake, 146-170 mg/kg/day for 1100 ppm; 410 mg/kg/day for 3000 ppm).

As described in the Method 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 1800 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 tumourigenic dose levels administered to male rats in the 2-year bioassays, which were 38 and 78 mg/kg/day for 900 and 1800 ppm mg/kg/day metofluthrin, respectively, and 73 and 154 mg/kg/day for 1500 and 3000 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 during the study.

Table 10. Summary of findings in chimeric mouse study

Experiment I. Donor ID; BD87				
Groups	Control	Metofluthrin 1800 ppm	Momfluorothrin 3000 ppm	hEGF 600 μg/kg
Number of animals examined	5	5	3[a]	5
Average Chemical intake (mg/kg/day)	0	272	410	0.6
Liver weight Absolute (g)	2.78±0.36	2.89±0.32	2.36±0.51	2.75±0.25
(fold control)	**(1.00)**	**(1.04)**	**(0.85)**	**(0.99)**
Liver weight Relative (g/body weight x 100)	13.47±1.80	14.43±2.13	11.83±1.43	13.78±1.13
(fold control)	**(1.00)**	**(1.07)**	**(0.88)**	**(1.02)**
Replicative DNA synthesis in human hepatocytes (%)	11.59±6.77	4.63±2.03	6.71±6.76	21.18±9.03
(fold control)	**(1.00)**	**(0.40)**	**(0.58)**	**(1.83)**
Human CYP2B6 mRNA (% of control average)	100±18.93	111±11.90	136±17.70*	124±18.24
(fold control)	**(1.00)**	**(1.11)**	**(1.36)**	**(1.24)**
Mouse Cyp2b10 mRNA (% of control average)	100±33.49	424±118.90**	1692±600.04*	154±26.17*
(fold control)	**(1.00)**	**(4.24)**	**(16.92)**	**(1.54)**

Experiment II. Donor ID; BD85				
Groups	Control	Metofluthrin 1800 ppm	Momfluorothrin 1100 ppm	hEGF 600 μg/kg
Number of animals examined	5	3[b]	8	5
Average Chemical intake (mg/kg/day)	0	239	170	0.6
Liver weight Absolute (g)	2.30±0.20	2.14±0.08	2.17±0.21	2.53±0.23
(fold control)	**(1.00)**	**(0.93)**	**(0.94)**	**(1.10)**
Liver weight Relative (g/body weight x 100)	11.71±0.79	11.72±0.68	11.19±0.67	12.94±0.87*
(fold control)	**(1.00)**	**(1.00)**	**(0.96)**	**(1.11)**
Replicative DNA synthesis in human hepatocytes (%)	7.44±4.00	5.23±3.08	4.59±1.61	12.22±5.51
(fold control)	**(1.00)**	**(0.70)**	**(0.62)**	**(1.64)**
Human CYP2B6 mRNA (% of control average)	100±12.90	137±14.75*	93±12.77	135±41.88
(fold control)	**(1.00)**	**(1.37)**	**(0.93)**	**(1.35)**
Mouse Cyp2b10 mRNA (% of control average)	100±55.9	281±136.5*	266±105.9**	112±27.2
(fold control)	**(1.00)**	**(2.81)**	**(2.66)**	**(1.12)**

Experiment III. Donor ID; BD195				
Groups	Control	Metofluthrin 1800 ppm	Momfluorothrin 1100 ppm	hEGF 600 μg/kg
Number of animals examined	5	8	5	5
Average Chemical intake (mg/kg/day)	0	285	146	0.6
Liver weight Absolute (g)	2.63±0.40	2.61±0.34	2.61±0.40	3.21±0.39*
(fold control)	**(1.00)**	**(0.99)**	**(0.99)**	**(1.22)**
Liver weight Relative (g/body weight x 100)	11.98±1.59	12.44±1.68	11.94±1.21	14.94±2.00*
(fold control)	**(1.00)**	**(1.04)**	**(1.00)**	**(1.25)**
Replicative DNA synthesis in human hepatocytes (%)	8.37±4.53	5.01±1.76	4.55±1.32	15.46±5.61
(fold control)	**(1.00)**	**(0.60)**	**(0.54)**	**(1.85)**
Human CYP2B6 mRNA (% of control average)	100±14.79	121±19.78*	86±16.29	120±29.02
(fold control)	**(1.00)**	**(1.21)**	**(0.86)**	**(1.20)**
Mouse Cyp2b10 mRNA (% of control average)	100±26.9	305±96.6**	191±14.1**	122±55.5
(fold control)	**(1.00)**	**(3.05)**	**(1.91)**	**(1.22)**

Results are presented as mean±SD, and values in parenthesis are fold change of control. Values significantly different from control are: * $p<0.05$ and ** $p<0.01$. a: 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. b: 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. Table is adapted from Okuda *et al*., (2017b).

CYP2B gene expression

Under these study conditions, increased levels of CYP2B6 mRNA were observed in human hepatocytes, but the effects were relatively weak, being 1.1-1.4 fold of control in animals given 1800 ppm metofluthrin and 1.4 fold of control in animals given 3000 ppm momfluorothrin. Although serum or liver concentrations of the test chemicals were not examined in the present study, significant increases in Cyp2b10 mRNA expression mouse hepatocytes (Table 10) demonstrated that treatment with metofluthrin or momfluorothrin significantly stimulated CAR in the mouse hepatocytes of the chimeric mice.

Replicative DNA synthesis

Under these conditions, treatment with metofluthrin or momfluorothrin did not result in any increases in replicative DNA synthesis of human hepatocytes (Table 10, Fig. 16A). However, as a positive control, hEGF treatment increased replicative DNA synthesis in human hepatocytes (Fig. 16A and 16B) though statistical significance was not observed due to the small number of animals examined (Table 10, Fig. 16A). When the data from all three experiments were combined, statistically significant increased replicative DNA synthesis was observed in the hEGF group (Fig. 16B). The BrdU labelling index was only determined in human hepatocytes and not in mouse hepatocytes in which the rate of replicative DNA synthesis was high even in controls (Fig. 17C and 17D), and thus it was difficult to compare between control and treatment animals. 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.

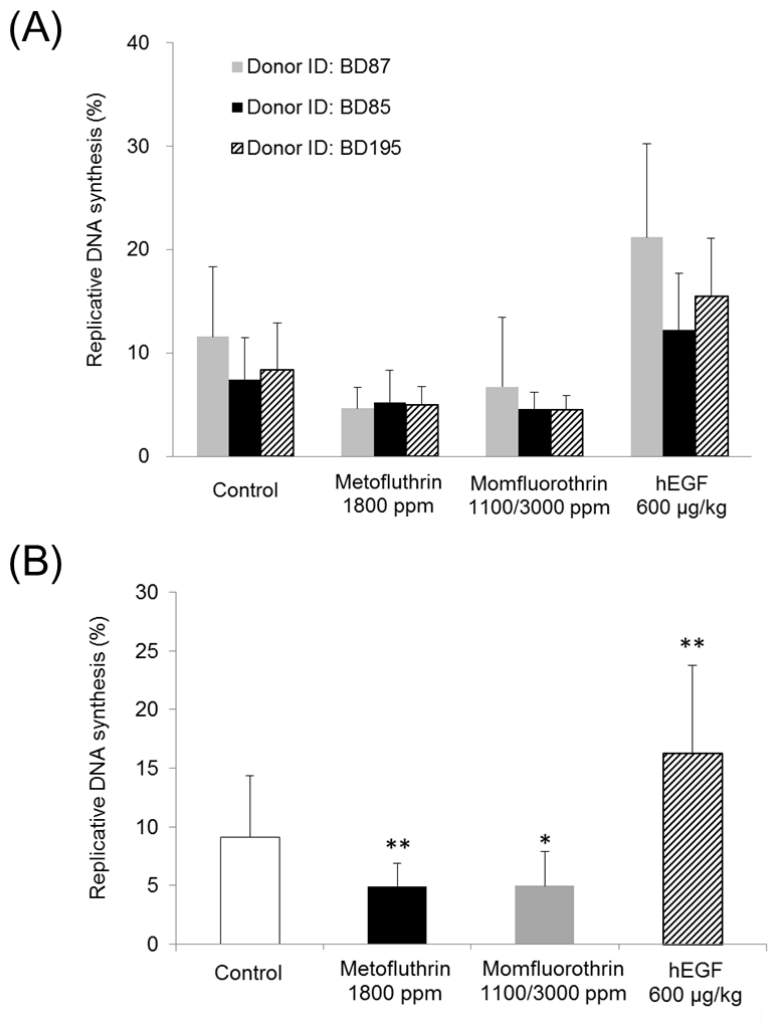

Figure 16. Effect of metofluthrin, momfluorothrin and hEGF on replicative DNA synthesis in human hepatocytes of chimeric mice. Replicative DNA synthesis as determined by BrdU labeling index is presented for individual (A) and average (B) of three experiments in chimeric mice from three different donors. Results are presented as mean ± SD (A, n=3-8 mice per experiment per group; B, n=3 experiments per group). Figure is adapted from Okuda *et al*., (2017b).

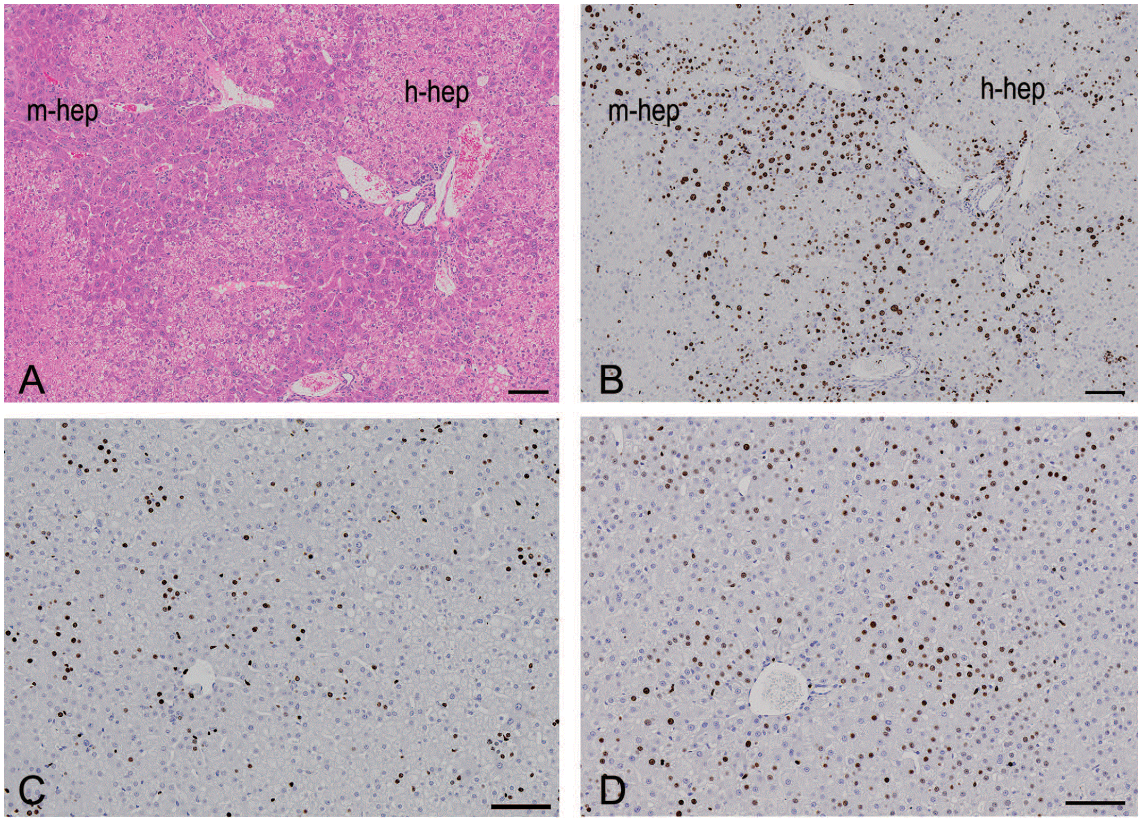

Figure 17. Liver histology and DNA synthesis in chimeric mice.

(A) Hematoxylin and eosin staining of appearance of livers of the control chimeric mice, with h-heps and m-heps representing human hepatocytes and mouse hepatocytes, respectively. (B-D) Immunohistochemistory for BrdU in the control animal (serial section of figure 17A (B), human hepatocytes area in the control animal (C) and human hepatocytes area in the hEGF treatment group (D). Scale bars are 100 μm. Figure is adapted from Okuda *et al*., (2017b).

Discussions

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 CAR KO rat study, CAR knockdown hepatocyte study using RNAi and hepatic global gene expression analysis (Okuda *et al*., 2017a). 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 1000 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.

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 3000 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, 5~1000 μM for Z-CMCA) would cover these expected concentrations in the liver. The treatment of rat hepatocytes with 1000 μM momfluorothrin and human hepatocytes with 1000 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*., 2017a). Thus, these findings suggest that the plasma concentrations of these chemicals in the carcinogenicity study with momfluorothrin were less than 1000 μM.

The treatment of rat hepatocytes with 500 and 1000 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 5-1000 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.

For human hepatocytes, in contrast to hHGF, replicative DNA synthesis was not increased by treatment with 500 and 1000 μM NaPB, 1-1000 μM momfluorothrin or 5-1000 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.

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 (See Fig. 1). As

described in chapter 1, 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 11. This includes not only the data for the effects of momfluorothrin in the rat, but also the available data for humans.

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 (Fig. 14). 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.

Table 11. Comparison of key and associative events on MOA for liver tumorigenesis of momfluorothrin in rats and humans (Table is adapted from Okuda *et al*., (2017b)).

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

As the stimulation of replicative DNA synthesis is the critical key event in the proposed MOA for momfluorothrin-induced rat hepatocellular tumor formation (Okuda *et al*., 2017a), the effect of momfluorothrin on replicative DNA synthesis has been studied in cultured rat and human hepatocytes (Fig. 15). 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 10, Fig. 17).

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). As examination of the available data demonstrates that the MOA for momfluorothrin-induced rat liver tumor 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 (Fig. 18). 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 tumors 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 tumors, 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.

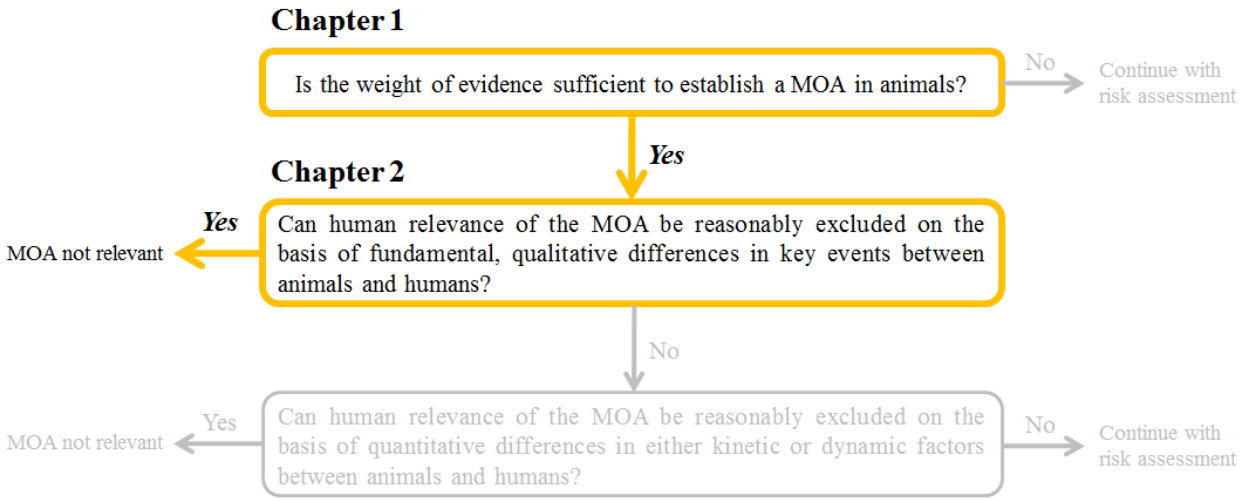

Figure 18. The conclusion of the human risk assessment in the rat liver tumors induced by momfluorothrin based on the IPCS framework.

Conclusions

The obtained data demonstrated that the MOA for momfluorothrin-induced rat hepatocellular tumors was mediated by CAR activation in chapter 1. 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 tumor formation could occur in human liver, the available *in vitro* and *in vivo* experimental data demonstrated that human hepatocytes appear to be refractory to the mitogenic effects of momfluorothrin in chapter 2. In addition, no stimulation of replicative DNA synthesis by NaPB and metofluthrin was demonstrated *in vivo* in the study utilizing chimeric mice with human hepatocytes. Therefore, these data suggested that CAR activators including momfluorothrin have no carcinogenic risk for humans.

Since pyrethroids, 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 (Tsuji *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).

Acknowledgements

The author deeply appreciates Tomoya Yamada Ph.D., senior scientist in Sumitomo Chemical Company, and Professor Yoshimasa Nakamura, the primary mentor of the doctorate degree program in Okayama University, for providing the expert technical advises throughout this research project.

The author also appreciates Professors Yoshiyuki Murata and Yoshinobu Kimura, the secondary mentor of the doctorate degree program in Okayama University, for providing the technical advises.

Finally, I also thank the other contributors to this research project from Sumitomo Chemical Company, Ltd., and Sumika Technoservice Corporation.

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