Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

Toxicology Research

RSCPublishing

ARTICLE

Lack of effect of metofluthrin and sodium phenobarbital on replicative DNA synthesis and Ki-67 mRNA expression in cultured human hepatocytes

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Tomoya Yamada[a,1], Hiroko Kikumoto[a], Brian G. Lake[b], and Satoshi Kawamura[a]

High doses of metofluthrin have been shown to produce hepatocellular tumours in rats. Previous *in vivo* and *in vitro* mode of action (MOA) studies have demonstrated that metofluthrin induces hepatic microsomal cytochrome P450 (CYP) 2B enzymes and hepatocellular replicative DNA synthesis in rats, suggesting that the MOA for liver tumour formation is similar to that of other rodent non-genotoxic hepatocarcinogens that are constitutive androstane receptor (CAR) activators. To evaluate potential human carcinogenic risk of metofluthrin, in this study the effect of metofluthrin (7.7-770 µM) on replicative DNA synthesis (as determined by BrdU labeling) was examined in cultured human hepatocytes from four different donors. The effect of sodium phenobarbital (NaPB), a well-known rodent hepatocarcinogen with a CAR-mediated MOA, was also investigated. In addition, the effect of metofluthrin on expression of Ki-67, CYP2B6 and CAR mRNAs in cultured human and rat hepatocytes was examined. Treatment with 10 and 100 ng/mL hepatocyte growth factor (HGF) produced a concentration-dependent increase in BrdU labeling in human hepatocyte preparations. However, no increase in BrdU labeling was observed after treatment with metofluthrin or NaPB. Treatment with HGF significantly increased Ki-67 mRNA expression in both human and rat hepatocytes. However, while metofluthrin increased Ki-67 mRNA expression in rat hepatocytes, treatment with metofluthrin and NaPB had no effect on Ki-67 mRNA expression in human hepatocytes. Treatment with NaPB produced an increase in CYP2B6 mRNA levels in human hepatocytes, with metofluthrin also producing a small effect. Neither metofluthrin nor NaPB significantly changed CAR mRNA expression levels in both cultured rat and human hepatocytes. Thus, while metofluthrin and NaPB could activate CAR in cultured human hepatocytes, neither compound increased BrdU labeling and Ki-67 mRNA expression. As human hepatocytes are refractory to the mitogenic effects of metofluthrin, in contrast to rat hepatocytes, the data suggest that the MOA for metofluthrin-induced rat liver tumour formation is not relevant for humans and hence that metofluthrin does not pose a carcinogenic hazard for humans.

1. Introduction

In recent years, frameworks for analysing the modes of action (MOAs) by which chemicals produce tumours in rodents and the relevance of such tumour data for human risk have been developed through the International Life Sciences Institute (ILSI) [1-3] and the International Programme on Chemical Safety (IPCS) [4, 5]. Metofluthrin (CAS-No.240494-70-6;2,3,5,6-tetrafluoro-4-methoxymethyl-benzyl*EZ*)-(1*RS*,3*RS*;1*RS*,3*SR*)-2,2-dimethyl-3-(prop-1-enyl) cyclopropanecarboxylate) is a Type I pyrethroid insecticide for use in pest control [6, 7]. To examine its carcinogenic potential, carcinogenicity studies were conducted where rats and mice were fed diets containing high concentrations of metofluthrin. While the expected main exposure route of metofluthrin in humans is inhalation, for toxicity studies using experimental animals, higher exposure levels were achieved by employing dietary exposure rather than inhalation exposure. Two-year treatment with high doses of metofluthrin (900 and 1800 ppm) produced hepatocellular tumours in HanBrl:WIST rats [8]. In contrast, metofluthrin was not carcinogenic in the liver or any other tissue in male or female CD-1 mice when administered at dietary levels of 100, 1000, and 2500 (reduced to 1750 during treatment period because of toxicity) ppm [8]. Metofluthrin was negative

in a number of genotoxicity assays, both *in vitro* and *in vivo*, at doses up to either the limit dose or the limit of solubility, including the reverse mutation test in bacterial systems (*Salmonella typhimurium TA100, TA98, TA1535,* and *TA1537* and *Escherichia coli WP2uvrA*) and the *in vitro* chromosomal test in mice. While the findings of these genotoxicity assays have not been published, Regulatory Agencies have concluded that metofluthin is not genotoxic [9, 10]. Thus the formation of rat liver tumors by metofluthrin must be due to a non-genotoxic MOA.

Previous MOA studies have demonstrated that metofluthrin produces liver hypertrophy, induces hepatic microsomal cytochrome P450 (CYP) 2B enzymes and increases hepatocellular proliferation in rats, suggesting that the MOA for liver tumour formation is similar to that of some other constitutive androstane receptor (CAR) activators such as sodium phenobarbital (sodium phenobarbitone; NaPB) [8, 11]. Moreover, alternative MOAs for metofluthrin-induced rat liver tumour formation including cytotoxicity, activation of the peroxisome proliferator-activated receptor alpha (PPARα), accumulation of iron and hormonal perturbation have been excluded [8, 11]. It is thus considered that metofluthrin induces liver tumours in rats by increasing hepatocellular proliferation via CAR activation.

The activation of CAR in rodent liver results in a pleiotropic response [12-16]. The key and associative events in the MOA for liver tumour formation by phenobarbital and related compounds have recently been described by Elcombe et al. (2014)[13]. Key events comprise activation of CAR, altered gene expression specific to CAR activation, increased cell proliferation, development of altered hepatic foci and the formation of liver tumours [13]. Associative events include the induction of cytochrome P450 (CYP) enzymes (in particular, the CYP2B subfamily enzymes), liver hypertrophy (increased liver weight and hepatocellular hypertrophy) and inhibition of apoptosis [13]. In terms of the human relevance of the MOA for phenobarbital-induced rodent liver tumour formation, a key species difference is that while phenobarbital stimulates replicative DNA synthesis in rodent hepatocytes, such effects are not observed in cultured human hepatocytes [13]. Since increased cell proliferation is a necessary key event in the MOA for NaPB-induced rodent liver tumours, the lack of this key event in humans strongly supports the conclusion that the rodent findings are not relevant to humans. Furthermore, while NaPB and related compounds can be non-genotoxic carcinogens and tumour promoters in rodents, a number of epidemiological studies have demonstrated that in human subjects receiving NaPB for many years, at doses producing plasma concentrations similar to those which are carcinogenic in rodents, there is no evidence of increased liver tumour risk [13, 17-20].

To evaluate the relevance of the MOA for metofluthrin-induced rat liver tumours for humans, we have previously reported data on species differences in the hepatic effects of NaPB and metofluthrin [21]. Both NaPB and metofluthrin induced CYP2B enzymes in cultured rat and human hepatocytes (only a single concentration of 50 μM was examined). While both compounds induced replicative DNA synthesis in cultured rat hepatocytes (10-1000 μM was examined) neither metofluthrin nor NaPB induced replicative DNA synthesis in cultured human hepatocytes. However, these studies were conducted with only two preparations of cultured human hepatocytes [21].

Table 1. Sources and details of human hepatocyte preparations used

Experiments	Donor lot number	Gender	Ethnicity	Age	Smoking	Alcohol use	Drug use	Couse of death	Supplier
Effects of metofluthrin on BrdU incorporation and CYP2B6, Ki-67 and CAR mRNA levels	LMP	Female	Caucasian	38	Yes	Yes	Unknown	Cerebrovascular accident	Celsis IVT
	IPH	Female	Caucasian	52	No	No	No	Cerebrovascular accident	Celsis IVT
	LLA	Male	Caucasian	26	Yes	Yes	Unknown	Head trauma	Celsis IVT
	QOQ	Male	Caucasian	66	Yes	Yes	No	Cerebrovascular accident	Celsis IVT
Effects of hepatocellular growth factor on Ki-67 mRNA levels	Hu1552	Female	Caucasian	38	No	No	No	Not available	Gibco
	Hu8127	Female	Caucasian	31	Yes	Yes	Yes	Cardiovascular event post heart transplant	Gibco
	Hu8164	Male	Caucasian	23	Yes	Yes	Yes	Head trauma	Gibco

The objective of the present study was to obtain additional data employing cultured human hepatocytes to further support our previous conclusion that the MOA for metofluthrin-induced rat liver tumour formation is not relevant for humans [8, 21]. The effects of metofluthrin and NaPB on hepatocellular proliferation and CYP2B6 mRNA expression were evaluated in cultured human hepatocytes from four subjects. Furthermore, to confirm species differences in susceptibility to hepatocellular proliferation the effect of metofluthrin on Ki-67 mRNA levels, as an additional relevant marker for cell proliferation [22, 23], was also investigated in cultured human and rat hepatocytes.

2. Materials and methods

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

2.1. Test chemicals

Metofluthrin (Lot No. PK-020301G, purity 96.6%) was provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan) and sodium phenobarbital (NaPB, Lot No. KLM4036, purity 98.0%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Hepatocytes

Information on the donors of the hepatocyte preparations used is presented in Table 1. Cryopreserved human hepatocytes were obtained from Celsis IVT (MD, USA) and Gibco (CA, USA). In the replicative DNA synthesis studies, a total of four human hepatocyte preparations from different two female and two male donors were used. Because of a lack of availability of these human hepatocyte preparations for the study determining the effect of hepatocyte growth factor (HGF) on Ki-67 mRNA levels, three human hepatocyte preparations from other donors were used. According to the suppliers instructions, cryopreserved human hepatocytes were thawed at 37 ºC for 1 min, transferred into 20 mL in Williams' medium E supplemented with 20 μM/mL L-glutamine, 1 nM/mL bovine insulin, 10 nM/mL dexamethasone, 100 μM/mL nicotinamide, 20 μM/mL L-ascorbic acid, 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.

Rat hepatocytes were obtained from male Wistar rats (BrlHan:WIST@Jcl(GALAS), aged 5 weeks, purchased from CLEA Japan Inc. (Fuji Breeding Center, Shizuoka, Japan) by a modified two-step collagenase digestion method described previously [21].

2.3. Assay of replicative DNA synthesis

Human hepatocytes were plated at a density of 3.5 x 10^4 cells/well per 100 μL of medium on 96-well plates coated with collagen I (AsahiTechnoGlass, Japan) and were cultured at 37 °C in a humidified incubator under an atmosphere of 95% air/5% carbon dioxide. After pre-incubation for 24 hours, human hepatocytes were incubated for 48 hours with serum free medium containing metofluthrin (7.7, 39, 77, 390, and 770 μM) or NaPB (100, 500, 750, or 1000 μM) with DMSO at a final concentration of 0.1% (v/v), or human recombinant HGF (1, 10, and 100 ng/mL). Since metofluthrin concentration in liver at 900–1800 ppm dose (liver tumor occurrence dose levels in 2-year bioassay) is estimated to be about 1-10 μM [11] and metofluthrin (10-1000 μM) increased replicative DNA synthesis without cytotoxicity in cultured rat hepatocytes [21], concentration of 7.7-770 μM metofluthrin were used in this experiment. The highest metofluthrin concentration used was thus >75 times that observed in the *in vivo* carcinogenicity study. For NaPB, plasma levels of PB in human subjects given therapeutic doses of 3-6 mg/kg range from 10-25 μg/mL [24], which are equivalent to plasma levels in rodents at hepatocarcinogenic dose levels. NaPB concentrations of 100 and 500 μM were used in the first two experiments for donors IPH and LLA, whereas concentrations, 500 and 750 μM NaPB and 500 and 1000 μM NaPB were examined in the subsequent two experiments with donors LMP and QOQ, respectively. Thus, 500 μM NaPB (equivalent to 127.6 μg/mL), which is about 5-fold higher than plasma levels of PB in human subjects given therapeutic doses (10-25 μg/mL, Monro 1993), was examined in the replicative DNA synthesis studies with human hepatocyte preparations from all four donors.

To activate DNA synthesis [25], 0.5 ng/mL EGF was added to all media including the control, metofluthrin, NaPB and HGF treatment groups. In addition, all media including control were supplemented with dimethyl sulfoxide (DMSO) at a final concentration of 0.1% (v/v) because DMSO was used as a vehicle to dissolve metofluthrin in the medium. The medium was changed at 24 hour intervals.

BrdU was added to the medium at the point of medium refreshment of chemical treatment at 24 hours. After a further 24 hours culture (labelling period), BrdU incorporation into DNA was determined using a Cell Proliferation ELISA BrdU (chemiluminescent) kit (Roche, Germany) according to the manufacturer's protocol as described previously [21, 26]. During this labelling period, BrdU is incorporated in place of thymidine into the DNA of cycling cells. After removing the labelling medium, the cells

are fixed, and the DNA is denatured in one step by adding FixDenat. After removing FixDenat, the anti-BrdU-POD antibody is added and then bound to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes are detected by the subsequent substrate reaction. The reaction product was quantified by measuring the light emission using a scanning multi-well luminometer (EnVision, PerkinElmer).

Assays were run in quadruplicate or octuplicate at each concentration of metofluthrin, NaPB, or HGF. The experiment was conducted twice with hepatocyte preparations from each donor. The proliferation rate was calculated from the luminescent intensity of hepatocytes treated with metofluthrin, NaPB, or HGF compared to control cultures (DMSO only treated). The measurements were conducted by Sumika Technoservice Corporation (Hyogo, Japan).

2.4 Assay of cell viability

Since 77-770 μM metofluthrin decreased replicative DNA synthesis in human hepatocytes from donor IPH (only one of two experiments with statistical significance) and donor QOQ was a low responder for both BrdU incorporation by HGF and CYP2B6 mRNA induction by NaPB (Fig.1), the cell viability of human hepatocyte preparations from these two donors was analyzed by the MTT assay, employing the same incubation conditions as for the replicative DNA synthesis experiments. Human hepatocytes were incubated at a density of 5×10^4 cells/well per 100 μL of medium on 96-well plates coated with collagen I (AsahiTechnoGlass). After pre-incubation for 24 hours, human hepatocytes were incubated for 48 hours in serum free medium containing metofluthrin (3.9, 39, 77, 390, or 770 μM) or NaPB (1000 μM) with DMSO at a final concentration of 0.1% (v/v). The media was supplemented with 0.5 ng/mL EGF to keep consistency with the culture conditions for the replicative DNA synthesis experiments. As a vehicle control, all media were supplemented with DMSO at a final concentration of 0.1% (v/v). The medium was changed at 24 hour intervals.

After the test chemical treatment, the medium was removed and medium containing 5 mg/mL of MTT (Dojindo laboratories, Kumamoto, Japan) was added to each well and the cells incubated for 4 hours at 37 ºC. The medium was removed, the cells washed with PBS and then 0.04 mol/L HCL / isopropanol (200 μL) was added to each well. The plates were shaken for 10 min on a micro mixer (confido-S202, FINEPCR, Seoul, Korea) and then read using a microplate reader (SH-1000 Lab, Corona Electric, Ibaraki, Japan) at a wavelength of 570 nm. The measurement was conducted by Sumika Technoservice Corporation.

2.5. Assay of mRNA induction

For assay of mRNA induction, human hepatocytes were plated at a density of 3.0×10^5 cells/well per 500 μL of medium on 24-well plates coated with collagen I (AsahiTechnoGlass). Rat hepatocytes were plated at a density of 6.0×10^4 cells/well per 500 μL of medium on 24-well plates coated with collagen I. After pre-incubation for 24 hours, human hepatocytes were incubated for 48 hours with serum free medium containing metofluthrin (3.9, 7.7, 39, 77, 390, or 770 μM) or NaPB (1000 μM) with DMSO at a final concentration of 0.1% (v/v), and rat hepatocytes were incubated for 48 hours with serum free medium containing metofluthrin (3.9, 7.7, 39, 77, 390, or 770 μM) with DMSO at a final concentration of 0.1% (v/v). In the experiment to evaluate the effect of HGF on Ki-67 mRNA levels, human and rat hepatocytes were incubated 48 hours with serum free medium containing 100 ng/mL HGF. To keep consistency with the culture conditions for the replicative DNA synthesis experiments, EGF (0.5 ng/mL) was supplemented in the all media. As a vehicle control, all media were supplemented with DMSO at a final concentration of 0.1% (v/v). The medium was changed at 24 hour intervals.

Table 2. Primer and probe sequences used for determination of CYP2B, Ki-67, CAR, and GAPDH mRNA levels

Target mRNA	Forward Primer	Reverse Primer	Probe	Product size
Human				
CYP2B6 [21]	CCCCAAGGACACAGAAGTATTTC	GATTGAAGGCGTCTGGTTTTTC	Not used	83bp
Ki-67 [34]	CTCCCTTAAGACGGCAGTGTATTAG	GAAGGCTCTGTCTCAGTATCTGAAGTT	AGCAAAAACGCCCAGGAACACCTACAA	115bp
CAR [c]	AAGAAGAGCTGATCCGGACACT	TGCACAAACTGTTCAAACATGGT	TTTTACCCAATAAGGCACCACCCACCTATG	73bp
GAPDH [34]	GACACCCACTCCTCCACCTTT	CATACCAGGAAATGAGCTTGACAA	CTGGCATTGCCCTCAACGACCA	79bp
Rat				
CYP2B1/2 [21,34]	GCTCAAGTACCCCCATGTCG	ATCAGTGTATGGCATTTTACTGCGG	Not used	109bp
Ki-67 [34]	GCAGACAAGCCTTCAGCAGTAA	TGGTACCATTGTCATCAATTTCAGT	CCAACATCAAGGCAAAGTGTGCGATC	94bp
CAR [c]	CCATCACCGGCCTTTCC	GCTGCACCATGAAAGTATTGATATCT	CCTGGCCCCCGTGTTGCCT	83bp
GAPDH [34]	GCTGCCTTCTCTTGTGACAAAGT	CTCAGCCTTGACTGTGCCATT	TGTTCCAGTATGATTCTACCCACGGCAAG	129bp

[c] The primer sets and probes are able to detect all known members of the CAR superfamily, i.e., transcript variants 1-15, and predicted transcript variants X1, X2 and X5 for human CAR; transcript variants 1-4, and predicted transcript variants X1, X2 and X3 for rat CAR.

At the end of the treatment period the medium was removed and the hepatocytes were washed with phosphate buffered saline (PBS). Total RNA from hepatocytes was extracted using Isogen solution (Nippon Gene) and RNeasy Mini Kits (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 -80ºC 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 manufacturer's instructions. The reaction mixture (20 μL) containing 10x RT Buffer containing total RNA (10–100 ng) (2 μL), 25 x dNTP mix (0.8 μL), 10x RT Random Primers (2 μL), 1 U/μL RNase Inhibitor (1μL) and 2.5U/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 needed for real-time PCR assays.

Quantitative real-time PCR assays for CYP2B (CYP2B6 for human, CYP2B1/2 for rat), Ki-67 and CAR mRNA were performed following the instruction manual of the PCR system (7500 Fast Real-Time PCR System, Applied Biosystems). In addition, levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined as an internal control. The primer sets are listed in Table 2. For the quantitation of Ki-67, CAR and GAPDH mRNA, the reaction mixture (25 μL) containing 2x TaqMan Universal Master Mix (Applied Biosystems) (12.5 μL), each primer (Forward and Reverse, 0.2 μM each), probe (0.2 μM) and cDNA (2 μL of 1/4 diluted solution). After incubation at 50 ºC for 2 min and 95 ºC for 10 min, the PCR reaction was performed for 40 cycles: denaturation at 95 ºC for 15s, anneal and extension at 60 ºC for 1 min. For the quantitation of CYP2B mRNA, the reaction mixture (25 μL) containing 2x Power SYBR Green PCR Master Mix (Applied Biosystems) (12.5 μL), each primer (Forward and Reverse, 0.4 μM each), and cDNA (2 μL of 1/4 diluted solution). After incubation at 95 ºC for 10 min, the PCR reaction was performed for 40 cycles: denaturation at 95 ºC for 30s, anneal at 61 ºC for 30s and extension at 72 ºC for 45s. The Ki-67, CYP2B and CAR mRNA levels were normalized to those of GAPDH mRNA. Each primer set did not form primer dimer or by-products and showed a single sharp peak under these conditions. The measurement was conducted by Sumika Technoservice Corporation.

Assays were run in duplicate or triplicate at each metofluthrin or NaPB concentration, except for hepatocytes from donor QOQ where, owing to low levels of DNA, pooled samples were analysed.

2.6. 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 test was employed for evaluation with $p \leq 0.05$ and 0.01 as the levels of significance.

3. Results

3.1. Effect on cell proliferation in cultured human hepatocytes

Human hepatocytes from four donors (donors: IPH, LLA, LMP, and QOQ; Table 1) were employed to determine the effects of metofluthrin and NaPB on replicative DNA synthesis. The assay was repeated twice with hepatocytes from each donor. Apart from the exceptions described below, good reproducibility was obtained ($R^2 \geq 0.91$, Fig. 1).

HGF statistically significantly increased replicative DNA synthesis in a concentration-dependent manner in all human hepatocyte preparations except for donor LMP where, unlike the first experiment, the second experiment did not show statistical significance due to a large variation between replicate wells. Of the four donors, QOQ was a low responder to HGF compared to the other three donors. However, all four of the human hepatocyte preparations studied displayed the ability to increase DNA synthesis in response to treatment with a mitogenic agent.

In contrast to HGF, neither metofluthrin nor NaPB treatment increased replicative DNA synthesis in any of the four human hepatocyte preparations examined. In the second experiment with hepatocytes from donor IPH, treatment with 77-770 μM metofluthrin significantly decreased replicative DNA synthesis. However, no reduction in replicative DNA synthesis was observed in the first experiment with hepatocytes from donor IPH indicating a lack of reproducibility of this apparent inhibitory effect.

3.2. Effect on cell viability in cultured hepatocytes

The effect of metofluthrin and NaPB on cell viability was determined in two human hepatocyte preparations from donors IPH and QOQ employing the MTT assay. No significant effect on cell viability was observed in hepatocyte preparations from both donors, even at a concentration of 770 μM metofluthrin or 1000 μM NaPB (Fig. 2). Furthermore, light microscopic examination of the cultured hepatocyte preparations from donors LLA and LMP revealed no differences after treatment with either metofluthrin or NaPB. These results demonstrate that metofluthrin at concentrations up to 770 μM and NaPB at concentrations up to 1000 μM are not cytotoxic to human hepatocytes and hence the lack of any increase in replicative DNA synthesis by metofluthrin and NaPB is not attributable to low cell viability.

3.3. Effects on CYP2B6, Ki-67, and CAR mRNA expression in cultured human and rat hepatocytes

Human hepatocytes from four donors (IPH, LLA, LMP, and QOQ; Table 1) were also employed to examine the effects of metofluthrin and NaPB on CYP2B6 mRNA expression levels determined by RT-PCR (Fig. 3). In the hepatocytes from three donors (IPH, LLA, and LMP), the treatment with 1000 μM NaPB produced a statistically significant 5-fold or more increase in CYP2B6 mRNA levels. In contrast, one donor QOQ showed only a small increase (1.7-fold control) without statistical significance in CYP2B6

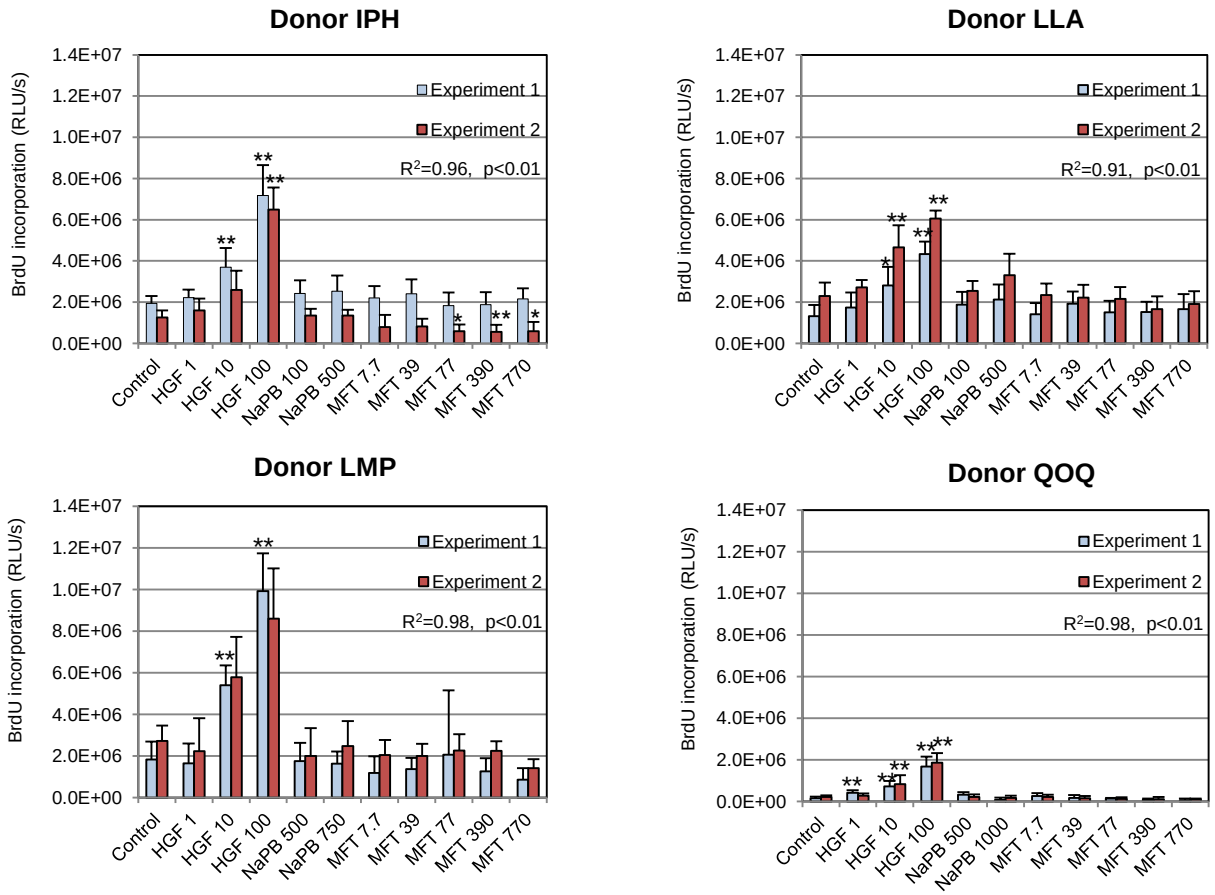

Figure 1. Effect of HGF, NaPB, and metofluthrin (MFT) on replicative DNA synthesis in cultured human hepatocytes from four donors. Human hepatocytes (donors IPH, LLA, LMP, and QOQ) were treated with 1-100 ng/mL HGF, 100-1000 μM NaPB, and 7.7-770 μM metofluthrin (MFT) for 48 hours and replicative DNA synthesis was determined by BrdU incorporation over the last 24 hours of culture. Results are presented as mean ± SD of 2-8 replicate wells per treatment in units of luminescence as relative light units per second (RLU/s). Data are presented for two replicate experiments (1 and 2) for each human hepatocyte preparation. Pearson correlation coefficients between two experiments were analyzed and coefficient of determination (R^2) and p-values are presented. Values significantly different from control (DMSO only treated) are: *p<0.05 and **p<0.01.

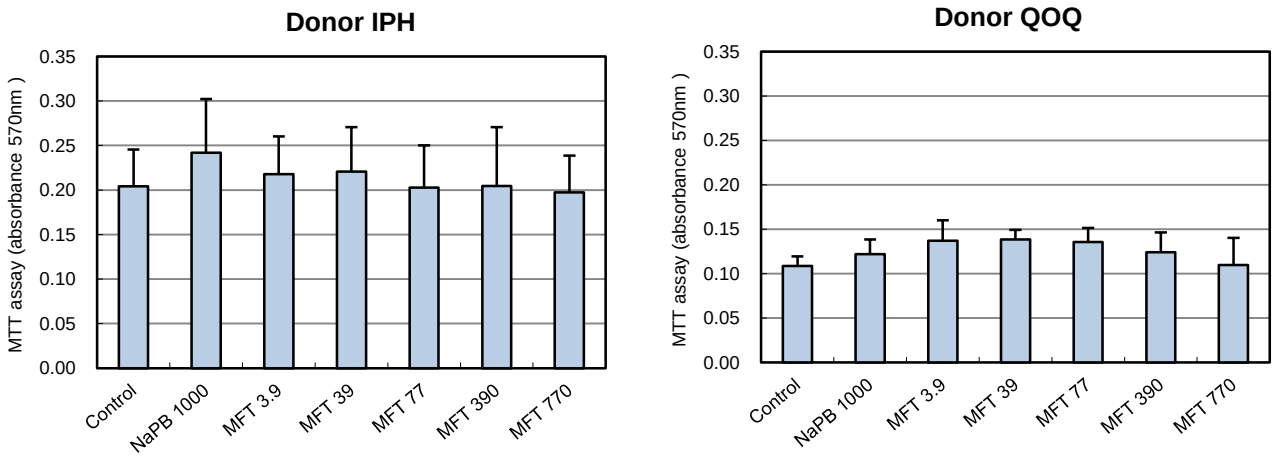

Figure 2. Effect of NaPB and metofluthrin (MFT) on cell viability (MTT assay) in cultured human hepatocytes. Results are presented as mean ± SD of 12 replicate wells. No values were statistically significantly different (all $p>0.05$) from control (DMSO only treated).

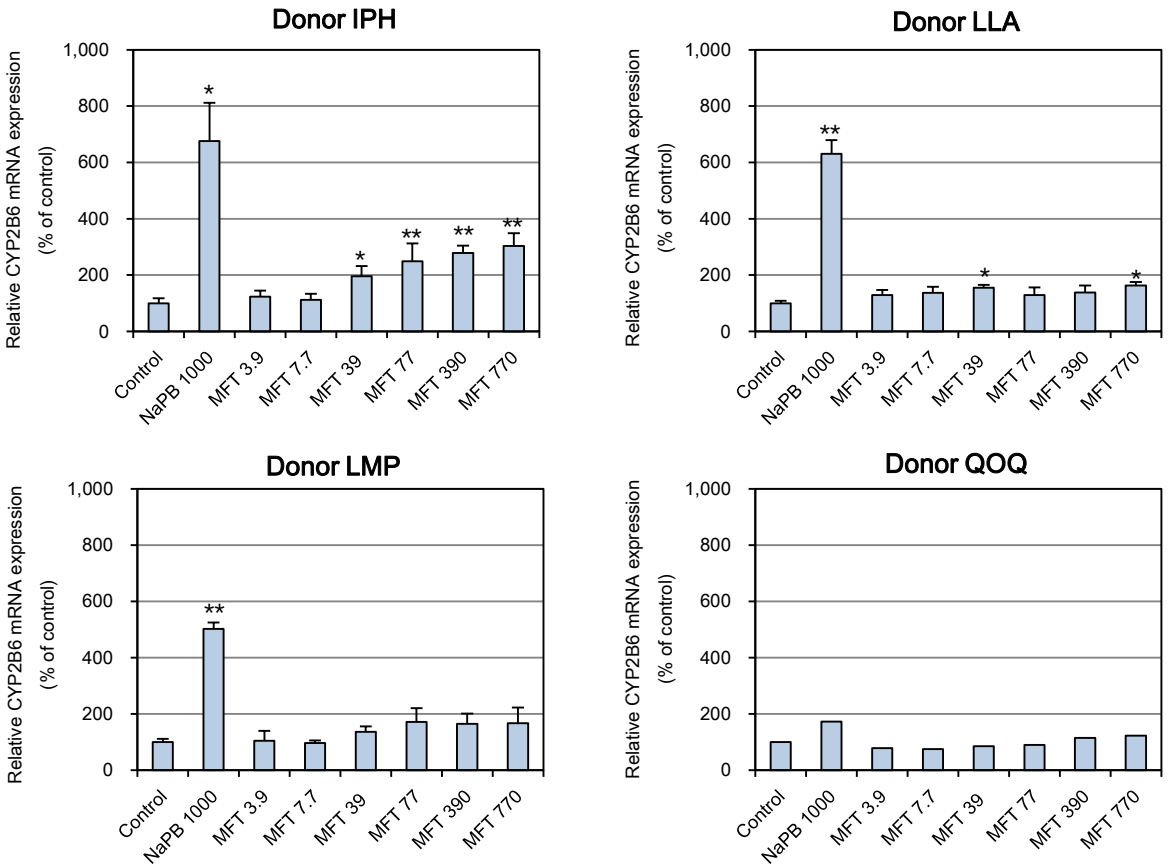

Figure 3. Effect of NaPB and metofluthrin (MFT) on CYP2B6 mRNA expression in cultured human hepatocytes. Human hepatocytes (donors IPH, LLA, LMP, and QOQ) were treated with 1000 μM NaPB or 3.9-770 μM metofluthrin for 48 hours and CYP2B6 mRNA was determined by quantitative real-time PCR. Results are presented as mean ± SD of triplicate wells per treatment, except for hepatocytes from donor QOQ where owing to low levels of DNA only pooled samples were analysed. Values significantly different from control (DMSO only treated) are: *$p<0.05$ and **$p<0.01$.

mRNA levels by treatment with 1000 μM NaPB.

In hepatocytes from donor IPH, metofluthrin significantly increased CYP2B6 mRNA levels in a concentration-dependent manner and at concentrations of 39 and 770 μM significantly increased CYP2B6 mRNA levels in hepatocytes from donor LLA. The treatment of hepatocytes from donors LMP and QOQ with metofluthrin resulted in concentration-dependent but not statistically significant increases in CYP2B6 mRNA levels. The increases in CYP2B6 mRNA levels in hepatocytes from donors LMP and QOQ treated with 770 μM metofluthrin were 1.6- and 1.2-fold control, respectively.

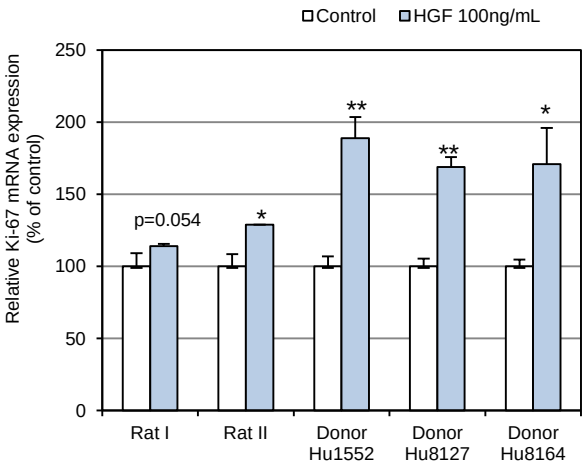

Figure 4. Evaluation of effect of HGF on Ki-67 mRNA levels in rat and human hepatocytes. Rat hepatocytes from two animals and human hepatocytes from three donors (Hu1552, Hu8127, and Hu8164) were treated with 100 ng/mL HGF for 48 hours and Ki-67 mRNA was determined by quantitative real-time PCR. Mean values ± SD from triplicate wells per treatment are presented. Values significantly different from control are: *p<0.05 and **p<0.01. The p-value for statistical analysis is also presented for Rat I.

Figure 4 shows the effect of HGF on Ki-67 mRNA levels determined by RT-PCR in rat and human hepatocytes. Under the culture conditions used in this study, HGF significantly increased Ki-67 mRNA levels in rat hepatocytes in one experiment and produced a small non-statistically significant increase (p=0.054, 1.14-fold control) in another experiment. Treatment with HGF produced significant increases in Ki-67 mRNA levels in human hepatocytes from donors Hu1552, Hu8127 and Hu8164. Thus, as an additional marker of replicative DNA synthesis, mRNA expression levels of the cell proliferation-related gene Ki-67 were determined by

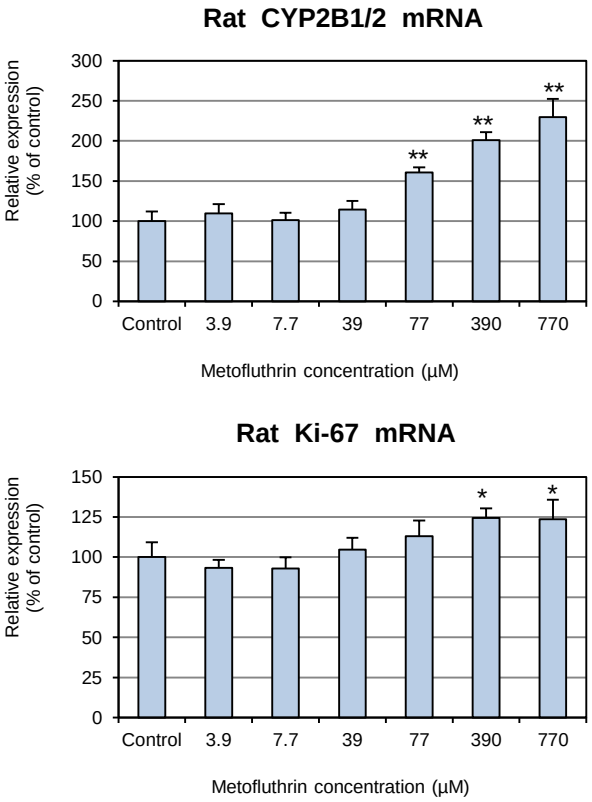

Figure 5. Effect of metofluthrin on CYP2B1/2 and Ki-67 mRNA expression in cultured rat hepatocytes. Rat hepatocytes from single animal were treated with 3.9-770 μM metofluthrin for 48 hours and CYP2B1/2 and Ki-67 mRNA was determined by quantitative real-time PCR. Results are presented as mean ± SD of triplicate wells per treatment. Values significantly different from control (DMSO only treated) are: *p<0.05 and **p<0.01.

RT-PCR in rat and human hepatocytes treated with metofluthrin. Metofluthrin treatment significantly increased Ki-67 mRNA levels as well as CYP2B1/2 mRNA levels in a concentration-related manor in rat hepatocytes (Fig. 5). In contrast, in human hepatocytes from four donors, neither metofluthrin at concentrations up to 770 μM nor NaPB at concentrations up to 1000 μM produced any statistically significant alterations in Ki-67 mRNA levels (Fig. 6). A large variation was observed in control Ki-67 mRNA levels in human hepatocytes from donors IPH and LMP (Fig. 6). In metofluthrin-treated hepatocytes from donors IPH and LMP, there appeared to be a trend towards decreased Ki-67 mRNA levels (Fig. 6). Since CYP2B6 and Ki-67 mRNA levels were determined in the same total RNA samples obtained from the cultured rat and human hepatocyte preparations, Pearson correlation coefficients between Ki-67 and CYP2B6 mRNA levels in control and metofluthrin-treated hepatocytes were determined in rat

hepatocytes and in hepatocytes from each human donor (Fig. 7). In rat hepatocytes, Ki-67 mRNA levels and CYP2B1/2 mRNA levels were positively correlated (R^2=0.893). In contrast, in human hepatocytes from IPH, LLA, and QOQ, no clear correlation was observed between these mRNA levels (R^2=0.38, 0.10 and 0.21, respectively). For human hepatocytes from donor LMP, while no positive correlation was observed, a weak negative correlation was observed (R^2=0.46).

CAR mRNA expression levels were also determined in the same total RNA samples obtained from the rat and human cultured hepatocyte preparations. Neither metofluthrin nor NaPB significantly affected CAR mRNA expression levels in cultured rat or human hepatocytes (Fig. 8).

4. Discussion

Previous studies have established a robust MOA for metofluthrin-induced rat liver tumor formation [8, 11]. This MOA is similar to that established for the CAR activator phenobarbital [13]. The stimulation of replicative DNA synthesis *via* CAR activation is a critical key event in the proposed MOA for metofluthrin-induced rat liver tumour formation [8]. Therefore, to assess the human relevance of the rodent MOA for metofluthrin-induced liver tumor formation, it is important to evaluate the effect of metofluthrin on replicative DNA synthesis in human liver.

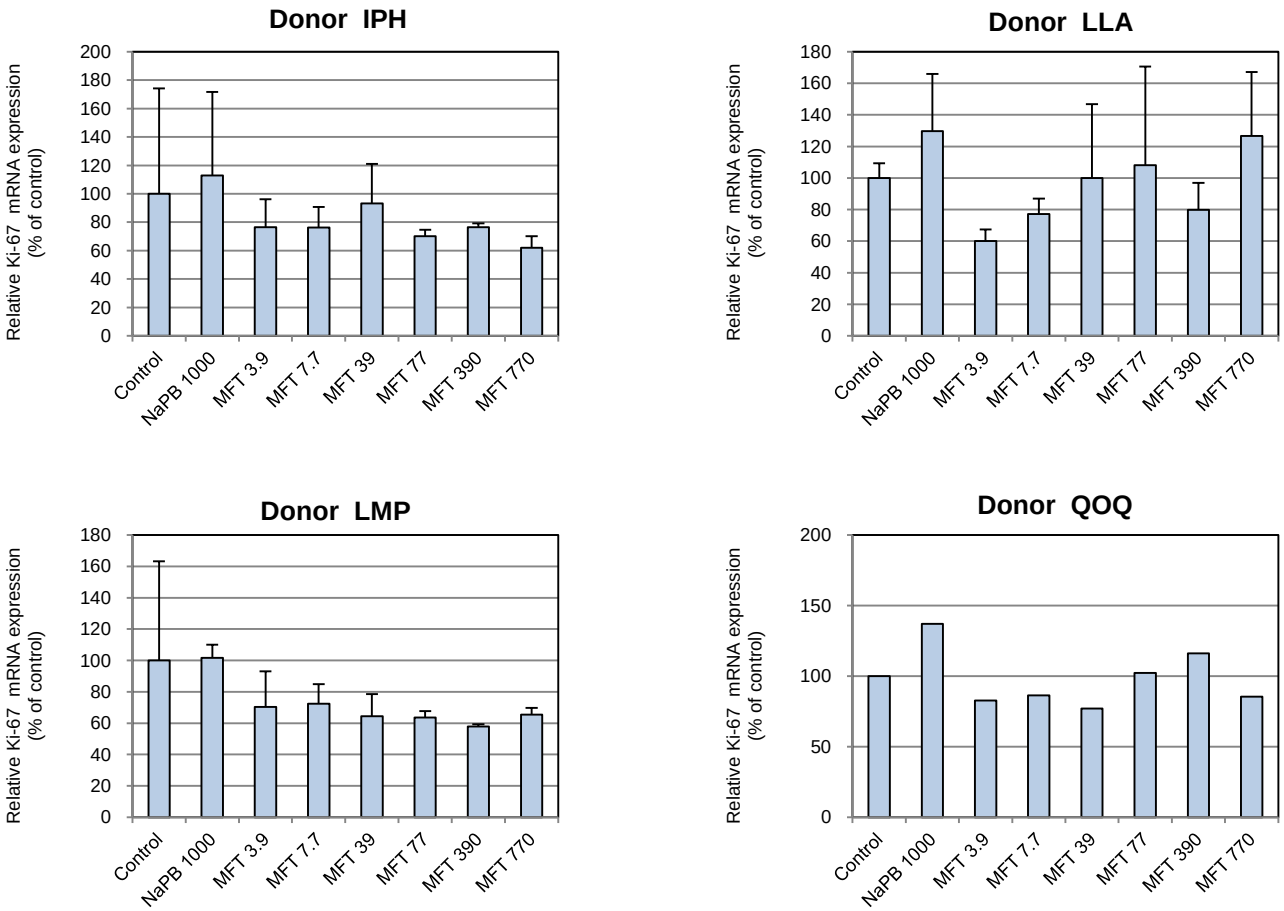

Figure 6. Effect of NaPB and metofluthrin (MFT) on Ki-67 mRNA expression in cultured human hepatocytes. Human hepatocytes (donors IPH, LLA, LMP, and QOQ) were treated with 1000 μM NaPB or 3.9-770 μM metofluthrin (MFT) for 48 hours and Ki-67 mRNA was determined by quantitative real-time PCR. Results are presented as mean ± SD of triplicate wells per treatment, except for hepatocytes from donor QOQ where owing to low levels of DNA only pooled samples were analysed. No values were significantly different (all p>0.05) from control (DMSO only treated).

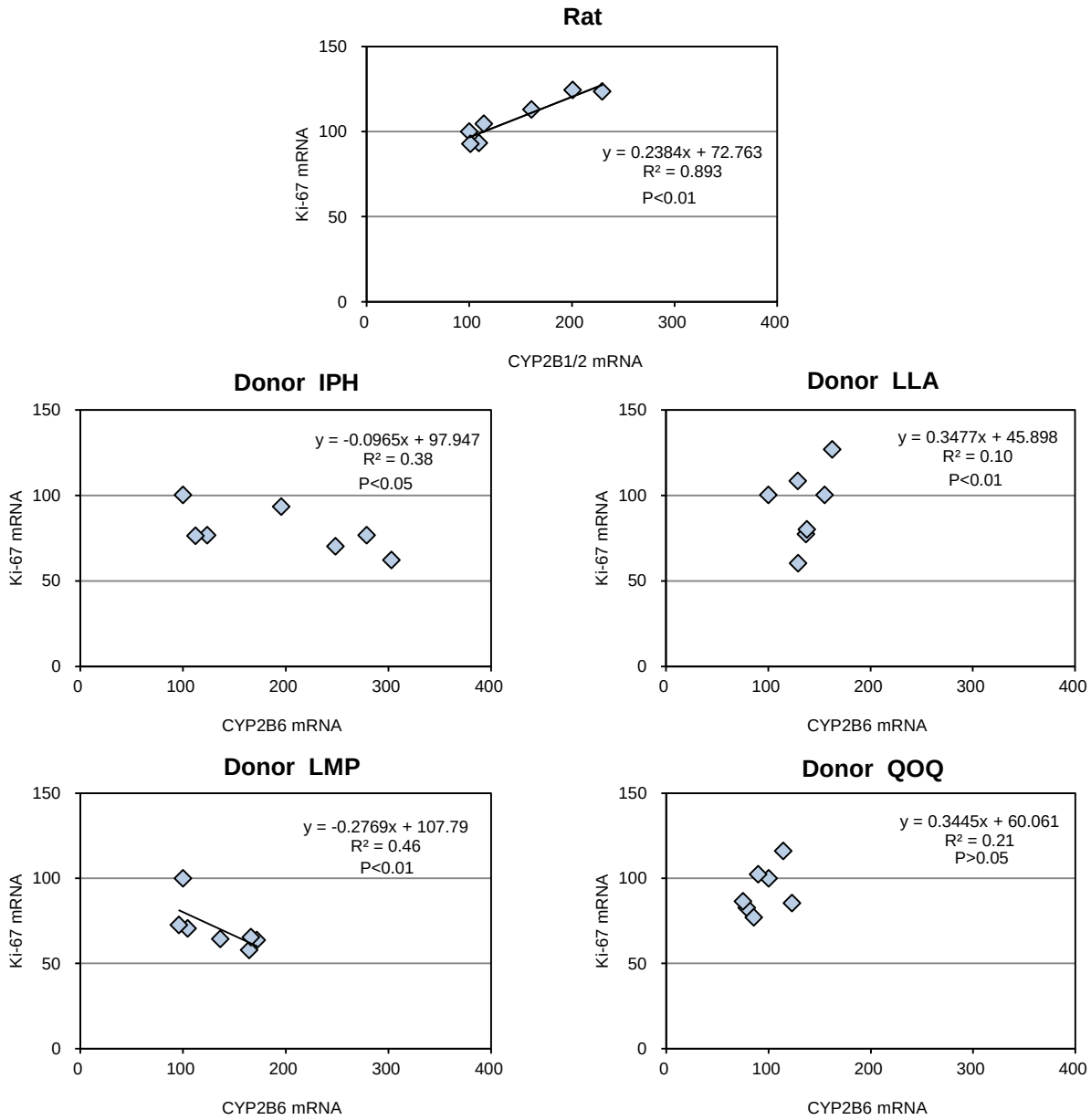

Figure 7. Scatter plots of mean values of Ki-67 and CYP2B mRNA levels per each concentration in cultured rat and human hepatocytes treated with metofluthrin (0, 3.9, 7.7, 39, 77, 390, or 770 μM). Data presented in Figure 5 are plotted for rat, and data presented in Figures 3 and 6 are plotted for each human donor. Pearson correlation coefficients between Ki-67 and CYP2B mRNA levels were analyzed and Primary regression equation (y=ax+b), coefficient of determination (R^2) and p-values are presented.

Metofluthrin concentrations in rat liver at tumorigenic dose levels of 900 and 1800 ppm (chemical intake, 40-96 mg/kg/day) were around 1-10 μM and significant increases in replicative DNA synthesis in cultured rat hepatocytes were observed at concentrations of 10 μM and greater [21]. Expected human exposure levels to metofluthrin are less than 0.001 mg/kg/day [8]. In this study, human hepatocytes were treated with 7.7-770 μM metofluthrin to evaluate the effect of metofluthrin at concentrations much greater than those required to produce liver tumours in rats and orders of magnitude above anticipated human exposure to metofluthrin.

A number of studies have shown that the CAR is present in

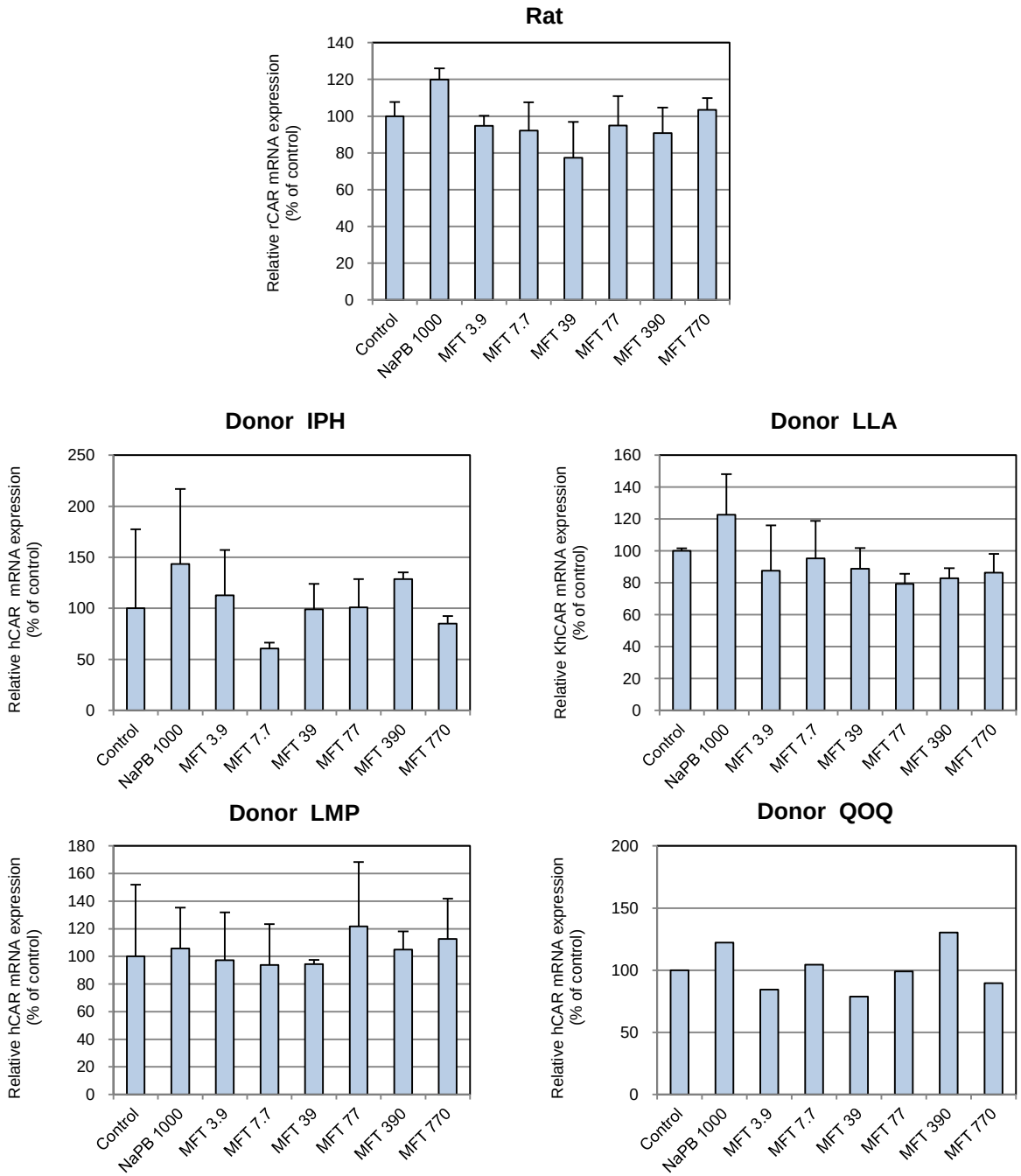

Figure 8. Effect of NaPB and metofluthrin (MFT) on CAR mRNA expression in cultured rat and human hepatocytes. Human (donors IPH, LLA, LMP, and QOQ) and rat hepatocytes were treated with 1000 μM NaPB or 3.9-770 μM metofluthrin (MFT) for 48 hours and CAR mRNA was determined by quantitative real-time PCR. Results are presented as mean ± SD of triplicate wells per treatment, except for hepatocytes from donor QOQ where owing to low levels of DNA only pooled samples were analysed. No values were significantly different (all $p>0.05$) from control (DMSO only treated).

human liver and that the receptor can be activated by drugs and other compounds [27, 28]. The induction of CYP2B enzymes serves as a marker for CAR activation [15]. In agreement with previous studies [28-30], the treatment of human hepatocytes with 1000 μM NaPB resulted in increases in CYP2B6 mRNA levels ranging from 1.7 to >6 fold, thus confirming the functional viability of the human hepatocytes used in these experiments to CYP2B inducers. In the present study, metofluthrin produced increases in CYP2B6 mRNA levels in human hepatocytes from all four donors. However, the effect of metofluthrin was less marked than that observed with NaPB, with statistically significant increases only being observed at some concentrations of metofluthrin examined in donor IPH and LLA. Overall,

these results suggest that metofluthrin can activate CAR in cultured human hepatocytes. However, while CYP2B6 mRNA levels were increased in cultured human hepatocytes, CAR mRNA levels were not changed by treatment with either metofluthrin or NaPB.

While the potential for compounds to increase cell proliferation in rodent liver can be examined by performing *in vivo* studies, compounds can also be tested for mitogenic potential *in vitro* by conducting studies using primary hepatocyte cultures. For example, a number of compounds that can activate either the CAR or the PPARα in rodent liver can induce replicative DNA synthesis in cultured rat and mouse hepatocytes [15, 26, 31-33]. We have already demonstrated that metofluthrin can increase replicative DNA synthesis in cultured rat hepatocytes *in vitro* [21] as well as *in vivo* [11]. Although we have also examined the effect of metofluthrin on replicative DNA synthesis in human hepatocytes, the study was limited to investigations with hepatocytes from only two donors [21]. The results of this previous study are thus greatly extended by the present data with human hepatocytes from an additional four donors where effects on both replicative DNA synthesis and Ki-67 mRNA expression have been determined.

As HGF is known to increase DNA synthesis in human hepatocytes [21, 25], it was used as a positive control in this study. HGF produced a concentration-dependent increase in DNA synthesis in human hepatocyte preparations from all four donors, confirming the functional viability of the human hepatocyte preparations used in this study to a mitogenic agent.

In contrast to the effect of HGF, both metofluthrin and NaPB did not stimulate replicative DNA synthesis in cultured human hepatocytes from all four donors examined. NaPB is a model CAR activator and known rodent liver mitogen[13,15]. In the MOA established for NaPB and related compounds, the key species difference between rodents and humans is the lack of effect on replicative DNA synthesis in human hepatocytes. The data obtained in the present study thus supports the results of previous limited investigations where NaPB has been shown not to stimulate replicative DNA synthesis in cultured human hepatocytes[21,33]. Moreover, in a recent study NaPB was shown not to stimulate replicative DNA synthesis in an *in vivo* study using chimeric mice with human hepatocytes[34].

While 77-770 μM metofluthrin decreased replicative DNA synthesis in human hepatocytes from donor IPH in only one of the two experiments performed, the results of the MTT assay demonstrated that metofluthrin at concentrations up to 770 μM had no effect on cell viability. Thus, metofluthrin was not cytotoxic to human hepatocytes, consistent with previous findings in both *in vivo* and *in vitro* studies [11, 21]. In addition, metofluthrin is not a genotoxic agent [9, 10]. Overall, as metofluthrin is not cytotoxic or genotoxic, the decreases in replicative DNA synthesis observed in only one of the two experiments performed with hepatocytes from donor IPH is unlikely to have any toxicological significance. This conclusion is supported by the results obtained with three other donors in this study and two additional donors in the previous study [21] where metofluthrin did not decrease replicative DNA synthesis in cultured human hepatocytes.

Ki-67 is an established cell proliferation marker[22, 23]. Under the experimental conditions used in this study, HGF (100 ng/mL) significantly increased Ki-67 mRNA levels in cultured rat and human hepatocytes. The effect of metofluthrin on Ki-67 mRNA levels was also examined. In cultured rat hepatocytes, metofluthrin activated CAR as demonstrated by increased CYP2B1/2 mRNA levels and also increased Ki-67 mRNA levels. In addition, a positive correlation of Ki-67 and CYP2B1/2 mRNA levels suggests that CAR activation is attributable to the increased Ki-67 mRNA expression in rat hepatocytes. In contrast, neither metofluthrin nor NaPB increased Ki-67 mRNA levels in human hepatocytes. A slight negative correlation between CYP2B6 and Ki-67 mRNA levels was observed in metofluthrin-treated human hepatocytes from donor LMP. However, this small effect has no biological significance, since no decrease in replicative DNA synthesis was observed in hepatocytes from this donor. Ki-67 mRNA levels (a biomarker of cell proliferation) were positively correlated with CYP2B1/2 mRNA levels (a biomarker of CAR activation) in metofluthrin-treated rat hepatocytes, whereas Ki-67 mRNA levels and CYP2B6 mRNA levels were not correlated in human hepatocytes, supporting the conclusion that CAR activation by metofluthrin increases hepatocyte proliferation only in rat and not in human hepatocytes.

The difference in response between rat and human hepatocytes to metofluthrin and NaPB cannot be explained by differences in CAR expression levels, because CAR expression levels appear to be similar between rat and human hepatocytes[34]. Furthermore, in this study CAR mRNA expression levels were not changed by metofluthrin treatment in both cultured rat and human hepatocytes. The lack of effect of metofluthrin and NaPB on replicative DNA synthesis in cultured human hepatocytes is in agreement with a recent *in vivo* study where NaPB did not increase hepatic replicative DNA synthesis in chimeric mice where the host mouse hepatocytes were replaced with human hepatocytes in the livers[34]. These findings suggest that species differences exist in the signalling of CAR target genes, particularly for genes associated with hepatocellular proliferation.

Table 3 Comparison of key and associative events on MOA for liver tumourigenesis of metofluthrin in rats and humans

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 [11]	Probable at high doses (inferred from induction of CYP2B enzymes in cultured hepatocytes [21, d])
Induction of CYP2B (A)	Direct experimental evidence *in vivo* [d] and *in vitro* in cultured hepatocytes [21,d]	Probable at high doses (Yes in cultured hepatocytes [21, d])
Hypertrophy (A)	Direct experimental evidence *in vivo* [11]	Possible at very high doses based on studies in human subjects given anticonvulsant drugs [37,38]
Increased hepatocellular proliferation (K)	Direct experimental evidence *in vivo* [d] and *in vitro* in cultured hepatocytes [21, d]	Not predicted (No in cultured hepatocytes [21, d]) (No in chimeric mouse with human hepatocyte given sodium phenobarbital [34])
Altered hepatic foci (K)	Direct experimental evidence *in vivo* [11]	Not predicted
Liver tumours	Yes [11]	Not predicted

Reference; [d] this study.

In assessing the relevance of an animal MOA data to humans, a concordance Table has been suggested as being of considerable value [2, 4]. Such a Table is presented in Table 3 which summarises the available rat and human data for the key and associative events in the proposed MOA for metofluthrin-induced rat liver tumour formation. Most data in rats are derived from previous studies [11, 21]. Additional evidence for increased cell proliferation was obtained in the present study where metofluthrin increased Ki-67 mRNA levels in cultured rat hepatocytes. For humans, as described above, high doses of metofluthrin could activate CAR and produce CYP2B6 enzyme induction in liver [21]. 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 [35, 36]. Thus, by comparison with the effects of such anticonvulsant drugs, at high doses metofluthrin also could have the potential to produce hypertrophy in human liver. However, a key species difference is that while metofluthrin is clearly a mitogenic agent in the rat, as demonstrated by both *in vivo* [11] and *in vitro* [21] studies, metofluthrin does not stimulate replicative DNA synthesis in cultured human hepatocytes in previous [21] and in the present studies and Ki-67 mRNA levels in cultured human hepatocytes in the present study. Overall, while some of the key (activation of the CAR) and associative (CYP2B enzyme induction and hepatocellular hypertrophy) events in the MOA for metofluthrin-induced rat liver tumour formation could occur in human liver, the available experimental data demonstrate that human hepatocytes are refractory to the mitogenic effects of metofluthrin. Hence, it is therefore reasonable to conclude that the proposed MOA for metofluthrin-induced rat liver tumour formation is not plausible for humans.

The data obtained in these studies with the CAR activator metofluthrin are in agreement with literature data on other CAR activators and with compounds that can activate the PPARα in rodent liver. Studies with other CAR activators and PPARα activators, including some hypolipidaemic drugs, have shown that these compounds stimulate replicative DNA synthesis in cultured rodent hepatocytes but not in cultured human hepatocytes [13, 15, 26, 32, 33, 37], and they also do not stimulate replicative DNA synthesis in chimeric mice with human hepatocytes [34, 38]. Metofluthrin thus produces similar effects on replicative DNA synthesis in rat and human liver to those produced by a number of other non-genotoxic agents which can produce liver tumours in rodents. The key species difference between rodents and humans appears to be that human hepatocytes are refractory to the mitogenic effects of such agents [13].

Conflict of interest

Author Professor Brian G. Lake consults for Sumitomo Chemical Company.

Funding

This work was supported by the Sumitomo Chemical Co., Ltd.

Acknowledgements

The authors thank Professor Samuel M. Cohen (University of Nebraska Medical Center, Omaha, Nebraska, USA) for valuable scientific discussions; and Ms. Miwa Kondo, Keiko Tanaka, Youko Obara, and Mr. Yu Okuda for their technical assistance. We also thank the other contributors to this research project from Sumitomo Chemical Co., Ltd., Sumitomo Chemical (UK) PLC, and Sumika Technoservice Corporation.

Notes and references

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

[b]Centre for Toxicology, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, United Kingdom

[1]To whom correspondence should be addressed.
Environmental Health Science Laboratory, Sumitomo Chemical Company, Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-8558, Japan
Phone : +81-6-6466-5363, Fax: +81-6-6466-5354.
Email: yamadat8@sc.sumitomo-chem.co.jp

Keywords:

liver tumour; mode of action; cytochrome P450; cell proliferation; human risk assessment

References

1. S. M. Cohen, J. Klaunig, M. E. Meek, R. N. Hill, T. Pastoor, L. Lehman-McKeeman, J. Bucher, D. G. Longfellow, J. Seed, V. Dellarco, P. Fenner-Crisp and D. Patton, *Toxicol. Sci.*, 2004, **78**, 181-186.
2. M. E. Meek, J. R. Bucher, S. M. Cohen, V. Dellarco, R. N. Hill, L. D. Lehman-McKeeman, D. G. Longfellow, T. Pastoor, J. Seed and D. E. Patton, *Crit. Rev. Toxicol.*, 2003, **33**, 591-653.
3. J. Seed, E. W. Carney, R. A. Corley, K. M. Crofton, J. M. DeSesso, P. M. Foster, R. Kavlock, G. Kimmel, J. Klaunig, M. E. Meek, R. J. Preston, W. Slikker, Jr., S. Tabacova, G. M. Williams, J. Wiltse, R. T. Zoeller, P. Fenner-Crisp and D. E. Patton, *Crit. Rev. Toxicol.*, 2005, **35**, 664-672.
4. A. R. Boobis, S. M. Cohen, V. Dellarco, D. McGregor, M. E. Meek, C. Vickers, D. Willcocks and W. Farland, *Crit. Rev. Toxicol.*, 2006, **36**, 781-792.
5. C. Sonich-Mullin, R. Fielder, J. Wiltse, K. Baetcke, J. Dempsey, P. Fenner-Crisp, D. Grant, M. Hartley, A. Knaap, D. Kroese, I. Mangelsdorf, E. Meek, J. M. Rice and M. Younes, *Regul. Toxicol. Pharmacol.*, 2001, **34**, 146-152.
6. J. R. Lucas, Y. Shono, T. Iwasaki, T. Ishiwatari, N. Spero and G. Benzon, *J. Am. Mosq. Control Assoc.*, 2007, **23**, 47-54.
7. K. Ujihara, T. Mori, T. Iwasaki, M. Sugano, Y. Shono and N. Matsuo, *Biosci. Biotechnol. Biochem.*, 2004, **68**, 170-174.
8. T. Yamada, S. Uwagawa, Y. Okuno, S. M. Cohen and H. Kaneko, *Toxicol. Sci.*, 2009, **108**, 59-68.
9. UK, *METOFLUTHRIN (PT 18), Assessment Report. Finalised in the Standing Committee on Biocidal Products at its meeting in May 2010 in view of its inclusion in Annex I to Directive 98/8/EC* 2010.
10. US.EPA, *METOFLUTHRIN: Second Report of the Cancer Assessment Review Committee. Memorandum from J.Kidwell to K. Harper and M. Suarez. Office of Pesticide Programs. TXR No.:0054668*, 2007.
11. Y. Deguchi, T. Yamada, Y. Hirose, H. Nagahori, M. Kushida, K. Sumida, T. Sukata, Y. Tomigahara, K. Nishioka, S. Uwagawa, S. Kawamura and Y. Okuno, *Toxicol. Sci.*, 2009, **108**, 69-80.
12. S. M. Cohen, *Toxicol. Pathol.*, 2010, **38**, 487-501.
13. C. R. Elcombe, R. C. Peffer, D. C. Wolf, J. Bailey, R. Bars, D. Bell, R. C. Cattley, S. S. Ferguson, D. Geter, A. Goetz, J. I. Goodman, S. Hester, A. Jacobs, C. J. Omiecinski, R. Schoeny, W. Xie and B. G. Lake, *Crit. Rev. Toxicol.*, 2014, **44**, 64-82.
14. M. P. Holsapple, H. C. Pitot, S. M. Cohen, A. R. Boobis, J. E. Klaunig, T. Pastoor, V. L. Dellarco and Y. P. Dragan, *Toxicol. Sci.*, 2006, **89**, 51-56.
15. B. G. Lake, *Xenobiotica*, 2009, **39**, 582-596.
16. E. S. Tien and M. Negishi, *Xenobiotica*, 2006, **36**, 1152-1163.
17. G. D. Friedman, S.-F. Jiang, N. Udaltsova, C. P. Quesenberry, J. Chan and L. A. Habel, *Int. J. Cancer*, 2009, **125**, 2173-2178.
18. C. La Vecchia and E. Negri, *Eur. J. Cancer Prev.*, 2014, **23**, 1-7.
19. J. H. Olsen, J. D. Boice, Jr., J. P. Jensen and J. F. Fraumeni, Jr., *J. Natl. Cancer Inst.*, 1989, **81**, 803-808.
20. J. H. Olsen, G. Schulgen, J. D. Boice, Jr., J. Whysner, L. B. Travis, G. M. Williams, F. B. Johnson and J. O. McGee, *Cancer Res.*, 1995, **55**, 294-297.
21. Y. Hirose, H. Nagahori, T. Yamada, Y. Deguchi, Y. Tomigahara, K. Nishioka, S. Uwagawa, S. Kawamura, N. Isobe, B. G. Lake and Y. Okuno, *Toxicology*, 2009, **258**, 64-69.
22. M. J. Iatropoulos and G. M. Williams, *Exp. Toxicol. Pathol.*, 1996, **48**, 175-181.
23. T. Scholzen and J. Gerdes, *J. Cell. Physiol.*, 2000, **182**, 311-322.
24. A. Monro, *Regul. Toxicol. Pharmacol.*, 1993, **18**, 115-135.
25. D. M. Runge, D. Runge, K. Dorko, L. A. Pisarov, K. Leckel, V. E. Kostrubsky, D. Thomas, S. C. Strom and G. K. Michalopoulos, *J. Hepatol.*, 1999, **30**, 265-274.
26. V. Goll, E. Alexandre, C. Viollon-Abadie, L. Nicod, D. Jaeck and L. Richert, *Toxicol. Appl. Pharmacol.*, 1999, **160**, 21-32.
27. J. T. Moore, L. B. Moore, J. M. Maglich and S. A. Kliewer, *Biochim. Biophys. Acta*, 2003, **1619**, 235-238.
28. H. Wang, S. Faucette, R. Moore, T. Sueyoshi, M. Negishi and E. LeCluyse, *J. Biol. Chem.*, 2004, **279**, 29295-29301.
29. S. Gerbal-Chaloin, J. M. Pascussi, L. Pichard-Garcia, M. Daujat, F. Waechter, J. M. Fabre, N. Carrere and P. Maurel, *Drug Metab. Dispos.*, 2001, **29**, 242-251.
30. R. J. Price, A. M. Giddings, M. P. Scott, D. G. Walters, C. C. Capen, T. G. Osimitz and B. G. Lake, *Toxicology*, 2008, **243**, 84-95.

31. S. C. Hasmall and R. A. Roberts, *Pharmacol. Ther.*, 1999, **82**, 63-70.
32. J. E. Klaunig, M. A. Babich, K. P. Baetcke, J. C. Cook, J. C. Corton, R. M. David, J. G. DeLuca, D. Y. Lai, R. H. McKee, J. M. Peters, R. A. Roberts and P. A. Fenner-Crisp, *Crit. Rev. Toxicol.*, 2003, **33**, 655-780.
33. W. Parzefall, E. Erber, R. Sedivy and R. Schulte-Hermann, *Cancer Res.*, 1991, **51**, 1143-1147.
34. T. Yamada, Y. Okuda, M. Kushida, K. Sumida, H. Takeuchi, H. Nagahori, T. Fukuda, B. G. Lake, S. M. Cohen and S. Kawamura, *Toxicol. Sci.*, 2014, **142**, 137-157.
35. H. W. Aiges, F. Daum, M. Olson, E. Kahn and S. Teichberg, *The Journal of Pediatrics*, 1980, **97**, 22-26.
36. H. I. Pirttiaho, E. A. Sotaniemi, J. T. Ahokas and U. Pitkänen, *Br. J. Clin. Pharmacol.*, 1978, **6**, 273-278.
37. J. C. Corton, M. L. Cunningham, B. T. Hummer, C. Lau, B. Meek, J. M. Peters, J. A. Popp, L. Rhomberg, J. Seed and J. E. Klaunig, *Crit. Rev. Toxicol.*, 2014, **44**, 1-49.
38. C. Tateno, T. Yamamoto, R. Utoh, C. Yamasaki, Y. Ishida, Y. Myoken, K. Oofusa, M. Okada, N. Tsutsui and K. Yoshizato, *Toxicologic Pathology [Epub ahead of print] DOI: 10.1177/0192623314544378*, 2014.