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Featured article
Invited review

Application of humanized mice to toxicology studies: Evaluation of the human relevance of the mode of action for rodent liver tumor formation by activators of the constitutive androstane receptor (CAR)

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Abstract: The constitutive androstane receptor (CAR)-mediated mode of action (MOA) for phenobarbital (PB)-induced rodent liver tumor formation has been established, with increased hepatocyte proliferation, which is a key event in tumor formation. Previous studies have demonstrated that PB and other CAR-activators stimulate proliferation in cultured rodent hepatocytes, but not in cultured human hepatocytes. However, in the genetically humanized CAR and pregnane X receptor (PXR) mouse (hCAR/hPXR mouse, downstream genes are still mouse), PB increased hepatocyte proliferation and tumor production *in vivo*. In contrast to the hCAR/hPXR mouse, studies with chimeric mice with human hepatocytes (PXB-mouse, both receptor and downstream genes are human) demonstrated that PB did not increase human hepatocyte proliferation *in vivo*. PB increased hepatocyte proliferation in a chimeric mouse model with rat hepatocytes, indicating that the lack of human hepatocyte proliferation is not due to any functional defect in the chimeric mouse liver environment. Gene expression analysis demonstrated that the downstream genes of CAR/PXR activation were similar in hCAR/hPXR and CD-1 mice, but differed from those observed in chimeric mice with human hepatocytes. These findings strongly support the conclusion that the MOA for CAR-mediated rodent liver tumor formation is qualitatively implausible for humans. Indeed, epidemiological studies have found no causal link between PB and human liver tumors. There are many similarities with respect to hepatic effects and species differences between rodent CAR and peroxisome proliferator-activated receptor α activators. Based on our research, the chimeric mouse with human hepatocytes (PXB-mouse) is reliable for human cancer risk assessment of test chemicals.
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Key words: cell proliferation, constitutive androstane receptor, cultured hepatocytes, mode of action, human relevance, humanized models

Introduction: The History of Chimeric Mouse with Human Hepatocytes (PXB-mouse)

Although the best method for assessing the safety of chemicals and drugs would be by testing them in humans directly, it is unethical or impractical for chemicals, especially pesticides or industrial chemicals. Therefore, the safety evaluation of chemicals is mainly dependent on animal testing. While these animal studies provide valuable information on possible hazards of test chemicals, considering the differences between human and rodent physiology, the predictive value of these rodent studies has limitations[1–5]. *In vitro* study systems, such as human precision-cut liver slices, hepatic microsomes, or primary hepatocytes, have been widely used and potentially provide useful information for predicting actual human *in vivo* metabolic profiles of test chemicals[6]. While cultured hepatocytes are considered the gold standard *in vitro* systems for many applications[6], they cannot be employed for long-term studies owing to time-dependent de-differentiation in culture. Arakawa *et al*. reported that constitutive androstane receptor (CAR) mRNA expression levels in two-dimensional cultured human hepatocytes were unstable between days 2 and 7 of culture period[7].

To overcome the weaknesses of the *in vitro* systems described above or to confirm the findings of *in vitro* systems, moving towards "humanizing" laboratory animal species came with the use of embryonic stem cells and the technological breakthrough of capability to delete the gene encoding for the animal homologue of a particular gene and

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transfecting the human homologue into the mouse genome[8]. Since biological reaction of the *in vivo* system is complex (i.e., network or crosstalk of cellular signaling), multiple animal gene knock-out and subsequent human gene knock-ins are needed to understand complex biological reactions. Because of technological complexity, genetically humanizing replaces only a few genes (mostly one or two genes), and thus a whole-cell replacement model is strongly preferred.

In order to generate an animal model that closely mirrors human patterns of metabolism and toxicity, a significant replacement of host liver cells with human hepatocytes would clearly be of great advantage[8, 9]. Consequently, chimeric mouse models with human hepatocytes, in which most mouse hepatocytes were replaced by transferred human hepatocytes, were developed. We used PXB-mouse® constructed by PhoenixBio Co., Ltd. (Higashi-Hiroshima, Japan) based on their described characteristics[9, 10]. Foster *et al*. stated that the uPA$^{+/+}$ severe combined immunodeficient (SCID) mouse system has been the most widely assessed in terms of similarity of drug-exposure in the human condition, and was shown to exhibit a considerably more human-like absorption, distribution, metabolism, and excretion (ADME) profile than their non-chimeric murine controls[8].

Characterization and application of chimeric mice with human hepatocytes (PXB-mouse) has recently been summarized in an excellent review by Tateno and Kojima[9]. Detailed methods and protocols for producing these chimeric mice are shown in a chapter of the book Hepatocyte Transplantation[11]. Albumin enhancer promoter-driven urokinase plasminogen activator transgenic mice (uPA-Tg mice) were produced in 1990 to investigate the physiological role of uPA *in vivo*. The mouse liver was damaged by high expression of uPA and could be repopulated by transplanting healthy mouse hepatocytes via spleen. uPA, a serine protease produced in mouse hepatocytes and secreted extracellularly in the uPA-Tg mice, is known to digest the extracellular matrix in the liver and trigger hepatocyte growth after partial hepatectomy, and has a role in activating hepatocyte growth factor. Thus, uPA induces engraftment of transplanted hepatocytes and stimulates the growth of engrafted hepatocytes. The uPA-Tg mice were crossed with immunodeficient mice and transplanted with rat hepatocytes, resulting in successful rat hepatocyte-chimeric mouse production in 1995[12]. Subsequently, human liver chimeric mice were generated using uPA/RAG2$^{-/-}$, uPA/SCID, Fah$^{-/-}$/Rag2$^{-/-}$/Il2rg$^{-/-}$, and herpes simplex virus type-1 thymidine kinase-NOG (TK-NOG) mice. However, the repopulation index (RI) of these models was 10%–70%. In 2004, Tateno *et al*. succeeded in producing highly repopulated humanized chimeric mice with an RI of more than 70% stably using uPA/SCID mice (PXB-mouse)[13]. These highly repopulated chimeric mice can be used as a humanized model for infection studies of hepatitis B virus (HBV) and hepatitis C virus (HCV), or to predict human metabolism and toxicity (reviewed by Tateno and Kojima, 2020)[9]. Gene expression levels were compared between hepatocytes from uPA/SCID mice and hepatocytes from human liver by microarray analysis, revealing that 82% of transcripts were expressed in both the hepatocytes within a 2-fold range difference[14].

However, uPA/SCID mice have four disadvantages: 1) human hepatocyte RI in mouse liver is decreased due to deletion of the uPA transgene by homologous recombination; 2) kidney disorders are likely to develop; 3) body size is small; and 4) hemizygotes cannot be used as hosts as they undergo more frequent homologous recombination than homozygotes. To overcome these disadvantages, Tateno *et al.* established a novel host strain that has a transgene containing albumin promoter/enhancer-driven urokinase-type plasminogen activator cDNA and has an SCID background (cDNA-uPA/SCID)[15]. The chimeric hemizygote cDNA-uPA/SCID mice (also known as PXB-mouse) showed a constant increase in body weight and human hepatocyte RI since there was no deletion of uPA genes and no kidney disorders. Furthermore, similar to uPA/SCID chimeric mice, hemizygous cDNA-uPA/SCID chimeric mice were successfully infected with HBV and HCV. Microarray analysis demonstrated that gene expression levels in the liver were similar between hepatocytes from uPA/SCID-chimeric mice and cDNA-uPA/SCID-chimeric mice[15]. Tateno and Kojima concluded that PXB-mouse livers show nearly normal morphology and express most genes at similar level to those expressed by normal human liver[9]. The hemizygous cDNA-uPA/SCID mice are useful hosts for producing chimeric mice for use in long-term studies, including hepatitis virus infection analysis or drug toxicity studies[15]. For transplantation, usually frozen pediatric hepatocytes (6-months-old to 14-years-old) are used as donor cells for chimeric mice because hepatocytes from younger donors have superior growth after transplantation than hepatocytes from older donors[16].

The chimeric mouse livers were characterized morphologically (Fig. 1A) and histologically (Fig. 1B) with respect to the extent of chimerism, containing both white and red areas (Fig. 1A). 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 represented colonies of transgene-deleted host hepatocytes, as reported previously[17].

In addition to the *in vivo* chimeric mouse system, Tateno *et al.* succeeded in isolating 1–2 × 10^8 hepatocytes using a two-step collagenase perfusion method from a 12-to 20-week old PXB-mouse liver in which human hepatocytes (1–10 × 10^5 cells) were transplanted into cDNA-uPA/SCID mice between 2 and 4 weeks of age. They refer to hepatocytes as PXB-cells®. Human hepatocytes proliferated up to 2,000-fold in mouse liver from transplantation to isolation. Since fresh human hepatocytes are known to be the most useful cells for *in vitro* human studies of metabolism and chemical toxicity, PXB-cells made available fresh human hepatocytes from the same donor on demand for at least 5 years[9]. Since PXB-cells retain high gene expression of cytochrome P450 (CYP), uridine diphosphate glucuronosyltransferase, and transporters[18], PXB-cells could be a novel *in vitro* tool for metabolism and toxicity studies.

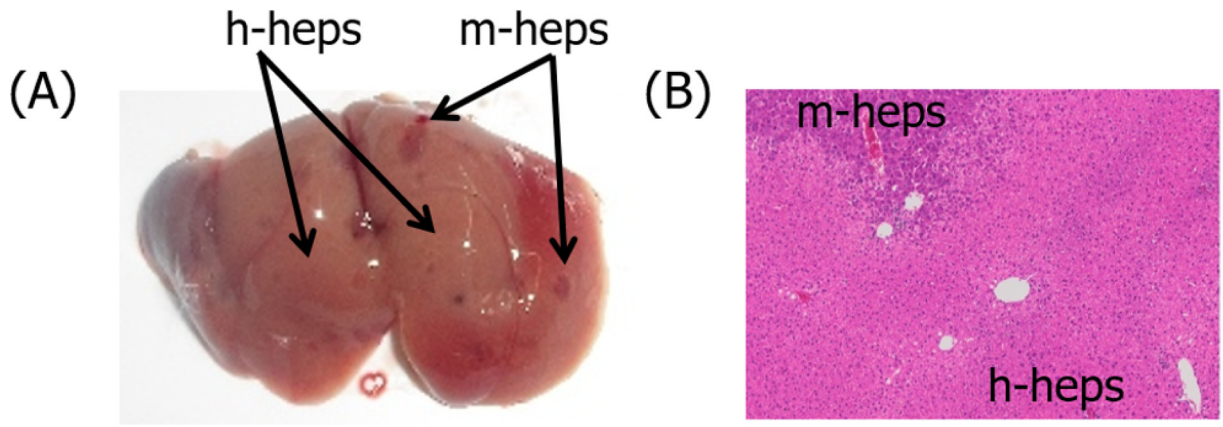

Fig. 1. Liver gross pathology and histology in chimeric mice. Photographs present gross (A) and histological (B) appearance of livers of control chimeric mice, with h-heps and m-heps representing human hepatocytes and mouse hepatocytes, respectively. (From Yamada *et al.*, 2014, with permission)[19].

Applications of Chimeric Mice with Human Hepatocytes in Studying Xenobiotic Metabolism and Toxicity

According to Tateno and Kojima, total 203 papers utilizing several types of chimeric mice or PXB-cells were published by October 2019, describing efficacy studies on HBV or HCV agents, or drug metabolism and pharmacokinetics studies including ADME, drug-drug interaction, and liver toxicity studies using drugs or chemicals (papers on studies that did not use chemicals or drugs are not included in this number)[9]. We used chimeric mice with human hepatocytes (PXB-mouse; both uPA/SCID chimeric mice and cDNA-uPA/SCID-chimeric mice) to investigate the hepatic effects of some nongenotoxic constitutive androstane receptor (CAR) activators (i.e., phenobarbital, metofluthrin, and momfluorothrin)[19, 20]. This was the first challenge in the application of this chimeric model for the evaluation of human relevance of the mode of action (MOA) for rodent liver tumor formation by activators of the CAR. The details are discussed in a later section of this review.

Prior to conducting the study with CAR-activators, the proliferation activity of the transplanted human hepatocytes was examined using a hepatocyte mitogen, human epidermal growth factor (hEGF). The treatment of chimeric mice with hEGF (150 μg/kg four times a day, i.p., for 2 days) significantly increased replicative DNA synthesis (RDS) [determined as 5-bromo-2'-deoxyuridine (BrdU) labeling] assessed with a marker of proliferation, *KI-67* (*MKI-67*) mRNA levels, in human hepatocytes of chimeric mice[19]. Some BrdU-positive cells were detected in the areas of human hepatocytes in the control animals (Fig. 2B). The BrdU labeling index was only determined in human hepatocytes and not in mouse hepatocytes, in which the rate of RDS was relatively high even in controls (Fig. 2B), making it difficult to compare the control and treatment animals in mouse hepatocytes of the chimeric mice. The cause of the high spontaneous RDS in mouse hepatocytes is unclear but may be related to the induced synthesis of DNA and/or hepatocyte damage by expression of uPA with consequent regeneration[20].

Furthermore, in two separate experiments, treatment of cultured human hepatocytes from chimeric mice (PXB-cells) with 100 ng/mL hEGF resulted in significant increases in RDS[19]. These data clearly demonstrated that the transplanted human hepatocytes in the chimeric mice were responsive to hEGF. Based on these findings, we decided to employ this chimeric model to evaluate the human relevance of the MOA for rodent liver tumor formation by CAR-activators.

Evaluation of MOA for Chemical-induced Liver Tumor Formation in Rodents

Since liver is the most common site of tumor formation in rodent carcinogenicity studies of non-genotoxic compounds[3, 21–23], evaluation of the human relevance of chemical-induced liver tumor production in rodents is very important to correctly protect humans from health risks. To avoid misclassifying chemicals as possible human carcinogens due to the limitations of long-term bioassays, it has become imperative to undertake an MOA analysis[24]. Consequently, MOA studies can help assist regulatory decision-making[25]. For example, MOA data are now frequently employed to help ascertain the human relevance of tumors produced in rodents by nongenotoxic carcinogens, including liver[26–29] and lung tumors[30, 31].

A framework for MOA analysis of rodent tumor and non-tumor toxicity, together with assessment of human relevance, was established by the International Life Sciences Institute (ILSI) (supported by the United States Environmental Protection Agency (US.EPA) and Health Canada) and the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) and has been described in a number of publications[32–40].

For carcinogenicity, the first stage is to evaluate wheth-

er it is possible to establish an MOA for tumor formation in experimental animals by identifying a series of key and associative events using a weight-of-evidence approach based on the modified Bradford Hill considerations[32–35, 40, 41]. A key event is defined as an empirically observable causal precursor step to the adverse outcome, which is a necessary element of the MOA[41]. 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 are considered biological processes that are not causal or necessary key events for the MOA, but are reliable indicators or markers for the key events[41]. Associative events can often be used as surrogate markers for a key event in an MOA evaluation or as indicators of exposure to a xenobiotic that has stimulated the molecular initiating event or a key event. Once a robust MOA is established, the key and associative events are compared, first qualitatively and then quantitatively between effects in experimental animals and humans[41].

MOAs have been established for tumor formation by nongenotoxic chemicals in various rodent tissues. For example, a recent analysis of 411 unique agrochemicals that have been evaluated for carcinogenicity by the US.EPA and the European Chemicals Agency (ECHA) identified 170 chemicals as non-genotoxic carcinogens. These chemicals produced 340 cases of treatment-related tumor formation, of which MOAs or MOA networks could be identified in 224 instances[3]. Further development of innovative test methods and enhanced understanding of carcinogenic processes will permit a better understanding of tumor formation in rodents and an evaluation of the relevance of such rodent tumors to humans[2–4, 42].

A number of MOAs have been established for liver tumor formation, both in humans and in rodent models, which are identified as two major categories, "DNA reactivity" and "Increased cell proliferation"[43, 44]. For the Increased cell proliferation MOAs, constitutive androstane receptor (CAR) activation, peroxisome proliferator-activated receptor alpha (PPARα) activation, aryl hydrocarbon receptor (AhR) activation, estrogen receptor activation, hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors (Statins), and porphyrias are known as receptor mediated MOAs. In contrast, non-receptor-mediated MOAs include cytotoxicity, infection, metal overload (e.g., iron and copper), and increased apoptosis (e.g., fumonisin B1). Inherited disorders leading to cytotoxicity (e.g., porphyrias, α-1-antitrypsin deficiency, etc.) are also recognized as MOAs for liver tumor formation[43, 44].

MOA for Liver Tumor Formation by Phenobarbital and Other CAR-activators

CAR is a nuclear receptor involved in all phases of drug metabolism and disposition, and has recently been implicated in energy metabolism, tumor progression, and cancer therapy[45, 46]. Phenobarbital (PB) is a non-genotoxic drug known as barbiturate anticonvulsants/hypnotics and is known to activate CAR by a ligand-independent mechanism[47]. The carcinogenicity of PB and/or its sodium salt (sodium phenobarbital; presented as PB in this review) was investigated by oral administration in multiple studies in mice and several studies in rats[48, 49]. PB consistently produced hepatocellular adenomas and carcinomas in multiple mouse strains. Hepatocellular adenomas were produced in rats after lifetime exposure in one study[50]. In contrast to mice and rats, PB did not produce liver tumors in Syrian hamsters[48, 49].

In 2001, the International Agency for Research on Cancer (IARC) concluded that PB is "*possibly carcinogenic to human (Group 2B)*" as there is sufficient evidence in experimental animals for the carcinogenicity of PB[49]. Because of the extensive therapeutic use of PB in humans as a sedative, hypnotic, and anti-epileptic agent for many years, data from a number of epidemiological studies are available. In contrast to the 2001 IARC conclusion, epidemiological studies, including a more recent analysis (IARC only evaluated epidemiological data up to 1995 and more recent analyses are also available), have found no causal links between PB and human liver tumors[47–49, 51, 52].

Apart from PB, many other chemicals have also been identified as rodent liver tumor producers with CAR-mediated MOA[27, 53]. According to our more recent critical analysis of available data, at least 21 chemicals have been established for having the CAR activation MOA for mouse and/or rat liver tumor formation[5]. In such situations, evaluation of the human relevance of the established CAR-mediated MOA for rodent liver tumor formation by PB and other CAR-activators would be very important for risk management of these chemicals.

Based on an evaluation of the literature, the key and associative events[41] for the CAR-mediated MOA for PB-induced rodent liver tumor formation were established by Elcombe *et al*[47]. CAR activation, altered gene expression specific to CAR activation, increased cell proliferation, clonal expansion leading to altered hepatic foci, and ultimately liver tumor formation are considered the key events, as they constitute necessary steps in the MOA (Fig. 3)[47]. In addition, induction of hepatic CYP2B enzymes and liver hypertrophy (i.e., increase in liver weight and hepatocellular centrilobular or panlobular hypertrophy) are considered associative events and represent reliable markers of CAR activation[47].

CAR-dependent Hepatocyte Proliferation

Increased cell proliferation represents an essential preneoplastic step in carcinogenesis by most non-genotoxic substances[26, 54, 55]. Studies employing mice lacking hepatic CAR (i.e., CAR knockout (KO) mice) have demonstrated the crucial role of hepatic CAR in mouse liver tumor formation for chemicals acting by this MOA. Unlike wild-type mice, the treatment of CAR KO mice with PB did not result in increased liver weight, liver centrilobular hepatocellular hypertrophy, induction of Cyp2b subfamily enzymes, he-

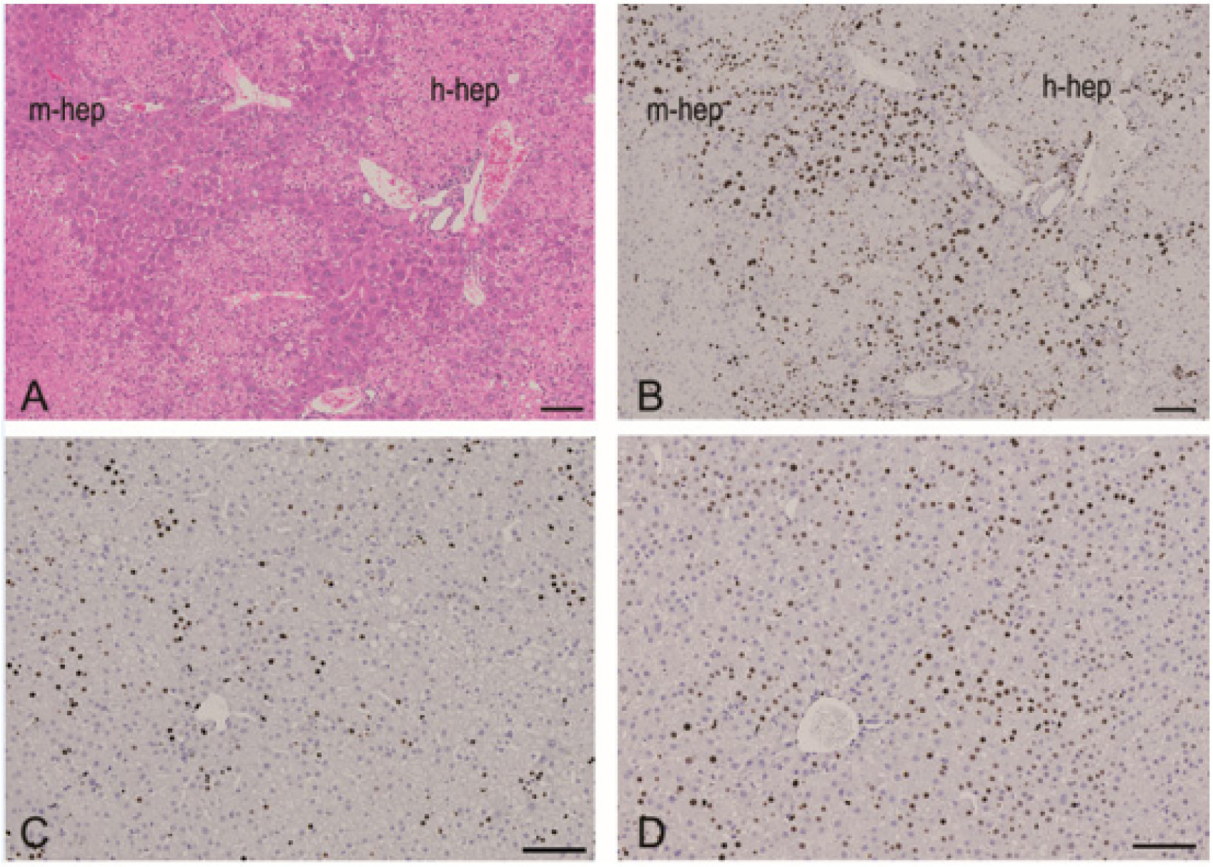

Fig. 2. Liver histology and DNA synthesis in chimeric mice treated with hEGF. Hematoxylin and eosin staining (A) and immunohistochemistory for BrdU (B) of livers of the control chimeric mice, with h-heps and m-heps representing human hepatocytes and mouse hepatocytes, respectively (A and B are serial sections). Immunohistochemistry for BrdU in human hepatocyte area of the control animal (C) and hEGF-treated animal (D). Scale bars are 100 µm. (From Okuda *et al.*, 2017)[20].

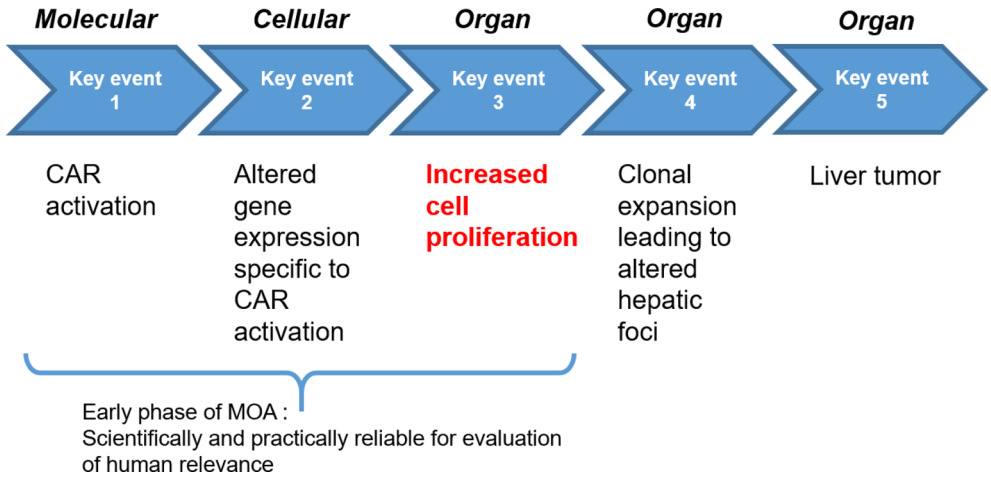

Fig. 3. Key events for rodent liver tumor formation by PB and other CAR-activators. Based on an evaluation of literature data, the key events for the CAR-mediated MOA for PB-induced rodent liver tumor formation were established in Elcombe *et al.* (2014)[47]. Since increased cell proliferation represents an essential preneoplastic step at an early phase of treatment in carcinogenesis by most nongenotoxic substances[26, 54, 55], it is the pivotal endpoint for evaluation of human relevance of the MOA for rodent liver tumor formation.

patocyte RDS, and following initiation with the genotoxic agent diethylnitrosamine (DEN) did not promote liver tumor formation[56–59].

CAR is present in human liver and can be activated by PB and other drugs and compounds[47, 60, 61]. To address possible human relevance, we determined the effects of PB and other CAR-activators (e.g., metofluthrin and momfluorothrin, pyrethroid insecticides that produced liver tumors in rats after long-term and high-dose treatment)[27, 62–64] on hepatocyte RDS in three experimental models: *in vitro* studies with cultured human hepatocytes[20, 65–67], together with *in vivo* studies with transgenic mice containing human hepatic CAR and PXR[68] and/or in chimeric mice with human hepatocytes[19, 20]. Because the lack of proper ADME properties resulting from cellular disconnection from the circulatory and other organ systems may make the assessment of chemical exposure results difficult with primary hepatocyte cultures[6], it is important to conduct *in vivo* studies to confirm the findings obtained from the *in vitro* studies. The findings of these three models are summarized below.

Studies in cultured human hepatocytes

As mentioned above, primary cultures of animal and human primary hepatocytes have been extensively used for *in vitro* testing (e.g., cytotoxicity, CYP enzyme induction, and RDS studies) as they can maintain functional activities for at least 24-72 h[6]. While PB has been shown to stimulate RDS in cultured mouse and rat hepatocytes, many studies from different laboratories have demonstrated that PB does not increase RDS in cultured human hepatocytes[20, 65–67, 69–74]. In addition to studies with PB, a number of other nongenotoxic rodent CAR-activators, including benfluralin, metazachlor, metofluthrin, momfluorothrin, the natural pyrethrins, nitrapyrin, and sedaxane, and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), have also shown no increased RDS in cultured human hepatocytes[20, 65, 66, 71–77]. While the above studies were performed with monolayer cultures (i.e., two-dimensional cultures), a study by Plummer *et al.* examined the effect of PB on RDS in a three-dimensional culture system and showed no increased RDS in cultured human hepatocytes[78]. These findings are consistent with those of epidemiological studies showing no increased risk of liver tumors with PB exposure[47–49, 51, 52].

Studies in transgenic mice with human CAR or both CAR and PXR

Treatment of hCAR mice with 500 ppm PB for 1 week resulted in increased hepatocyte proliferation[56]. Luisier *et al.* demonstrated that male hCAR/hPXR mice dosed with 0.05% PB in drinking water for 91 days increased relative liver weight, hepatocyte hypertrophy, and induction of *Cyp2b10* mRNA levels, together with a transient induction of DNA replication and mitotic genes in hepatocytes[79]. Recently, Haines *et al.* treated C57BL/6J wild-type and hCAR/hPXR mice with diets containing 186, 496, 654, and 984 ppm PB for 7 days. In the wild-type mice, a statistically significant dose-dependent increase in hepatocyte RDS was observed at all PB dose levels examined. However, in the hCAR/hPXR mice, 186 ppm PB treatment did not increase hepatocyte RDS significantly, while 496–984 ppm PB did increase hepatocyte RDS statistically significantly. These effects were less marked than those observed in wild-type mice. Finally, in a recent study, the treatment of hCAR/hPXR mice with 1,000 ppm PB in the diet for 7 days resulted in significant increase in relative liver weight, hepatocyte RDS, *Cyp2b10*, and *Cyp3a11* mRNA level, and in mRNA levels of some cell cycling genes, namely *Mki67*, *Mdm2*, *Pcna*, and *Gadd45β*[68]. Contrarily, one study involving intraperitoneal injection of 80 mg/kg/d PB for 4 days in hCAR/hPXR mice did not show significant effect on hepatocyte RDS[80]. This apparent lack of effect is most likely attributable to the treatment time and/or dose levels of PB administered. Overall, these studies demonstrate that the treatment of either hCAR or hCAR/hPXR mice with PB can result in increased hepatocyte RDS, although the effects of PB treatment are less marked in hCAR/hPXR mice than in wild-type mice[68, 69].

Braeuning *et al.* performed an initiation/promotion study in which wild-type and hCAR/hPXR mice were administered with a single dose of DEN followed by treatment with 500 ppm PB in the diet for 40 weeks. While tumor incidence assessed, either multiplicity or tumor volume • fraction was less marked in the hCAR/hPXR mice, and PB promoted DEN-initiated liver tumors in both wild-type and hCAR/hPXR mice[81]. Based on these findings, these authors suggested that PB-induced liver tumor formation in rodents could be relevant for humans[81–83]. Consequently, these findings raised controversy to the conclusion of the 2010 workshop that the MOA for PB-induced rodent liver tumors is not relevant to humans[47]. However, Bae *et al.* recently speculated that, although more detailed studies are needed, CAR may function as a tumor suppressor by suppressing liver cancer stem cell (LCSC) activity or hindering de-differentiation of differentiated cells into LCSCs, as well as inhibiting the key markers for LCSCs such as CD133[84].

For interpreting findings from the hCAR and hCAR/hPXR mice, it should be noted that in these transgenic mouse models, the human receptor(s) operate in a mouse hepatocyte environment. The downstream genes acted upon by CAR are those of the mouse, not humans[5, 27, 29, 85], indicating that the findings from hCAR and hCAR/hPXR mice do not appropriately reflect human responses. Towards better understanding of these controversial findings, it is very important to evaluate the effects of PB on human hepatocyte proliferation in a chimeric mouse model in which human receptor(s) operate in a human hepatocyte environment.

Studies in chimeric mice with human hepatocytes (PXB mouse)

As shown in Table 1, uPA/SCID mice were employed for the evaluation of PB[19], and in subsequent investigations, the cDNA-uPA/SCID mouse model has been used in studies with metofluthrin and momfluorothrin[20]. Metofluthrin and momfluorothrin are pyrethroid insecticides that induced

Table 1. Effect of Some Chemicals on Replicative DNA Synthesis in Chimeric Mice with Human Hepatocytes

Chemical	Information of chemical (Use, activity, liver carcinogenic dose and animals)	Type of chimeric mouse	Number of donors (age, ethnicity, sex)	Dose levels, method and periods of test chemical or positive control	Hepatocyte replicative DNA synthesis[a]	References
Phenobarbital[b]	• Barbiturate anticonvulsants/ hypnotics • CAR activator • Liver carcinogenic dose: 65–70 mg/kg/day in mice	uPA/SCID	1 (2-year-old Hispanic female)	0, 500, 1,000 and 1,500 ppm in diet for 7 days (chemical intake: 69, 150 and 230 mg/kg/day)	Not increased (BRDU, *MKI-67* mRNA, *PCNA* mRNA)	19, 47
				hEGF (positive control): 150 μg/kg, intraperitoneal injection at four times a day, for 2 days	Increased (BRDU, *MKI-67* mRNA)	
Metofluthrin	• Pyrethroid insecticide • CAR activator • Liver carcinogenic dose: 900 and 1800 ppm (chemical intake: 38–47 and 78–96 mg/kg/day, respectively) in rats	cDNA- uPA/SCID	3 (2-year-old Hispanic female, 2-year-old Caucasian male, and 5-year-old African American male)	0 and 1,800 ppm (chemical intake: 239–285 mg/kg/day)	Not increased (BRDU)	20, 27, 62, 63
				hEGF (positive control): 150 μg/kg, intraperitoneal injection four times a day, for 2 days (Sharing data with momfluorothrin)	Increased (BRDU)	
Momfluorothrin[c]	• Pyrethroid insecticide • CAR activator • Liver carcinogenic dose: 1,500 and 3,000 ppm (chemical intake: 73 and 154 mg/kg/day, respectively) in rats	cDNA- uPA/SCID	3 (2-year-old Hispanic female, 2-year-old Caucasian male, and 5-year-old African American male)	0 and 3,000 ppm in diet for 7 days to 1 donor (chemical intake: 410 mg/kg/day) 0 and 1,100 ppm in diet for 7 days to 2 donors (chemical intake: 170 and 146 mg/kg/day, respectively)	Not increased (BRDU)	27, 29, 64
				hEGF (positive control): 150 μg/kg, intraperitoneal injection four times a day, for 2 days (Sharing data with metofluthrin)	Increased (BRDU)	
Fenofibrate	• Hypolipidemic agents • PPARα activator • Liver carcinogenic dose: 200 mg/kg/day in mice	uPA/SCID	3 (9-month-old Caucasian male, 4-year-old Caucasian female and 6-year-old African-American female)	0, 30 and 300 mg/kg/day, by gavage for 4 days	Not increased (BRDU, *CYCLIN-B1* mRNA, *CDK1* mRNA)	86, 87
5-aminolevulinic acid	• Porphyrinogenic compound to induce porphyria-mediated cytotoxicity • Endogenous non-proteinogenic amino acid • No carcinogenicity data	cDNA-uPA/SCID	1 (2-year-old Hispanic female)	Experiment I : 0 and 7,000 ppm in diet for 28 days (chemical intake: 686 mg/kg/day)[d] Experiment II:0, 3,500 and 5,000 ppm in diet for 28 days (chemical intake: 381 and 537 mg/kg/day)	Increased at 7,000 ppm (BRDU) Increased at 3,500 and 5,000 ppm (BRDU)	91

[a]Hepatocyte replicative DNA synthesis was determined by 5-bromo-2'-deoxyuridine (BRDU) labeling and mRNA expression levels (*MKI67*, *PCNA*, *CYCLIN-B1*, or *CDK1*).

[b]Chimeric mice were also dosed with 2,500 ppm phenobarbital, but were not examined due to early death. Data at 1,500 ppm in chimeric mice were obtained from two surviving animals.

[c]Since five of eight animals died during treatment at 3,000 ppm, data were evaluated in three surviving animals. Thus, two other experiments using different donors were conducted at 1,100 ppm.

[d]Since four of seven animals found dead or moribund during treatment at 7,000 ppm, data were evaluated in three surviving animals.

liver tumors in rats with CAR-mediated MOA[27, 62–64]. Previous studies with cultured hepatocytes have demonstrated that metofluthrin[65, 66] and momfluorothrin[20] increased rat hepatocyte RDS, but not in human hepatocytes, which is strongly consistent with the results of PB.

Studies with cultured human hepatocytes have demonstrated that while hepatocyte RDS can be increased by treatment with growth factors such as EGF or hepatocyte growth factor (HGF), no chemicals (e.g., drugs or agrochemicals) have been reported to induce RDS in human hepatocytes[5]. As mentioned in an earlier section, we demonstrated that treatment with EGF enhances RDS in the human hepatocytes of chimeric mice (both uPA/SCID and cDNA-uPA/SCID mouse models), thus demonstrating that the transplanted human hepatocytes in chimeric mice can respond to a hepatocyte mitogen[19, 20]. In the human hepatocytes of chimeric mice treated with 1500 ppm PB, cytosolic glycogen areas were decreased and the size of the cells was slightly increased in the centrilobular area (Fig. 4A and B). These hepatic changes suggest that human-originated hepatocytes exhibited slight hypertrophic changes after PB treatment, the effect being less marked than that observed in WH rats and CD-1 mice[19]. Electron microscopic evaluation in chimeric mice treated with 1500 ppm PB revealed that an increase in smooth endoplasmic reticulum was observed in the human-originated hepatocytes (Fig. 4C and D), which supported the light microscopic changes. In contrast to the EGF response, as shown in Table 1, human hepatocytes in the chimeric mouse model did not respond to CAR-activators with increased RDS in rats (PB[19, 62, 64], metofluthrin[62] and momfluorothrin[64])[19, 20]. Based on data from the chimeric mouse model and cultured human hepatocytes, the ECHA concluded that a classification for carcinogenicity was not justified for metofluthrin and momfluorothrin[27].

More recently, we provided data showing that treatment with 1,000 ppm PB significantly increased RDS, together with a small increase in *MKI67* mRNA levels, in chimeric rat hepatocyte mice[68], which is consistent with a large number of previous *in vivo* and *in vitro* studies in rats showing increased RDS. Hence, the chimeric mice with transplanted rat hepatocytes retained the original characteristics of normal rat hepatocytes by increasing RDS in response to stimulation by the CAR-activator PB[68]. These results strongly support the conclusion that the lack of proliferation of human hepatocytes in the chimeric mouse model is not due to any functional defect in the mouse liver environment, but because human hepatocytes are truly refractory to the mitogenic effects of PB and other rodent CAR-activators, as observed in cultured human hepatocyte studies.

Since increased cell proliferation represents an essential pre-neoplastic step in carcinogenesis by non-genotoxic substances[26, 54, 55], the absence of any mitogenic effects of CAR-activators in human hepatocytes strongly suggests that these compounds will not produce liver tumors in humans. This is strongly consistent with the findings from the *in vitro* cultured human hepatocyte system[20, 65–67, 69–74] and epidemiological studies showing no increased risk of liver tumors[47–49, 51, 52].

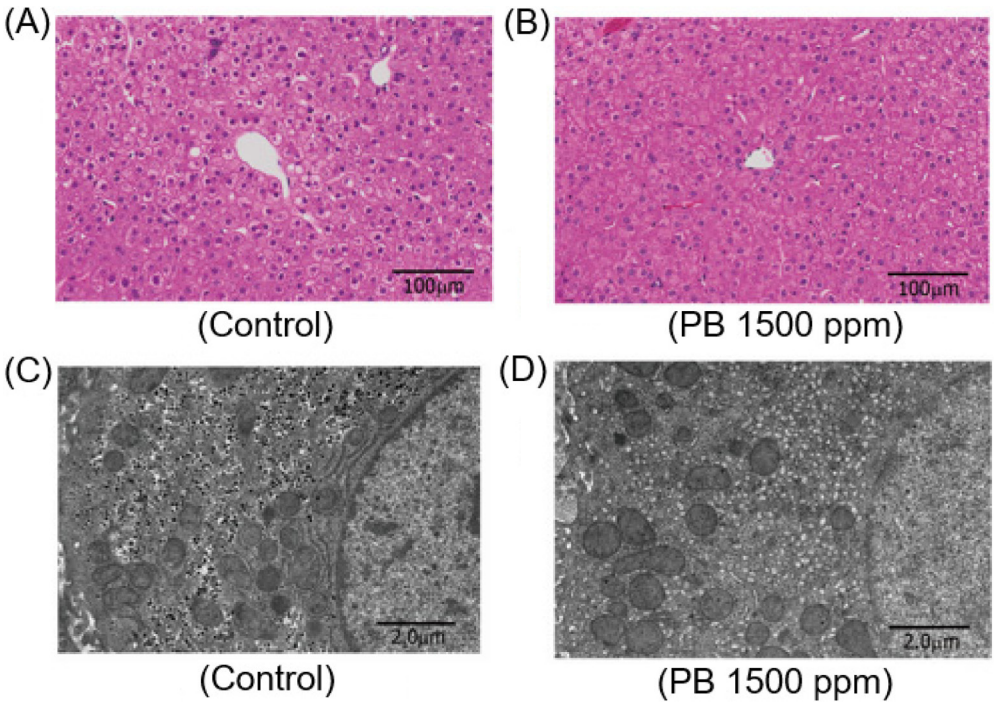

Fig. 4. Liver histology in chimeric mice treated with PB. Histopathology (A, B) and ultrastructure (C, D) of human hepatocyte-originated areas of chimeric mice given 0 (A, C) and 1500 ppm (B, D) PB are also presented. Centrilobular hepatocellular hypertrophy (B) and proliferation of the smooth endoplasmic reticulum (D) was observed in PB-treated chimeric mice. (From Yamada *et al*., 2014, with permission)[19].

In addition to the effects of rodent CAR-activators, the PPARα-activator fenofibrate was also shown to be a mitogenic agent in mouse hepatocytes but not in human hepatocytes of the chimeric mice[86, 87] (Table 1), which is consistent with the previous conclusion that PPARα-activated MOA for rodent liver tumor formation is not relevant to humans[28]. Furthermore, gene expression analysis in chimeric mice (cDNA-uPA/SCID) treated with fenofibrate suggested that PPARα may have a suppressive effect on DNA synthesis in human hepatocytes[88].

In a recent study using the same chimeric human hepatocyte mouse model (PXB-mouse, cDNA-uPA/SCID), treatment with KMTR2 (an anti-human tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) monoclonal antibody) was shown to induce hepatotoxicity and apoptosis in human hepatocytes, which is associated with an upregulation of cell cycle-related functions likely representing cellular regeneration[89]. Ishida *et al.* reported that the cytotoxicity of aflatoxin B1 was detected by using histological examination and biochemical analysis in chimeric mice with human hepatocytes (PXB-mouse, cDNA-uPA/SCID), although hepatocyte proliferation was not examined[90]. More recently, Eguchi *et al.* evaluated the utility of the PXB mouse as an *in vivo* experimental model to evaluate the key events of the porphyria-mediated cytotoxicity MOA in humans, using 5-aminolevulinic acid, a representative porphyrinogenic compound[91]. They concluded that the PXB mouse is a useful model for evaluating the key events of porphyria-mediated cytotoxicity MOA in humans[91].

Overall, these data demonstrate that functional availability in this chimeric mouse model is not only for receptor-mediated MOA but also for cytotoxicity/regeneration-MOA. Therefore, the chimeric mouse with human hepatocytes (PXB-mouse) is a reliable model for evaluating human relevance of MOA for rodent liver tumor formation of test chemicals.

Comparative Gene Expression Analysis in Humanized Models Treated with PB

Based on the increases in hepatocyte proliferation and liver tumors in the hCAR/hPXR mice, Braeuning *et al.* suggested that PB-induced liver tumor formation in rodents could be relevant for humans[81, 83, 92]. As described above, other laboratories also demonstrated similar findings of increased hepatocyte RDS in hCAR and hCAR/hPXR mice. Therefore, these hCAR or hCAR/hPXR mice may provide experimentally correct responses to treatment with CAR-activators; however, this genetically humanized model is of questionable biological significance regarding human relevance. As the hCAR operates in a mouse hepatocyte environment, including downstream genes and their activation[85], caution is needed to extrapolate the results of this animal model to humans[5, 27, 29]. To further investigate the details of CAR-activated signaling in hCAR/hPXR mice compared to chimeric mice, we evaluated global gene expression in the livers of PB-treated chimeric mice and hCAR/hPXR mice[68, 93].

Wnt/β-catenin signaling is a useful pathway for evaluating tumorigenicity in humans and rodents. The Wnt/β-catenin signaling pathway regulates key aspects of mammalian cell biology, with aberrant Wnt pathway activation leading to β-catenin stabilization, which can result in tumor formation in the liver and other organs[94, 95]. Dong *et al.* demonstrated that activation of β-catenin and CAR in mice resulted in liver tumor formation, with mouse liver tumors having a conserved gene expression signature with those observed in some human hepatocellular carcinomas[94]. Thus, we focused on the effects of PB on Wnt/β-catenin signaling in chimeric mice with human hepatocytes and hCAR/hPXR mice.

We first conducted comprehensive analyses of DNA methylation, hydroxymethylation, and gene expression using microarrays of hepatic genes in CD-1 mice treated with PB for 1 week, in chimeric human hepatocyte mice treated with PB for 1 week (both models revealed similar serum PB levels after 7-day PB treatment; approximately 70 μg/mL), and in liver adenomas from a DEN/PB initiation/promotion study[93]. Nine cell proliferation/growth-related genes (*Abcc4, Apoa1, Cblb, Ccdc85b, Cdk5r1, Dlg1, Egfr, Prg4 and Tff1*) were commonly observed in both the livers of CD-1 mice treated with PB for 7 days and also in the liver adenomas from the DEN/PB study, and thus these genes are considered as candidate genes responsible for early events in PB-induced liver tumor induction; with effects on a large number of genes related to the Wnt/β-catenin signaling pathway being observed. In contrast to the CD-1 mice, chimeric mice with human hepatocytes treated with PB for 7 days had no effect on these nine genes and fewer effects on Wnt/β-catenin signaling pathway genes[93].

Recently, we further analyzed the effect of PB on hepatic gene expression pattern in the hCAR/hPXR mice (48 μg/mL), CD-1 mice (43 μg/mL), chimeric mice with human hepatocytes (27 and 75 μg/mL), and liver adenomas from the DEN/PB study (15 μg/mL) (values in parentheses are plasma PB concentrations after treatment)[68]. The data demonstrate that the gene expression pattern of Wnt/β-catenin signaling in the livers from the hCAR/hPXR mice clustered closely with those of the liver tumor samples from C3H mice. However, the gene expression pattern of Wnt/β-catenin signaling in the chimeric mice with human hepatocytes was clearly different from those of hCAR/hPXR mice, CD-1 mice, and the liver tumor samples, even at higher PB serum concentrations[68].

Overall, unlike mouse hepatocytes, exposure of human hepatocytes to nongenotoxic CAR-activators appears to have little effect on the genes associated with the Wnt/β-catenin signaling pathway[68, 93]. These findings support our consideration that although the hCAR/hPXR genes have been inserted genetically, the downstream genes are still mouse and may be the basis for the increased hepatocyte RDS, Wnt/β-catenin signaling, and tumor production observed in hCAR/hPXR mouse studies.

Conclusion

As described above, data from transgenic mice with either human CAR or human CAR and PXR are not useful for evaluating the human relevance of liver tumorigenesis because the human nuclear receptors function in a mouse hepatocyte environment[5, 27, 29, 85]. Thus, the data obtained from these models were similar to those obtained from wild-type mice (Fig. 5). In contrast, data from chimeric mice with human hepatocytes are consistent with the findings of *in vitro* cultured human hepatocytes, where CAR-activators do not stimulate RDS in human hepatocytes, which is distinctly different from rodents. In addition to hepatocyte RDS, global gene expression analysis demonstrated clear differences in the effects of PB on gene expression between chimeric mice and hCAR/hPXR mice. These findings suggest that the chimeric mouse model is reliable for studies investigating the human relevance of the hepatic effects of rodent CAR-activators on liver tumorigenesis, whereas the hCAR/hPXR mouse is not[5].

Current applications of the chimeric mouse model in studies investigating the human relevance of the hepatic effects of test chemicals on liver tumorigenesis are limited to short-term studies as described above[19, 20, 86]. The ultimate test of carcinogenicity assessment in humans using this chimeric model may be a long-term carcinogenicity study. However, to our knowledge, such a study has not been performed and would be technically very difficult. In addition to the very high cost, several preliminary studies would have to be performed, such as determining the long-term survival rate and suitable dose levels (MTD; maximum tolerated dose). Considering the adverse outcome pathway concept, investigation of the effect on hepatocyte replication (i.e., observable causal precursor step of tumor formation)[5, 41] is sufficient for decision making in the safety assessment of test chemicals with CAR-mediated rodent liver tumor production. This is consistent with the suggested carcinogenicity assessment process by Cohen *et al*., where a transition from the bioassay to a decision-tree matrix that can be applied to a broader range of chemicals, with better predictivity, based on the premise that cancer is the consequence of DNA coding errors that arise either directly from mutagenic events or indirectly from sustained cell proliferation[2].

Furthermore, as with PPARα activators, after global acceptance that CAR-mediated rodent liver tumor production is not relevant to humans, it would not be necessary to perform studies in either cultured human hepatocytes or in chimeric with human hepatocytes[5].

Overall, the available data demonstrate that the established MOA for rodent liver tumor formation by PB and other CAR-activators is qualitatively not plausible for humans, which is consistent with previous evaluations[5, 27, 29, 47]. This conclusion is supported by data from several human epidemiological studies showing no increased risk of liver or

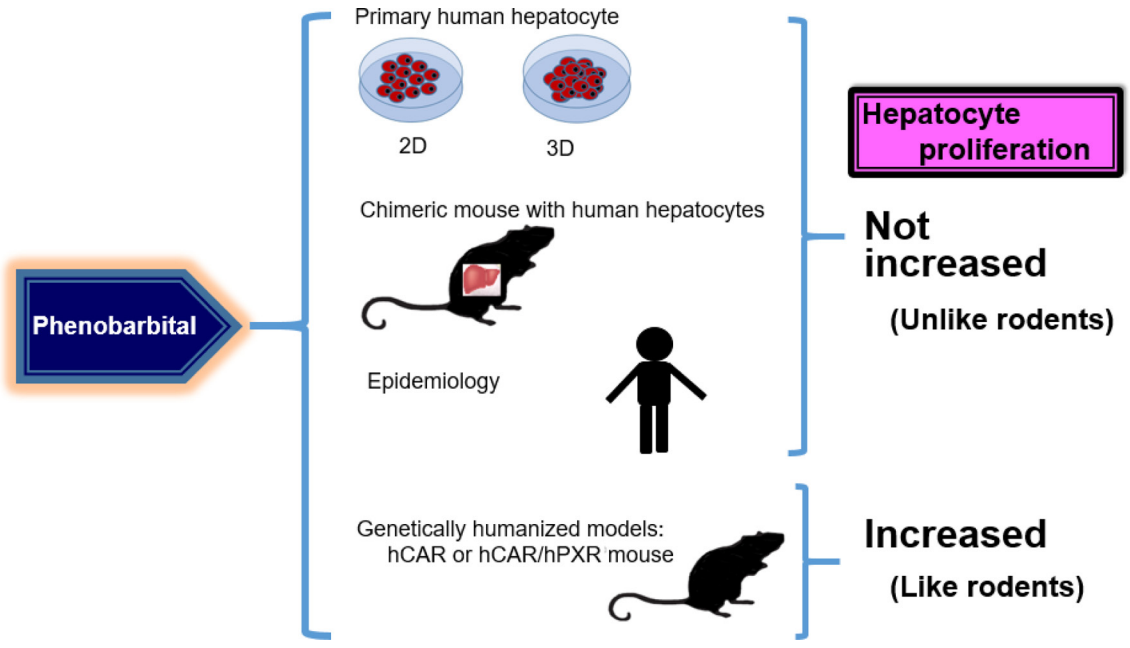

Fig. 5. Overall summary for effects of phenobarbital on human hepatocyte proliferation in different experimental models and epidemiological studies. The data obtained from transgenic mice with either human CAR or human CAR and PXR (hCAR or hCAR/hPXR mouse) showed increased hepatocyte proliferation, similar to that obtained in wild-type mice or rats. In contrast, data from the chimeric mice with human hepatocytes are consistent with the findings with *in vitro* cultured human hepatocyte studies where CAR-activators do not increase hepatocyte proliferation, distinctly different from wild-type mice or rats. The data from the *in vitro* cultured human hepatocyte studies and the chimeric mice with human hepatocytes are consistent with the data from a number of human epidemiological studies showing no increased risk of liver or other tumors. 2D: a two-dimensional culture system. 3D: a three-dimensional culture system.

other tumors in individuals exposed to CAR-activators such as PB[5]. These findings are similar to the hepatic effects and species differences of PPARα activators[5]. As pointed out by Lake, based on current knowledge, the 2001 IARC classification of PB (i.e., "*possibly carcinogenic to human (Group 2B)*") would appear to be outdated[29]. Regarding humanized models, some (but not all) humanized models appear to be useful for the prediction of human responses to chemical exposure. For correct prediction, as discussed in this review, we should select suitable humanized model(s) based on their characterization. To my understanding, the accumulated experimental data suggest that the chimeric mouse with human hepatocytes (PXB-mouse) is very reliable for prediction of short-term effects on hepatocyte replication irrespective of mitogenic- or cytotoxicity/regeneration-MOA. Thus, this model would be valuable for the prediction of liver carcinogenicity of test chemicals, especially human-specific metabolites.

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