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Phenobarbital-mediated tumor promotion in transgenic mice with humanized CAR and PXR

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Running title: tumor promotion in hCAR/hPXR mice by PB

ABSTRACT

The nuclear receptors CAR (constitutive androstane receptor) and possibly PXR (pregnane X receptor) mediate the hepatic effects of phenobarbital (PB) and similarly-acting compounds. Although PB is a potent non-genotoxic tumor promoter in rodent liver, epidemiological data from epilepsy patients treated with phenobarbital do not show a specific role of PB in human liver cancer risk. That points to species differences in the susceptibility to tumor promotion by PB, which might be attributed to divergent functions of the PB receptors CAR and PXR in mice and humans. In the present study, male transgenic mice expressing human CAR and PXR were used to detect possible differences between wild type and humanized mice in their response to CAR activation in a tumor initiation/promotion experiment with a single injection of the tumor initiator N-nitrosodiethylamine preceding chronic PB treatment for 10 months. Analysis of liver tumor burden revealed that PB strongly promoted the outgrowth of hepatocellular adenoma driven by activated β-catenin in wild type mice, whereas the tumor-promoting effect of PB was much less pronounced in the humanized group. In conclusion, the present findings demonstrate that human CAR and PXR support tumor promotion by PB in mouse liver, but to a significantly lesser extent than the wild type murine receptors.

Key words: liver tumor; non-genotoxic carcinogen; HCC; constitutive androstane receptor; pregnane X receptor

INTRODUCTION

Phenobarbital (PB), also known as phenobarbitone, is a long-acting barbiturate that has been used in human medicine mainly as anticonvulsant, often in combination with phenytoin and other therapeutics. PB is a well-established non-genotoxic hepatocarcinogen in rodents (Lee, 2000; Whysner *et al.*, 1996) and there is toxicological concern about the relevance of this tumor-promoting effect for humans. Several large epidemiological studies on epileptics who received prolonged treatment with PB, phenytoin and other anticonvulsants have been conducted which, on the one hand, have provided little evidence that PB and phenytoin are carcinogenic to humans, but cannot, on the other hand, rule out this possibility (Clemmesen and Hjalgrim-Jensen, 1978; Lamminpaa *et al.*, 2002; Olsen *et al.*, 1989; Olsen *et al.*, 1995).

The precise mechanism of the liver tumor promoting activity of PB in rodents is not fully understood. The experimental evidence points towards a possible role of PB interfering with gap junction-mediated intercellular communication since the liver tumor promotional activity of the barbiturate is lost in mice deficient for connexin32, the major gap junctional protein in mouse liver (Moennikes *et al.*, 2000). A second important protein clearly involved in liver tumor promotion in mice is β-catenin: PB strongly selects during the promotional phase for hepatocytes carrying activating mutations of the *Ctnnb1* gene, encoding a mutant and constitutively activated version of the transcription factor β-catenin (Aydinlik *et al.*, 2001). The conditional knockout of *Ctnnb1* in mouse hepatocytes eradicates PB-mediated tumor promotion (Rignall *et al.*, 2011). A third important mechanism is the activation of nuclear receptors: PB activates the constitutive androstane receptor (CAR) and, to a lesser extent, also the pregnane X receptor (PXR). CAR plays a pivotal role in the tumor promotional activity of PB, since the emergence of liver tumors can no longer be promoted by PB in CAR-deficient mice (Yamamoto *et al.*, 2004). Finally, liver tumor susceptibility factors play an important role: mice of strains with high susceptibility to chemically induced hepatocarcinogenesis and a high prevalence of spontaneous liver tumors, such as C3H or B6C3F1, are also more susceptible to PB-mediated tumor promotion than mice of resistant

strains, such as C57BL (Bursch *et al.*, 2004; Diwan *et al.*, 1986; Jones *et al.*, 2009). The lack of a significant carcinogenic effect of PB in humans may thus have different reasons: (1) due to toxicokinetic reasons, the human PB dose level at the therapeutic dose (1-4 mg/kg body weight per day; Lagenstein, 1983) may be too low to produce the significant carcinogenic effects seen in rodents, where daily dose levels >80mg/kg body weight are generally used in rodent initiation/promotion studies. (2) Alternatively, toxicodynamic differences may render humans less susceptible to the tumor promoting effects of PB. One of the reasons for reduced susceptibility of humans could be related to differences in the structure and function of the human CAR/PXR relative to the murine receptors. However, we have very recently published the results of a study where the transcriptional as well as the proliferative responses to PB were studied in a double humanized (CAR^h-PXR^h) mouse model and only minor differences in response were observed when compared to those of wildtype mice (Luisier *et al.*, 2014). We have now used the same strains of CAR^h-PXR^h and wildtype mice to comparatively investigate the tumor promoting efficacy of PB in a long-term initiation/promotion study. The results of the study demonstrate that liver tumor formation can be promoted by PB in both wildtype and CAR^h-PXR^h humanized mice, but to a significantly lesser extent in the humanized model.

MATERIALS AND METHODS

Animal experiments. Male C57BL/6-*Nr1i2*$^{tm1(NR1I2)Arte}$*Nr1i3*$^{tm1(NR1I3)Arte}$ mice expressing human CAR and PXR (in the following referred to as hCAR/hPXR mice) and wild type (WT) C57BL/6 control mice were purchased from Taconic (Cologne, Germany). The animals (15 mice per each of the four groups; i.e. WT, WT plus phenobarbital, hCAR/hPXR, hCAR/hPXR plus phenobarbital) were kept individually in type II long cages and received a single intraperitoneal injection of 90mg/kg body weight N-diethylnitrosamine (DEN; dissolved in 0.9% NaCl solution) at 6 weeks of age. Starting one week later, mice were fed either control diet or a diet containing 0.05% (w/v) phenobarbital (Ssniff, Soest, Germany) for 40 weeks. For this purpose, phenobarbital (Sigma, Taufkirchen, Germany) had been incorporated into a rat/mouse maintenance diet (Ssniff; catalog no. V1534) via a premixture. Mice had access to food and tap water ad libitum. They were kept at 22±2°C room temperature and 55±10% relative humidity on a 12h dark/light cycle and sacrificed in a time window between 9 and 11 a.m. to avoid circadian variations. For analysis of short term DEN effects, two additional mice of each genotype were injected with DEN as described above and sacrificed 3h later. Following sacrifice, livers were excised, weighed, and immediately frozen on dry ice. A schematic representation of the animal experiments is presented in Figure 1. Animal weight was initially assessed on a weekly basis, with an extension of the intervals to 4 weeks towards the end of the study. Mice were inspected daily for overall appearance, posture, and motion. Animals received humane care and protocols complied with institutional guidelines.

Histology and immunostaining. Cryostat sections (10µm thickness) were used for all stainings. O^6-ethylguanine was visualized following fixation in -20°C methanol for 30min using an antibody against O^6-ethylguanine (1:500 dilution; gift from Dr. J. Thomale, Essen, Germany) in combination with a Cy3-conjugated goat anti-mouse secondary antibody (1:100; Dianova, Hamburg, Germany) according to the methodology described previously (Rignall *et al.*, 2011). Immunohistochemical staining of glutamine synthetase (GS) was carried out on formaldehyde-fixed slices according to standard methods (Braeuning *et al.*, 2010), using a primary antibody against GS (1:1000; Sigma) together with an appropriate horseradish

peroxidase (HRP)-conjugated secondary antibody (1:100; Dako, Glostrup, Denmark) with 3-amino-9-ethylcarbazole/H_2O_2 as substrates. Nuclei were counterstained by hematoxylin. Histochemical staining for glucose-6-phosphatase activity was performed according to Wachstein and Meisel (1957).

Tumor quantification. Liver tumor burden was measured as the area fraction (equivalent to the volume fraction) of glucose-6-phosphatase-altered lesions using an Axio Imager light microscope (Imager.M1; Zeiss, Göttingen, Germany) with AxioVision software Rel.4.5 (Zeiss). Number and size of glucose-6-phosphatase lesions per cm^3 of liver tissue were calculated according to Campbell *et al.* (1982). For each mouse, the right, left lateral, and caudate lobes of the liver were analyzed using three slices per liver lobe with at least 20 sections distance between the individual slices. Immunohistochemical staining for GS and E-cadherin was assessed on parallel slices.

Mutation analysis. Tumor tissue samples were punched out of GS-stained cryostat sections by the use of a sharpened cannula as previously described (Braeuning *et al.*, 2010). Following proteinase K digestion of the isolates, a 248bp fragment of the *Ctnnb1* gene containing the mutation hotspots in exon 3 was amplified by PCR using the primer pair *Ctnnb1*-fwd: 5'-ACTCTGTTTTTACAGCTGACC-3' and *Ctnnb1*_rev: 5'-TTTACCAGCTACTTGCTCTTG-3' (rev). PCR products were analyzed for mutations by dideoxy sequencing of both strands.

Preparation of hepatic microsomal fractions. Microsomes were prepared from snap frozen liver tissue (3 pools of 3-4 mice per genotype/treatment) as previously described (Meehan *et al.*, 1988). Microsomes were stored at -70°C until required. Microsomal protein concentrations were determined using the Biorad Protein Assay Reagent (Bio-Rad Labs, Herts, UK).

Immunoblotting. Immunoblot analysis was carried as previously described using polyclonal antisera raised against human POR (Smith *et al.*, 1994), rat cytochrome P450 (CYP) 2B1, CYP2C6, CYP3A1, and CYP2D1 (Forrester *et al.*, 1992). 15µg of hepatic microsomal protein was loaded per lane, and recombinant POR, CYP2B10, CYP2C9,

CYP2D22, and CYP3A11 proteins generated in house were used as standards. Immunoreactive proteins were detected using polyclonal goat anti-rabbit HRP-conjugated immunoglobulins as secondary antibody (Dako, Ely, UK), and visualized using Immobilon chemiluminescent HRP substrate (Millipore, Watford, UK) and a FUJIFILM LAS-3000 mini imaging system (Fujifilm UK, UK). GRP78 was used as a loading control. Densitometric analysis was performed using Multi Gauge V2.2 software (Fujifilm UK).

Gene expression analysis. Trizol reagent (Life technologies, Darmstadt, Germany) was used to isolate total RNA, which was transcribed by avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) as previously described (Ganzenberg *et al.*, 2013). Gene expression analyses were carried out on a LightCycler system by the use of the FastStart DNA master SYBR Green I kit (Roche, Mannheim, Germany) and the following primer pairs: 18s rRNA_fwd 5'-CGGCTACCACATCCAAGGAA-3'; 18s rRNA_rev 5'-GCTGGAATTACCGCGGCT-3'; Cyp2b10_fwd 5'-TACTCCTATTCCATGTCTCCAAA-3'; Cyp2b10_rev 5'-TCCAGAAGTCTCTTTTCACATGT-3'; Cyp2c_fwd 5'-CTCCCTCCTGGCCCCAC-3'; Cyp2c_rev 5'-GGAGCACAGCTCAGGATGAA-3'; Cyp2d_fwd 5'-GACATCCCGTGACATCGAAGTAC-3'; Cyp2d_rev 5'-CAGCAGGGAGGTGAAGAAGAGG-3'; Cyp3a_fwd 5'-TCACAGCCCAGTCAATTATCTT-3'; Cyp3a_rev 5'-GGAATCATCACTGTTGACCCT-3'. Values were normalized to the expression of the housekeeping gene 18s rRNA according to Pfaffl (2001).

Phenobarbital determination. The concentration of phenobarbital in mouse liver homogenate was measured by protein precipitation followed by reverse phase LC-MS/MS. Mouse liver homogenates were prepared by weighing mouse livers and preparing a 1 in 5 homogenate with milli-Q deionized water. Standards were prepared by adding a series of phenobarbital solutions to blank control mouse liver homogenates. The samples and standards (100µl) were extracted with acetonitrile (400µl), then centrifuged at 13,000 rpm for 10 minutes. 50µl of the supernatant was transferred to 96 well plates and diluted with 200µl 50/50 (v/v) acetonitrile/water. The samples were injected onto a Shimadzu UFLC-20AD System coupled to an ABSciex Triple Quad 6500 Mass Spectrometer. The mass

spectrometer was operated in electrospray, negative ion mode and the MRM transition 230.989>187.000 was monitored. The UFLC column was Kinetex C18XB 50 x 2.1mm, 2.6µm, maintained at 40°C. Mobile phase A was 10mM ammonium formate in water and mobile phase B was 0.1% formic acid in acetonitrile. A gradient was run from 20% mobile phase B to 98% mobile phase B over 0.3 minutes and held at 98% mobile phase B until 1.2 minutes, at a flow rate of 0.55ml/min. The total run time was 2 minutes. Linearity was demonstrated over the range 0.05-10µg/ml in mouse liver homogenate.

   Statistical analysis. For analysis of possible genotype and/or treatment effects ANOVA analyses with Bonferroni's correction were performed using GraphPad Prism Software (Graph Pad Software, La Jolla, CA, USA). Hepatic PB level data were analyzed using Student's t-test. Differences were considered significant when $p < 0.05$.

RESULTS

Animal and organ weight

Mice were given a single i.p. injection of DEN at 6 weeks of age, followed by 40 weeks of tumor promotion by PB (Figure 1). In the absence of PB, body weight gain of the animals throughout the experiment did not significantly differ between the two genotypes. However, it was slightly different between the WT and hCAR/hPXR groups in the presence of the tumor promoter (Figure 2A): on the one hand, PB-treated WT mice exhibited a more pronounced weight gain than controls, whereas, on the other hand, the body weight of PB-treated hCAR/hPXR mice remained lower than that of non-PB-treated hCAR/hPXR mice. Thus, body weight of PB-treated WT mice was significantly higher, as compared to PB-treated hCAR/hPXR mice throughout the second half of the experiment (Figure 2A; values at the end of the experiment: WT+PB: 38.1g ±1.1g, hCAR/hPXR+PB: 35.4g ±0.8g). However, even though statistically significant, animals did not show signs of toxicity or illness, indicating that this finding might not be of major biological relevance. PB significantly increased the liver to body weight ratio in both genotypes (Figure 2B). The mean relative liver weight of hCAR/hPXR mice was significantly higher, as compared to WT animals, in both the non-PB (6.28% vs. 5.53% of body weight) and the PB (8.55% vs. 7.72%) group (Figure 2B). Lipid droplet accumulation was observed in PB-treated mice from both genotypes, with the more pronounced effect observed in hCAR/hPXR mice (Figure 2C; see also images in Figure 5).

Expression and induction of CAR target genes

Expression of model CAR target genes related to drug-metabolism enzymes was assessed at the mRNA and protein levels. The mRNAs encoding the CYP isoforms 2b10, 2c, and 3a were expressed at higher basal levels in hCAR/hPXR mice (Figure 3A). Induction upon PB treatment was seen in both genotypes. Cyp2b10 (and, slightly failing our criteria for statistical significance, Cyp2c) was induced up to higher levels in WT as compared to hCAR/hPXR mice by PB. By contrast, Cyp3a induction by PB was weak in WT mice, whereas a very pronounced response was observed in the humanized mice (Figure 3A). Induction of CYPs

by PB was also detected by Western blotting. CYP2B and CYP2C proteins were comparably induced by PB in mice from both genotypes. Resembling the mRNA results, CYP3A induction was stronger in the humanized mice (Figure 3B).

Tumor quantification

Tumor burden of WT and hCAR/hPXR mice was quantified. In the absence of PB, the liver tumor incidence was 46.7% (7 out of 15) in WT and 80% (12/15) and in hCAR/hPXR mice. Treatment with PB increased the tumor incidence to 100% (14/14 and 15/15, respectively) in mice from both genotypes. The hepatic tumor volume fraction, measured as G6Pase-altered lesions, was below 0.25% in the non-PB groups, irrespective of the genotype. Treatment with PB led to elevated mean hepatic tumor volume fractions in both WT (6.53%) and hCAR/hPXR (2.25%) mice (Figure 4A), an effect which was statistically significant for WT, but not for hCAR/hPXR mice. Tumor burden of PB-treated WT mice was significantly higher than tumor burden of PB-treated hCAR/hPXR mice (Figure 4A). The calculated liver tumor multiplicity was low in the non-PB groups and significantly elevated upon PB treatment in mice from both genotypes (Figure 4B). Again, the values obtained for the PB-treated WT group were significantly higher than for the PB-treated hCAR/hPXR group (123 lesions/cm^3 vs. 64 lesions/cm^3; Figure 4B). Grouping of tumors according to their size revealed that the appearance of very small lesions (<0.5mm in diameter) was significantly induced by PB in both genotypes, without statistically significant differences between humanized mice and controls (Figure 4C). The incidence of medium size tumors with diameters between 0.5mm and 1.6mm was also significantly increased by PB in mice from both genotypes, but the response of WT mice was significantly more pronounced, as compared to hCAR/hPXR mice (Figure 4C). Similarly, large tumors (>1.6mm) were significantly more frequent in PB-treated WT mice, as compared to WT controls, whereas no such significant increase was seen in hCAR/hPXR mice treated with PB (Figure 4C).

Phenotypically, the vast majority of tumors (>90%) in livers of mice from both genotypes were eosinophilic, glutamine synthetase-positive well-differentiated hepatocellular adenoma

(Figure 5). This tumor phenotype is indicative of an activation of the Wnt/β-catenin signaling pathway and expected for a protocol that consists of treatment of 6 weeks old mice according an established DEN/PB regimen (Aydinlik *et al.*, 2001; Hailfinger *et al.*, 2006). A minor fraction of tumors were basophilic, glutamine synthetase-negative hepatocellular adenoma. No carcinomas were observed in the study. To validate the activation of the β-catenin in the tumors, mutation analyses for *Ctnnb1* (encoding β-catenin) were conducted. Tumors were randomly chosen from PB-treated animals and analyzed as described in the methods section. As expected, activating mutations in exon 3 of *Ctnnb1* were detected at high frequency in tumors from both genotypes, demonstrating that PB promotes the growth of same type of tumors in WT and hCAR/hPXR mice (Table 1).

DNA adduct formation by DEN

The different response of WT and hCAR/hPXR mice might be, in principle, due to differences in either the tumor initiation or tumor promotion process. To exclude an influence of initiation, formation of O^6-ethylguanine, a major DNA adduct formed by DEN, in mouse livers of both genotypes was analyzed 3h after administration of DEN. As evident from Figure 6, O^6-ethylguanine was detected in perivenous hepatocytes. This is consistent with previous findings (Rignall *et al.*, 2011) and with the theory of a preferential perivenous metabolic activation of DEN by perivenously-expressed cytochrome P450 enzymes. No remarkable differences between WT and hCAR/hPXR mice were observed regarding the formation of O^6-ethylguanine (Figure 6).

Analysis of hepatic PB levels

It has been recently shown that administration of PB to hCAR/hPXR mice via the drinking water for up to 91 days leads to slightly elevated levels of PB in liver tissue of these mice, as compared to WT controls (Luisier *et al.*, 2014). Therefore, tissue levels of PB were also analyzed in livers from WT and humanized mice from the present study. Indeed, the mean hepatic PB levels were significantly higher in mice with humanized CAR/PXR (20.6µg/g liver

vs. 11.9µg/g liver; Figure 7A). These data allowed us to normalize the tumorigenic response of each animal to its individual hepatic PB levels (Figure 7B). Based on this correlation, the overall difference in tumor response between mice of the two genotypes becomes even more obvious. In WT animals, PB tissue levels modestly correlated with hepatic tumor volume fraction (correlation coefficient r^2=0.40; p=0.02), whereas no such correlation was visible in the hCAR/hPXR group.

DISCUSSION

The present data demonstrate pronounced tumor promotion by PB in WT mice. Tumor response in our experiment closely resembles findings from a previous study conducted in our laboratory, where mice with similar genetic background had been treated according to the same protocol, resulting in a hepatic tumor volume fraction of approximately 5% (present study: 6.5%) and a tumor multiplicity of approximately 100 lesions/cm^3 (present study: 123/cm^3) after 36 weeks of tumor promotion (Marx-Stoelting *et al.*, 2009). Tumor response in PB-treated hCAR/hPXR mice was significantly less pronounced, regarding tumor volume fraction as well as tumor multiplicity. Furthermore, size class distribution of tumors shows that the most pronounced genotype differences are observed in the medium and large tumor size classes. Altogether, these data consistently indicate a reduction of tumor promotional activity of PB in the hCAR/hPXR model.

While being a potent liver tumor promoter in rodents, epidemiological data do not provide sufficient evidence for a liver tumor-promoting effect of PB in humans (see introduction). The present data demonstrate that liver tumor promotion by PB is significantly more pronounced in WT mice, as compared to transgenic mice which express the human versions of the nuclear receptors CAR and PXR. This is rather surprising, since in a recent study dealing with PB effects in hCAR/hPXR mice following shorter exposure times (and in the absence of the tumor initiator DEN), no fundamental differences between the response of WT and the receptor-humanized mice were detected regarding the induction of CAR/PXR downstream genes related to drug metabolism or hepatocyte proliferation when exposed to different doses of PB via the drinking water for up to 90 days (Luisier *et al.*, 2014). This was also the case in the present study. These results are, however, at variance with another study, which did not detect a proliferative response of hCAR hepatocytes following administration of PB after intraperitoneal administration of PB at a dose of 80mg/kg body weight per day for 4 days (Ross *et al.*, 2010). Similarly, sulfoxaflor treatment of mice for 7 days resulted in a slight increase in hepatocellular proliferation in WT, but not in hCAR/hPXR mice (LeBaron *et al.*, 2013). The reasons for this discrepancy are not fully understood: In the case of sulfoxaflor,

differences in the affinity of the compound for mouse and human CAR might exist which have influenced the outcome of the study. However, in the case of PB, neither the different routes of administration nor the rather slight differences in dosing and duration can serve as an obvious explanation. A more recent study from the same laboratory, using an alternate route of administration (0.1% PB in the diet for 7 days) to generate higher systemic exposure revealed that hCAR/hPXR mice respond to PB with hepatocellular replicative DNA synthesis (discussed in Luisier *et al.* (2014)). Nonetheless, the short-term effects of PB, i.e. hepatocyte hypertrophy, induction of drug-metabolizing enzymes, and transient hyperplasia of the liver, are not necessarily good quantitative surrogates for a tumor-promoting activity. This is evident from the comparison of tumor promotion (higher in WT mice) and drug metabolism induction (slight differences between genotypes, but in principle similar) in the present study, as well as from the comparison of the data in Luisier *et al.* (2014) with the present data on tumor promotion. PB selects for the outgrowth of liver tumors with mutationally activated β-catenin (Aydinlik *et al.*, 2001); accordingly, tumor formation in mice with a conditional hepatocyte-specific knockout of the β-catenin gene *Ctnnb1* cannot be promoted by PB (Rignall *et al.*, 2011). However, a considerable induction of drug-metabolizing enzymes by PB can be achieved in these mice (Braeuning *et al.*, 2011; Braeuning *et al.*, 2009). Moreover, the regulation of CYP induction and proliferation by PB is uncoupled in mice with hepatocyte-specific knockout of *Ctnnb1* (Braeuning *et al.*, 2011).

This study for the first time provides clear evidence that species differences in the receptors CAR and/or PXR affect tumor promotion by PB in the liver. The observed differences become even more pronounced when the data are normalized to hepatic PB levels, which were higher in hCAR/hPXR mice as compared to WT animals. This view assumes that the two PB tissue concentrations are below the plateau of the biological response to PB, an assumption which is substantiated by the results on CYP induction in the recent study by Geter et al. (2014), where PB was administered at different doses for up to 7 days. The reason for the different hepatic PB levels is not known. In principle, it might be related either to a differential intake of PB, or to differences in metabolism and/or elimination. The first

scenario would imply an increased food intake of the hCAR/hPXR mice. This cannot be fully excluded, since food uptake has not been measured in the present study. However, the fact that hCAR/hPXR mice treated with PB gained weight slightly slower than their WT counterparts suggests that the hCAR/hPXR mice most likely did not consume significantly more food than the mice from the WT+PB group. Therefore, the differences are most likely to be attributed to metabolism or excretion. However, the rather minor alterations in CYP expression between the two genotypes do not provide clear evidence for major differences in PB metabolism.

To estimate a possible human risk, exposure levels of mice and humans have to be compared (Table 2). Food intake was not measured in the present study. Therefore, the exact dose of PB of the animals is not known. However, based on published data, C57BL/6 mice consume approximately 5g of diet per 30g mouse and day (Bachmanov *et al.*, 2002). From this we can calculate an average PB dose of approximately 80mg/kg body weight per day for the mice in our present study (PB concentration 0.05% in diet). The human exposure at the therapeutic level is 1-4mg/kg body weight per day. At first glance, this suggests a large difference in exposure level. However, when scaled allometrically, the murine dose of 80mg/kg body weight per day equals a human dose of approximately 11.5mg/kg body weight per day, which is not much higher than therapeutic dosage. With respect to biological activity in the liver, serum and liver tissue concentrations of PB are the more relevant measures for comparison. The mean liver concentrations of PB in the present study were 11.9µg/g liver in WT mice and 20.6µg/g in hCAR/hPXR mice. PB serum levels were not determined. However, in a recent study (Luisier *et al.*, 2014), where PB was given in drinking water at a concentration of 0.05% to mice of the same strain, average liver levels at steady state in WT mice were 15.1µg/g and average serum levels were 17.8µg/ml. Similar serum levels of approximately 15µg/ml were observed in male CD1 mice given PB via the diet at a level of 75mg/kg body weight per day (Geter *et al.*, 2014). For comparison, the therapeutic steady state serum level of PB as an anticonvulsant in humans is commonly reported as 15-70µg/ml (e.g. see Houghton *et al.* (1975)). We were not able to find data on human PB liver levels at

the therapeutic dose, but, based on the reported partition coefficient of liver/serum of 2.25 in humans (El-Masri and Portier, 1998), the liver steady state levels would be approximately two-fold those of the serum level. This indicates that the serum and liver levels in humans at the therapeutic dose are in the same range or even higher than those observed in the mouse treated with a dose of PB where tumor promotional activity of the drug is seen. In fact, short-term effects of PB comparable to those observed in the mouse have been reported also for humans, i.e. the induction in the levels and activities of drug-metabolizing enzymes in livers of epileptics (Sotaniemi *et al.*, 1978) as well as in cultured primary human hepatocytes (Madan *et al.*, 2003).

In summary, the present data do not absolve PB of being a tumor promoter with possible relevance to humans. Phenobarbital-mediated tumor promotion clearly occurs in mouse liver expressing the human CAR and PXR receptors, but, regarding both the resulting tumor multiplicities and tumor volume fractions, at a much lower intensity than in WT animals. The combination of elevated PB levels and reduced tumor burden in the hCAR/hPXR mice does not support the assumption that the human receptors are responsible for toxicokinetic alterations which might markedly reduce tumor promotion by PB between in hCAR/hPXR mice. Rather, toxicodynamic factors such as species differences in receptor function might play a role, especially since the therapeutic PB concentrations in humans are not substantially different from the concentrations in mice. "Humanized" mouse strains serve as very attractive experimental tools to investigate possible species differences in response to agents that exert their effects through the respective receptors. Caution is appropriate, however, since the human receptors function in a mouse-based heterologous system where gene regulatory protein interactions may differ from human hepatocytes. Nonetheless, the models might be very useful to analyze the effects of species-specific ligands and interspecies differences in receptor function.

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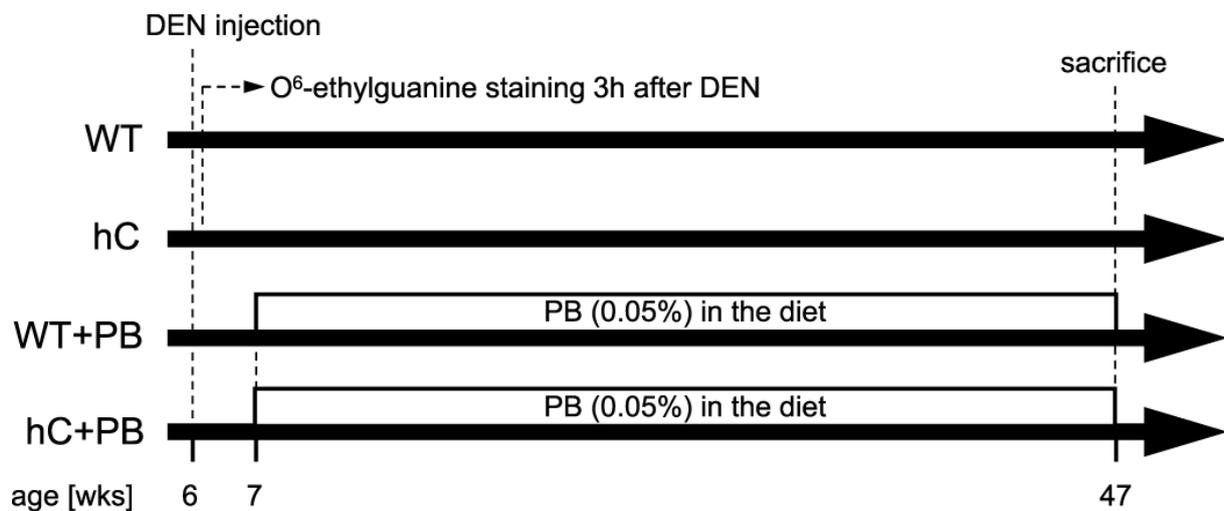
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Fig. 1. Schematic delineation of the experimental setup of the tumor initiation/promotion study in mice with humanized CAR and PXR (hC) and wild type (WT) controls. Mice were injected at 6 weeks of age with a single i.p. dose of 90µg/g body weight N-nitrosodiethylamine (DEN), followed by chronic treatment with phenobarbital (PB; 0.05% in the diet) for 40 weeks. The initiation/promotion experiment was conducted with15 mice per group. One mouse from the WT+PB group died 2 days after DEN injection, thus downsizing this group to 14 mice. The formation of DNA adducts following the application of DEN was analyzed in livers of two additional mice from each genotype 3h after DEN injection.
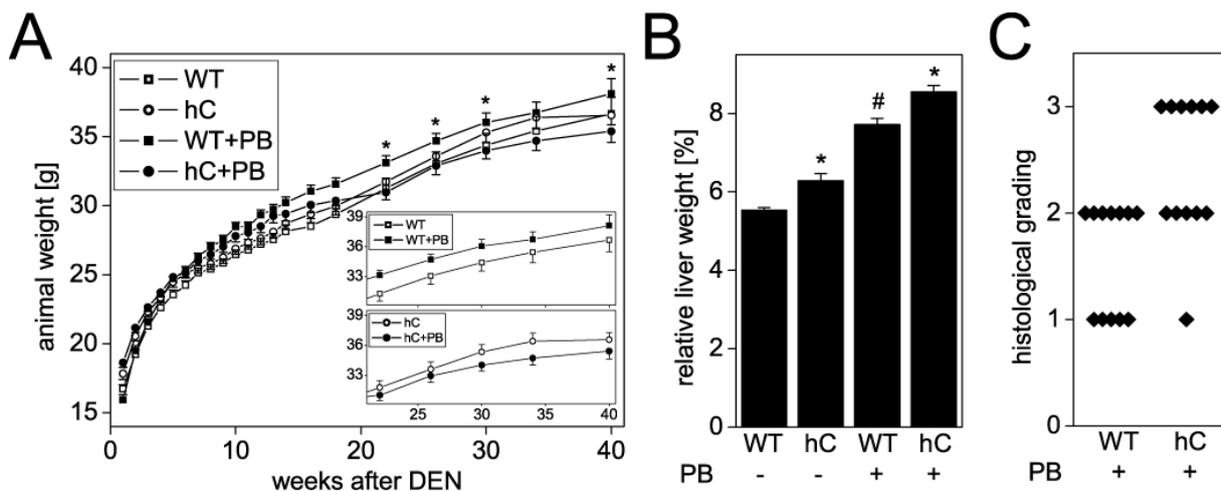

Fig. 2. Time course of animal weight gain during the experiment **(A)** and relative liver weight (liver weight in % of total body weight) of animals at sacrifice **(B)**. Animals were weighed at the respective time points; liver weight was determined at sacrifice. Mean +SEM of n=14-15 animals per group are shown. Statistical significance (p<0.05) is indicated as follows:

*significant genotype effect in PB-treated mice; #significant PB treatment and genotype effect.

(C) Histological grading of lipid droplet accumulation in liver tissue. 0, none; 1, weak; 2, moderate; 3, strong. 12 mice per group were analyzed. See also Figure 5 for comparison. Abbreviations: WT, wild type; hC, hCAR/hPXR; PB, PB-treated.
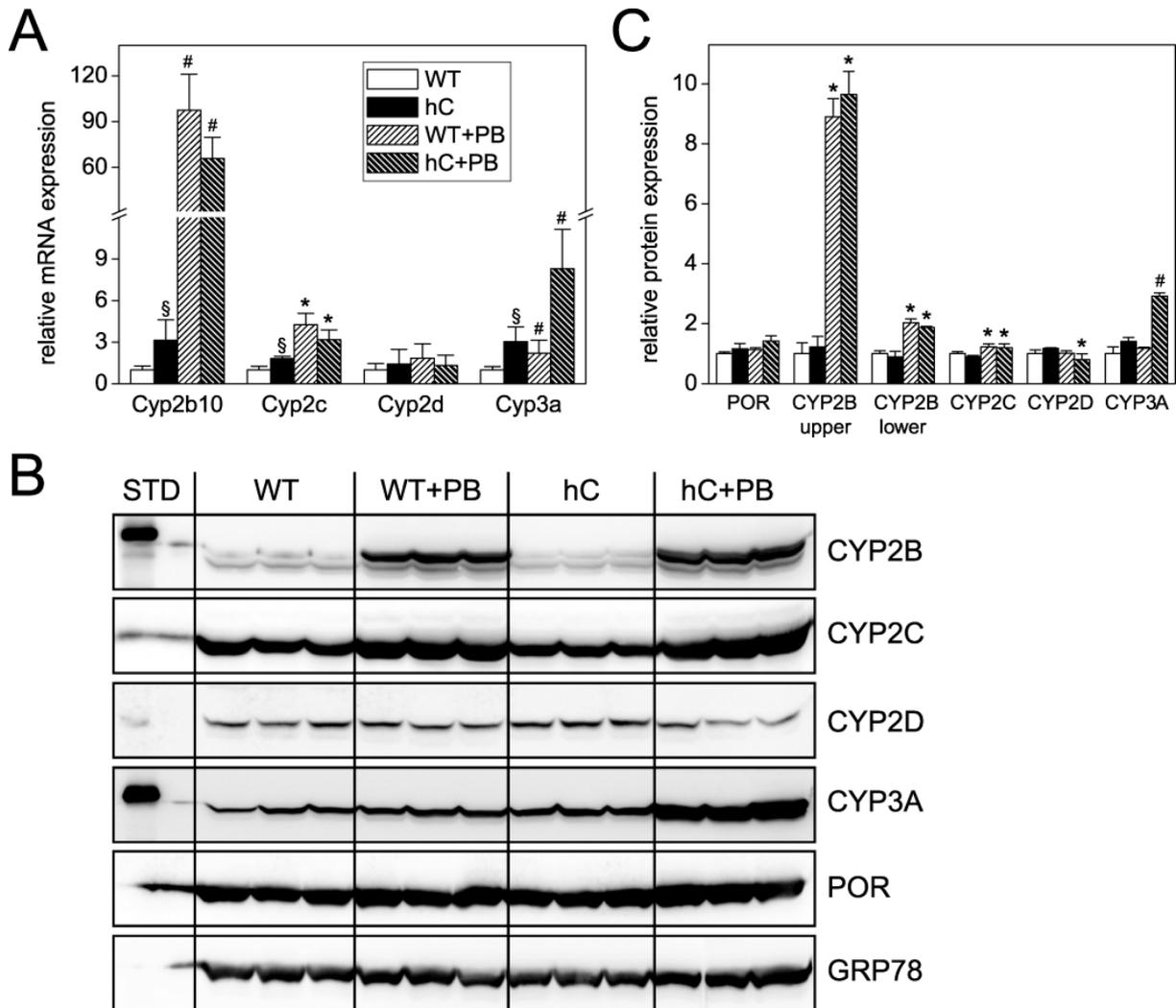

Fig. 3. PB-induced expression of drug-metabolizing enzymes in WT and hCAR/hPXR mice. Expression at the mRNA **(A)** and protein levels **(B)** was assessed by real-time RT-PCR and Western blotting, respectively. **(C)** Densitometric analysis of protein data from **(B)**. Gene expression data depict the mean +SD of n=5 randomly selected mice per group. For protein analysis, pooled tissue from n=3-4 different mice was used for each lane. Statistical significance (p<0.05) is indicated as follows: *significant genotype effect in PB-treated mice; #significant PB treatment and genotype effect; §significant genotype effect in untreated mice. Abbreviations: WT, wild type; hC, hCAR/hPXR; PB, PB-treated; STD, protein standard.
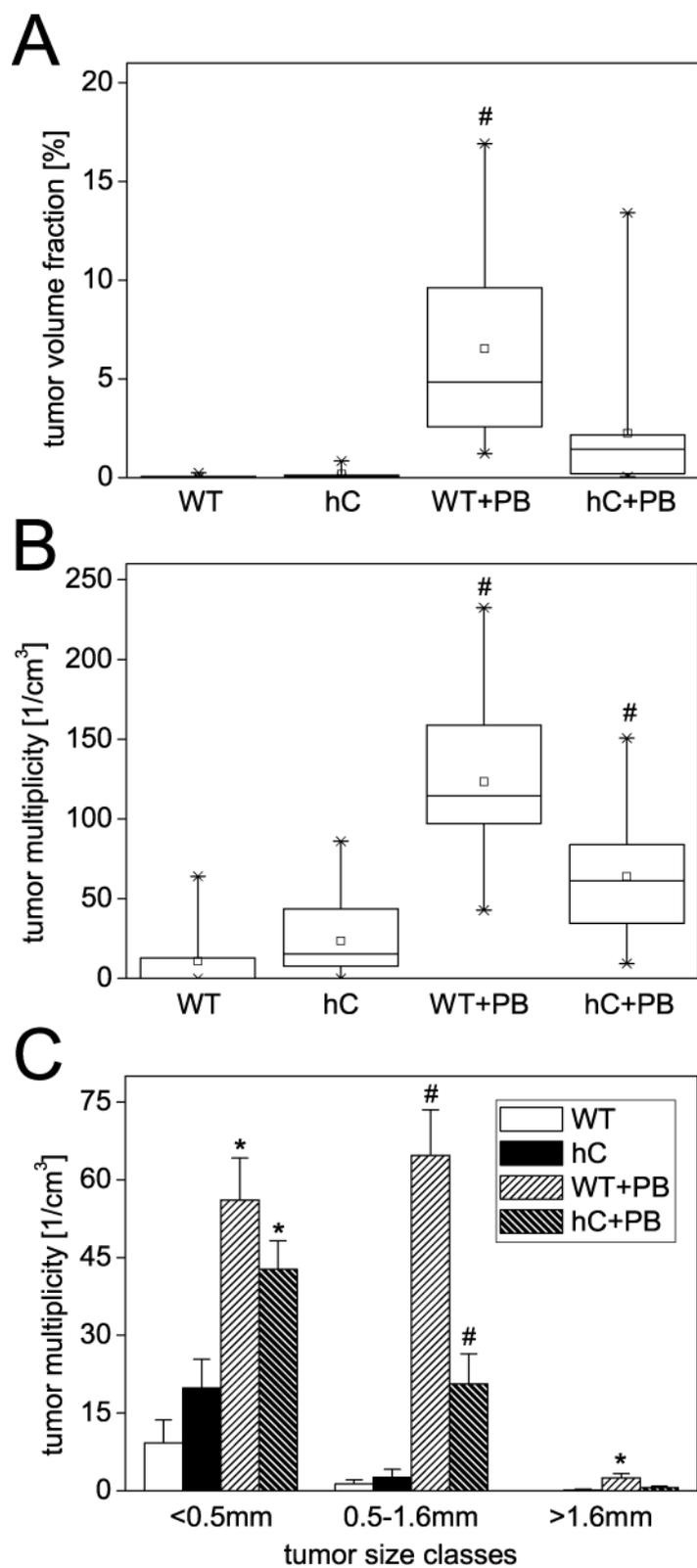
Fig. 4. Analysis of tumor burden. Hepatic tumor volume fractions **(A)** of the different treatment groups and liver tumor multiplicities **(B)** were assessed. **(C)** Size class distribution of liver tumors. Box charts **(A-B)** or mean +SEM **(C)** of n=14-15 animals per group are shown. Statistical significance (p<0.05) is indicated as follows: * significant genotype effect in PB-

treated mice; # significant PB treatment and genotype effect. Abbreviations: WT, wild type; hC, hCAR/hPXR; PB, PB-treated.
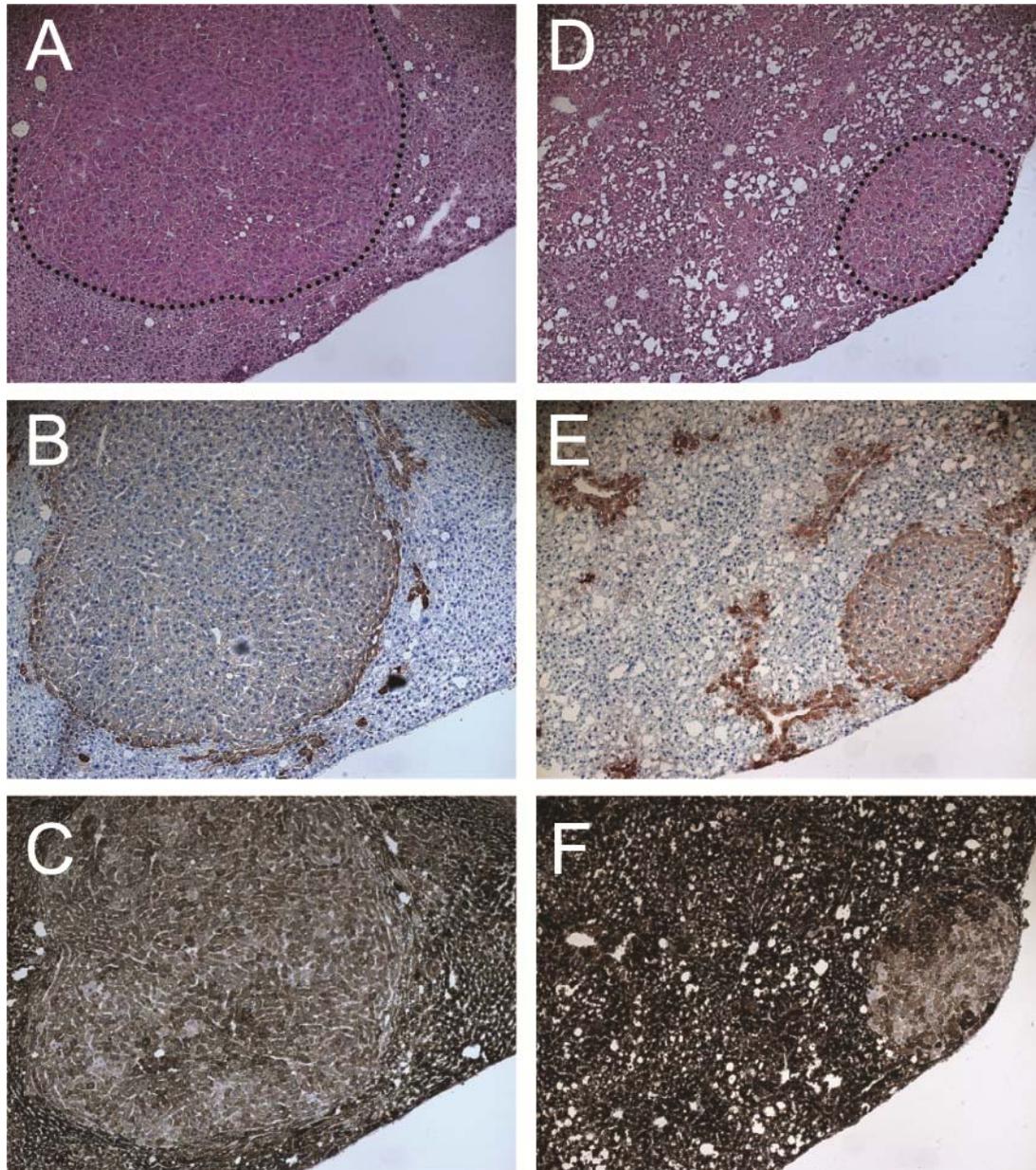

Fig. 5. Tumor phenotype in PB-treated WT **(A-C)** and hCAR/hPXR **(D-F)** mice. Representative images of hematoxylin/eosin staining **(A,D)**, immunoreactivity for the β-catenin marker GS **(B,E)**, and activity of the tumor marker G6Pase **(C,F)** are depicted. Please note the accumulation of lipid droplets in tumor-surrounding normal tissue in the hCAR/hPXR liver visible as 'holes' in the slices.
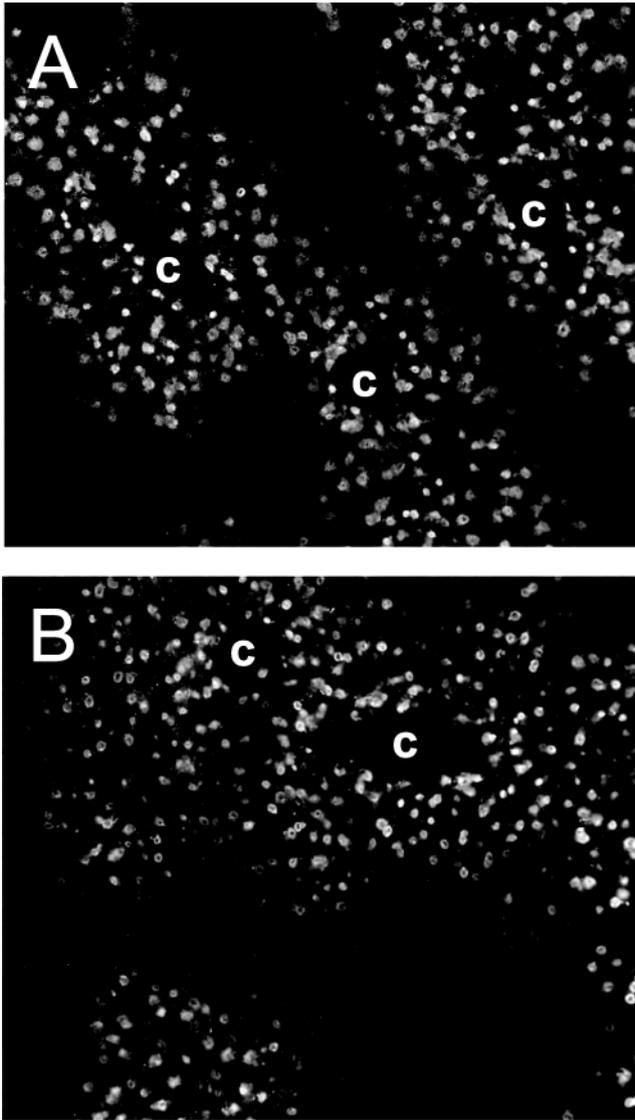
Fig. 6. DNA adduct formation following administration of DEN. Representative images of immunofluorescent staining of O^6-etyhlguanine in the liver of a WT **(A)** and a hCAR/hPXR **(B)** mouse are shown. Please note the preferential staining of hepatocyte nuclei surrounding the central veins (c), whereas hepatocytes near the portal triad are not affected by the treatment. Staining was performed on liver slices from animals sacrificed 3h after DEN injection (see also scheme in Fig. 1).
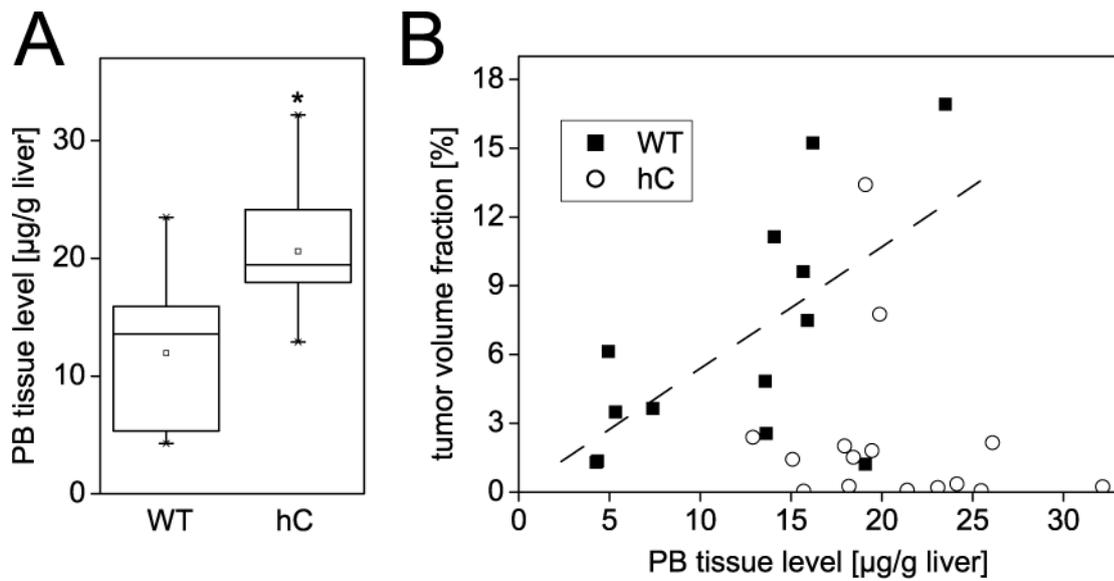
Fig. 7. PB levels in liver tissue. **(A)** Hepatic levels of PB were measured in liver tissue of WT and hCAR/hPXR mice at termination of the experiment after 40 weeks of PB exposure. Box charts (n=13-15 mice per group) are depicted. Statistical significance (p<0.05) is indicated as follows: * significant genotype effect in PB-treated mice. **(B)** Correlation of PB levels from **(A)** with hepatic tumor volume fractions (see Figure 4). The dashed line illustrates the correlation of the two parameters in WT mice. Abbreviations: WT, wild type; hC, hCAR/hPXR.

Table 1.

***Ctnnb1* mutations in tumors from WT and hCAR/hPXR mice.**

	tumor origin	
amino acid exchange	WT	hCAR/hPXR
D32G	1	2
S33P	3	2
S33A	0	1
I35S	2	0
S37P	4	1
T41A	2	1

none detected	0	1

Types of mutations and detected mutation frequency are shown. Total numbers of tumors analyzed were n=12 (WT) and n=8 (hCAR/hPXR). All analyzed tumors were from PB-treated mice.

Table 2.

Interspecies comparison of PB dose, plasma and liver levels.

parameter	mouse	human
dose [mg/kg body weight/ day]	~80 [1, estimated]	1-4 [2]
allometrically scaled dose *(exp. 0.75)*	---	11.5
plasma [µg/ml]	17.8 [3] / 15 [4]	15-70 [5]
liver levels [µg/g]	11.9 - 20.6 [1] / 15.1 [3]	33-157 [6, estimated]

Abbreviations : WT, wild type ; hC, humanized CAR/PXR; references: (1) present study; (2) Lagenstein, 1983; (3) Luisier *et al.*, 2014; (4) Geter *et al.*, 2014; (5) Houghton *et al.*, 1975; (6) El-Masri and Portier, 1998.