Drug Metabolism and Disposition, Mini-review

Distinct roles of the sister nuclear receptors PXR and CAR in liver cancer development

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Abbreviations: AP-1, activator protein 1; CAR, constitutively active receptor/constitutive androstane receptor; CCL2, /C-C motif chemokine 2; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CXCL2, chemokine (C-X-C motif) ligand 2; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transformation; FGF, fibroblast growth factor; FOXA, the class A of forkhead box transcription factors; FOXO, the class O of forkhead box transcription factors; CXCL2, chemokine (C-X-C motif) ligand 2; HNF4α, hepatocyte nuclear factor-4α; IL-6, interleukin-6; KRT8/18, keratin 8/18; LBD, ligand-binding domains; LEF, lymphoid enhancer factor; MCP-1, monocyte chemotactic protein-1; MOA, mode of action; NF-κB, nuclear factor-kappa B, NR, nuclear receptor; P450, cytochrome P450; PCN, pregnenolone 16α-carbonitrile; PCNA, proliferating cell nuclear antigen; PPARα, peroxisome proliferator-activated receptor α; PPI, protein–protein interaction; PXR, pregnane X receptor; TCF, T cell factor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; TEAD, TEA-domain
family members; TGF, transforming growth factor; TNF, tumor necrosis factor; YAP, Yes-associated protein.
Abstract

Pregnane X receptor (PXR) and constitutively active receptor/constitutive androstane receptor (CAR) are xenobiotic-responsive transcription factors belonging to the same nuclear receptor gene subfamily (NR1I) and highly expressed in the liver. These receptors are activated by a variety of chemicals and play pivotal roles in many liver functions, including xenobiotic metabolism and disposition. Phenobarbital, an enzyme inducer and liver tumor promoter, activates both rodent and human CAR but causes liver tumors only in rodents. Although the precise mechanism for phenobarbital/CAR-mediated liver tumor formation remains to be established, intracellular pathways, including the Hippo pathway/YAP-TEAD system and β-catenin signaling, seem to be involved. In contrast to CAR, previous findings by our group suggest that PXR activation does not promote hepatocyte proliferation but it enhances the proliferation induced by various stimuli. Moreover, and surprisingly, PXR may have antitumor effects in both rodents and humans by targeting inflammatory cytokine signals, angiogenesis and epithelial-mesenchymal transition. In this review, we summarize the current knowledge on the associations of PXR and CAR with hepatocyte proliferation and liver tumorigenesis and their molecular mechanisms and species differences.

Significance statement

Pregnane X receptor (PXR) and constitutively active receptor/constitutive androstane receptor (CAR) have very similar functions in the gene regulation associated with xenobiotic disposition, as suggested by their identification as xenosensors for enzyme induction. In contrast, recent reports clearly suggest that these receptors play distinct roles in the control of hepatocyte proliferation and liver cancer development. Understanding these differences at the molecular level may help us evaluate the human safety of chemical compounds and develop novel drugs targeting liver cancers.
1. Introduction

Pregnane X receptor (PXR) and constitutively active receptor/constitutive androstanereceptor (CAR) (also known as NR1I2 and NR1I3, respectively) are members of the nuclear receptor (NR) gene superfamily. Among the NR members, PXR and CAR are well known as xenobiotic-responsive receptors that function as xenosensors (Willson and Kliewer, 2002; Handschin and Meyer, 2003; Timsit and Negishi, 2007). They are highly expressed in the liver and intestine, which are frequently exposed to xenobiotic compounds, including drugs, foods, food additives, and food contaminants (Willson and Kliewer, 2002; Handschin and Meyer, 2003; Timsit and Negishi, 2007). To protect the body from these chemicals, these receptors coordinately play pivotal roles in xenobiotic metabolism and disposition by regulating the expression of genes encoding drug-metabolizing enzymes and drug transporters (Willson and Kliewer, 2002; Handschin and Meyer, 2003; Timsit and Negishi, 2007). While CYP3A and CYP2B subfamily members of cytochrome P450s (P450s) are typical enzymes whose expression is regulated by PXR and CAR, respectively, the expression of other (sub)family P450s and phase II enzymes, such as UDP-glucuronosyltransferases, sulfotransferases, and glutathione S-transferases, is also regulated by PXR and CAR (Willson and Kliewer, 2002; Handschin and Meyer, 2003; Timsit and Negishi, 2007).

In addition to drug metabolism and disposition, PXR and CAR are also involved in various physiological and pathophysiological systems in the liver and other tissues, including glucose, lipid and energy homeostasis (Kodama et al., 2004; Ding et al., 2006; Nakamura et al., 2007; Roth et al., 2008), immunological and inflammatory responses (Kakizaki et al., 2008; Cave et al., 2016; Klepsch et al., 2019), and cell proliferation and hepatocarcinogenesis (Yoshinari, 2019; Shizu and Yoshinari, 2020). Compared to the roles of PXR and CAR in drug metabolism and disposition, the mechanism of these additional functions of PXR and CAR has not been fully elucidated. However, protein–protein interactions (PPIs) with other transcription factors, such as FOXO (the class O of forkhead box transcription factors) and FOXA (the class A of forkhead box transcription factors) family transcription factors, seem to be involved in the regulatory process by PXR and CAR (Konno et al., 2008; Pavek, 2016;
Mackowiak et al., 2018; Yoshinari, 2019), rather than their direct binding to the promoter
dependencies of the target genes, which is thought to be the primary mechanism regulating
P450 gene expression. In addition, species differences in the “extra” functions of PXR and
CAR remain to be evaluated.

Among the receptors' functions beyond xenobiotic disposition, we focus on their
roles in hepatocarcinogenesis in this mini-review. In the regulation of gene expression
associated with drug metabolism and disposition, PXR and CAR are very similar, sister-like
NRs. In contrast, for chemical carcinogenesis, information on the role of PXR is very limited
compared to CAR. This is mainly because the CAR activator phenobarbital is also well-known
to be a liver tumor promoter (Whysner et al., 1996; IARC, 2001; Elcombe et al., 2014). Since

tumor formation is one of the most serious adverse effects of chemicals, such as drug
candidates and pesticides, understanding its mechanism and evaluating whether the results
can be extrapolated to humans are very important for chemical safety assessment. In addition,
PXR is activated by a large number of structurally unrelated compounds. These facts indicate
that clarifying the roles of not only CAR but also PXR in chemical carcinogenesis is of great
importance.

2. Chemical activation of CAR and PXR and its species differences

It is well-known that species differences are often observed in xenobiotic-mediated P450
induction between humans and rodents. The identification of PXR and CAR as the main
regulators of P450 induction has demonstrated that most of the species differences are
explained by the species differences in the structures of ligand-binding domains (LBDs) of
these receptors (Kretschmer and Baldwin, 2005; Buchman et al., 2018). For example,
pregnenolone 16α-carbonitrile (PCN) activates mouse PXR but not human PXR, whereas
SR12813 and rifampicin bind to human but not mouse PXR (Bertilsson et al., 1998; Kliewer et
al., 1998; Kretschmer and Baldwin, 2005; Buchman et al., 2018).

6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
(CITCO) is a human CAR ligand but it is not a mouse CAR ligand, while
1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) acts as a mouse but not human CAR ligand (Moore et al., 2000; Tzameli et al., 2000; Maglich et al., 2003).

Phenobarbital is one of the classical inducers of drug-metabolizing enzymes (Remmer and Merker, 1963). While phenobarbital-dependent P450 induction was found to be CAR-dependent (Honkakoski et al., 1998; Wei et al., 2000), later studies have demonstrated that this anti-epileptic drug is unique in terms of CAR and PXR activation. Phenobarbital does not bind to CAR (Li et al., 2019) and indirectly activates it by inhibiting epidermal growth factor receptor-dependent cellular signals involving mitogen-activated protein kinase kinase and extracellular signal-regulated kinase, the protein phosphatase PP2A and the adaptor protein RACK1 (Yoshinari et al., 2003; Koike et al., 2007; Mutoh et al., 2009; Osabe and Negishi, 2011; Mutoh et al., 2013; Negishi, 2017). These findings about the indirect activation of CAR by phenobarbital are consistent with the fact that this drug induces P450s in various species with little species difference. In addition, phenobarbital has been reported as a human PXR ligand, indicating that phenobarbital is a dual activator of CAR and PXR in humans (Li et al., 2019). There is no information on whether phenobarbital can activate PXR via an indirect mechanism.

3. Mechanism for CAR-dependent hepatocarcinogenesis in rodents

Phenobarbital is a well-known inducer of drug-metabolizing enzymes and was demonstrated to activate CAR via an indirect mechanism (Mutoh et al., 2013; Negishi, 2017). Since phenobarbital is also known as a nongenotoxic carcinogen and liver tumor promoter (Whysner et al., 1996; IARC, 2001; Elcombe et al., 2014), the role of CAR in the phenobarbital-dependent liver tumorigenesis was intensively investigated. In 2004, Yamamoto et al. clearly demonstrated that CAR is essential for phenobarbital-induced liver tumor formation using a 2-step hepatocarcinogenesis model and Car-null mice with the tumor initiator diethylnitrosamine (Yamamoto et al., 2004). Huang et al. also demonstrated that CAR was required for liver tumor formation induced by phenobarbital and the murine CAR ligand TCPOBOP and suggested that induction of Mdm2 expression was a key event for
CAR-dependent liver tumorigenesis (Huang et al., 2005). It should be noted that TCPOBOP activates mouse CAR but not rat CAR and it does not promote liver tumors in rats (Poland et al., 1981; Diwan et al., 1992).

Studies on the role of CAR in hepatocyte proliferation have identified several CAR target genes involved in proliferation. For example, MYC and its target FOXM1 were demonstrated to be key transcription factors for TCPOBOP-induced expression of cell cycle-related genes, including Cdc2, cyclins, Mcm members, and Cdc20 (Blanco-Bose et al., 2008). In addition, a series of experiments reported by Dr. Columbano’s group using TCPOBOP have provided valuable information on the mechanism of CAR-mediated hepatocyte hyperplasia. TCPOBOP-induced hepatocyte proliferation in mice was independent of proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Ledda-Columbano et al., 1998), and of the induced expression of c-Fos and c-Jun (Columbano et al., 1997). Although TCPOBOP treatment increased cyclin D expression as an early event during hepatocyte proliferation in mice (Ledda-Columbano et al., 2000), cyclin D1 was not essential for TCPOBOP-induced murine hepatocyte proliferation since the numbers of proliferating cells were comparable between wild-type mice and mice lacking cyclin D1 (Ledda-Columbano et al., 2002). They also demonstrated that the expression of Gadd45b, which encodes a modulator of the p53 tumor suppressor protein, was induced by TCPOBOP treatment in mouse livers (Columbano et al., 2005). Finally, they demonstrated the possible involvement of the Hippo pathway and its effector protein Yes-associated protein (YAP) (Kowalik et al., 2011; Perra et al., 2014), which acts as a transcriptional coactivator for TEA-domain family members (TEAD) to regulate cell proliferation and antiapoptotic pathways during carcinogenesis (Pan, 2010; Harvey et al., 2013; Wang et al., 2021).

The Hippo pathway is a signaling pathway involving multiple kinases, including mammalian Ste20-like kinases MST1/2 and large tumor suppressor kinases LATS1/2, the latter of which phosphorylates YAP, leading to its degradation in the cytoplasm (Pan, 2010; Lin et al., 2013; Wang et al., 2021). In normal livers, the pathway is activated, and thus YAP is...
inactivated (Zhou et al., 2009; Song et al., 2010), but in human liver cancers and other carcinomas, the signal was disrupted, and YAP phosphorylation was prevented, increasing the nuclear levels of transcriptionally active YAP (Zender et al., 2006; Zheng et al., 2011; Harvey et al., 2013). However, the causal relationship between YAP activation and CAR-dependent hepatocyte proliferation and/or liver hyperplasia remains unclear.

As described above, phenobarbital activates both human and rodent CAR through an indirect mechanism to cause enzyme induction. In contrast, it is believed that the liver tumor-promoting effects of phenobarbital are rodent-specific and that phenobarbital does not directly act as a tumor promoter in humans (Fig. 1) (IARC, 2001; Elcombe et al., 2014). How does the phenobarbital-activated CAR regulate proliferating signals selectively in rodent hepatocytes? Although promoter binding is the main gene-regulating mechanism of NRs, including CAR, this mechanism seems to show little species difference because their DNA-binding domains are often highly conserved among species. On the other hand, the LBDs of CAR and PXR show large species differences, as illustrated by their ligand selectivity. Since PPI is another important mechanism for NRs, including CAR, to control gene expression through an indirect mechanism (i.e., via another transcription factor), CAR may regulate proliferating signals by PPI with another transcription factor through its LBD (Shizu and Yoshinari, 2020).

Based on these facts and reports from Dr. Columbano’s group, we investigated functional crosstalk between CAR and YAP (Abe et al., 2018). We confirmed that TCPOBOP treatment of mice significantly increased the expression of cell cycle-related genes as well as CAR target genes and that the treatment induced YAP accumulation in the nucleus and YAP target gene expression, including \textit{Birc5}, \textit{Ankrd1}, and \textit{Myc}. In addition, we found that cotreatment with the YAP inhibitor verteporfin (Liu-Chittenden et al., 2012; Gibault et al., 2016; Wei and Li, 2020) suppressed TCPOBOP-induced hepatomegaly and tended to suppress the expression of \textit{Ankrd1} and \textit{Mcm2}.

To further investigate the role of YAP in CAR-dependent hepatocyte proliferation, in vitro systems, such as primary hepatocytes, could be useful. However, it is very difficult to
investigate CAR functions in detail in primary hepatocytes because CAR expression levels quickly decline after the preparation of primary cultures. To overcome this problem, we expressed mouse CAR using adenovirus in AML-12 cells, which are derived from mouse hepatocytes (Abe et al., 2018). With this system of CAR expression, we observed enhanced CAR target gene expression and CAR-dependent proliferation. More importantly, CAR expression enhanced YAP target gene expression. Using this in vitro system, we investigated the role of YAP in CAR-dependent hepatocyte proliferation and demonstrated that YAP knockdown with siRNA or verteporfin treatment suppressed CAR-dependent cell proliferation and YAP target gene expression. Since YAP acts as a coactivator for TEAD to regulate the expression of genes associated with cell proliferation and anti-apoptosis (Zhou et al., 2009; Pan, 2010; Song et al., 2010; Lin et al., 2013; Wang et al., 2021), we investigated whether CAR could affect the gene transcription mediated by TEAD/YAP and found that CAR enhanced TEAD/YAP-mediated gene expression in in vitro reporter gene assays. These results suggest that YAP activation is crucial, at least in mice, for hepatocyte proliferation induced by CAR activators.

The crucial role of β-catenin signaling in phenobarbital-induced liver tumorigenesis has been demonstrated by Drs. Schwarz and Braeuning (Braeuning and Pavek, 2020). β-Catenin has two major functions: it mediates intracellular signaling of cell adhesion associated with cadherin and works as a coactivator for various transcription factors, including T cell factor (TCF)/lymphoid enhancer factor (LEF) family members, in the Wnt/β-catenin signaling pathway (Garcia-Lezana et al., 2021). β-Catenin is often aberrantly activated in many tumors, including hepatocellular carcinoma in humans (Wong et al., 2001; Nault et al., 2017; Rebouissou and Nault, 2020; Garcia-Lezana et al., 2021) and in liver tumors in mice induced by diethylnitrosamine and phenobarbital (Aydinlik et al., 2001). The group of Schwarz and Braeuning demonstrated that phenobarbital did not promote tumor formation in mice with liver-specific knockout of β-catenin after initiation with diethylnitrosamine (Rignall et al., 2011), suggesting its critical role in CAR-mediated liver tumor formation, although another group reported that β-catenin knockout increased the number of liver tumors formed with a
diethylnitrosamine/phenobarbital model (Awuah et al., 2012). Another report demonstrated that TCPOBOP treatment-induced hepatocyte proliferation was significantly reduced in mice lacking β-catenin in a liver-specific manner compared to wild-type mice, without affecting the induced expression of Cyp2b10 in the liver (Ganzenberg et al., 2013). Taken together, the results presented to date strongly suggest that β-catenin signaling and the Hippo pathway/YAP-TEAD system are key signaling pathways controlling CAR-mediated hepatocyte proliferation and liver tumor development, although a precise molecular mechanism(s) remains to be established.

4. Role of PXR in hepatocyte proliferation

Although chemical activation of PXR, as well as CAR, causes hepatocellular hypertrophy and hepatomegaly in rodents, which are thought to be associated with enzyme induction (Maronpot et al., 2010), it remains unclear whether PXR activation could induce hepatocyte proliferation and liver tumors. Staudinger et al. demonstrated that repeated administration of the representative PXR ligand PCN at a relatively high dose increased the number of proliferating cell nuclear antigen (PCNA)-positive cells in mouse livers, suggesting PXR-mediated cell cycle progression of hepatocytes (Staudinger et al., 2001). In addition, recovery after partial hepatectomy was delayed in PXR-knockout mice (Dai et al., 2008). These results suggested that PXR was a positive regulator of murine hepatocyte proliferation. In contrast, PXR overexpression suppressed the growth of neuroblastoma cells (Misawa et al., 2005) and colon cancer cells (Ouyang et al., 2010). In addition, rifampicin activation of PXR reduced the proliferation of human hepatoma-derived HepG2 cells through cell cycle arrest in G1 phase, and siRNA-mediated knockdown of PXR prevented rifampicin-induced suppression of the cell cycle (Zhuang et al., 2011). These results suggested that PXR is a negative regulator of cell cycle progression. Moreover, the tumor suppressor protein p53 and cyclin-dependent kinase 2 functionally interact with PXR to negatively regulate its function through direct binding or phosphorylation (Lin et al., 2008; Elias et al., 2013). These results clearly indicate that PXR plays a role in cell cycle regulation, but these findings are
To evaluate the role of PXR in hepatocyte proliferation, our group performed a series of experiments and found that PXR has a unique function in hepatocyte proliferation, at least in mice: PXR activation by itself does not induce the proliferation of murine hepatocytes. It further promotes proliferation induced by different types of stimuli, including CAR and peroxisome proliferator-activated receptor α (PPARα) activation, fibroblast growth factor 19 (FGF19), epidermal growth factor (EGF), and liver injury (Shizu et al., 2013; Shizu et al., 2016).

Mice were intraperitoneally treated with PCN or TCPOBOP alone or in combination and hepatocyte proliferation was measured 48 h after treatment (Shizu et al., 2013). The results demonstrated that TCPOBOP administration induced hepatocyte proliferation very strongly, whereas PCN alone had no effect. Surprisingly, a much stronger induction of hepatocyte proliferation was observed in mice treated with both TCPOBOP and PCN than in mice treated with TCPOBOP alone. This stimulating effect of PCN was not observed in Pxr-null mice. Interestingly, the mRNA levels of Cyp2b10, a representative CAR target gene, were comparable between the TCPOBOP group and the TCPOBOP+PCN group. Similar effects of PCN administration were observed in experiments using the PPARα agonist Wy-14643, which causes hepatocyte proliferation in rodents and acts as a CAR agonist (Klaunig et al., 2003; Gonzalez and Shah, 2008). In addition, the antifungal agent imazalil, which was found to be a mouse PXR activator in our laboratory, also enhanced TCPOBOP-dependent hepatocyte proliferation (Yoshimaru et al., 2018). These results suggested that although PXR is unable to induce hepatocyte proliferation in mice, it enhances CAR- or PPARα-dependent hepatocyte proliferation by an indirect mechanism and does not just enhance CAR or PPARα functions.

The results implied that PXR activation could enhance hepatocyte proliferation induced by various types of stimuli. We thus tested this idea using a mouse model of hepatocyte proliferation during regeneration after liver injury induced with carbon tetrachloride (CCl₄) (Shizu et al., 2016). PCN was administered to mice 24 h after CCl₄ treatment, and
hepatocyte proliferation was evaluated at various time points. The results demonstrated that the peak proliferation (e.g., number of Ki-67-positive cells and the mRNA levels of cell cycle-related genes) was shifted by PCN treatment to earlier time points compared to mice treated with CCl₄ alone, and PCN treatment significantly increased the number of mitotic-stage hepatocytes at 48 h after CCl₄ treatment. Again, this PCN-dependent stimulation of hepatocyte proliferation was not observed in Pxr-null mice. PCN treatment also increased the number of proliferating hepatocytes observed in mice treated with recombinant FGF19. In addition, adenoviral expression of mouse PXR and PCN treatment enhanced EGF-induced proliferation of murine hepatocyte-derived AML cells. These results indicate that PXR enhances hepatocyte proliferation induced by not only chemical substances but also growth factors.

Our group has proposed a possible mechanism for this unique function of PXR on hepatocyte proliferation, where PXR downregulates the expression of a battery of proteins that negatively regulate cell cycle progression based on the following findings (Shizu et al., 2013; Shizu et al., 2016; Yoshinari, 2019). PCN treatment increased the RNA content of quiescent hepatocytes and decreased the hepatic mRNA levels of genes associated with G0/G1 and G1/S checkpoints, including Cdkn1b (p27), Rbl2 (p130), Cdkn1a (p21), Cdkn1c (p57) and Cdkn2a (p19), in wild-type but not Pxr-null mice (Shizu et al., 2013; Shizu et al., 2016). These results are consistent with our findings on the PCN-induced acceleration of hepatocyte proliferation in liver injury model mice (Shizu et al., 2016) and the fact that in p27-deficient mice, long-term phenobarbital treatment following diethylnitrosamine, a representative tumor initiator, significantly increases the number of PCNA-positive hepatocytes and promotes liver tumor formation compared to wild-type mice (Sun et al., 2008). Moreover, we demonstrated that ligand-activated PXR prevented gene expression induced by FOXO3 in AML12 cells and that PCN treatment decreased the mRNA levels of FOXO target genes in wild-type mice but not Pxr-null mice (Shizu et al., 2016). FOXO family members play crucial roles in the regulation of cell cycle suppressor genes (Chen et al., 2006; van der Vos and Coffer, 2011). We thus investigated the influence of PXR activation on the
FOXO3-dependent gene expression of Rbl2 as a model gene for the negative regulators of the cell cycle and found that PXR interacted with FOXO3 to suppress the FOXO3-mediated Rbl2 expression (Shizu et al., 2016). These results suggest that PXR reduces the expression of cell cycle suppressor genes in the liver to increase hepatocytes' sensitivity to various types of proliferating stimuli.

A functional interaction between PXR and YAP was reported by Dr. Bi’s group (Jiang et al., 2019). They demonstrated that PCN treatment of wild-type but not Pxr-null mice increased the number of Ki-67-positive cells and SOX9+ hepatocyte-like cells around the periportal area in the liver and that PXR interacted with YAP by coimmunoprecipitation. Importantly, the distribution of proliferating cells after PCN treatment is different from that observed in the liver of mice treated with phenobarbital or TCPOBOP, which is more evenly observed (Sun et al., 2008; Shizu et al., 2013). These results suggest that PXR activation results in YAP activation to induce the proliferation of a population of cells, at least in murine livers.

Our group has also demonstrated functional crosstalk between PXR and YAP (Abe et al., 2019). PCN treatment of mice increased nuclear YAP levels as much as in the mice treated with TCPOBOP. However, the extent of induced expression of YAP target genes and cell cycle-related genes was much less than that observed in TCPOBOP-treated mice, although human PXR enhanced YAP/TEAD-mediated reporter gene expression in an in vitro system. Taken together with the results by Dr. Bi’s group, the data suggest that PXR interacts with YAP in murine hepatocytes as does CAR, but the consequence is quite different from that observed with CAR, although PXR and CAR act very similarly in gene regulation related to xenobiotic disposition. These differences should be elucidated in future studies.

5. Role of PXR in liver tumorigenesis

Based on our idea about the association between PXR and hepatocyte proliferation, it was thought that PXR could enhance liver tumorigenesis by stimulating hepatocyte proliferation induced by chemical carcinogens, especially those known to be liver tumor promoters such as...
phenobarbital. Our recent findings using 2-stage liver carcinogenesis model mice suggest that this hypothesis is not correct (Shizu et al., 2021).

Male C3H mice were treated with diethylnitrosamine and then with phenobarbital and/or PCN for 20 or 35 weeks to investigate the influence of PCN treatment on preneoplastic lesions as well as liver tumor formation, respectively (Shizu et al., 2021). Immunohistochemical staining of keratin 8/18 (KRT8/18), markers of preneoplastic changes in mouse livers (Kakehashi et al., 2010; Kawai et al., 2010), indicates that phenobarbital markedly increased the areas of KRT8/18-positive hepatocytes and that PCN cotreatment tended to further increase these areas after 20-week treatment. In contrast, the results obtained for the size and frequency of liver tumors after 35 weeks of treatment were surprising. Carcinoma or eosinophilic adenoma was observed in all of the mice treated with phenobarbital, as expected. In mice treated with phenobarbital and PCN, all of the mice also produced carcinoma or acidophilic adenoma, but the size of the carcinoma (mean diameter) and the number of the carcinomas/eosinophilic adenomas was significantly less than that in mice treated with phenobarbital only. In addition, PCNA-positive areas were also significantly reduced by PCN cotreatment. As expected, PCN treatment alone produced neither preneoplastic lesions nor liver tumor. These results suggest that PXR has antitumor effects, at least in CAR-mediated hepatocarcinogenesis models, although it stimulates CAR-mediated hepatocyte proliferation and increases the incidence of preneoplastic lesions during the early phase of carcinogenesis (Fig. 2).

The mechanism underlying the antitumor effects of PXR is currently unknown. One possible mechanism may involve the anti-inflammatory activity of PXR since it is well established that proinflammatory cytokines, including transforming growth factor (TGF)-β, IL-6, TNF-α, monocyte chemotactic protein-1 (MCP-1)/C-C motif chemokine 2 (CCL2), FGF-2, and vascular endothelial growth factor play various roles in the formation of hepatocellular carcinoma, including the promotion of cell survival, cell proliferation, epithelial-mesenchymal transformation (EMT), and angiogenesis (Sun and Karin, 2013; Yang et al., 2019; Rico Montanari et al., 2021).
There are many reports showing that PXR prevents or diminishes the signals and expression of these cytokines. In human hepatocytes, treatment with PXR ligands, including rifampicin and clotrimazole, suppressed 12-O-tetradecanoyl-phorbol-13-acetate-induced expression of nuclear factor-kappa B (NF-kB) target genes such as IL-2, cyclooxygenase-2, NF-kB inhibitor α, and TNF-α (Zhou et al., 2006). Similarly, in primary cultured hepatocytes prepared from wild-type mice, PCN treatment reduced lipopolysaccharide-induced expression of IL-1β, IL-6, and TNF-α, but suppression was not observed in hepatocytes from Pxr-null mice (Sun et al., 2015). Our group has also reported that PXR suppresses the function of NF-kB and activator protein 1 (AP-1) to downregulate the expression of chemokines CCL2 and chemokine (C-X-C motif) ligand 2 (CXCL2) in liver injury model mice through coactivator competition (Okamura et al., 2020). We also reported that PCN treatment attenuated the concanavalin A-induced expression of Nos2, Ccl2, and Cxcl2 and ameliorated concanavalin A-induced liver injury in mice (Kodama et al., 2017). Hu et al. (2010) proposed that SUMOylation of PXR is involved in the PXR-dependent repression of inflammatory signals.

Interestingly, it has also been reported that treatment with proinflammatory cytokines reduces the expression of PXR-target genes such as CYP3A4 and Cyp3a11 in vivo and in vitro (Teng and Piquette-Miller, 2005; Gu et al., 2006; Zhou et al., 2006; Yang et al., 2010; Okamura et al., 2019). These results strongly suggest that PXR and inflammatory signals involving NF-kB and/or AP-1 mutually repress each other. This repressive crosstalk may thus be associated with the antitumor activity of PXR observed in our study (Shizu et al., 2021).

According to our recent findings and related literature, we have proposed that PXR negatively regulates EMT during hepatocarcinogenesis (Shizu et al., 2021). EMT is a crucial event in the dissemination of cancer cells during the progression of hepatocellular carcinoma (van Zijl et al., 2009). It induces the transition of epithelial cells to the mesenchymal phenotype and leads to invasion/migration and the metastasis of transformed cells (van Zijl et al., 2009; Giannelli et al., 2016). Thus, PXR activation may attenuate liver cancer progression by preventing EMT in liver cancer cells. In contrast, activation of PXR in HepG2 cells induced
EMT by downregulating the gene expression of hepatocyte nuclear factor-4α (HNF4α), a key NR involved in liver function (Kodama et al., 2015). HNF4α has been reported to induce a transition to an epithelial phenotype of liver cancer cells, and the downregulation of HNF4α is important for the progression of liver cancer cells (Lazarevich et al., 2004). These results suggest that PXR activation in liver cancer cells may induce the progression of liver tumors by inducing migration and metastasis.

Recently, our group reported that PCN treatment in hepatocarcinogenesis model mice with diethylnitrosamine and phenobarbital clearly attenuated liver cancer progression (Shizu et al., 2021). Basically, EMT of hepatocytes is controlled by cytokine signaling from hepatic stellate cells, Kupffer cells and/or other inflammatory cells (Costa-Silva et al., 2015; Giannelli et al., 2016; Ma et al., 2018; Yan et al., 2018). In addition, the expression levels and transcriptional activity of PXR in liver cancer cells were downregulated (Kotiya et al., 2016), and PXR activation was reported to prevent the activation of hepatic stellate cells and TGFβ secretion (Haughton et al., 2006) and, as mentioned above, inhibit the expression of inflammatory cytokines (Zhou et al., 2006; Hu et al., 2010; Sun et al., 2015; Kodama et al., 2017; Okamura et al., 2020), which is closely related to EMT in hepatocellular carcinoma (Yan et al., 2018). Taken together, PXR may control liver cancer progression by regulating cytokine signals in the tumor microenvironment.

Using 2-step hepatocarcinogenesis model mice as described above, we demonstrated that PCN treatment alone neither increased the expression of cytokeratin-8/-18, murine markers for preneoplastic lesions (Kakehashi et al., 2010; Kawai et al., 2010) at 20 weeks nor induced tumor formation at 35 weeks (Shizu et al., 2021). These results are consistent with our findings that PXR activation alone did not induce the proliferation of mouse hepatocytes in vivo (Shizu et al., 2013; Shizu et al., 2016). Taken together, in contrast to CAR, PXR activation has no liver tumor-promoting effects, although these two receptors have very similar functions during the detoxification of chemicals by regulating the expression of genes encoding drug-metabolizing enzymes and transporters (Fig. 3).
6. CAR and PXR in human liver cancer

6.1. CAR

Although phenobarbital activates both rodent and human CAR to cause enzyme induction in these species, there seem to be clear species differences in phenobarbital-mediated liver tumor formation. While the experimental data clearly indicate that phenobarbital induces liver tumors through CAR in rodents (Whysner et al., 1996; IARC, 2001; Yamamoto et al., 2004; Huang et al., 2005; Elcombe et al., 2014), the epidemiological data strongly suggest that the drug and its related compounds have little association with liver cancer in humans (IARC, 2001; La Vecchia and Negri, 2014; Stritzelberger et al., 2021). Thus, CAR-dependent liver tumor formation, i.e., liver tumor-promoting effects of phenobarbital, is considered to be rodent-specific. These species differences are supported by the results of in vitro and in vivo experiments considering the mode of action (MOA) of CAR-dependent liver tumorigenesis. The MOA proposed for phenobarbital-induced liver tumors is as follows: 1) CAR activation, 2) gene expression alteration specific to CAR activation, 3) increased cell proliferation, 4) formation of altered hepatic foci, and finally, 5) liver tumor development (Elcombe et al., 2014; Yamada et al., 2021). There are a number of associated events, including epigenetic changes, hepatic induction of CYP2B subfamily enzymes, liver hypertrophy, decreased apoptosis, and the inhibition of gap junctions (Elcombe et al., 2014; Yamada et al., 2021).

Considering this MOA, species differences in CAR-mediated liver tumorigenesis are thought to be due to a lack of proliferation of human hepatocytes (Elcombe et al., 2014; Yamada et al., 2021). Thus, many MOA analyses were performed for liver tumor formation in mice and rats with CAR activators, and their human relevance was evaluated by investigating replicative DNA synthesis in rodent and human hepatocytes. The results are comprehensively summarized and reviewed by Yamada et al. (2021). All of the compounds induced DNA synthesis in rodent hepatocytes but not in human hepatocytes, although positive compounds such as EGF and HGF increased DNA synthesis in both rat and human hepatocytes (Yamada et al., 2021).

In addition to in vitro studies, in vivo studies with receptor- or liver-humanized mice
were employed to investigate the human relevance of CAR-mediated hepatocyte proliferation and liver tumor development. In mice in which murine CAR and PXR are replaced with human counterparts, phenobarbital treatment induced hepatic P450 expression (both CYP2B10 and CYP3A11), hepatomegaly, and centrilobular hepatocyte hypertrophy but not DNA synthesis, as indicated by BrdU labeling (Ross et al., 2010). Another study using the same chimeric mice prolonged phenobarbital treatment-induced gene expression related to DNA replication and the cell cycle as well as a proliferation marker gene (Luisier et al., 2014). Moreover, in a two-stage carcinogenesis study with CAR/PXR-humanized mice, phenobarbital treatment increased the number of liver tumors formed, although the extent was much lower than that in wild-type mice (Braeuning et al., 2014). Chimeric mice with humanized livers were also used to demonstrate that phenobarbital did not induce replicative DNA synthesis and had no effects on the mRNA levels of cell cycle-related genes in human hepatocytes, although it induced CYP2B and CYP3A expression (Yamada et al., 2014).

6.2. PXR

Information about the role of PXR in human liver cancers is very limited except that reported using cultured cell lines and humanized animal models. In one report, an association was investigated between the use of anti-tuberculosis drugs, including isoniazid and rifampicin, the latter of which is a representative human PXR ligand, and the risk of hepatocellular carcinoma development in Taiwanese patients with cirrhosis (Lim et al., 2015). The results indicate that treatment with isoniazid and rifampicin for a long time or at high doses is associated with an increased risk of hepatocellular carcinoma development, although whether PXR activation is involved is unclear.

On the other hand, rifampicin was suggested to exhibit antitumor effects through its antiangiogenic properties (Shichiri et al., 2009; Shichiri and Tanaka, 2010). Treatment with low-dose rifampicin among patients with hepatitis C virus-related cirrhosis who are at a high risk of liver cancer resulted in only a single occurrence of hepatocellular carcinoma during the follow-up period (up to 187 months with 149 months of treatment with rifampicin) (Shichiri et
The authors also demonstrated that rifampicin treatment suppressed tumor growth in xenograft model mice implanted with human colon cancer CW-2 cells, as well as angiogenesis in mice implanted with human sarcoma 180 cells (Shichiri et al., 2009). In addition, rifampicin treatment suppressed the expression of genes associated with growth, migration and angiogenesis in cultured human dermal microvascular endothelial cells but not in nonendothelial cells, including HepG2 cells. Since angiogenesis is necessary for the progression and metastasis of most cancer cells and its inhibition is a key therapeutic strategy for liver cancers (Kerbel and Folkman, 2002; Carmeliet, 2005; Zhu et al., 2011; Zhu et al., 2020), additional studies are necessary to evaluate the role of PXR in angiogenesis in liver cancer development and to determine whether PXR can be a novel therapeutic target for liver cancer treatment.

7. Conclusion and Future Perspectives

In accordance with the fact that PXR and CAR are members of the same subfamily (NR1I) of the NR gene superfamily, numerous studies reported to date clearly indicate that these receptors are closely related in their structures and functions in the gene regulation associated with xenobiotic metabolism and disposition. In contrast, studies on the role of these receptors in hepatocyte proliferation and hepatocarcinogenesis strongly suggest that they have distinct roles in liver cancer development (Fig. 2). As phenobarbital is known to be a liver tumor promoter, CAR is a positive regulator of liver cancer development, although it seems rodent-specific, and CAR activation in humans is not associated with liver tumorigenesis. In contrast, we think that PXR activation might negatively regulate liver cancer development through its anti-inflammatory, antiangiogenic and/or anti-EMT activity. To establish the roles of PXR and CAR in liver cancer development at the molecular level, to provide information about the risk assessment of chemicals that produce liver tumors in rodent carcinogenicity tests and to clarify whether PXR can be a therapeutic target for liver cancer, there remain many open questions to be answered: Why and how do PXR and CAR differentially regulate liver tumorigenesis? How does CAR regulate YAP activity? How does
PXR suppress liver cancer development? What is a PXR target gene or protein in terms of its antitumor effects?

To answer these questions and to investigate the detailed mechanisms of hepatocyte proliferation and liver tumorigenesis, animal models are valuable and definitely needed. However, we should keep in mind that there are clearly species differences in the structures of PXR and CAR, especially in their LBDs, which may be important for PPIs with other proteins (Konno et al., 2008; Pavek, 2016; Mackowiak et al., 2018; Yoshinari, 2019), in the doubling time of hepatocytes (Taub, 2004; Michalopoulos, 2007; Utoh et al., 2010; Van Haele et al., 2019), and in CAR-mediated liver tumor promotion (Shizu and Yoshinari, 2020). Chimeric mice with humanized liver (Katoh et al., 2008; Yoshizato and Tateno, 2009; Tsuge, 2021) and recent advances in techniques for hepatocyte culture, including microphysiological systems (Ishida, 2018; Kyffin et al., 2018; Polidoro et al., 2021), may help to resolve these questions.

Conflicts of interest

The authors declare no conflicts of interest.

Authorship Contributions

Participated in research design: Yoshinari and Shizu.
Conducted experiments: Yoshinari and Shizu.
Performed data analysis: Yoshinari and Shizu.
Wrote the manuscript: Yoshinari and Shizu.

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**Legends to figures**

**Figure 1. Species differences in phenobarbital-induced/CAR-mediated liver tumor development.** Although phenobarbital activates both rodent and human CAR to induce the expression of genes associated with xenobiotic metabolism and disposition (Honkakoski et al.,
1998; Wei et al., 2000), it induces hepatocyte proliferation and liver tumor formation only in rodents (IARC, 2001; Elcombe et al., 2014).

**Figure 2. Distinct roles of CAR and PXR in liver tumor development in rodents.** CAR activation induces hepatocyte proliferation and consequently liver tumor formation in rodents (IARC, 2001; Yamamoto et al., 2004; Huang et al., 2005; Elcombe et al., 2014). Studies with mouse models suggest that PXR activation enhances CAR-mediated hepatocyte proliferation but suppresses CAR-mediated liver cancer development (Shizu et al., 2013; Shizu et al., 2016; Shizu et al., 2021).

**Figure 3. Similarity and dissimilarity of the roles of CAR and PXR in enzyme induction and liver tumor development.** While CAR and PXR have very similar functions in the gene expression associated with xenobiotic metabolism and disposition (Willson and Kliwer, 2002; Handschin and Meyer, 2003; Timsit and Negishi, 2007), they act as dissimilar regulators for liver tumor development. Studies with rodent models indicate that only CAR but not PXR is associated with liver tumor formation (IARC, 2001; Yamamoto et al., 2004; Huang et al., 2005; Elcombe et al., 2014; Shizu et al., 2021).
Figure 1

**Rodent liver**

- Phenobarbital → Activation → CAR → Enzyme induction (↑)
- Phenobarbital → Activation → CAR → Proliferation (↑)
- Phenobarbital → Activation → CAR → Tumor formation (↑)

**Human liver**

- Phenobarbital → Activation → CAR → Enzyme induction (↑)
- Phenobarbital → Activation → CAR → Proliferation (✗)
- Phenobarbital → Activation → CAR → Tumor formation (✗)
Figure 3

**Enzyme induction**

- **CAR** → Activation → Cyp2b
- **PXR** → Activation → Cyp3a

**Carcinogenesis**

- **CAR** → Activation → Proliferation → Anti-apoptosis → Tumor formation
- **PXR** → Anti-apoptosis