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DRUG METABOLISM & DISPOSITION

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Special Section on Xenobiotic Receptors—Minireview

Regulation of Nuclear Receptors PXR and CAR by Small Molecules and Signal Crosstalk: Roles in Drug Metabolism and Beyond

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ABSTRACT

Pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are ligand-activated transcription factors that regulate the expression of drug metabolizing enzymes and drug transporters. Since their discoveries, they have been studied as important factors for regulating processes related to drug efficacy, drug toxicity, and drug-drug interactions. However, their vast ligand-binding profiles extend into additional spaces, such as endogenously produced chemicals, microbiome metabolites, dietary compounds, and environmental pollutants. Therefore, PXR and CAR can respond to an enormous abundance of stimuli, resulting in significant shifts in metabolic programs and physiologic homeostasis. Naturally, PXR and CAR have been implicated in various diseases related to homeostatic perturbations, such as inflammatory bowel disorders, diabetes, and certain cancers. Recent findings have injected the field with new signaling mechanisms and tools to dissect the complex PXR and CAR biology and have strengthened the potential for future PXR and CAR modulators in the clinic. Here, we describe the historical and ongoing importance of PXR and CAR in drug metabolism pathways and how this history has evolved into new mechanisms that regulate and are regulated by these xenobiotic receptors, with a specific focus on small molecule ligands. To effectively convey the impact of newly emerging research, we have arranged five diverse and representative key recent advances, four specific challenges, and four perspectives on future directions.

SIGNIFICANCE STATEMENT

PXR and CAR are key transcription factors that regulate homeostatic detoxification of the liver and intestines. Diverse chemicals bind to these nuclear receptors, triggering their transcriptional tuning of the cellular metabolic response. This minireview revisits the importance of PXR and CAR in pharmaceutical drug responses and highlights recent results with implications beyond drug metabolism.

Introduction

It has been estimated that the research and development costs of a single drug are approximately 2.6 billion US dollars, and the time between initiation of a drug discovery program and resulting clinical approval is roughly a decade. Furthermore, only ∼10% of drugs that enter clinical testing will eventually be approved (DiMasi et al., 2016). Clinical trial failures occur for numerous reasons, including scientific factors (e.g., safety issues or lack of efficacy) and procedural faults (e.g., insufficient funding, failure to follow regulatory guidance, or problems with patient recruitment, enrollment, and retention) (Fogel, 2018). Maximizing efficiency is clearly a major financial and temporal concern in drug development processes, both scientifically and administratively. Physiologic disposition of drugs largely determines clinical efficacy and is therefore an important component in the drug design process (Zhang and Tang, 2018). Enhanced knowledge of drug metabolism and elimination pathways has led to safer drugs, but evaluation methods are in continual optimization. In this review, we will discuss the roles of two nuclear receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), in regulating drug disposition processes, how their biologic activities are integrated into drug development platforms, and potential for PXR and CAR modulators in drug metabolism and human diseases.

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ABBREVIATIONS: AF-1, activation function 1; AF-2, activation function 2; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2, 1-b][1, 3]thiazole-5-carbaldehyde-O-(3, 4-dichlorobenzyl)oxime; CYP, cytochrome P450; CYP3A4, cytochrome P450 family 3 subfamily A member 4; DBD, DNA-binding domain; FDA, US Food and Drug Administration; GTEx, Genotype-Tissue Expression; IBD, inflammatory bowel disease; LBD, ligand-binding domain; MDR1, multidrug resistance protein 1; PBREM, phenobarbital-responsive enhancer module; PCN, pregnenolone 16α-carbonitrile; PXR, pregnane X receptor; RXR, retinoid X receptor; SPA70, specific PXR antagonist 70; SRC-1, steroid receptor coactivator 1; TCPOBOP, 1, 4-bis[2-(3, 5-dichloropyridyloxy)] benzene; Teff, effector T cell; WT, wild-type; XREM, xenobiotic-responsive enhancer module.

In seeking drug approval, the US Food and Drug Administration (FDA) requires evaluation of drug-drug interaction potential by "(1) identifying the principal routes of the drug's elimination; (2) estimating the contribution of enzymes and transporters to the drug's disposition; and (3) characterizing the effect of the drug on enzymes and transporters" (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions). Drug candidates should be assayed for interaction with, metabolism by, and inhibition or induction of various metabolism-related proteins, which occurs mainly in liver and intestine. Importantly, PXR and CAR are primarily expressed in the liver and gastrointestinal tract (Nishimura et al., 2004; Petrick and Klaassen, 2007) and are key transcription factors that govern the expression of genes encoding drug metabolizing enzymes including cytochrome P450s (CYPs) (Forman et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999; Sueyoshi et al., 1999), UDP-glycosyltransferases (Sugatani et al., 2001, 2004), glutathione-S-transferases (Knight et al., 2008), sulfotransferases (Yetti et al., 2018), and drug transporters such as multidrug resistance protein 1 (MDR1) (Geick et al., 2001; Synold et al., 2001; Burk et al., 2005). A regulated gene set of particular note is the CYP3A family due to its known activity on the majority of clinically applied drugs (Zhai et al., 2022). PXR and CAR binding to chemicals results in subsequent nuclear translocation, binding to target gene promoters, and enhanced transcription. The receptors have large, flexible ligand binding pockets (particularly in the case of PXR) and display great promiscuity in their ligand binding profiles (Buchman et al., 2018). Because diverse chemicals can activate PXR and CAR and the downstream drug metabolism pathways, evaluation of gene induction through these mechanisms is standard in drug development pipelines (Jones et al., 2017).

PXR and CAR are recognized as key players in drug safety and efficacy, and their primary links to major metabolic enzymes had a profound impact on drug development strategies. However, a combination of historical and newly emerging data suggests these receptors also influence biology in additional manners. These new findings are grounded in the traditional drug metabolism knowledge but come to light in specific contexts. Thus, it seems that these receptors that are nearing three decades since their discoveries (1994 for CAR and 1998 for PXR) still have fresh biologic insights to give. Because the drug responsive roles of PXR and CAR have recently been reviewed extensively elsewhere (Elmeliegy et al., 2020; Hall et al., 2021; Skandalaki et al., 2021; Honkakoski, 2022; Karpale et al., 2022; Liu et al., 2022; Stanley and Wolf, 2022), in the following sections we will briefly describe the continuing importance of PXR and CAR in drug development efforts and then focus on the natural evolution of studies beyond drug metabolism with an emphasis on recent reports.

Brief Historical Perspective

PXR and CAR are members 2 and 3 of nuclear receptor subfamily 1, group I (NR1I2 and NR1I3, respectively). PXR was first reported in 1998 as a main regulator of CYP family 3 subfamily A member 4 (CYP3A4) transcription in response to structurally diverse chemicals (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). CAR was first described in 1994 and later found to be a transcriptional regulator of CYP2B6 (Baes et al., 1994; Sueyoshi et al., 1999; Xie et al., 2000b). Subsequent studies identified numerous additional target genes for both receptors. PXR and CAR have the conventional modular nuclear receptor structure consisting of activation function 1 (AF-1), DNA-binding domain (DBD), hinge, ligand-binding domain (LBD), and activation function 2 (AF-2) (Fig. 1A). They also use the basic receptor transcriptional regulatory mechanisms with the general steps of ligand binding, nuclear translocation, dimerization with retinoid X receptor (RXR), binding to response elements in target gene promoters, recruitment of nuclear receptor coactivator (e.g., steroid receptor coactivator 1 [SRC-1]), and recruitment of transcription machinery (Baes et al., 1994; Choi et al., 1997; Bertilsson et al., 1998; Lehmann et al., 1998; Muangmoonchai et al., 2001) (Fig. 1B). Both receptors can bind to phenobarbital-responsive enhancer module (PBREM) sites or xenobiotic-responsive enhancer module (XREM) sites, but CAR binds preferentially to PBREM and PXR binds preferentially to XREM (Honkakoski et al., 1998; Goodwin et al., 1999; Goodwin et al., 2001, 2002). Furthermore, since PXR and CAR can both be triggered by the same ligands, activation of redundant sets of genes can lead to the generation of coordinated processes for the elimination of toxins (Moore et al., 2000; Xie et al., 2000b).

While PXR and CAR are both generally classified as xenobiotic receptors, PXR is significantly more promiscuous in ligand binding than CAR and is more often discussed in drug development programs (Moore et al., 2000, 2002; Chai et al., 2020). The difference can be explained by the size of the ligand binding pockets (1200–1600 $Å^3$ for PXR *versus*. ~600 $Å^3$ for CAR), and the apparent flexibility of the "floor" of the PXR ligand binding pocket (Buchman et al., 2018). Accordingly, most CAR ligands bind PXR, but not vice versa (Moore et al., 2000). Even ligands that were once thought to be CAR-selective, such as 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO), have been found to be PXR binders (Maglich et al., 2003; Lin et al., 2020a). Because of the apparent overlapping cellular roles of PXR and CAR, more selective molecules are important to biologically differentiate the two receptors, and recent efforts have yielded such chemicals, such as DL5050 (Liang et al., 2019). Conversely, inverse agonists are relatively abundant for CAR, but not so for PXR. Inverse agonists bind to a receptor and induce recruitment of corepressor rather than coactivator, thereby reducing target gene expression. The high ligand-independent activity of CAR compared with PXR makes CAR a good target for inverse agonists (Kublbeck et al., 2011; Carazo and Pavek, 2015; Cherian et al., 2015, 2016, 2018). Representative ligands for each receptor are shown in Figs. 2 and 3.

A major concern of in vivo drug metabolism studies is species-specific responses. Preclinical drug efficacy and safety studies rely heavily on rodent models, but these models have inherently different metabolic responses to xenobiotics. The prototypical PXR agonist rifampicin effectively activates human, but not mouse, PXR, and pregnenolone 16α-carbonitrile (PCN) activates mouse, but not human, PXR. The potent mouse CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) has little effect on human CAR, and the human CAR agonist CITCO only weakly activates mouse CAR (Moore et al., 2000; Omiecinski et al., 2011). These selective effects make metabolic events such as drug-drug interactions difficult to predict. Accordingly, substantial work has been aimed at humanizing drug metabolism responses in mice. The first humanized PXR model used genetic ablation of the mouse *Pxr* gene followed by transgenic overexpression of human PXR from a liver-specific promoter, resulting in a loss of PCN-mediated CYP induction and gain of rifampicin response (Xie et al., 2000a; Staudinger et al., 2001). Various methods have since been used to generate transgenic models with different PXR or CAR expression patterns and human receptors coupled with human CYP3A (Wei et al., 2000; Robertson et al., 2003; Zhang et al., 2003; Saini et al., 2004; Yu et al., 2005; Cheung et al., 2006; Gong et al., 2006; Ma et al., 2007a, 2008; Scheer et al., 2008; Igarashi et al., 2012; Ly et al., 2017; Niu et al., 2018). These models have been instrumental in our understanding of physiologic drug metabolism pathways as well as diseases associated with pathway modulation; for instance, early experiments in *Pxr*-null or *Car*-null mice showed that PXR and CAR are involved in acetaminophen-induced liver toxicity (Zhang et al., 2002; Guo et al., 2004).

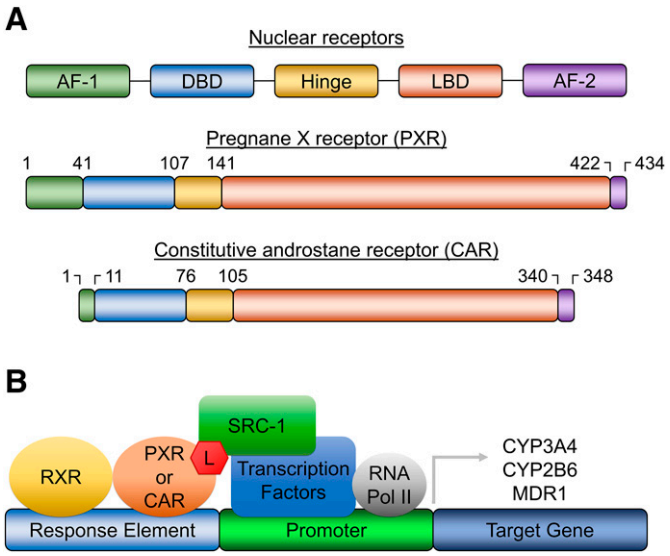

Fig. 1. (A) General schematic of nuclear receptor domains and schematics of PXR and CAR domains with numbered amino acid positions. (B) Schematic of PXR and CAR transcriptional transactivation. PXR and CAR are activated by ligands (L), resulting in heterodimerization with RXR, recruitment of coactivator (SRC-1), assembly of additional transcription factors, and transcription by RNA polymerase II.

Although PXR and CAR are often studied in the context of drug metabolism, they are also implicated in various homeostatic processes and diseases. This is not surprising, as their ligand-binding diversity applies to endobiotics as well as xenobiotics, and their downstream gene products alter cellular chemical makeups. For example, PXR and CAR prevent liver damage from endogenous chemicals such as bile acids by regulating the expression of genes involved in biosynthesis, transport, and metabolism of these substances (Staudinger et al., 2001; Zhang et al., 2004). Additional physiologic processes and diseases associated with PXR and CAR include glucose metabolism, obesity, diabetes, intestinal barrier function, inflammatory bowel disease (IBD), and general liver physiology and associated diseases such as alcoholic liver disease (Ma et al., 2007b; Shah et al., 2007; Gao et al., 2009; Spruiell et al., 2014; Venkatesh et al., 2014; Oladimeji et al., 2017; Uehara et al., 2019; Cai et al., 2021). Positive and negative regulation of PXR and CAR have both been found to have either physiologic detriments or benefits, highlighting the importance of both overactivity and underactivity in specific contexts. Collectively, these published results strongly indicate value for PXR- and CAR-targeting small molecules in modulating drug metabolism responses as well as treating diseases.

Key Recent Advances

To give a broad overview of current research in the PXR and CAR fields, we have arranged five vignettes to highlight assorted recent findings with significant implications beyond drug responses. While the scientific impacts of the findings are yet to be fully realized, we believe that these developments will shape future studies of the xenobiotic receptors in multiple directions.

Vignette 1: Identification of a Biologically Active PXR Inverse Agonist. Because of its xenobiotic-sensing function, PXR activation can induce undesirable physiologic effects such as drug-drug interactions or drug inefficacy. Therefore, PXR antagonists or inverse agonists may have significant therapeutic value as administered codrugs (Mani et al., 2013; Hall et al., 2021). Enhancing drug efficacy by cotreating with inhibitors of metabolic enzymes is a common practice, exhibited by Pfizer's SARS-CoV-2 drug Paxlovid, which is a combination of a direct acting antiviral and the CYP3A inhibitor ritonavir (Mahase, 2021). However, inhibition of the upstream transcription factors is not currently a clinically used practice, and PXR antagonists have historically been difficult to identify because of PXR's propensity to be activated when bound by ligands. Until recently, only a few chemicals had been studied as PXR antagonists, and these compounds were nonspecific and inactive in vivo (Wang et al., 2007; Mani et al., 2013; Poulton et al., 2013). In 2017, Lin et al. reported the inverse agonist SPA70 (specific PXR antagonist 70), which inhibits basal PXR activity and blocks activation by exogenously added PXR agonists. SPA70 is highly specific to PXR over other nuclear receptors, exhibits little cytotoxicity, enhances activity of chemotherapeutic agents, and importantly, is active in

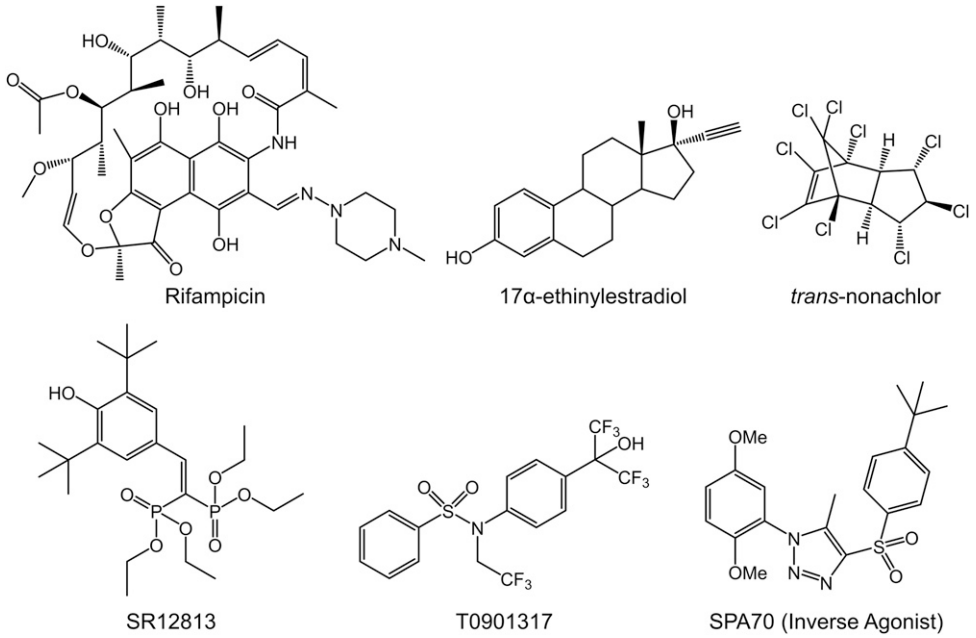

Fig. 2. Representative xenobiotic ligands for human PXR. All ligands are agonists with the exception of SPA70, which is an inverse agonist.

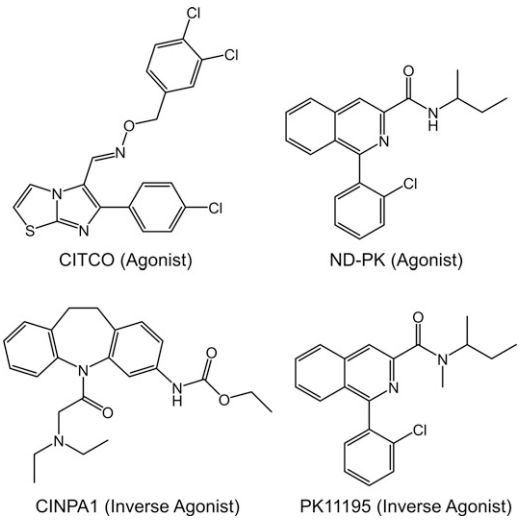

Fig. 3. Representative xenobiotic ligands for human CAR. The chemical structures of two agonists and two inverse agonists are shown. PK11195 is a synthetic inverse agonist that is metabolically converted by CYP3A4 in hepatocytes to the agonist ND-PK (Mackowiak et al., 2017).

humanized PXR mouse models (Lin et al., 2017a,b). SPA70 has since been used in various studies and has been shown to prevent PXR-mediated hemorrhagic shock-induced liver injury (Xie et al., 2019).

Vignette 2: Combinations of Environmental Chemicals Can Synergistically Bind PXR. Humans are constantly exposed to a variety of xenobiotic molecules such as pharmaceuticals, plant components, cosmetics, fragrances, toxic materials, metals, food preservatives, and environmental chemicals. Humans are potentially exposed to millions of xenobiotic substances during their lifespans. While physiologic metabolism processes render many of these chemicals less toxic, there are numerous examples of metabolic events making xenobiotics more chemically reactive, toxic, or carcinogenic (Wogan et al., 2004; Idle and Gonzalez, 2007; Patterson et al., 2010). In 2007, the United States Environmental Protection Agency described the "ToxCast" program to develop methods and assays to forecast toxicity of environmental chemicals (Dix et al., 2007), and in 2010, PXR was found to be associated with in vivo perturbations by these chemicals that could potentially lead to human disease (Judson et al., 2010; Kortagere et al., 2010). Importantly, it was shown in 2015 that a synthetic estrogen and a pesticide chemical cooperatively bind to PXR, leading to PXR activation. Both chemicals in this study (17α-ethinylestradiol and *trans*-nonachlor) bind only weakly to PXR when used alone, but together, they make a potent PXR-binding "cocktail" (Delfosse et al., 2015). This cocktail model was later expanded to additional molecules and demonstrates that chemical mixtures may alter physiology at concentrations where individual components are considered safe (Delfosse et al., 2021).

Vignette 3: PXR Is Responsive to Natural Products of the Human Gut Microbiome. The human gut microbiome contains hundreds of commensal microbial species with biochemical capabilities that help regulate normal digestive function (Qin et al., 2010; Javdan et al., 2020). These processes generate a diverse array of metabolites that are often distinct from human-derived metabolites and can thus impact human physiology in unique manners (Backhed et al., 2005; Koppel et al., 2017). Because of its intestinal residence and ligand-binding promiscuity, PXR was hypothesized to respond to such metabolites. The ensuing 2014 study focused on indole and its metabolites that are exclusively produced by intestinal bacteria and found that both CAR and PXR are activated by these compounds, but PXR responds to a greater extent. Through this mechanism, PXR was found to protect against inflammatory intestinal injury (Venkatesh et al., 2014), strengthening the line of evidence connecting PXR to IBD (Cheng et al., 2012). In two 2020 follow up studies, synthetic indole derivatives were reported as noncytotoxic PXR agonists that reduce colitis in an acute colitis mouse model. Not only was this impactful for a novel method of PXR ligand discovery, but it was a proof-of-concept study for a general "microbial metabolite mimicry" drug discovery method (Dvořák et al., 2020a,b; Illes et al., 2020). Consistent with these results, a separate independent 2021 report found that PXR transcriptional activity is strongly affected by the absence of gut microbes (Barretto et al., 2021). The collective results pinpoint PXR-microbiome interplay as a potential mechanism for unexpected drug-drug or food-drug interactions.

Vignette 4: PXR and CAR Physically Interact in a Mutually Inhibitory Manner. PXR and CAR have long been known to have both shared and distinct gene targets, resulting in a dual xenobiotic sensing mechanism with a partially overlapping metabolic response (Xie et al., 2000b; Li et al., 2015; Cui and Klaassen, 2016). However, unexpected findings over the past two decades suggested that PXR and CAR have a complex interreceptor relationship (Staudinger et al., 2001; Saini et al., 2005; Park et al., 2012). For example, detoxifying enzymes and transporters were found to be upregulated in *Pxr*-null compared with wild-type (WT) mice, and this upregulation was lost when CAR was also deleted (Saini et al., 2005). Data from PXR, CAR, and additional nuclear receptors indicated that receptors may modulate the activities of other receptors by competing for common cofactors, such as coactivators (Pavek, 2016). In 2022, Bwayi et al. reported the surprising observation that PXR and CAR physically interact with each other, resulting in their mutual inhibition. Cellular and biophysical results indicated that the interaction occurs at the RXR dimerization interface on the LBD of each receptor, and the PXR-CAR heterodimer could be disrupted by coexpression of RXR, thereby restoring PXR and CAR activities (Bwayi et al., 2022). This finding gives mechanistic insights into a historical observation and opens new avenues to study receptor crosstalk in xenobiotic responses. Because of the observed inhibitory mechanism, we might expect that the combined activity of PXR and CAR in vivo depends on the expression level of each receptor. Specifically, higher PXR:CAR ratio may result in a dominantly PXR xenobiotic response with corresponding repression of the CAR program, and the reverse may be expected of a higher CAR:PXR ratio.

Vignette 5: CAR Regulates Intestinal CD4+ Effector T (T_{eff}) Cell Homeostasis. MDR1 is a well-characterized transmembrane transporter found in many normal human tissues and malignancies. MDR1 is implicated in innate and acquired drug resistance and plays a key role in drug distribution and excretion (Chen et al., 1994; Smit et al., 1998), and its gastrointestinal expression has long been known to be influenced by PXR and CAR activities (Geick et al., 2001; Synold et al., 2001; Burk et al., 2005). In 2017, MDR1 was shown to regulate efflux of toxic bile acids from T_{eff} cells resident in the small intestine lamina propria. Without MDR1, intestinal T_{eff} cells incurred oxidative stress, resulting in an inflammatory phenotype that induced Crohn's disease-like ileitis (Cao et al., 2017). In 2021, CAR activation by bile acids was identified as the source of MDR1 expression in these T_{eff} cells, and CAR activation also resulted in increased T_{eff} detoxifying enzymes and anti-inflammatory cytokines, thereby protecting the small intestine against bile acid-induced toxicity and inflammation. Accordingly, CAR deficiency in T_{eff} cells caused ileitis as in the MDR1-deficient cells (Chen et al., 2021). These studies highlight the importance of CAR in maintaining homeostasis of cells other than the normally discussed metabolic cells like hepatocytes

and enterocytes and further strengthens CAR as a vital player in IBD.

Current Challenges and Knowledge Gaps

Remarkable progress has been made in understanding the roles of PXR and CAR in maintaining physiologic homeostasis. However, significant questions and blockades are still prevalent in the fields. Although we cannot fully describe the present challenges in PXR and CAR research, we have identified four significant areas for future improvement.

Challenge 1: Dissection of Mechanisms of PXR Agonism Versus Antagonism. As a xenobiotic receptor, PXR binds a large variety of chemicals with low affinities to produce a large metabolic response (Stanley et al., 2006). Integration of PXR evaluation into drug development pipelines has led to a significant decrease in PXR activators in the clinic (Yu et al., 2018; Hall et al., 2021). Current strategies rely on chemically modifying lead compounds to retain potency for the intended target while reducing PXR binding, which is costly and time-consuming (Hall et al., 2021). An alternative approach to this process could be cotreating with a PXR antagonist, but development of such inhibitors comes with its own challenges. Since SPA70 was reported as a specific PXR inverse agonist, early chemical derivatization efforts yielded only agonists and weaker antagonists (Lin et al., 2017b; Li et al., 2021a). The difficulty in achieving PXR inhibition can be visualized in three representative molecules (Fig. 4). SPA70, SJC2, and SJB7 are highly chemically similar synthetic compounds that bind PXR LBD with similar potency. However, SPA70 is an inverse agonist, SJC2 is a neutral antagonist, and SJB7 is an agonist. A similar phenomenon has been shown for CAR, where the inverse agonist PK11195 is metabolically converted by CYP3A4 in hepatocytes to the CAR agonist ND-PK (Fig. 3) (Mackowiak et al., 2017). Pinpointing the mechanisms of such biologic differences is not trivial, and the insights may be limited to each specific pharmacophore. Huber et al. have performed mutagenesis and quantitative molecular dynamics studies to confront this problem for PXR and identified distinct AF-2 conformations adopted by each molecule class (agonist, neutral antagonist, inverse agonist) (Huber et al., 2021). Combinatorial chemistry, biology, biochemical, structural, and computational efforts will be necessary to fully characterize the structure-activity relationship and generalize results to multiple chemical scaffolds.

Challenge 2: Chemical Selectivity Between Human PXR/CAR and Mouse PXR/CAR. Studies to differentiate the biologic roles of PXR and CAR are commonly performed in knockout mouse models or cell lines. However, genetic removal of one receptor drastically alters the activity of the remaining receptor (Saini et al., 2005; Bwayi et al., 2022). A multitude of genes are either up- or downregulated in PXR-knockout HepaRG cells compared with WT cells, signifying a shift in cellular homeostasis in response to PXR depletion. Enzymes such as CYP3A4, CYP2B6, and CYP2C9 are increased upon PXR knockout, and this has been shown to be a result of PXR-CAR interactions (Bwayi et al., 2022). To obtain more physiologically relevant results, pharmacological manipulation of the receptors in their natural settings is preferable. The field has generated ligands specific to PXR over CAR (e.g., rifampicin as agonist and SPA70 as inverse agonist), but CAR ligands are generally PXR binders. CITCO, once believed to be a CAR-specific agonist, was found to be a PXR agonist (Lin et al., 2020a), and the CAR inverse agonist CINPA1 was also found to be a weak PXR agonist at high concentrations (Cherian et al., 2015; Jeske et al., 2017; Toporova et al., 2020). Although some specificity can be derived from tuning the ligand concentration (e.g., using a low concentration of CITCO that will theoretically activate CAR but not PXR), this method may not be reliable due to different cell systems having different CAR-to-PXR ratios that will change the ligand response. Importantly, the CITCO analog DL5050 was recently shown to have markedly reduced PXR activity and enhanced CAR activity compared with CITCO (Liang et al., 2019). Development of highly specific chemicals is both important and challenging, as seemingly minor effects in routine profiling assays, such as HepG2-based PXR- or CAR-sensitive luciferase reporters, can translate to larger activation events in more physiologically relevant models like primary human hepatocytes. Efforts are further confounded by species selectivity, and chemicals should be tested across additional species to avoid potentially contaminating results in humanized mouse models by activation or inhibition of a mouse receptor that is still present.

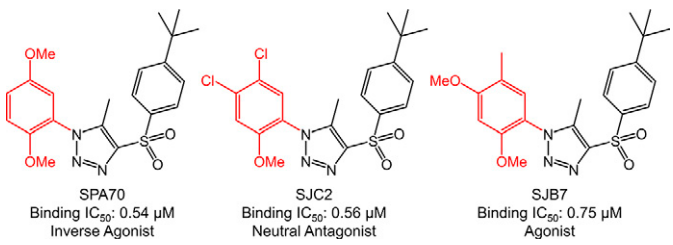

Fig. 4. Structurally similar compounds have varying PXR activities. Three related compounds are shown that have similar PXR LBD binding potencies but different cellular outcomes (inverse agonist, neutral antagonist, or agonist). PXR LBD binding IC_{50} values are from Lin et al. (2017b).

Challenge 3: Sexually Dimorphic PXR and CAR Activities. Sex influences a multitude of physiologic processes and diseases, including the incidence of disease and treatment outcome (Credendino et al., 2020). Women experience adverse drug reactions nearly twice as often as men, and this can be attributed, at least in part, to differences in pharmacokinetics (Moyer et al., 2019; Zucker and Prendergast, 2020). The National Institutes of Health (NIH) Revitalization Act of 1993 required that women be included in clinical drug research, but it was not until 2015 that the NIH released a policy expanding the sex factor into vertebrate animal studies (Waltz et al., 2021). It has long been known that PXR and CAR transcriptional networks are subject to sex biases, resulting in gender-dependent expression of genes such as the CYP3A family (Yoshinari et al., 2001; Hernandez et al., 2009; Thangavel et al., 2011, 2013). Interestingly, the 2021 study on the PXR-microbiome interaction referenced in "Vignette 3" revealed that "most microbiota-sensitive genes were PXR-dependent in the liver in males, but not in females" (Barretto et al., 2021). A host of studies has indicated the importance of analyzing the PXR and CAR networks in sex-dependent fashions due to their sexually dimorphic natures, thereby complicating in vivo studies of drug metabolism and PXR/CAR homeostatic regulation. In fact, even primary hepatocytes derived from humans revealed sex-dependent differences in PXR activity, exhibiting the importance of analyzing cells extracted from both males and females (Thangavel et al., 2011, 2013). Future studies should fully consider the biologic outcomes of PXR and CAR modulation on in vivo and ex vivo models.

Challenge 4: Context-Specific PXR and CAR Modulation. Both positive and negative modulation of PXR and CAR have potential therapeutic applications, depending on context. For instance, PXR agonists may be useful for treatment of IBD while antagonists may help increase codrug efficacy. The therapeutic benefit of PXR agonists can be illustrated by rifaximin, a nonorally bioavailable rifampicin analog that achieves high intestinal concentrations due to poor uptake. Rifaximin is an antibiotic approved for treatment of IBD; however, a series of experiments performed in PXR humanized mouse models suggests that the therapeutic effect may be due to rifaximin-mediated PXR activation (Ma et al., 2007b; Cheng et al., 2010; Mencarelli et al., 2011). Furthermore, treatment of nonhumanized mice with the mouse-specific PXR agonist PCN are protected from colitis in an acute colitis model (Shah

et al., 2007). PXR antagonists, on the other hand, would be beneficial in cases of liver injury or drug inefficacy. PXR activation was previously shown to sensitize humanized mice to hemorrhagic shock-induced liver injury, and the antagonist SPA70 attenuated the effect (Xie et al., 2019). Additionally, Lin et al. previously found that SPA70 enhanced activity of the known PXR agonist paclitaxel on a PXR-expressing colon cancer cell model, indicating that PXR inhibition can increase the availability of a chemotherapy drug (Lin et al., 2017b). An interesting aspect of therapeutic considerations could be disease-related dysregulation of PXR and CAR pathways. While PXR is normally only expressed in tissues such as liver and intestine, it becomes highly expressed in tumors of additional tissues, such as the pancreas [Fig. 5; RNA-seq data derived from the Genotype-Tissue Expression (GTEx) Project and the cBio Cancer Genomics Portal (Cerami et al., 2012; Gao et al., 2013)]. Therefore, treatments directed at pancreatic cancers may benefit from cotreatment with a PXR antagonist. CAR expression and pharmacological activation has been linked to liver tumorigenesis in both suppressive and stimulatory directions, indicating a critical balance between CAR activity and cell proliferation (Huang et al., 2005; Li et al., 2022). These examples highlight the importance of contextual studies. Future elucidations of the interplay between xenobiotic receptor activities and specific physiologic contexts will allow drugs to be developed to modulate PXR and CAR at precise biologic interfaces.

Perspective on Future Directions

The future of xenobiotic receptor research is open to numerous directions, from classic drug metabolism modulation to discovery of new biologic pathways. This is evident in the representative recent discoveries and challenges described above. To narrow the expansive view of possibilities, we describe below four specific examples of future explorations.

Perspective 1: Further Evaluation of PXR-Microbiome and CAR-Microbiome Interplay. The first studies of PXR agonism by intestinal microbial metabolites focused on indole and its derivatives, which are products of tryptophan metabolism (Venkatesh et al., 2014; Dvořák et al., 2020a; Illes et al., 2020). Additional classes of microbial metabolites, such as bile acids, short-chain fatty acids, branched-chain amino acids, and trimethylamine N-oxide are possible sources of intestinal PXR modulation (Agus et al., 2021), and bile acids have previously been shown to be agonists of both PXR and CAR (Staudinger et al., 2001; Chen et al., 2021). Therefore, additional metabolites may be PXR or CAR activators. Structural studies could shed light on the binding mechanisms and aid in predicting the binding potential of other metabolites, but the small, hydrophobic nature of the currently known binders coupled with the micromolar range binding affinities may make structure determination difficult. Though the concept of "microbial metabolite mimicry" was previously used to synthesize indole-based PXR agonists, the same process could theoretically be used to derive antagonists, although we have discussed the historical difficulty in obtaining PXR antagonists. Importantly, various microbe-derived molecules have been implicated in the pathogenesis of metabolic disorders (Agus et al., 2021). Because the PXR-microbiome and CAR-bile acid relationships were found to regulate intestinal barrier integrity by two distinct mechanisms (Venkatesh et al., 2014; Chen et al., 2021), we may consider the possibility that PXR or CAR interactions with microbial products play parts in said metabolic disorders.

Perspective 2: Expansion of the PXR Ligand Cocktail Model. Observations that two chemicals can synergistically bind and activate PXR open the door for new xenobiotic interaction investigations. While the current results are limited to a subset of pharmaceutical compounds and environmental pollutants (Delfosse et al., 2015; Delfosse et al., 2021), one can envision endless combinatorial studies. With the

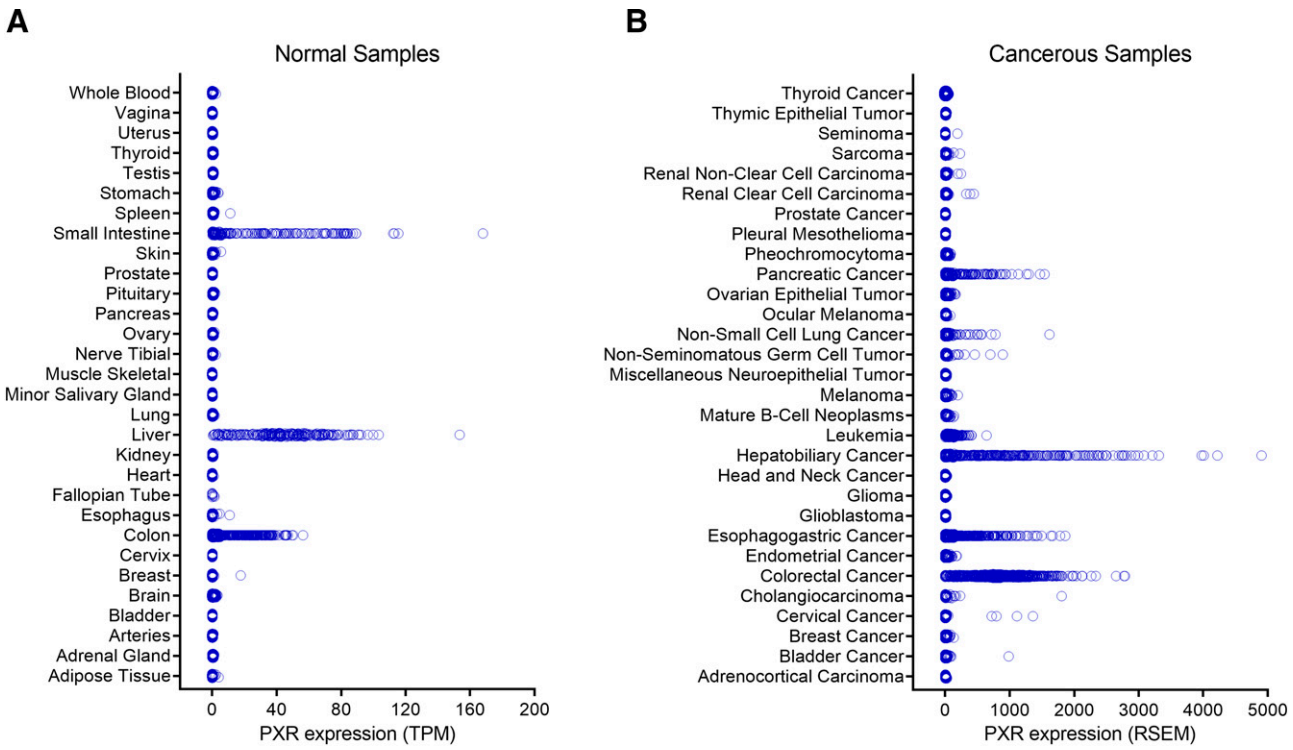

Fig. 5. PXR is differentially expressed upon cancer development in certain tissues. RNA-seq data are presented for the expression of PXR in (A) normal and (B) cancerous patient samples. Normal samples are from the Genotype-Tissue Expression (GTEx) Project, and cancerous samples are from the cBio Cancer Genomics Portal. Data were extracted from GTEx Portal and cBioPortal on 07/13/2022 and are presented in their respective native database units: GTEx data are presented as transcripts per million (TPM), and cBioPortal data are presented as RNA-seq by expectation maximization (RSEM).

demonstration that PXR can bind metabolites of resident intestinal bacteria (Venkatesh et al., 2014), an enticing prospect is the analysis of microbial metabolite combinations with xenobiotic substances. For example, can a drug such as 17α-ethinylestradiol synergistically bind and activate PXR with a metabolite such as indole, as it does with *trans*-nonachlor? Binding events like these could have profound effects on drug pharmacokinetics that are difficult to foresee because they would be influenced by factors such as diet, opening the door to questions of drug interactions with dietary supplements or various other nutritional sources. Furthermore, the previous reports focused on synergistic PXR agonists, but can the same approach be used to discover antagonistic cocktails? The synergistic cocktail model is a significant finding and will likely have great impact on future studies, including the realm of drug-drug interactions.

Perspective 3: Translational Assessment of PXR Antagonists in Disease Models. Lin et al. described SPA70 as a specific PXR antagonist that blocks PXR activation in a humanized mouse model (Lin et al., 2017b), and SPA70 has been used in multiple studies to exhibit antagonism of diverse PXR agonists (Li et al., 2019; Creusot et al., 2020; Lin et al., 2020a; Li et al., 2021b). Importantly, SPA70 was shown to protect humanized PXR mice from hemorrhagic shock-induced liver injury, indicating a potential translational prospect of PXR antagonists (Xie et al., 2019). This potential has been previously discussed, but no chemicals have yet demonstrated activity in humans. The discovery of SPA70 represents a major step forward, allowing future studies of the benefits of PXR antagonism in preventing drug-drug interactions, enhancing drug efficacy, and abrogating PXR-mediated homeostatic perturbations such as cholestasis, hypercholesterolemia, inflammation, and hepatic steatosis (Mani et al., 2013). While chemistry efforts to improve the druglike properties of SPA70 continue, alternative routes can also be exploited to achieve reduction of PXR activity. In 2015, conjugation of a ligand for a protein of interest to a ligand for an E3 ubiquitin ligase was demonstrated as an effective method to induce proteasomal degradation of a target (Winter et al., 2015). This approach has been applied to various proteins, including nuclear receptors (Bondeson et al., 2015), and is an alternative to chemical inhibition of protein activity. Exploring this pathway for PXR and CAR may yield highly potent and selective degraders, as such molecules have been shown to specifically degrade target proteins, even those differing by a single amino acid (Nabet et al., 2018; Alabi et al., 2021). To facilitate the development of PXR and CAR degraders, Lin et al. and Huber et al. have developed tools for this methodology, both in a general context and for PXR specifically (Lin et al., 2020b,c; Lin and Chen, 2021; Huber et al., 2022).

Perspective 4: Context-Specific PXR and CAR Regulations. Two major confounding factors in PXR and CAR studies were discussed above (species and sex differences), and additional factors are involved, such as those alluded to before (microbiome composition and the newly identified PXR-CAR interaction). Navigating these contextual relationships is critical to fully describe the biologic outcomes of modulating xenobiotic receptors, and consideration of new methods and models will aid in exploring the biology. While there is potential for a diversity of future studies, we will use the case of CAR in liver tumorigenesis as an example. In 2005, it was reported that chronic CAR activation in mice resulted in liver carcinogenesis (Huang et al., 2005). However, a 2022 report indicated that in humans, CAR suppresses hepatocellular carcinoma development – a stark contrast from the earlier experiments (Li et al., 2022). Clearly, the physiologic environment profoundly impacts the outcomes, and although the 2022 study was well-performed, the authors noted that the in vivo model was not of truly human origin because it was an extrahepatic xenograft in nude mice. Models such as those that remove native mouse hepatocytes and repopulate the liver with human hepatocytes may be useful to generate a more humanized xenobiotic response. These models have been shown to express a range of human xenobiotic response genes, including PXR and CAR, and to respond to inducers such as rifampicin and phenobarbital (Dandri et al., 2001; Tateno et al., 2004; Azuma et al., 2007). Future development efforts may focus on improving the translation of PXR and CAR activities from rodents to humans and deciphering how specific physiologic conditions modify PXR and CAR outputs.

Conclusions

We are excited to contribute this minireview to the "Xenobiotic Receptors" special section in honor of *Drug Metabolism and Disposition*'s 50th anniversary and to celebrate Dr. Wen Xie's scientific contributions to the field as a recipient of the Richard Okita Award in Drug Metabolism and Disposition. Dr. Xie's contributions have been paramount to the success of the field, beginning with his development of the first humanized PXR mouse model as a postdoctoral fellow under Dr. Ronald M. Evans (Xie et al., 2000a). The following 20+ years brought a wealth of knowledge and swaths of new researchers to the field of xenobiotic receptors. PubMed keyword searches reveal >2600 and >1600 articles for PXR and CAR, respectively, at the time of this review, and amazing new findings continue to unfold. Analysis of xenobiotic receptor responses have led to more efficacious drugs and new pathways to therapeutically exploit. Recent discoveries combined with expansion of historical paradigms promises a bright future for xenobiotic receptor research.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Poudel, Huber, and Chen.

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