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REGULATION OF *CYP3A* GENE TRANSCRIPTION BY THE PREGNANE X RECEPTOR*

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■ **Abstract** The pregnane X receptor (PXR) is a promiscuous nuclear receptor that has evolved to protect the body from toxic chemicals. PXR is activated by a structurally diverse collection of xenobiotics, including several widely used prescription drugs. Various lipophilic compounds produced by the body, such as bile acids and steroids, also activate PXR. PXR stimulates the transcription of cytochrome P450 3A monooxygenases and other genes involved in the detoxification and elimination of these potentially harmful chemicals. Assays that detect PXR activation have important implications for the design of future drugs in two respects. On the one hand, PXR activation assays can be used to determine whether candidate drugs are likely to induce *CYP3A* gene expression and interact with other medicines. On the other hand, PXR agonists may prove useful in the treatment of diseases in which toxic metabolites accumulate, such as cholestatic liver disease.

OVERVIEW

The Cytochrome P450 Superfamily

Cytochromes P450 (CYP) are a superfamily of heme-thiolate proteins that play a central role in the oxidative, peroxidative, and reductive metabolism of an immense

*ABBREVIATIONS CYP, cytochrome P450; PXR, pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; LCA, lithocholic acid; ER6, everted repeat with a 6 nucleotide spacer; DRn, direct repeat with a n nucleotide spacer; DBD, DNA-binding domain; LBD, ligand-binding domain; CAR, constitutive androstane receptor; SPA, scintillation proximity assay; RXRα, 9-cis retinoic acid receptor α; XREM, xenobiotic-responsive enhancer module.

array of endogenous compounds; this array includes fatty acids, steroids, leukotrienes, prostaglandins, bile acids, biogenic amines, and fat-soluble vitamins. Thus, CYPs are considered important in the maintenance of steady-state levels of many compounds involved in diverse cellular processes. In addition, many of these enzymes are responsible for the detoxification of foreign or xenobiotic chemicals such as drugs, carcinogens, and environmental contaminants (1). Xenobiotic metabolizing CYPs catalyze the first step (Phase I) in the biotransformation of lipophilic substrates to more hydrophilic derivatives, thereby facilitating their excretion. In some cases, the metabolites produced in this process are potentially carcinogenic, mutagenic, or toxic (2, 3).

CYP Expression is Inducible by Xenobiotics

The CYP superfamily is ancient and has existed for over 1.5 billion years (3, 4). The CYP monooxygenase system probably evolved to metabolize steroids required for the maintenance of membrane integrity, and subsequently, it assumed a role in the breakdown of foreign compounds (5). Prior to the discovery of cytochrome P450, it was understood that the efficacy of many drugs was determined by the speed at which they were metabolized by the body. The activities of drug-metabolizing enzymes in the liver were markedly increased when animals were exposed to hormones, drugs, insecticides, and carcinogens. This enzyme induction accelerated the biotransformation of drugs and reduced their "duration and intensity" (6). The nature of the enzymatic activity induced was characteristic of the type of inducer. This suggested that multiple enzyme systems existed and, moreover, that the regulation of expression of these proteins was subject to divergent control mechanisms (7).

It is now known that many CYPs involved in xenobiotic metabolism have highly inducible patterns of expression. Frequently, the inducer is a substrate for the inducible enzyme and, as such, enhances its own metabolism. Elevated expression of the detoxifying enzyme persists only as long as the inducer/substrate remains in the organism (8). The xenobiotic induction of human drug-metabolizing enzymes underlies many reported drug interactions and is of considerable importance for patients subject to combination drug therapy (9).

THE *CYP3A* SUBFAMILY

CYP3A Expression and Catalytic Activity

METABOLISM OF XENOBIOTICS For over 40 years certain steroid hormones or hormone derivatives were understood to exhibit a protective effect against various types of intoxication. These "catatoxic steroids" did not have to possess any classic hormone properties to afford protection against drugs or toxins. Seminal studies by Seyle in the early 1970s identified the synthetic pregnenolone derivative pregnenolone 16α-carbonitrile (PCN) as one of the most potent catatoxic steroids

tested (10). This steroid was the prototype inducer for a novel group of P450s (11), the CYP3A subfamily.

To date, approximately 40 members of the *CYP3A* subfamily have been identified (11a). In humans, the predominant CYP expressed in adult liver and small intestine is CYP3A4 (12, 13). It is estimated that CYP3A4 is involved in the metabolism of approximately 60% of all therapeutic drugs and is reported to be at the center of many clinically important drug interactions (9, 12–14). In general, CYP3A4 substrates are large ($M_r > 300$), lipophilic molecules. The structural diversity exhibited by CYP3A4 substrates is striking and includes antimycotics, macrolide antibiotics, contraceptive steroids, antiviral agents, and calcium channel blockers (9, 12–14).

METABOLISM OF ENDOGENOUS COMPOUNDS Thus far, the major endogenous CYP3A4 substrates identified are steroids. In adult humans, the primary pathway for inactivation of testosterone, progesterone, androstenedione, and cortisol appears to be CYP3A4-mediated 6β-hydroxylation. CYP3A4 is also reported to be active in testosterone 2β- and 15β-, progesterone 16α- and 2β-, and 17β-estradiol 2- and 4-hydroxylation (15–20).

In addition to steroid hydroxylation, CYP3A4 is active in the 6-hydroxylation of the bile acids, lithocholic acid (LCA) and taurochenodeoxycholic acid (21, 22), and is reported to be the major catalyst of microsomal 25-hydroxylation of 5α-cholestane-3α, 7α, 12α-triol, an alternative pathway for side-chain degradation in the biosynthesis of bile (23). Rodent CYP3A subfamily members also catalyze the 6-hydroxylation of LCA (24).

Induction of *CYP3A* Genes by Structurally Diverse Compounds

One of the most interesting facets of the *CYP3A* subfamily is the ability of structurally unrelated chemicals to stimulate the transcription of these genes. Early studies, performed in vivo and in primary cultures of rat hepatocytes, demonstrated that *CYP3A23* expression was highly inducible by the antiglucocorticoid PCN, and, paradoxically, by dexamethasone, a potent glucocorticoid (25, 26). Schuetz and Guzelian (26, 27) demonstrated that in cultured rat hepatocytes the time- and concentration-dependent induction of *CYP3A23* by dexamethasone did not parallel activation of a typical glucocorticoid-responsive gene, tyrosine aminotransferase. In addition, activation of the tyrosine aminotransferase gene by dexamethasone was antagonized by PCN, while induction of *CYP3A23* by glucocorticoids was synergistically enhanced by the presence of this antiglucocorticoid (27, 28). This led to the hypothesis that the induction of *CYP3A23* by glucocorticoids was mediated by a mechanism distinct from that of the classical glucocorticoid receptor (GR; NR3C1) pathway (27).

It is now clear that rat *CYP3A* genes, predominantly *CYP3A23*, are inducible by an array of compounds, including steroids such as PCN, dexamethasone, betamethasone, hydrocorticosone, α-methylprednisolone, mifepristone (RU486), and

spironolactone, the antibiotic triacetyloleandomycin, antifungal drugs, polychlorinated binphenyls, organochloride pesticides, and the barbiturate phenobarbital (29–35). Induction of the human *CYP3A4* gene by xenobiotics is also well documented. One of the most effective inducers of *CYP3A4* expression, both in vivo and in vitro, is the macrocyclic antibiotic rifampicin (34, 36–38). Interestingly, although the *CYP3A4* and rabbit *CYP3A6* genes are strongly induced by rifampicin, *CYP3A23* expression is not efficiently activated by this drug (29, 34, 39, 40). Conversely, PCN is an efficacious activator of *CYP3A* expression in rats and mice but not humans (29, 34). Additionally, *CYP3A4* expression is inducible by steroids, notably dexamethasone, RU486, spironolactone, and cyproterone acetate, the antifungal agent clotrimazole, the anticonvulsant phenytoin, the nonsteroidal anti-inflammatory drug phenylbutazone, the proton pump inhibitors omeprazole and lansoprazole, and the anticancer agent paclitaxel (29, 34, 38, 41–45).

Xenobiotic Response Elements in *CYP3A* Genes

Until recently, the molecular mechanisms by which structurally diverse xenobiotics transcriptionally activate *CYP3A* genes have been unresolved. Guzelian and coworkers utilized a modified matrigel culture system to identify a region in the *CYP3A23* proximal promoter (bases −220 to −56 relative to the transcription initiation site) that conferred responsiveness to dexamethasone and PCN when linked to a heterologous thymidine kinase promoter (28). In agreement with earlier reports (26, 27), the dose-dependent induction of *CYP3A23*-reporter gene constructs by dexamethasone was distinct from that of a classical GR-mediated response (28). DNase I footprint analysis of this region identified three sites that were capable of forming complexes with nuclear proteins. Notably, GR did not bind directly to these sites (46, 47). Embedded within these three regions were binding motifs known to be recognized by members of the nuclear receptor superfamily of ligand-activated transcription factors (46–50). Thus, site A (bases −110 to −91) was over 80% identical to the consensus binding site for the orphan nuclear receptor hepatocyte nuclear factor-4 (HNF-4α; NR2A1), site B (also known as DexRE-1, bases −136 to −118) comprised a direct repeat (DR) of the AGTTCA motif separated by three nucleotides (DR3), and site C (DexRE-2, −169 to −144) contained an imperfect nuclear receptor binding site that could be viewed as either an everted repeat with a 6 nucleotide spacer (ER6) or a direct repeat with a 4 nucleotide spacer (DR4) (46, 47). The ability of nuclear receptors to interact with these elements was demonstrated in a number of reports (51–55). Mutation analysis suggested that site B was central to the steroid-responsiveness of this unit; disruption of either nuclear receptor half-site in this motif destroyed dexamethasone and PCN induction or reporter gene activity (46, 47, 53). Furthermore, Quattrochi et al. (46, 53) reported that the isolated site B was capable of conferring dexamethasone- and PCN-inducibility on a minimal thymidine kinase promoter in rat hepatocytes.

In an extension of these studies, Barwick et al. (56) examined xenobiotic activation of the human *CYP3A4* and rabbit *CYP3A6* genes. The 5′-flanking regions of *CYP3A4* (bases −179 to −35), *CYP3A6* (bases −186 to −37), and *CYP3A23* (bases −220 to −56) were linked to a heterologous promoter and reporter gene and transiently transfected into primary cultures of rat and rabbit hepatocytes. When transfected into rat hepatocytes, dexamethasone and PCN, but not rifampicin, activated each chimeric *CYP3A*-reporter gene construct. However, in primary cultures of rabbit hepatocytes, rifampicin and dexamethasone, but not PCN, transactivated the *CYP3A* reporter gene constructs. These observations led to the hypothesis that the host cellular environment, rather than the structure of the gene, determines the species-specific pattern of *CYP3A* inducibility. The xenobiotic responsiveness was mapped to a conserved 18-bp motif spanning nucleotides −170 to −153 and −177 to −160 of *CYP3A4* and *CYP3A6*, respectively (56). This element contained two copies of an $AG^{G}/_{T}TCA$ hexamer organized as ER6.

The Pregnane X Receptor (PXR)

IDENTIFICATION OF THE NUCLEAR RECEPTOR PXR PXR is a member of the nuclear receptor superfamily, which includes the steroid, retinoid, and thyroid hormone receptors. Members of this family function as ligand-activated transcription factors and have critical roles in nearly every aspect of development and adult physiology. Family members share a common domain structure that includes a highly conserved DNA binding domain (DBD) with two zinc fingers. The DBD targets the receptor to short stretches of DNA, termed response elements, in the regulatory regions of target genes. The carboxy-terminal region of the nuclear receptors includes the conserved ligand-binding domain (LBD). The LBD serves as the docking site for ligands and also contains dimerization motifs and transcriptional activation domains such as the activation function 2 (AF-2) helix. The binding of a ligand to the LBD results in a conformational change in the AF-2 helix; this change allows the nuclear receptor to interact with accessory proteins and activate the expression of target genes (48, 57, 58).

Since nuclear receptors share conserved domains, it has been possible to isolate novel family members using sequence homology. Many of these receptors are orphans in the sense that their physiologically relevant ligands have yet to be determined (49, 50, 59–61). In 1997, a sequence representing a fragment of a novel mouse nuclear receptor first appeared in the Washington University expressed sequence tag database. Since then, clones representing the full-length mouse, rat, rabbit, and human orthologs of this receptor have been cloned by various groups and named the pregnane X receptor (PXR; NR1I2) based on their activation by various natural and synthetic pregnanes (Figure 1) (62–68). The human PXR has also been called the pregnane-activated receptor (PAR) and the steroid and xenobiotic receptor (SXR) (62, 63). PXR is most closely related to the constitutive androstane receptor (CAR; NR1I3) and the 1,25-dihydroxyvitamin D_3 receptor (VDR; NR1I1) (62–65).

Evidence that PXR Is a Key Regulator of *CYP3A* Induction by Xenobiotics

PXR EXPRESSION PATTERNS As an initial step in the characterization of PXR, the tissue-specific expression profiles of mouse, human, rat, and rabbit PXR were examined. In each species, PXR was abundantly expressed in liver and, to a lesser extent, colon and small intestine (62–68). Whereas mouse PXR mRNA was also detected in stomach and kidney, human PXR was not found in these tissues (62–65). Low-level expression of rat PXR was also found in lung (66). Importantly, these are the same tissues in which *CYP3A* genes are expressed and induced (25, 69–72).

The molecular determinants underlying the tissue-specific and developmental expression of PXR are yet to be resolved. However, studies performed in the fetal livers of HNF-4α-null mice show that expression of the *Pxr* gene was almost completely abolished in mice lacking function HNF-4α (73). Additionally, PXR and HNF-4α are both abundantly expressed in the liver, intestine, and kidney, which suggests that the *Pxr* gene may be directly regulated by HNF-4α (64, 74). The human and rodent *PXR* genes also appear to be hormonally regulated. Studies by Huss & Kasper (75) and Pascussi et al. (76) demonstrate that glucocorticoids promote the accumulation of PXR mRNA in a GR-dependent manner. The GR-mediated induction of PXR expression is a component of the well-documented induction of *CYP3A* expression by dexamethasone (75, 76). Regulation of *Pxr* by steroid hormones may also cause the elevated levels of PXR mRNA observed in the liver and ovary of perinatal mice (77).

XENOBIOTICS BIND AND ACTIVATE PXR In the absence of its cognate response elements, PXR activators were initially delineated using a chimeric system in which the LBD of mouse PXR (mPXR) was fused to the DBD of the GAL4 yeast transcription factor. An expression vector containing the chimeric GAL4-mPXR cDNA was cotransfected into CV-1 cells with $(UAS)_5$-tk-CAT, a reporter plasmid containing five copies of the GAL4 recognition motif linked to a minimal thymidine kinase promoter and CAT reporter gene. Surprisingly, the GAL4-mouse PXR chimera was activated by both glucocorticoids, including dexamethasone and dexamethasone t-butyl acetate, and antiglucocorticoids, such as PCN and RU486. Naturally occurring steroids, namely progesterone, pregnenolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 5β-pregnane-3,20-dione, were also identified as activators of mouse PXR. The ability of both dexamethasone and PCN to activate mouse PXR suggested that this receptor may play a role in the transcriptional regulation of *CYP3A* genes (64).

Although mouse, human, rat, and rabbit PXR are activated by several common compounds, notably 5α-pregnane-3,20-dione, the pharmacology of these receptors is strikingly divergent. For example, both human PXR and mouse PXR are effectively activated by dexamethasone *t*-butylacetate, RU486, and corticosterone, whereas human and rabbit PXR but not mouse or rat PXR are activated by rifampicin. Similarly, the bisphosphonate ester SR12813 is a potent and efficacious

activator of human and rabbit but not rat and mouse PXR (67). Conversely, PCN, dexamethasone, and 17α-hydroxypregnenolone are efficacious activators of mouse, but not human, PXR (63, 65, 67). The pharmacological differences between these receptors provide strong evidence that PXR is a key mediator of the xenobiotic induction of *CYP3A* genes because the mouse, rabbit, and human PXR activation profiles correlate well with *CYP3A* induction profiles in these three species (67). Thus, while rifampicin, for example, is an effective inducer of human *CYP3A4* and rabbit *CYP3A6*, it is a poor activator of rat or mouse *CYP3A* expression (29, 34, 39, 40). To date, two high-affinity human PXR ligands have been identified, namely hyperforin and SR12813 (67, 78, 79). Hyperforin is a putative antidepressant found in St. John's wort, a popular herbal remedy used for the treatment of a variety of indications, including depression and inflammation (80). A number of clinically important drug interactions have been reported in patients taking St. John's wort (81). In reporter gene assays, hyperforin activated human PXR with a half-maximal effective concentration (EC_{50}) of $\sim$25 nM. This compound was shown to bind the human PXR LBD with similar affinity and to be an effective inducer of *CYP3A4* expression in primary cultures of human hepatocytes (78). The identification of the cholesterol-lowering drug SR12813 as a potent PXR ligand has facilitated the development of a binding assay (67). This scintillation proximity assay (SPA) has demonstrated that the many structurally diverse compounds that activate PXR in cell-based assays do so through direct interactions with the receptor LBD. Importantly, SPA can be run in a high-throughput format and is therefore suitable for screening large numbers of compounds for their ability to activate PXR and, consequently, induce *CYP3A4* expression (67, 82). The removal of these compounds from the drug discovery process should greatly accelerate the development of novel and safer therapeutics. The structures of selected PXR ligands are shown in Figure 2.

The number of compounds identified as PXR ligands and *CYP3A* inducers continues to grow. Schuetz et al. (83) reported that the organochloride pesticides, trans-nonachlor and chlordane, polychlorinated biphenyls, the antimineralocorticoid spironolactone, and antiandrogen cyproterone acetate were capable of activating mouse PXR. Subsequently, Masuyama and coworkers (84) showed that the endocrine-disrupting chemicals, nonylphenol and phthalic acid, were mouse PXR ligands and *CYP3A* inducers. Recently, Synold et al. (85) demonstrated that the chemotherapeutic agent paclitaxel, a known *CYP3A4* inducer (45), was an efficacious human PXR ligand. Intriguingly, this report also documented the characterization of a potent PXR antagonist. Ecteinascidin-743 (ET-743), a marine-derived antineoplastic agent, antagonized the PXR-dependent activation of reporter gene constructs by SR12813 with a half-maximal inhibitory concentration (IC_{50}) of 3 nM. Moreover, induction of *CYP3A4* expression by SR12813 was completely blocked by ET-743 (85).

PXR BINDS TO XENOBIOTIC RESPONSE ELEMENTS IN *CYP3A* PROMOTERS The high degree of sequence homology within the DBDs of PXR, Xenopus orphan nuclear receptor-1 (xONR1; NR1I2), and VDR implied that these receptors may share

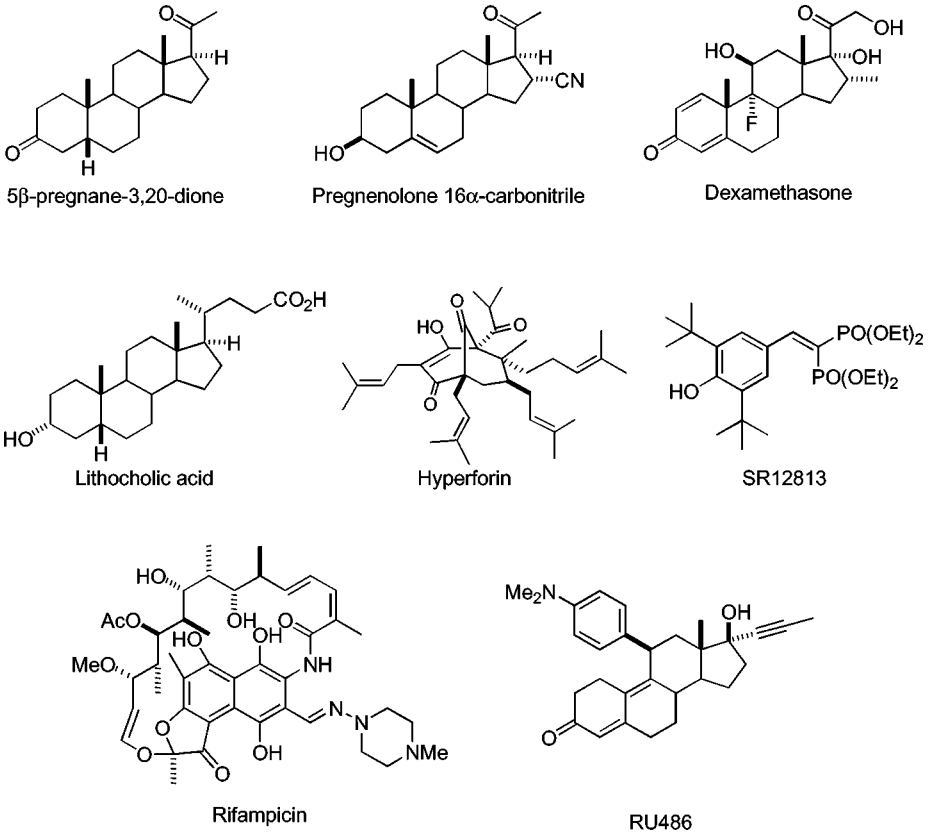

Figure 2 Chemical structures of selected xenobiotic and endogenous compounds known to activate PXR.

common DNA-binding characteristics. The xONR1 and VDR bind their cognate response elements (organized as a DR3 motif) as heterodimers with the 9-*cis* retinoic acid receptor α (RXRα; NR2B1) (48, 49). Mouse PXR was shown to be capable of efficiently binding the DR3 motif embedded in site B of the *CYP3A23* proximal promoter as a heterodimer with RXRα (Figure 3) (64). Moreover, multimerized *CYP3A23* DR-3 elements conferred mouse PXR-mediated dexamethasone- and PCN-responsiveness on a thymidine kinase-reporter gene

Figure 3 PXR binds to response elements in xenobiotic inducible genes. PXR binds as a heterodimer with RXRα to DR3, DR4, and ER6 elements in the regulatory regions of various *CYP3A* and *CYP2B* subfamily members. The DR4 and ER6 binding motifs from the human multidrug resistance 1 (MDR1) gene are also shown.

DIRECT REPEAT (DR) 3

CYP3A23	TGAACT TCA TGAACT	
	<-----	<-----
CYP3A2	TGAACT TTA TGAACT	
	<-----	<-----
CYP3A2	TGACCT TCT TGAGCT	
	<-----	<--x--
CYP3A4	TGAACT TGC TGACCC	
	<-----	<-----

EVERTED REPEAT (ER) 6

CYP3A23	TTAACT CAAAGG AGGTCA	
	<x----	----->
CYP3A4	TGAACT CAAAGG AGGTCA	
	<-----	----->
CYP3A5	TGAACT CAAAGG AGGTAA	
	<-----	----x>
CYP3A6	TGAACT CAGAGG AGGTCA	
	<-----	----->
CYP3A7	TTAACT CAATGG AGGTCA	
	<x----	----->
MDR1	TGAGAT TAAACA AGTTCA	
	<--xx-	----->

DIRECT REPEAT (DR) 4

CYP2B1 Cyp2b10	TGTACT TTCC TGACCT	
	<-x---	<-----
CYP2B6	TGTACT TTCC TGACCC	
	<-x---	<-----
CYP2B6	TGGACT TTCC TGAACC	
	<-x---	<-----
MDR1	TGAACT AACT TGACCT	
	<-----	<-----

construct (64). Subsequently, a number of groups demonstrated that PXR-RXRα heterodimers interacted with the ER6 motif in the proximal promoter region of the *CYP3A4* gene (62, 63, 65). Reporter gene constructs harboring multimerized copies of this element were activated by PXR in transient transfection assays (62, 63, 65). Although this ER6 element supports PXR-mediated transactivation, maximal induction of the *CYP3A4* promoter is dependent on a distal xenobiotic-responsive enhancer module (XREM) located ~8 kb upstream of the transcription initiation site (86). The XREM region contains an additional high-affinity PXR binding site (a DR3 element), which works in a coordinate manner with the ER6 element in the promoter proximal region of *CYP3A4*. Thus, in the context of the native *CYP3A4* promoter (bases −362 to +53) the proximal ER6 element has no inherent ability to promote PXR-mediated transcription in experiments performed in liver-derived cell lines. However, this element is required for maximal functionality of the XREM: Mutation of this element in the reporter gene constructs containing the XREM reduced PXR-responsiveness by ~50% (86). The XREM region and promoter proximal ER6 element appear to be functionally conserved in the human *CYP3A7* gene (87, 88).

Blumberg et al. (63) examined the ability of PXR-RXRα heterodimers to bind and activate a series of tester elements containing repeats of the $AG^{G}/_{T}TCA$ hexamer. In addition to DR3 and ER6 elements, the PXR-RXRα complex bound and activated DR4 and DR5 motifs. Functional DR4 elements have been characterized in the 5′-flanking regions of various *CYP2B* subfamily members and the multidrug resistance 1 (MDR1) gene (Figure 3) (89–92). The PXR-dependent activation of *CYP2B1*, *CYP2B6*, and *Cyp2b9* is mediated by the phenobarbital-responsive enhancer module region (PBREM) (89, 91–93). The PBREM is located ~2 kb upstream of the transcription initiation site and contains two DR4 motifs that bind both PXR-RXRα and CAR-RXRα heterodimers. The orphan nuclear receptor CAR mediates induction of *CYP2B* genes by phenobarbital and phenobarbital-like inducers (93). Notably, CAR is also implicated in the regulation of *CYP3A* and is reported to exert its effects on transcription through the PXR-responsive DR3 and ER6 elements in the 5′-flanking regions of these genes (89; 92; 94; B. Goodwin, E. Hodgson, C. Liddle, submitted for publication). The fact that PXR and CAR bind common response elements suggests that interplay between these two receptors is likely to be a central theme in the regulation of xenobiotic-inducible CYPs.

TARGETED DISRUPTION OF THE PXR GENE IN MICE Genetic evidence that PXR mediates xenobiotic induction of *CYP3A* genes was recently provided by two groups who developed mice that harbor a disrupted *Pxr* gene (96, 97). These mice were viable, fertile, bred with normal Mendelian distribution, and did not exhibit any overt phenotypic change. Extensive serum analysis did not reveal any significant changes in a number of parameters, including free- and HDL cholesterol, triglycerides, steroid hormones, transaminases, total bilirubin, and bile acids. However, the PXR-null mice failed to induce *Cyp3a11* expression when challenged with

PCN or dexamethasone (89, 96, 97). Interestingly, one group reported that *Cyp3a11* expression was elevated in the PXR-null mice compared to wild-type animals (97). The molecular basis for this observation is not clear, but it is possible that in the absence of PXR the nuclear receptor binding motifs in the *Cyp3a11* promoter are more accessible to factors with higher basal transcriptional activity, such as CAR. In support of this, PXR and CAR are reported to bind common response elements in the *CYP3A4*, *CYP3A23*, *CYP2B1*, *CYP2B6*, and *Cyp2b10* genes (discussed above) (89; 91; 92; 94; B. Goodwin, E. Hodgson, C. Liddle, submitted for publication).

TRANSGENIC MODELS Xie et al. (96) prepared transgenic mice that expressed either wild-type human PXR (Alb-PXR) or a constitutively active PXR (Alb-VP-PXR) under control of the liver-specific albumin promoter. Subsequently, the Alb-PXR transgenic animals were bred with PXR-null mice. As expected, "humanized" but not wild-type animals responded to the human-specific *CYP3A* inducer, rifampicin. Conversely, while PCN was an efficacious inducer of *Cyp3a11* in the wild-type mice, no induction was observed in the humanized animal (96). The Alb-VP-PXR transgenic mice exhibited constitutively high levels of *Cyp3a11* expression, which, in turn, resulted in reduced tribromoethanol-induced anesthesia and zoxazolamine paralysis when compared to wild-type animals. Interestingly, the Alb-VP-PXR animals exhibited growth retardation, hepatomegaly, and histologic liver toxicity, which suggests that sustained activation of PXR may be deleterious (96).

Role of PXR in Bile Acid Homeostasis

PXR REGULATES GENES INVOLVED IN BILE ACID SYNTHESIS, TRANSPORT, AND METABOLISM For over 20 years, it has been understood that treatment of rodents with PCN results in a profound suppression of the gene encoding cholesterol 7α-hydroxylase (Cyp7a1), the first and rate-limiting step in the conversion of cholesterol to bile acids (98–101). In PXR-null mice, the *Cyp7a1* gene is dysregulated in two respects. First, *Cyp7a1* mRNA levels in PXR-null mice were $\sim$50% of that in wild-type mice and, secondly, PCN treatment failed to suppress *Cyp7a1* expression, which demonstrates a dual role for PXR in both the basal expression and the repression of *Cyp7a1* (97). The PXR-dependent regulation of bile acid biosynthetic genes seemed to be confined to *Cyp7a1* because the expression of other genes involved in this cascade, namely sterol 27-hydroxylase (CYP27), oxysterol 7α-hydroxylase (CYP7B1), and oxysterol 12α-hydroxylase (CYP8B1), was unchanged (B. Goodwin, S.A. Kliewer, unpublished observations).

A systematic examination of hepatocellular transporter regulation revealed that the Na^+-independent organic anion transporter 2 (Oatp2) was inducible by PCN (J.L. Staudinger, C.D. Klaassen, unpublished observations). Oatp2 is a basolateral (sinusoidal) transporter that participates in the uptake of a wide range of amphipathic substrates, including bile acids and xenobiotics (102, 103). PCN treatment

strongly induced *Oatp2* expression in wild-type but not PXR-null mice, confirming that this gene is regulated in a PXR-dependent manner.

BILE ACIDS BIND AND ACTIVATE PXR Given the role of CYP7A1, CYP3A, and *OATP2* in bile acid synthesis, metabolism, and transport (discussed above), bile acids were screened using a cell-based reporter gene assay for their ability to activate PXR. Notably, LCA, a hydrophobic secondary bile acid formed in the intestine by the bacterial 7α-hydroxylation of chenodeoxycholic acid, was an efficacious activator of human PXR and also activated mouse PXR (22, 97). In addition, the LCA metabolite 3-keto-LCA effectively activated both human and mouse PXR. LCA and 3-keto-LCA bound human PXR in a SPA with IC_{50} values of 9 μM and 15 μM, respectively (97).

LCA is a toxic bile acid known to cause cholestasis, a condition characterized by the cessation or impairment of bile flow, which can give rise to nutritional imbalance related to malabsorption of lipids and fat-soluble vitamins (104–107). Moreover, the concomitant accumulation of bile acids and the toxins normally excreted in bile can cause irreversible liver damage. Importantly, in the livers of cholestatic patients and in rodent models of biliary cholestasis, the reported concentration of LCA (5–10 μM) (108) would be sufficient to activate PXR (22, 97). The fact that LCA could function as a PXR ligand led Staudinger et al. (97) to examine whether this bile acid could induce *Cyp3a11* and *Oatp2* expression in a PXR-dependent manner. Indeed, treatment of wild-type but not PXR-null mice with LCA resulted in a marked induction of both *Cyp3a11* and *Oatp2* expression (97). Taken together, these results suggest that PXR is capable of acting as a physiological sensor for LCA and/or LCA metabolites and of coordinately regulating gene expression so as to prevent the accumulation of these potentially harmful bile acids. Thus, PXR represses *Cyp7a1* expression, thereby blocking bile acid synthesis. At the same time, induction of *Oatp2* could increase uptake of LCA and other bile acids from sinusoidal blood into the hepatocyte where CYP3A-mediated hydroxylation could take place. The more polar bile acid metabolites could then be excreted in the feces or urine.

POTENTIAL UTILITY OF PXR IN THE TREATMENT OF CHOLESTASIS If PXR is involved in protecting the liver from potentially harmful bile acids, one would predict that PXR activation would alleviate or prevent the hepatoxicity caused by cholestatic bile acids, such as LCA. Indeed, almost 30 years ago Seyle first showed that PCN treatment blocked the hepatotoxicity and mortality caused by LCA treatment (109). Studies performed in PXR-null mice have demonstrated that PXR mediates the hepatoprotective effects of PCN (22, 97). Thus, as expected, treatment of wild-type and PXR-null mice with LCA resulted in severe liver damage as manifested by the appearance of necrotic foci and elevated serum markers of liver damage (22, 97). Cotreatment of animals with PCN profoundly reduced the detrimental effects of LCA in wild-type but not PXR-null mice (22, 97). Similarly, mice expressing a constitutively active PXR (Alb-VP-PXR) were resistant

to LCA-induced histologic liver damage (22). These data indicate that PXR plays a fundamental role in protecting the liver against pathophysiological levels of bile acids.

The urine from patients suffering from cholestatic liver disease contains elevated levels of 6α-hydroxylated bile acids (110). Because CYP3A4 is reported to be capable of catalyzing the 6α-hydroxylation of both LCA and taurochenodeoxycholic acid (21), induction of *CYP3A4* expression could account for the increased levels of 6α-hydroxylated bile acids excreted by these individuals. Thus, it is likely that this pathway is a relevant mechanism for reducing the levels of toxic bile acids in humans. Elevated levels of 6α-hydroxylated bile acids are also observed in healthy subjects treated with rifampicin (111). Interestingly, the use of rifampicin for treatment of the pruritus associated with cholestasis is well documented (e.g., 112, 113). In some instances, rifampicin treatment promoted remission of cholestasis (114–116). It is also notable that St. John's wort extracts are used as a Turkish folk medicine for a variety of hepatic disorders, including cholestasis (117). The molecular basis for the clinical effects of rifampicin on cholestasis has remained obscure. Based on the studies described above, the anticholestatic effects of rifampicin may be mediated by PXR, raising the intriguing possibility that potent PXR ligands could prove efficacious in the treatment of cholestasis.

Identification of Novel PXR Target Genes

To better understand the biology of PXR, it is important to delineate genes that are regulated by this receptor. Although PXR response elements in the regulatory regions of *Cyp7a1* and *Oatp2* are yet to be characterized, studies performed in PXR-null mice strongly suggest that these genes are directly regulated by PXR (97). Examination of the published literature reveals a number of potential PXR target genes. Among the genes reported to be regulated by known PXR ligands are *CYP1A1* (118), *CYP2C8* and *CYP2C9* (85, 119), *MDR1* (85, 90), multidrug resistance-associated protein-2 (MRP2) (120, 121), and members of UDP-glucuronosyltransferase (122), sulfotransferase (123–125), and carboxylesterase families (126). Collectively, the proteins encoded by these genes are involved in the detoxification of xenobiotics and bile acids, thus, reinforcing the protective role of PXR. The development of high-throughput differential gene expression profiling systems should greatly facilitate the identification of PXR target genes and aid the characterization of novel signaling pathways.

X-Ray Crystal Structure of the PXR LBD

How does PXR recognize so many different chemicals? Two recent high-resolution X-ray crystal structures of the human PXR LBD provide important insights into the molecular basis for this promiscuity. The structure of the human PXR LBD was determined in both the absence and presence of SR12813. Overall, the architecture of the human PXR LBD resembles that of other nuclear receptors (58, 127) . The LBD is composed of 10α helices and a five-stranded β-sheet

that fold to form a large hydrophobic cavity in the bottom of the molecule (Figure 4).

Despite its similarity in global fold to other nuclear receptors, the structure of the human PXR LBD also reveals several features that distinguish it from its much less promiscuous relatives. First, the human PXR ligand-binding pocket is large (>1100 Å^3) and spherical in shape, which may allow it to accommodate a variety of different ligands. The hydrophobic pocket may be accessible to ligands through a gated channel present between helices 7 and 10. The position of this ligand entry site is unique to PXR among those nuclear receptors for which LBD structures have been determined. Second, the ligand-binding cavity of human PXR is extremely hydrophobic. Twenty of the 28 residues that line the ligand-binding pocket are hydrophobic. Eight polar residues that offer the potential for hydrogen bonds are distributed evenly throughout the otherwise hydrophobic cavity. The character of the PXR ligand-binding pocket is in keeping with the nature of PXR ligands, which are generally hydrophobic and contain one or more functional groups capable of forming hydrogen bonds. Third, the PXR LBD contains $\sim$40 residues between helices 1 and 3 that are not found in most nuclear receptors. These residues contribute two additional strands of β-sheet to the 3-stranded β-sheet typically found in nuclear receptor LBDs and also define and extend the lower portion of the ligand-binding cavity. Finally, PXR is unique in having a stretch of 13 residues between helices 5 and 7 that loop out from the LBD, exposing a series of hydrophobic residues to solvent. This loop is connected to the existing ligand-binding cavity by a solvent-accessible pore and may allow the PXR ligand-binding pocket to expand to an even larger volume when confronted with large molecules. The position of this loop may provide the flexibility needed for PXR to bind to both relatively small (e.g., phenobarbital) and large (e.g., rifampicin, paclitaxel) ligands (127).

The features that permit the PXR LBD to bind to ligands in a promiscuous manner are highlighted by the co-crystal structure of PXR bound to SR12813 (Figure 4). A single molecule of SR12813 binds into the large hydrophobic pocket of the PXR LBD. Remarkably, SR12813 can dock into the large, spherical pocket in any of three different orientations. Although SR12813 occupies essentially the same space in each of the three orientations, each binding mode is facilitated by a different set of hydrogen bonds and hydrophobic interactions. Thus, PXR does not present a specific ligand-binding surface that drives a single binding mode. Rather, PXR offers a smooth, predominantly hydrophobic binding surface with evenly spaced hydrogen bond donors and acceptors that permit the binding of a variety of hydrophobic compounds as well as the binding of a single compound in multiple orientations. This binding mode stands in sharp contrast to the highly specific lock-and-key type of interactions characteristic of most other nuclear receptor-ligand interactions (127).

The availability of high-resolution PXR LBD crystal structures has also resulted in a much better understanding of the species-specific differences in PXR activation profiles. It is now possible to compare the residues that define the ligand-binding

pocket across species. Insight gleaned from the PXR LBD structure recently led to recognition that targeted mutation of only four polar residues that differ between the mouse and human PXR ligand-binding pockets effectively "humanized" the mouse PXR such that it gained responsiveness to SR12813. Thus, the precise positioning and nature of polar residues in the ligand-binding pocket is critical in defining the activation profile of PXR (127). Ultimately, a detailed molecular understanding of the PXR LBD structure may permit prospective, *in silico* screening—that is, through computer simulation and modeling—for drug candidates that activate the receptor.

SUMMARY AND FUTURE PERSPECTIVES

Tremendous progress has been made during the past several years in understanding the transcriptional regulation of CYP gene expression by xenobiotics. It is now established that the nuclear receptor, PXR, functions as a key regulator of *CYP3A* gene expression in a variety of species. Interestingly, many of the chemicals that activate PXR are also CYP3A substrates. Thus, PXR and the CYP3A hemoproteins appear to have evolved as part of a coupled system in order to detect and metabolize potentially toxic xenobiotics (Figure 5). In addition to *CYP3A*, PXR also regulates *CYP2B*, *CYP2C*, *MDR1*, and *Oatp2* gene expression. Thus, PXR activation results in the coordinate regulation of a variety of different genes involved in different aspects of xenobiotic metabolism.

The discovery of PXR has important pharmaceutical ramifications. We now know that activation of PXR in the liver and intestine represents the molecular basis for an important class of drug-drug interactions. In the past it was necessary to perform *CYP3A* induction studies in primary human hepatocytes in order to assess whether a clinical candidate was likely to interact with other drugs. This was a time consuming process that depended on the availability of high-quality human liver tissue. The availability of high-throughput binding assays and cell-based reporter assays for human PXR now makes it possible to rapidly screen candidate molecules in vitro for their *CYP3A* induction potential. Compounds that bind to or activate PXR can then be eliminated from the drug discovery process. The availability of high-resolution human PXR LBD crystal structures raises the possibility of testing drug candidates *in silico* for their potential to bind to PXR. Chemicals that are able to dock into the ligand-binding pocket of PXR could be eliminated at the very earliest stages of the drug discovery process, possibly even before any time is invested synthesizing them.

Finally, it may also be possible to exploit PXR as a drug target. Since PXR activation causes drug-drug interactions, this idea appears paradoxical. However, PXR represents an important component in the body's defense mechanism against hydrophobic xenobiotics and endogenous metabolites such as bile acids. As such, PXR may prove to be a powerful target for the treatment of diseases in which toxic

metabolites accumulate in the liver or intestine. Indeed, there is already evidence to suggest that PXR agonists such as rifampicin and St. John's wort are useful in the treatment of cholestasis, a disease characterized by impaired bile flow and the accumulation of toxic bile acids in the liver. These findings leave us with the exciting possibility that PXR agonists, with improved pharmacokinetic and pharmacodynamic properties, may prove useful in the widespread treatment of cholestasis and perhaps other human diseases.

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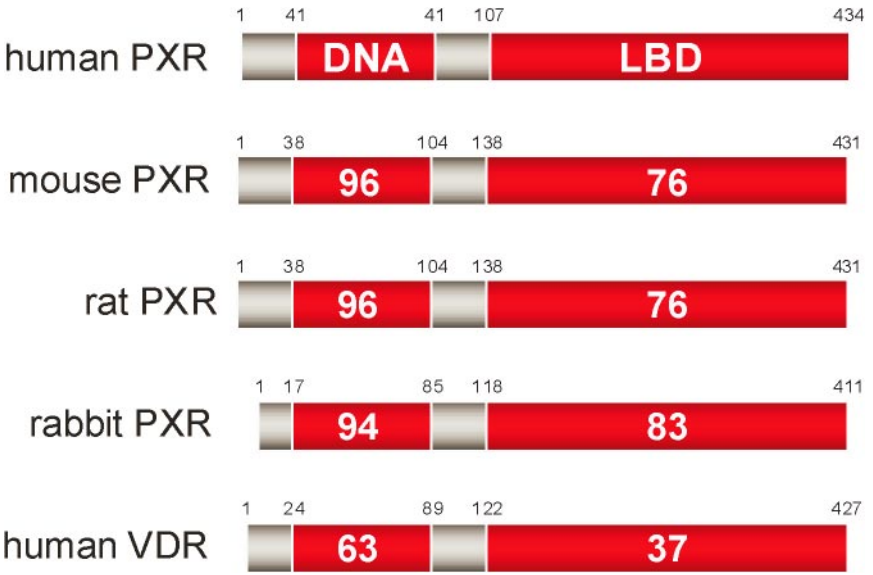

Figure 1 Sequence comparison of PXR among species. Alignment of the human, mouse, rat, and rabbit PXR and human vitamin D receptor (VDR). The similarity between the DNA-binding (DBD) and ligand-binding domains (LBD) is expressed as percent amino acid identity. Specific amino acid positions are also indicated.

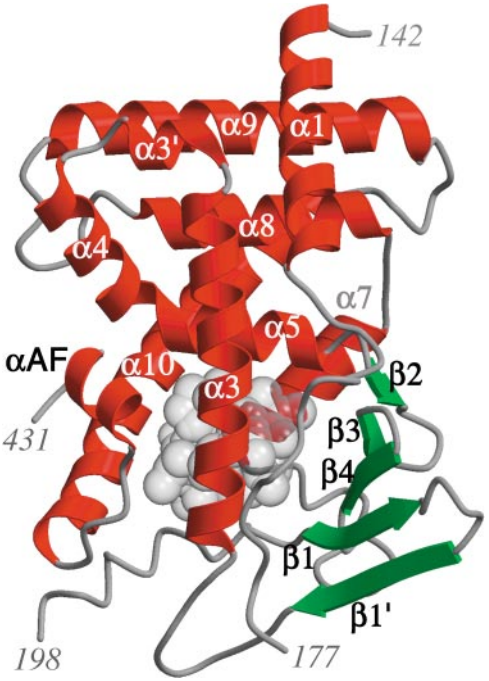

Figure 4 Crystal structure of the human PXR LBD. The X-ray crystal structure of the human PXR LBD is presented as a ribbon drawing. The alpha helices and beta sheets are shown in red and green, respectively. The ligand-binding pocket is outlined in gray. αAF, activation function 2 helix.

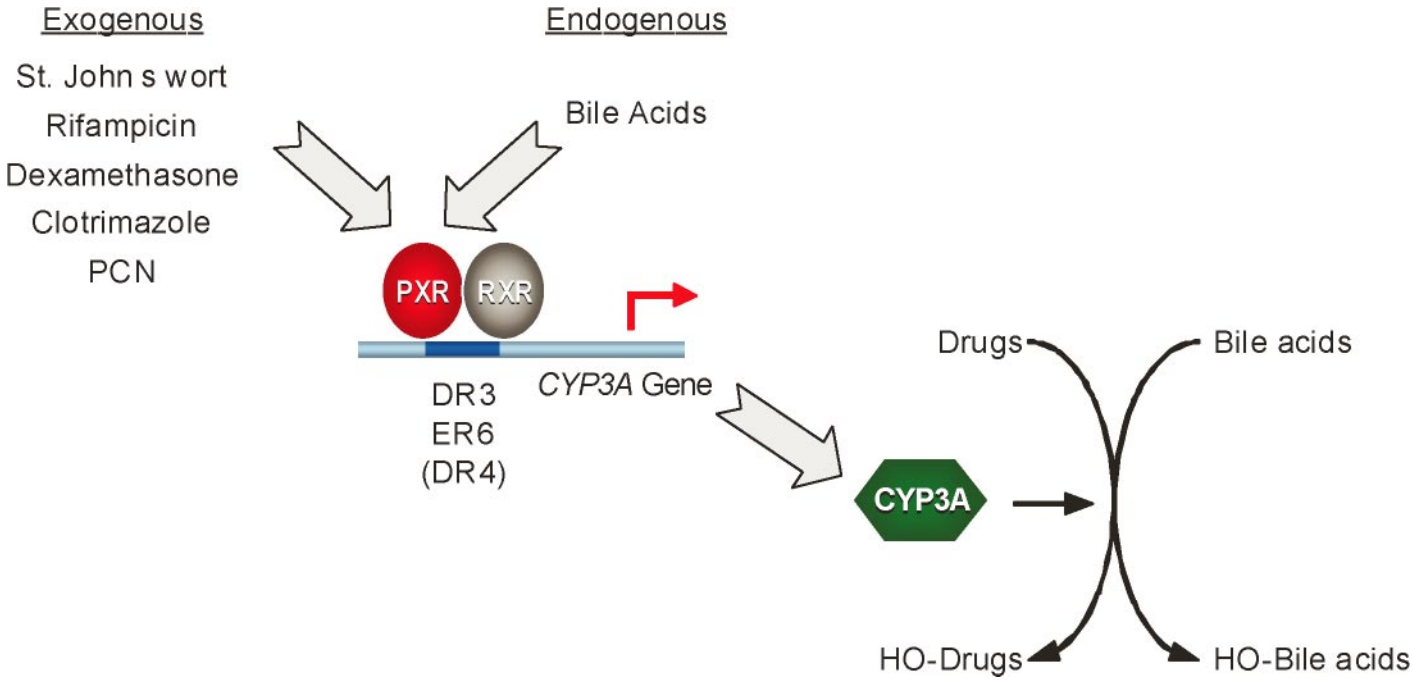

Figure 5 Role of PXR in xenobiotic and bile acid metabolism. PXR binds response elements in the regulatory regions of CYP3A genes as a heterodimer with RXRα. Activation of PXR by xenobiotics or bile acids induces expression of CYP3A, which in turn results in increased hydroxylation of potentially harmful drugs or bile acids.