0026-895X/05/6802-403–413$20.00
MOLECULAR PHARMACOLOGY
Vol. 68, No. 2
13292/3042220
Mol Pharmacol 68:403–413, 2005
Printed in U.S.A.

A Pregnane X Receptor Agonist with Unique Species-Dependent Stereoselectivity and Its Implications in Drug Development

Ying Mu, Corey R. J. Stephenson, Christopher Kendall, Simrat P. S. Saini, David Toma, Songrong Ren, Hongbo Cai, Stephen C. Strom, Billy W. Day, Peter Wipf, and Wen Xie

Center for Pharmacogenetics (Y.M., S.P.S.S., D.T., S.R., W.X.), Department of Pharmaceutical Sciences (Y.M., S.P.S.S., D.T., S.R., B.W.D., W.X.), Department of Chemistry (C.R.J.S., C.K., B.W.D., P.W.), Center for Chemical Methodologies and Library Development (C.R.J.S., C.K., P.W.), Department of Pathology (H.C., S.C.S.), and Department of Pharmacology (W.X.), University of Pittsburgh, Pittsburgh, Pennsylvania

Received March 28, 2005; accepted May 4, 2005

ABSTRACT

Pregnane X receptor (PXR) is an orphan nuclear receptor that regulates the expression of genes encoding drug-metabolizing enzymes and transporters. In addition to affecting drug metabolism, potent and selective PXR agonists may also have therapeutic potential by removing endogenous and exogenous toxins. In this article, we report the synthesis and identification of novel PXR agonists from a library of peptide isosteres. Compound **S20**, a *C*-cyclopropylalkylamide, was found to be a PXR agonist with both enantiomer- and species-specific selectivity. **S20** has three chiral carbons and was resolved into its two enantiomers. The individual **S20** enantiomers exhibited striking mouse/human-specific PXR activation, whereby enantiomer (+)-**S20** preferentially activated hPXR, and enantiomer (−)-**S20** was a better activator for mPXR. As a human PXR (hPXR) agonist, (+)-**S20** was more potent and efficacious than rifampicin. Mutagenesis studies revealed that the ligand binding domain residue Phe305 is critical for the preference for the (−)-**S20** enantiomer by the rodent PXR. Treatment of **S20** induced the expression of drug-metabolizing enzymes and transporters in reporter gene assays, in primary human hepatocytes, and in "humanized" hPXR transgenic mice. To our knowledge, **S20** represents the first compound whose enantiomers have opposite species preference in activating a xenobiotic receptor. The stereoselectivity may be used to guide the development of safer drugs to avoid drug-drug interactions or to achieve human-specific therapeutic effects when a xenobiotic receptor is being used as a drug target.

The orphan nuclear receptor pregnane X receptor (PXR; NR1I2) was originally identified as a species-specific xenosensor that regulates the expression of phase I cytochrome P450 enzymes (Blumberg et al., 1998; Kliewer et al., 1998; Xie et al., 2000a). Subsequent functional analysis revealed a much broader role of PXR in xenobiotic regulation. PXR can function as a master regulator in regulating phase I and II enzymes (Falkner et al., 2001; Sonoda et al., 2002; Xie et al., 2003), as well as members of the drug transporter family. This broad regulation is due to the presence of PXR-responsive elements in the promoter regions of many xenobiotic enzyme and transporter genes (for review, see Xie et al., 2004). This comprehensive role of PXR in xenobiotic regulation is supported by several DNA microarray-based gene profiling analyses performed in wild-type, transgenic, and knockout mouse models (Ueda et al., 2002; Rosenfeld et al., 2003). Although the initial functional characterization of PXR has focused on the liver and intestine, recent studies have revealed a broader extrahepatic role of PXR, such as the PXR-mediated regulation of P-glycoprotein expression and transport function at the blood-brain barrier (Bauer et al., 2004).

Even though PXR was identified as a "xenobiotic receptor", emerging evidence has suggested PXR as a potential thera-

This work was supported in part by National Institutes of Health grants ES012479 and CA107011 (to W.X.) and GM067082 (to P.W.). Y.M. is supported by an National Institutes of Health International Postdoctoral Fellowship (AT002029). Normal human hepatocytes were obtained through the Liver Tissue Procurement and Distribution System, Pittsburgh, Pennsylvania, which was funded by National Institutes of Health Contract N01-DK92310.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.013292.

ABBREVIATIONS: PXR, pregnane X receptor; LCA, lithocholic acid; CAR, constitutive androstane receptor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; FXR, farnesoid X receptor; LBD, ligand-binding domain; SAR, structure-activity relationship; hPXR, human PXR; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin; mPXR, mouse PXR; SRC, steroid receptor coactivator; rPXR, rat PXR; MRP2, multidrug resistance associated protein 2.

peutic target for several human diseases (Xie et al., 2004). This disease relevance is consistent with the notion that many of the PXR target genes are involved in the biotransformation and homeostasis of endogenous and exogenous chemicals that may influence physiological and pathological processes in mammals. For example, activation of PXR in mice has been shown to promote bilirubin clearance and prevent hyperbilirubinemia (Xie et al., 2003). This beneficial effect has been attributed to activation by PXR of bilirubin detoxifying genes, such as the conjugating enzymes UGT1A1 and the excretion transporter MRP2 (Sugatani et al., 2001; Kast et al., 2002; Huang et al., 2003; Xie et al., 2003).

PXR is also important for the prevention of bile acid toxicity. Bile acids are major by-products of cholesterol catabolism in the liver. Despite their beneficial role in solubilizing and absorbing lipids, accumulation of bile acids can cause irreversible liver damage, resulting in cholestasis. Both pharmacological and genetic activation of PXR in mice has been shown to confer resistance to lithocholic acid (LCA) hepatotoxicity (Staudinger et al., 2001; Xie et al., 2001). This protective effect may be due to a PXR-mediated combined induction of CYP3A (Xie et al., 2001) and the hydroxysteroid sulfotransferase (Sonoda et al., 2002). It is important to note that the constitutive androstane receptor (CAR) has also been shown to be important in the clearance of bilirubin and bile acids via overlapping yet distinct mechanisms (Huang et al., 2003; Xie et al., 2003; Saini et al., 2004). The vitamin D receptor-mediated induction of CYP3A is also suggested to promote bile acid detoxification when this receptor is activated by vitamin D receptor agonists or bile acids (Thummel et al., 2001; Makishima et al., 2002).

In this report, using the combination of chemical library development with functional assays, we report the identification of a novel class of PXR agonists from a library of synthetic peptide bond mimetics. **S20**, a *C*-cyclopropylalkylamide, was identified as an efficacious PXR agonist. It is interesting that although racemic **S20** activated both human PXR (hPXR) and mouse PXR (mPXR), the two enantiomers of **S20** exhibited striking species-specific PXR activation. This species-dependent stereoselectivity may provide guidance to avoid or to achieve species-specific xenobiotic receptor activation during pharmaceutical development.

Materials and Methods

Chemicals. The method for the synthesis of the chemical library has been described previously (Wipf and Kendall, 2001; Wipf et al., 2001, 2003). 1,4-bis[2-(3,5-Dichloropyridyloxy)] benzene (TCPOBOP) was a gift from Dr. Stephen Safe (Texas A&M, College Station, TX). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids and Transient Transfection. The reporter plasmids (tk-USA-Luc, tk-CYP3A4-Luc, tk-CYP3A23-Luc, tk-MRP2-Luc, and tk-EcRE-Luc) and the expression vectors [Gal-hPXR LBD, Gal-mPXR LBD, CAR, and farnesoid X receptor (FXR)] have been described previously (Xie et al., 2000b; Sonoda et al., 2002; Saini et al., 2004). The creation of rat PXR (rPXR) F305L and hPXR L308F mutants (gifts from Dr. Richard Kim) was described previously (Tirona et al., 2004). All reporter genes contain three copies of the corresponding response elements. Transfections were performed on 48-well plates. CV-1 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (Xie et al., 2000b). HepG2 cells were transfected using polyethylenimine polymer (kindly provided by Dr. Xiang Gao). Transfected cells were treated with appropriate compounds for 24 h before harvesting and assay for luciferase activity. Luciferase activity was normalized against the cotransfected and β-galactosidase activity. All transfections were performed in triplicate.

Human and Mouse Primary Hepatocyte Preparation and Treatment. Human livers were obtained through the Liver Tissue Procurement and Distribution System, and hepatocytes were isolated by three-step collagenase perfusion (Strom et al., 1996). Mouse hepatocytes were also prepared by collagenase perfusion. Cells were plated on gelatin-coated T25 flasks and maintained in hepatocyte maintenance medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) supplemented with 0.1 mM dexamethasone, 0.1 mM insulin, 50 μg/ml gentamicin, 50 ng/ml amphotericin, and incubated overnight. Cells were treated with appropriate drugs for 48 h before RNA harvest and Northern blot analysis. The drug treatment was reduced to 24 h when cycloheximide was added.

Animals and Drug Treatment. The PXR null mice and "humanized" hPXR transgenic mice have been described previously (Xie et al., 2000a). Mice were maintained with food and water available ad libitum. When necessary, mice were subjected to a single intraperitoneal injection of 50 mg/kg **S20** 24 h before sacrifice and tissue harvesting. The use of mice in this study complied with all relevant federal guidelines and institutional policies.

Northern Blot Analysis. Total RNA was isolated from mouse tissues or primary hepatocytes using the TRIzol reagent (Invitrogen). Northern blot analysis was performed as described previously (Xie et al., 2000a). The cDNA probe of the human CYP3A4 and UGT1A1 was cloned by reverse transcription-polymerase chain reaction from human liver mRNA. When necessary, quantification was performed with the NIH Image software.

Results

Identification of a PXR Agonists from a Combinatorial Chemical Library. We have recently reported the synthesis of a library of allylic amides, homoallylic amides, and *C*-cyclopropylalkylamides (Wipf and Kendall, 2001; Wipf et al., 2001, 2003; Janjic et al., 2005). The library was built to examine the scope of diversity-oriented chemical synthesis offered by the three-component aldimine condensation reaction, as well as to explore new scaffolds for biological activity. The effects of the library compounds on PXR were evaluated with a chimeric receptor in which the ligand-binding domain (LBD) of hPXR was fused to the DNA binding domain of the yeast transcription factor Gal4. The activity of PXR was determined using a Gal4-responsive reporter tk-UAS-Luc that contains three copies of the Gal4 binding site. The reporter gene -fold activations elicited by library compounds (at 10 μM) are summarized in Table 1. The chemical and biological information contained in Table 1 allows some general analysis of structure-activity relationship (SAR) for the library compounds as PXR agonists (see *Discussion*). It is noteworthy that **S20**, a *C*-cyclopropylalkylamide exhibited the highest reporter gene -fold activation among the 73 library compounds. **S20** has three asymmetric carbons and can be resolved into two enantiomers, (+)-**S20** and (−)-**S20** (Fig. 1, A and B). The carbon scaffold of **S20** is distinct from the chemical structures of known PXR agonists, such as the hPXR-specific RIF (Fig. 1C), and the rodent-specific pregnenolone-16α-carbonitrile (PCN) (Fig. 1D).

Species-Dependent Stereoselectivity of S20 in PXR Activation. The **S20** compound used for the initial screening was diastereomerically pure but contained an equal (racemic) mixture of the two enantiomers. Because the biological activity of a compound is often attributable to one enantio-

TABLE 1

hPXR activation of the library compounds

Corresponding structures are shown in Fig. 6. For hPXR activation assay, CV-1 cells were cotransfected with a chimeric receptor Gal-hPXR LBD and the tk-UAS-Luc reporter. Transfected cells were treated with the indicated compounds for 24 h before luciferase assay. Results are shown as -fold induction over vehicle controls and represent the mean ± S.D. from triplicate assays. Concentration of ligand was 10 μM.

Compound	C1-C2[a]	R^1	R^2	R^3	R^4	R^5	R^6	-Fold Activation
Allylic amides (racemic)								
S1		$P(O)Ph_2$	2-C_6H_4OMe	H	H	*n*-Bu		3.4 ± 0.20
S2		$P(O)Ph_2$	3-C_6H_4OMe	H	H	*n*-Bu		4.0 ± 0.45
S3		$P(O)Ph_2$	4-C_6H_4Cl	H	H	*n*-Bu		3.3 ± 0.06
S4		$P(O)Ph_2$	3-$C_6H_4NO_2$	H	H	*n*-Bu		4.1 ± 0.06
S5		$P(O)Ph_2$	4-$C_6H_4NO_2$	H	H	*n*-Bu		3.8 ± 0.21
S6		$P(O)Ph_2$	4-$C_6H_4CO_2Me$	$SiMe_3$	H	(*E*)-CH = CHC_6H_{13}		2.7 ± 0.35
S26		$P(O)Ph_2$	Ph	H	H	*n*-Bu		3.4 ± 0.40
S27		$P(O)Ph_2$	Ph	Et	H	Et		4.4 ± 0.15
S28		$P(O)Ph_2$	4-$C_6H_4CO_2Me$	H	H	*n*-Bu		3.5 ± 0.29
S29		$P(O)Ph_2$	Ph	H	H	$(CH_2)_2OSi(t\text{-Bu})Ph_2$		3.3 ± 0.56
S30		$P(O)Ph_2$	Ph	H	H	$(CH_2)_2CO_2Si(i\text{-Pr})_3$		1.6 ± 0.29
S31		$P(O)Ph_2$	Ph	H	H	$(CH_2)_2N(Ts)CO_2Et$		2.2 ± 0.26
S32		$P(O)Ph_2$	(*E*)-CH = CHPh	H	H	*n*-Bu		3.2 ± 0.17
S33		$P(O)Ph_2$	(*E*)-C(Me)=CHPh	H	H	*n*-Bu		4.0 ± 0.21
S34		$P(O)Ph_2$	C≡CPh	H	H	*n*-Bu		3.0 ± 0.25
S35		$P(O)Ph_2$	Ph	H	Me	*n*-Bu		3.4 ± 0.06
S36		$P(O)Ph_2$	Ph	Me	H	H		3.6 ± 0.21
S37		Ts	Ph	H	H	*n*-Bu		3.5 ± 0.12
S38		Ts	$(CH_2)_2Ph$	H	H	*n*-Bu		3.1 ± 0.17
C-Cyclopropylalkylamides (racemic)								
S7	*anti*	$P(O)Ph_2$	3-C_6H_4OMe	H	H	*n*-Bu		4.4 ± 0.69
S8	*anti*	$P(O)Ph_2$	4-C_6H_4Cl	H	H	*n*-Bu		2.7 ± 0.40
S12	*anti*	$P(O)Ph_2$	Ph	H	H	$(CH_2)_2OH$		1.7 ± 0.15
S13	*anti*	$P(O)Ph_2$	2-C_6H_4OMe	H	H	*n*-Bu		2.3 ± 0.15
S14	*anti*	$P(O)Ph_2$	Ph	H	H	$CH = CH_2$		4.2 ± 0.55
S15	*anti*	$P(O)Ph_2$	Ph	Me	H	$CH = CH_2$		4.2 ± 0.06
S16	*anti*	$P(O)Ph_2$	Ph	H	H	CO_2Me		1.9 ± 0.45
S17	*anti*	CO_2Bn	Ph	H	H	CO_2Me		2.4 ± 0.11
S18	*anti*	CO_2Bn	Ph	H	H	CONH*i*-Pr		3.6 ± 0.10
S19	*anti*	CO_2Bn	Ph	Me	H	CONH*i*-Pr		3.0 ± 0.36
S20	*syn*	CO_2Bn	Ph	H	Me	CONH*i*-Pr		5.4 ± 0.64
S39	*anti*	$P(O)Ph_2$	Ph	H	H	*n*-Bu		3.2 ± 0.21
S40	*syn*	$P(O)Ph_2$	Ph	H	H	*n*-Bu		3.3 ± 0.31
S41	*anti*	C(O)Ph	Ph	H	H	*n*-Bu		3.6 ± 0.23
S42	*anti*	C(O)-4-$C_6H_4NO_2$	Ph	H	H	*n*-Bu		2.8 ± 0.26
S43	*anti*	$P(O)Ph_2$	Ph	H	H	$(CH_2)_2OSi(t\text{-Bu})Ph_2$		2.5 ± 0.29
S44	*anti*	$P(O)Ph_2$	Ph	H	H	$(CH_2)_2CO_2Si(i\text{-Pr})_3$		2.1 ± 0.10
S45	*anti*	$P(O)Ph_2$	Ph	H	H	$(CH_2)_2N(Ts)CO_2Et$		0.6 ± 0.06
S46	*anti*	$P(O)Ph_2$	C≡CPh	H	H	*n*-Bu		3.6 ± 0.32
S47		$P(O)Ph_2$	Ph	Me	H	H		4.1 ± 0.23
S48	*syn*	$P(O)Ph_2$	Ph	H	Me	*n*-Bu		3.6 ± 0.25
S49	*anti*	$P(O)Ph_2$	Ph	Et	H	Et		3.7 ± 0.51
S50	*anti*	Ts	Ph	H	H	*n*-Bu		4.3 ± 0.32
S51	*syn*	Ts	Ph	H	H	*n*-Bu		4.5 ± 0.45
S52	*anti*	Ts	$(CH_2)_2Ph$	H	H	*n*-Bu		3.4 ± 0.35
C-Dicyclopropylalkylamides (racemic)								
S9		$P(O)Ph_2$	4-$C_6H_4CO_2Me$	Me	*n*-C_6H_{13}			3.1 ± 0.36
S10		C(O)-3,5-$C_6H_3(NO_2)_2$	4-$C_6H_4CO_2Me$	Me	*n*-C_6H_{13}			1.5 ± 0.12
S11		$P(O)Ph_2$	Ph	Me	$(CH_2)_2OH$			3.0 ± 0.29
C-Cyclopropylalkylamides (enantiomerically pure)								
S21		CO_2Bn	Ph	(*S*)-NHCH(Me)Ph				2.1 ± 0.20
S22		CO_2Bn	Ph	NH-4-C_6H_4Br				1.3 ± 0.10
S24		CO_2Bn	Ph	L-Phe-OMe				1.3 ± 0.06
S25		C(O)-4-C_6H_4Br	Ph	OMe				1.4 ± 0.15
S23		CO_2Bn	Ph	L-Phe-OMe				1.5 ± 0.10
Trisubstituted *C*-Cyclopropylalkylamides (racemic)								
S53		$P(O)Ph_2$	Ph	*n*-Bu	Me			2.9 ± 0.15
S54		C(O)-3,5-$C_6H_3(NO_2)_2$	Ph	*n*-Bu	Me			2.9 ± 0.15
S55		C(O)Ph	Ph	*n*-Bu	Me			3.8 ± 0.06
Homoallylic amides (racemic)								
S57	*syn*	$P(O)Ph_2$	Ph	Et	H	Et	H	3.0 ± 0.20
S58	*anti*	$P(O)Ph_2$	Ph	Et	H	Et	H	3.5 ± 0.35
S59	*anti*	$P(O)Ph_2$	4-$C_6H_4CO_2Me$	$(CH_2)_2OSi(t\text{-Bu})Ph_2$	H	H	H	2.7 ± 0.10
S60	*syn*	$P(O)Ph_2$	4-C_6H_4OMe	*n*-Bu	H	H	H	3.6 ± 0.06
S61	*syn*	Ts	Ph	$(CH_2)_2OSi(t\text{-Bu})Ph_2$	H	H	H	3.3 ± 0.55
S62	*anti*	$P(O)Ph_2$	Ph	$(CH_2)_2OSi(t\text{-Bu})Ph_2$	H	H	H	2.2 ± 0.20
S63	*syn*	C(O)-3,5-$C_6H_3(NO_2)_2$	Ph	*n*-Bu	H	H	H	3.2 ± 0.23
S64	*syn*	Ts	Ph	*n*-Bu	H	H	H	3.7 ± 0.53
S65		$P(O)Ph_2$	Ph	H	H	Me	H	3.5 ± 0.30
S66		$P(O)Ph_2$	Ph	Me	Me	H	Me	4.3 ± 0.55

TABLE 1 *Continued.*

Compound	C1-C2[a]	R^1	R^2	R^3	R^4	R^5	R^6	-Fold Activation
Propargylic amides (racemic)								
S67		$P(O)Ph_2$	Ph	*n*-Bu				3.8 ± 0.25
1,1-Dichlorocyclopropanes (racemic)								
S68		Ph	$4\text{-}C_6H_4O(CH_2)_2NMe_2$	Ph				0.9 ± 0.06
S69		$4\text{-}C_6H_4OMe$	$4\text{-}C_6H_4OBn$	Ph				2.1 ± 0.26
S70		Ph	Ph	Ph				1.2 ± 0.06
S71		H	Ph	Ph				2.9 ± 0.21
S72		Ph	$4\text{-}C_6H_4O(CH_2)_2Br$	$4\text{-}C_6H_4OMe$				2.3 ± 0.42
S73		$4\text{-}C_6H_4OMe$	$4\text{-}C_6H_4OBn$	$4\text{-}C_6H_4OMe$				1.8 ± 0.10

Ph, phenyl (C_6H_5); Me, methyl (CH_3); Et, ethyl (CH_2CH_3); Pr, propyl ($(CH_2)_2CH_3$); Bu, butyl ($(CH_2)_3CH_3$); Ts, *p*-toluenesulfonyl ($p\text{-}CH_3C_6H_4SO_2$); Bn, benzyl (CH_2Ph); Phe, phenylalanine.

[a] Relative stereochemistry.

mer of a racemate (Kupfer et al., 1977), the **S20** enantiomers were prepared separately and evaluated for PXR activation using the Gal-PXR transfection systems. The potential species preference was evaluated by comparing the reporter activity elicited by Gal-hPXR with that elicited by Gal-mPXR. Although the racemic **S20** activated both hPXR and mPXR, the activation of PXR by the two enantiomers was remarkably species-dependent (Fig. 2, A and B). As shown in Fig. 2A, when Gal-hPXR was used, enantiomer (+)-**S20** showed a modestly higher activity than the racemic **S20**. In sharp contrast, enantiomer (−)-**S20** was a weak hPXR activator, inducing the reporter only 2-fold. The opposite enantiomorphic preference was observed when Gal-mPXR was used. The activity of (+)-**S20** on mPXR was less than half of that of racemic **S20**, whereas (−)-**S20** was fully active (Fig. 2B). The stereoselectivity was further evaluated in primary hepatocyte cultures. In human hepatocytes, (+)-**S20** was a potent CYP3A4 mRNA inducer, whereas (−)-**S20** had little effect (Fig. 2C). When wild-type mouse hepatocytes were used (−)-**S20**, but not (+)-**S20**, induced CYP3A11 expression (Fig. 2D). RIF and PCN were included to verify the hepatocyte responsiveness. We conclude that the activation of hPXR and mPXR by **S20** shows opposing enantioselectivity. Note that the respective induction of CYP3A4 and CYP3A11 in the human and mouse hepatocytes was sustained in the presence of 1 μg/ml of the protein synthesis inhibitor cycloheximide (Fig. 2, E and F). This concentration of cycloheximide has been shown to inhibit protein synthesis in primary hepatocytes (Kotokorpi et al., 2004), suggesting that the **S20**-mediated CYP3A mRNA induction does not require new protein synthesis.

S20 Is an Efficacious PXR-Specific Agonist that Induces Coactivator Recruitment. The efficacy of racemic **S20** and its enantiomers as hPXR agonists was measured and compared with RIF using the Gal-hPXR transfection system. As shown in Fig. 3A, both **S20** and RIF activated

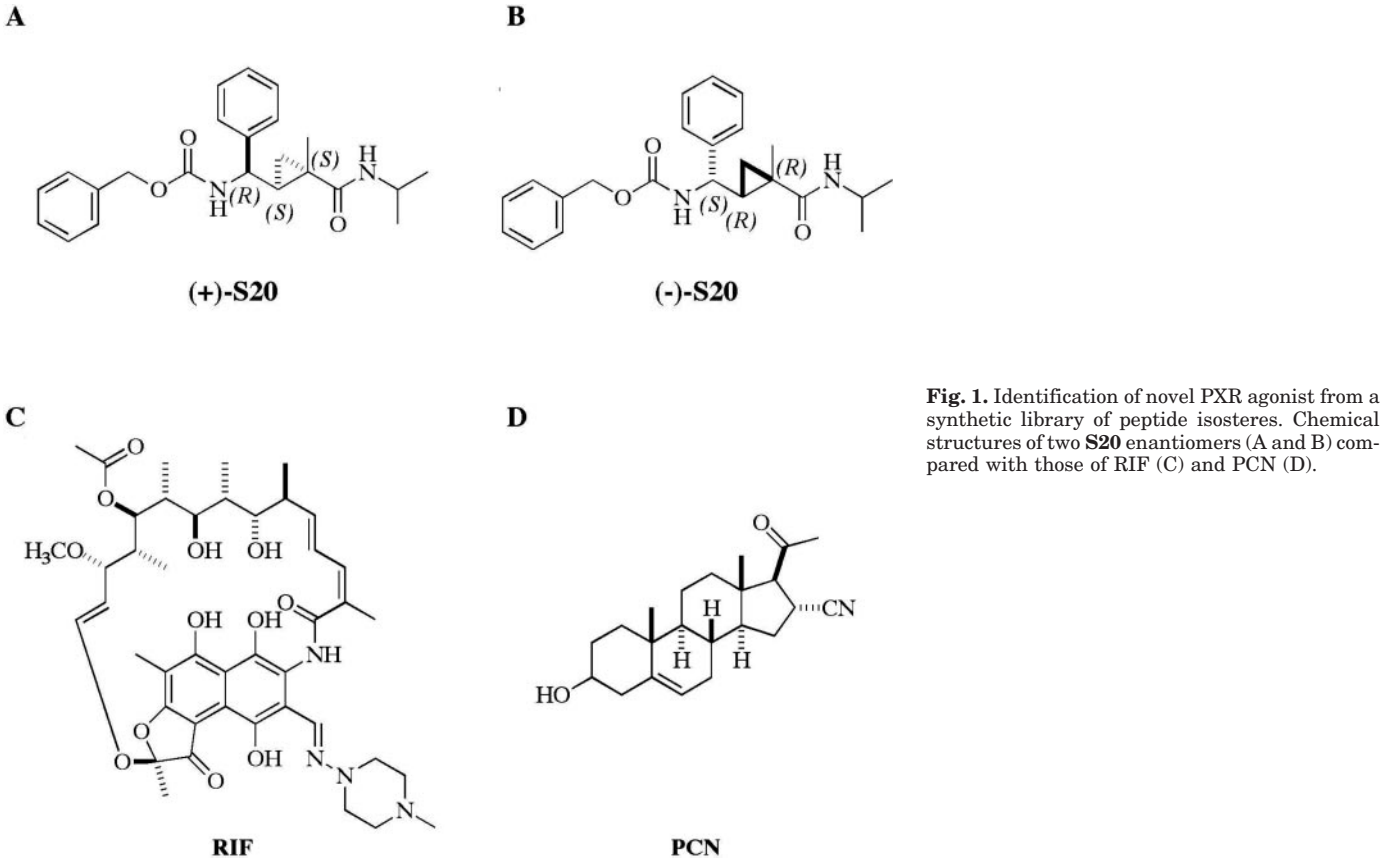

Fig. 1. Identification of novel PXR agonist from a synthetic library of peptide isosteres. Chemical structures of two **S20** enantiomers (A and B) compared with those of RIF (C) and PCN (D).

hPXR in a concentration-dependent manner. Compared with RIF, racemic **S20** was more potent but had a similar EC_{50} of 2 μM. The enantiomer (+)-**S20,** on the other hand, was not only more potent but also more efficacious than RIF, with an estimated EC_{50} of 400 nM. The superior potency and efficacy of (+)-**S20** compared with RIF was also confirmed when the wild-type hPXR and a PXR-responsive reporter gene (tk-CYP3A4-Luc) were used in the transfection (Fig. 3B). The ability of (−)-**S20** to activate mPXR was compared with that of PCN, a prototypical mPXR-specific agonist. Although (−)-**S20** is preferred by mPXR (Fig. 2B), this enantiomer had a similar potency and efficacy in activating the wild-type mPXR and inducing tk-CYP3A23-Luc, another PXR-responsive reporter gene (Fig. 3C). The approximate equivalency of (−)-**S20** in mPXR activation (compared with PCN) was also confirmed in an independent Gal-mPXR transfection assay (data not shown).

A hallmark of ligand-dependent activation of nuclear receptors is the recruitment of p160 nuclear receptor coactivators, such as the steroid receptor coactivator 1 (SRC-1). The Gal-hSRC-1 receptor interaction domain transfection system was used to examine whether treatment with **S20** caused interaction between hSRC-1 and PXR. As shown in Fig. 3D, cotransfection of VP-mPXR or VP-hPXR, in the absence of agonists, induced the reporter activity by 2- to 3-fold, consistent with our recent report (Saini et al., 2005). The PXR-hSRC-1 interaction was enhanced by the treatment with racemic **S20**. RIF and PCN were also analyzed to verify the responsiveness and specificity of this coactivator recruitment assay.

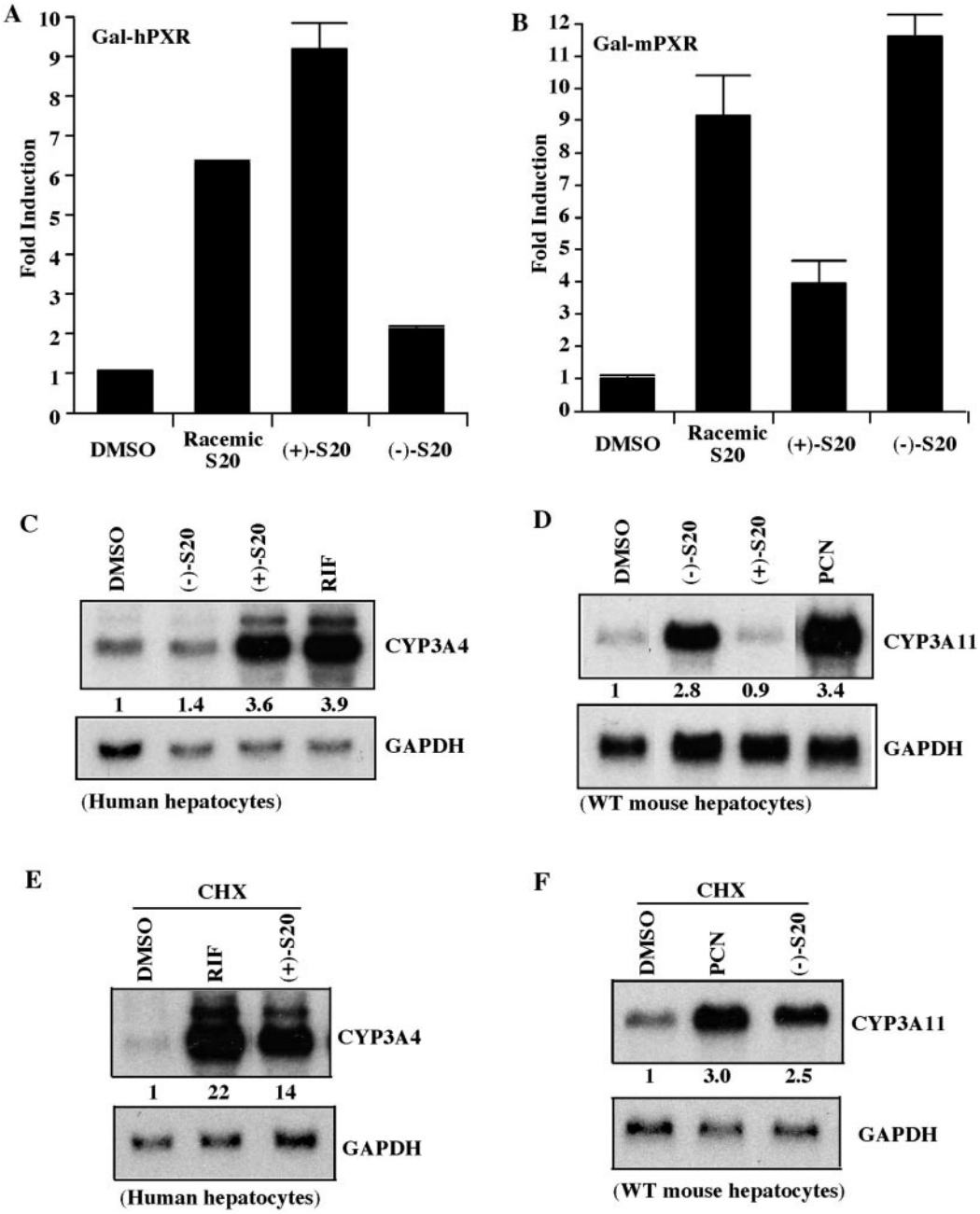

Fig. 2. Species-dependent stereoselectivity of **S20** in PXR activation. A and B, CV-1 cells were cotransfected with tk-UAS-Luc reporter and Gal-hPXR LBD (A) or Gal-mPXR LBD (B). Transfected cells were treated with indicated compounds at 10 μM for 24 h before luciferase assay. Results are shown as -fold induction over vehicle controls and represent the average and S.E. from triplicate assays. C, enantiomer (+)-**S20,** but not (−)-**S20** (10 μM each), induced CYP3A4 mRNA expression in human hepatocytes. RIF (10 μM) was used as a positive inducer. D, enantiomer (−)-**S20,** but not (+)-**S20** (20 μM each), induced CYP3A11 mRNA expression in wild-type mouse hepatocytes. PCN (10 μM) was used as a positive inducer. E and F, **S20** enantiomer-mediated induction of CYP3A in the human (E) and mouse (F) hepatocytes is sustained in the presence of the protein synthesis inhibitor cycloheximide (1 μg/ml).

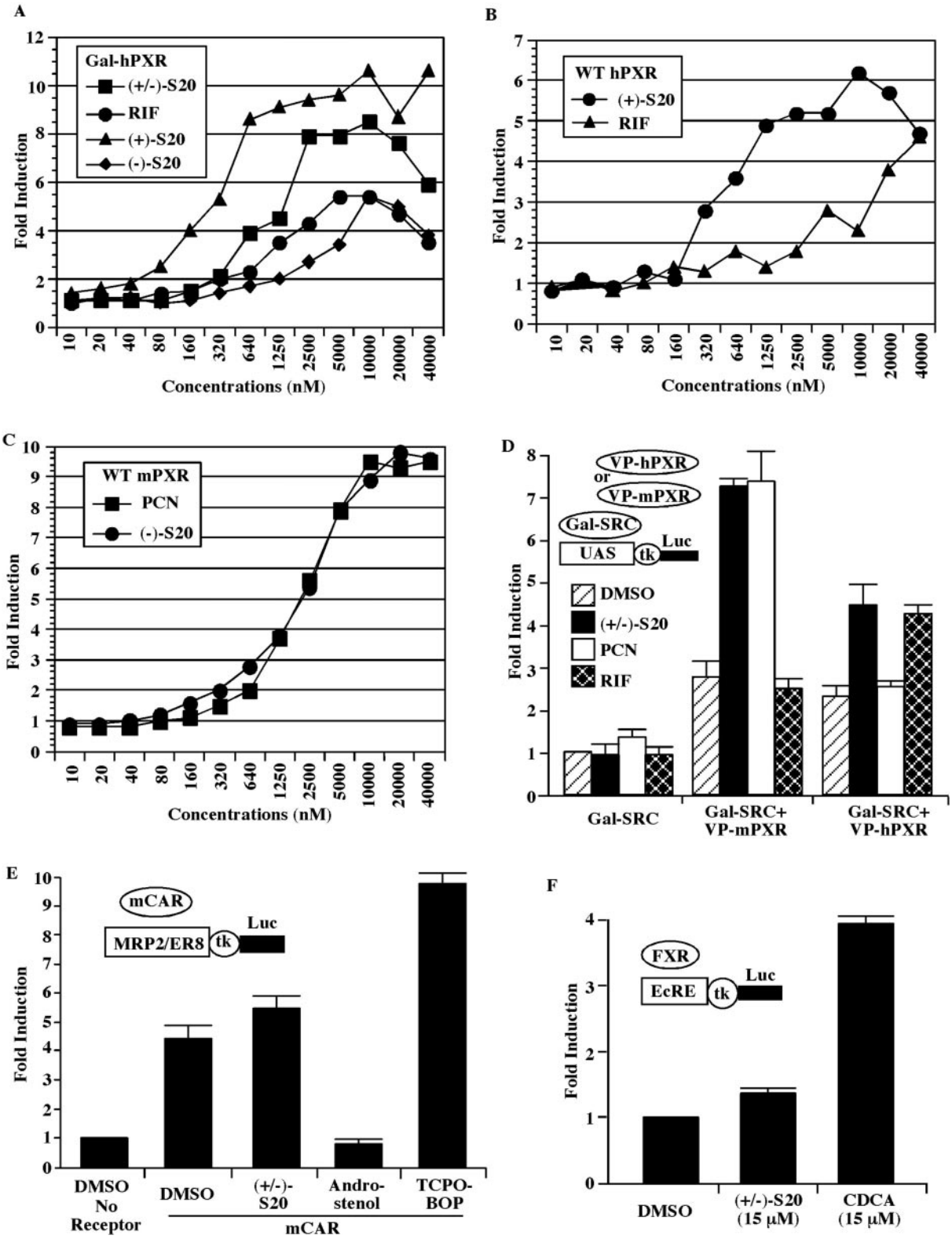

Fig. 3. S20 is an efficacious PXR-specific agonist that induces coactivator recruitment. A, enantiomer (+)-**S20** was a more potent and efficacious hPXR agonist than RIF. HepG2 cells were cotransfected with Gal-hPXR LBD and tk-UAS-Luc reporter before treatment with increasing concentrations of racemic or enantiomeric **S20** or RIF. Results are shown as -fold induction over vehicle controls and represent the average from triplicate assays. B, the superior potency and efficacy of (+)-**S20** compared with RIF were verified when the wild-type hPXR and a PXR-responsive tk-CYP3A4-Luc reporter gene were used to transfect CV-1 cells. C, enantiomer (−)-**S20** had a similar potency and efficacy to activate the wild-type mPXR and to induce the PXR responsive tk-CYP3A23-Luc reporter gene. Hep2 cells were used for transfection. D, ligand-independent and -dependent interactions between PXR LBD and hSRC-1. CV-1 cells were cotransfected with tk-UAS-Luc reporter and Gal-hSRC1-RID in the absence or presence of VP-mPXR or VP-hPXR expression vectors. Transfected cells were treated with vehicle or the indicated compounds for 24 h before luciferase assay. E, **S20** had no effect on CAR activity. CV-1 cells were transfected with the CAR-responsive tk-MRP2-Luc reporter alone or in the presence of mCAR expression vector before drug treatment. F, **S20** did not activate FXR. CV-1 cells were cotransfected with FXR and the FXR-responsive tk-EcRE-Luc reporter before drug treatment. Concentrations of ligands in D and E were, for **S20**, RIF, and PCN, 10 μM, for androstenol, 5 μM, and for TCPOBOP, 250 nM. Ligand concentrations in F are indicated.

S20 was a PXR-specific agonist, in that it had no effect on the activity of CAR and FXR. When mCAR was cotransfected with the CAR-responsive tk-MRP2-Luc reporter, racemic **S20** did not alter the constitutive activity of CAR (Fig. 3E). In the same transfection, CAR activity was inhibited by androstenol and increased by TCPOBOP as expected (Fig. 3E). Neither the racemic nor the enantiomerically pure **S20** activated hCAR in a similar transfection assay (data not shown). Likewise, FXR was activated by chenodeoxycholic acid (CDCA), as expected, but not by racemic **S20**, when the FXR-responsive tk-EcRE-Luc reporter was cotransfected (Fig. 3F).

S20 Induces the Expression of Drug-Metabolizing Enzymes and Transporters in Reporter Gene Assays, in Human Hepatocytes, and in "Humanized" Mice. PXR is known to regulate *CYP3A* genes (Blumberg et al., 1998; Kliewer et al., 1998). To examine whether or not the potential PXR ligands can bind and activate PXR to induce CYP3A gene expression, we performed an independent ligand activation assay using the full-length mPXR or hPXR and the CYP3A reporter gene tk-CYP3A4-Luc. This reporter contains three copies of the ER-6 type PXR response element derived from the *CYP3A4* gene (Blumberg et al., 1998). In both mPXR- and hPXR-transfected cells, the tk-CYP3A4 reporter was activated by racemic **S20** (Fig. 4A; hPXR data not shown). Activation of PXR by racemic **S20** also induced the expression of tk-MRP2-Luc (Fig. 4B), a PXR-responsive reporter that contains three copies of the ER-8 type element derived from the drug transporter MRP2 gene.

The capability of **S20** to induce PXR target phase I and II enzymes was also evaluated in human hepatocytes. As shown in Fig. 4C, compared with vehicle-treated cells, treatment of hepatocytes (from donor HH1052) with 5 μM racemic **S20** induced the mRNA expression of both CYP3A4 and UGT1A1

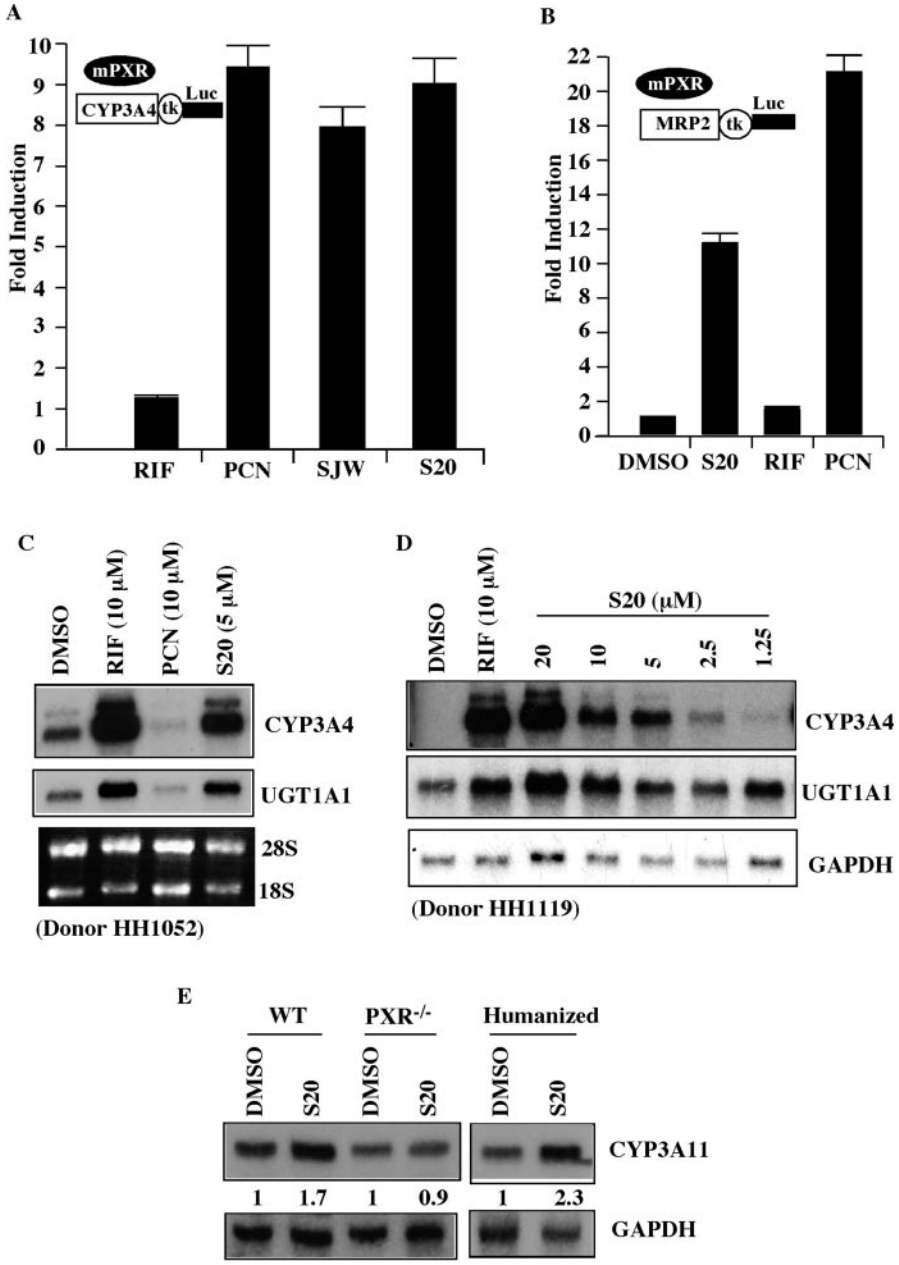

Fig. 4. S20 induces the expression of drug-metabolizing enzymes and transporters in reporter gene assays, in human hepatocytes, and in "humanized" mice. A, PXR-mediated activation of CYP3A4 reporter gene by **S20**. CV-1 cells were cotransfected with the PXR-responsive tk-CYP3A4-Luc reporter and mPXR expression vector. Transfected cells were treated with indicated compounds. Results are shown as -fold induction over vehicle controls and represent the average from triplicate assays. B, PXR-mediated activation of MRP2 reporter gene by **S20**. CV-1 cell were cotransfected with the PXR-responsive tk-MRP2-Luc reporter and expression vector for mPXR before ligand treatment. C, treatment of **S20** induced the mRNA expression of CYP3A4 and UGT1A1 in primary human hepatocytes. Cells were treated with compounds for 48 h before harvesting for total RNA and Northern blot analysis. The ethidium bromide staining of the agarose gel shows the equal loading. D, Northern blot analysis showing a concentration-dependent induction of CYP3A4 and UGT1A1 mRNA in another preparation of human hepatocytes. GAPDH probing was used as loading control. Concentrations of ligands were 10 μM for A and B except for St John's wort (SJW), which was 300 mg/ml or as indicated in C and D. E, PXR is necessary and sufficient to mediate the **S20**-induced CYP3A11 expression in mice. Mice of indicated genotypes were given a single intraperitoneal injection of vehicle or **S20** (50 mg/kg) 24 h before tissue harvesting. Total liver RNA was subjected to Northern blot analysis. All **S20** used in Fig. 4 was racemic.

as revealed by Northern blot analysis. The potency of induction was comparable with that induced by 10 μM RIF. As expected, PCN did not induce the expression of either enzyme. Concentration-dependent induction of CYP3A4 and UGT1A1 by **S20** was also observed in hepatocytes prepared from another donor (HH1119) (Fig. 4D). The lower basal expression of CYP3A4 in HH1119 compared with that in HH1052 is consistent with the known individual variation in the expression of this enzyme (Strom et al., 1996).

We previously reported the creation of PXR null and "humanized" hPXR transgenic mice (Xie et al., 2000a). The "humanized" mice were generated by introducing a liver-specific hPXR transgene into the mPXR null background (Xie et al., 2000a). The creation of these mice allowed for the evaluation of the need for PXR in the induction of drug-metabolizing enzymes by **S20** in vivo. As shown in Fig. 4E, treatment of wild-type mice with a single dose of **S20** (50 mg/kg body weight) induced the expression of CYP3A11 and this induction was abolished in PXR-null mice. In contrast, the CYP3A11 inducibility was restored in the "humanized" hPXR transgenic mice. Therefore, PXR is necessary for CYP3A11 induction by **S20**, and hPXR alone was sufficient to restore the regulation.

Structural Determinants of PXR in the S20 Enantioselectivity. The LBD of rPXR exhibits 98% amino acid homology with that of mPXR (Zhang et al., 1999). These two receptors share ligand profiles (data not shown). Tirona et al. (2004) recently reported the identification of amino acids in rPXR that determine species-specific rifampicin activation. The Phe305 of rPXR (conserved in mPXR) and its human counterpart Leu308, residues that are located within or are neighboring the flexible loop that forms part of the pore to the ligand-binding cavity, were found to be critical for the species-dependent ligand specificity (Tirona et al., 2004). We then used rPXR F305L and hPXR L308F mutants (Tirona et al., 2004) to examine whether the same residues are also important to determine the **S20** enantiospecificity. As shown in Fig. 5, the preference of rPXR to (−)-**S20** was completely abolished in the rPXR F305L mutant (Fig. 5A). In contrast, the L308F mutation had little effect on the hPXR's preference to (+)-**S20** (Fig. 5B).

Discussion

The species-dependent stereoselectivity of **S20** in PXR activation is of particular interest. Human and rodent PXRs are known to have overlapping yet distinct ligand profiles. This species-dependent ligand specificity is believed to be caused by the divergence of amino acid sequences in the LBDs of the human and mouse PXR receptors (Watkins et al., 2001; Tirona et al., 2004). Mutagenesis studies in the context of the highly conserved rPXR revealed that Phe305, a residue that is important for rifampicin specificity (Tirona et al., 2004), was also critical for the preference of rPXR for the (−)-**S20** enantiomer (Fig. 5A). Phe305 is located in the flexible loop that forms part of the pore to the ligand-binding cavity, based on the published crystal structure of hPXR LBD (Watkins et al., 2001). To our surprise, the corresponding L308F mutation had little effect on the preference of hPXR for the (+)-**S20** enantiomer (Fig. 5B). A direct **S20** enantiomer-PXR LBD docking modeling was not successful because of the flexible structures of the two enantiomers (M. Redinbo, personal communication). Nevertheless, the current findings represent, to our knowledge, the first example that an enantiomeric pair of compounds is capable of activating an orphan nuclear receptor in a species-specific manner.

Our findings are potentially implicated in drug development. From the perspective of pharmaceutical development, the conceivable benefits of this knowledge of stereoselectivity are: 1) for pharmaceutical agents whose therapeutic effects do not rely on PXR, the choice of PXR-neutral but therapeutically effective enantiomers may help to avoid untoward drug-drug interactions. The drug-induced enzyme production is the primary mechanism for the side effect of drug-drug interactions; and 2) for drugs whose therapeutic target is PXR (see below for discussion), a hPXR-specific enantiomer will be necessary to achieve intended therapeutic effects in humans. Thus, the identification of species- and enantiomer-specific ligands for PXR can be both desirable and valuable. This work takes a first step in this direction by demonstrating exquisite species-dependent enantiomer-based selectivity. Future studies are necessary to determine whether the stereoselectivity is applicable to other xenobiotic nuclear receptors, such as CAR, or orphan nuclear receptors in general.

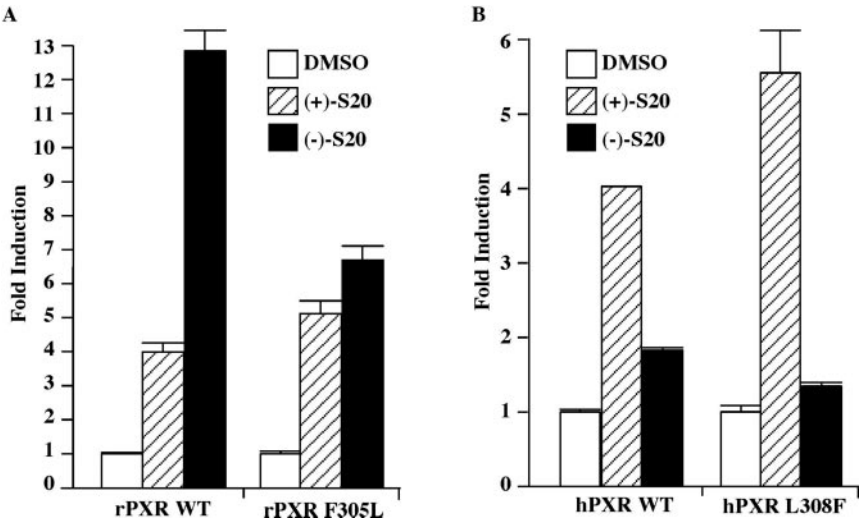

Fig. 5. Structural determinants of PXR in the **S20** enantioselectivity. HepG2 cells were transfected with tk-CYP3A23-Luc reporter gene, together with the wild-type and mutant rPXR (A) or hPXR (B). Transfected cells were treated with vehicle or the indicated **S20** enantiomers for 24 h before luciferase assay. Results are shown as -fold induction over vehicle controls and represent the average from triplicate assays.

The analysis of biological effects summarized in Table 1 and Fig. 6 allows some generalized SAR analysis for PXR agonists with the allylic amide and *C*-cyclopropylalkylamide scaffolds. Although both scaffolds contain some high-potency agonists (-fold induction >4; i.e., **S2**, **S4**, **S7**, **S14**, **S15**, **S20**, **S27**, **S33**, **S47**, **S50**, **S51**, and **S66**), the percentage of low potency agents (fold induction <2) is considerably higher for the *C*-cyclopropylalkylamides (i.e., **S10**, **S12**, **S16**, **S22**, **S23**, **S24**, **S25**, **S30**, **S45**, **S68**, **S70**, and **S73**) than for the allylic amides (i.e., **S30**), thereby making the *C*-cyclopropylalkylamide scaffold more responsive to SAR fine-tuning. It is interesting that **S20**, the most potent and, so far, the most intriguing PXR agonist, has a cyclopropylalkylamide scaffold. For structurally closely related allylic amides and *C*-cyclopropylalkylamides, consistent levels of agonism are observed (**S2** and **S7**). Homoallylic amides also show a relatively flat SAR; only **S66** qualifies as a high-potency agonist. Among the *C*-cyclopropylalkylamides, ester or extended chain amide substitution patterns at the cyclopropane ring are not well tolerated (for example, **S16**, **S22**, **S24**, **S25**, and **S45**), and neither are halogen substituents at the cyclopropane methylene group (**S68**, **S70**, and **S73**). In contrast, a broad range of *N*-substituents are among the most active compounds. Examples include $P(O)Ph_2$ in **S14** and **S47**, CO_2Bn in **S20**, and *p*-toluenesulfonyl (*p*-$CH_3C_6H_4SO_2$) in **S50**. The relative configuration of the cyclopropane ring and the amide methine carbon seems to play a minor role (for example, **S50** versus **S51**), which is not overly surprising because the C(1) terminus of the *C*-cyclopropylalkylamide scaffold is freely rotating and substituted with large aromatic groups that are likely to fold onto the target in a fashion to optimize hydrophobic interactions.

Although PXR was isolated as a "xenobiotic receptor" that regulates drug metabolism, accumulating evidence has implicated the role of PXR in the treatment and prevention of human diseases, such as hyperbilirubinemia and bile acid-associated cholestasis. Genetic activation of PXR in transgenic mice prevents hyperbilirubinemia and LCA-induced hepatotoxicity (Xie et al., 2001, 2003). Similar genetic and pharmacological studies suggest that activation of CAR is also protective against hyperbilirubinemia and LCA hepatotoxicity (Huang et al., 2003; Saini et al., 2004). The notion that activation of PXR and CAR may be medically beneficial has also been supported by clinical observations. For example, RIF has been shown to relieve pruritus in cholestatic liver disease by stimulating 6α-hydroxylation and elimination of bile acids (Wietholtz et al., 1996). This therapeutic effect of RIF is in agreement with the identification of RIF as a human-specific PXR activator and CYP3A4 inducer. Likewise, phenobarbital and the Chinese herbal remedy "Ying Zhi Huang" have been clinically used to treat neonatal hyperbilirubinemia (Valaes et al., 1980; Huang et al., 2004). This therapeutic effect has recently been attributed to the activation of CAR and subsequent induction of bilirubin detoxifying enzymes and transporters (Huang et al., 2003, 2004; Xie et al., 2003). It is our opinion that accumulating evidence, including animal studies and anecdotal clinical observation, is supporting the role of xenobiotic receptors as potential drug targets, and potent and selective PXR and CAR agonists may gain therapeutic value. However, it remains to be determined whether the **S20** enantiomers have the appropriate properties, such as pharmacokinetics and bioavailability, to serve as pharmacological agents in humans. The in vivo induction of CYP3A11 by **S20** in the "humanized" hPXR transgenic mice, although modest, was promising, in our opinion, in that it represents our first efforts with a compound from this series in animals, and **S20** retained activity. The dose and scheduling of treatment were those used previously for some of the known PXR agonists, such as PCN and RIF. The in vivo pharmacokinetic and pharmacodynamic properties of **S20** are still unknown; it is

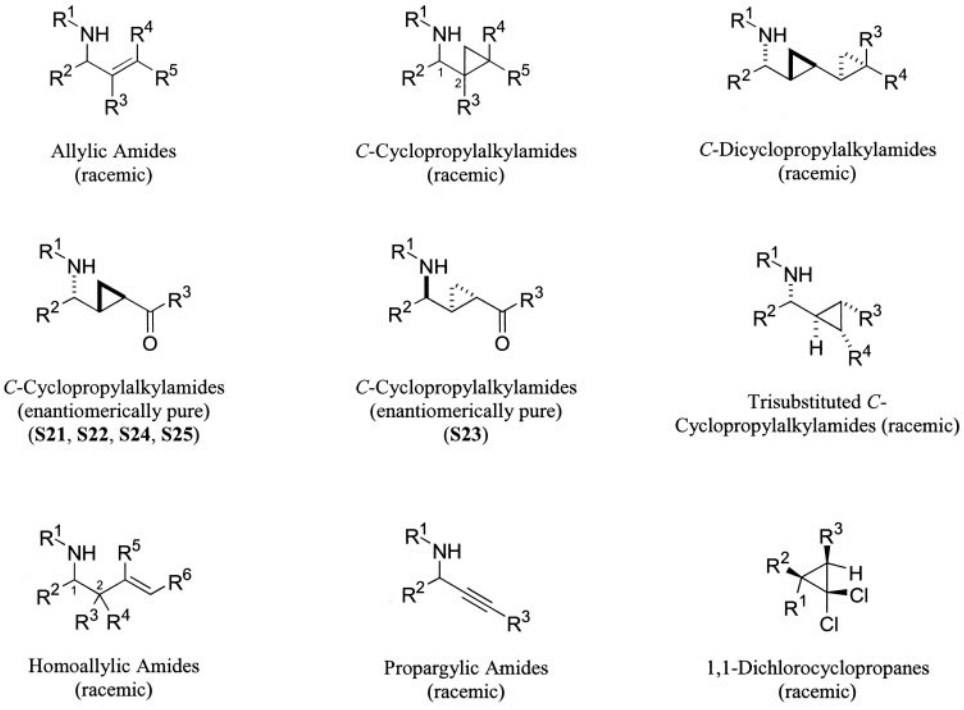

Fig. 6. Compound structures. See Table 1 for explanation.

likely that the regimen of treatment we chose is not optimal for target gene regulation.

The majority of the published reports on PXR ligands have focused on either endogenous chemicals, such as bile acids and their intermediates and vitamin K (Staudinger et al., 2001; Xie et al., 2001; Makishima et al., 2002; Dussault et al., 2003; Goodwin et al., 2003; Tabb et al., 2003) or existing xenobiotics, including drugs (e.g., paclitaxel, ritonavir) (Dussault et al., 2001; Synold et al., 2001), herbal medicines (e.g., St. John's wort) (Moore et al., 2000) and environmental xenobiotics (e.g., polychlorinated biphenyls, 1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene) (Schuetz et al., 1998; Wyde et al., 2003; Tabb et al., 2004), and pesticides (e.g., transnonachlor and chlordane) (Schuetz et al., 1998). The current study represents a strategy for PXR ligand identification by combining chemical library synthesis with functional evaluation using both cell cultures and whole animals. Although the results of the functional ligand assays are convincing, we have yet to perform ligand binding studies to demonstrate the direct binding of **S20** to PXR. We therefore cannot exclude the possibility that **S20** may activate PXR via indirect pathways. Future work will involve the development of a focused library of analogs to obtain sufficient structure-activity relationship data to better understand and increase the potency of PXR activation by this new ligand scaffold. Moreover, a crystal structure analysis of **S20**-bound PXR LBD would extend our understanding of the molecular basis for ligand recognition and enantiomer preference by PXR.

Although the enantiomer-specific metabolic profiles have been reported for many drugs, the current study represents the first example that enantiomers could have opposite species preference in activating PXR, an important regulator of drug-metabolizing enzymes. It is conceivable that the knowledge of stereoselectivity may be used to guide the development of safer drugs to avoid drug-drug interactions or to achieve human-specific drug effects when a xenobiotic receptor is being used as a therapeutic target.

Acknowledgments

We thank Dr. Mathew Redinbo for ligand docking analysis, Dr. Richard Kim (Vanderbilt University, Nashville, TN) for the gift of PXR mutant constructs, Dr. Xiang Gao (University of Pittsburgh, Pittsburgh, PA) for providing the polyethylenimine transfection reagent, Tom Jones for comments on the manuscript, and Jung Hoon Lee for expertise in chemical structures.

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Address correspondence to: Dr. Wen Xie, Center for Pharmacogenetics, Salk Hall 656, University of Pittsburgh, Pittsburgh, PA 15261. E-mail: wex6@pitt.edu