New Opportunities for Pregnane X Receptor (PXR) Targeting in Drug Development. Lessons from Enantio- and Species-Specific PXR Ligands Identified from A Discovery Library of Amino Acid Analogues

Peter Wipf[2,3,*], Haibiao Gong[1,2], Jelena M. Janjic[2], Song Li[1,2], Billy W. Day[2,3] and Wen Xie[1,2,*]

[1]*Center for Pharmacogenetics,* [2]*Department of Pharmaceutical Sciences, and* [3]*Department of Chemistry and Center for Chemical Methodologies and Library Development, University of Pittsburgh, Pittsburgh, PA 15260, USA*

Abstract: Nuclear hormone receptors (NHRs) are transcription factors that bind to lipophilic signaling molecules (ligands), and subsequently regulate the expression of target genes. Since NHR ligands have potential as therapeutic agents, one of the most active research areas in the NHR field is the synthesis and identification of small molecule ligands for NHRs. Wipf *et al*. have recently reported the creation of a discovery library using a new method for the synthesis of homoallylic amides, allylic amides and *C*-cyclopropylalkylamides. This article is intended to review the use of this discovery library to screen for activators for the pregnane X receptor (PXR), a master xenobiotic receptor that regulates the expression of Phase I and Phase II enzymes as well as drug transporters. Our screening of the discovery library identified potent PXR activators whose carbon scaffolds are distinct from the chemical structures of known PXR agonists. Moreover, we found that enantiomers of the same compound show a species-specific activation of PXR. The development of the discovery library and the implications of enantiospecificity of PXR ligand design represent the primary focus of this account.

Key Words: Nuclear hormone receptors, gene expression, small molecule modulators, peptide mimetics, PXR, pregnane X receptor, enantiospecific binding, species specificity, discovery library screening.

1. NUCLEAR HORMONE RECEPTORS (NHRS) AND THEIR LIGANDS

The NHR superfamily includes receptors for hormonal ligands such as steroids, retinoids, thyroid hormone and vitamin D [1]. NHRs are transcription factors that bind to lipophilic signaling molecules and subsequently regulate the expression of target genes. Most, if not all, NHRs share a common modular structure with a conserved N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Fig. **1A**). In addition to determining ligand specificity, the LBD contains a ligand-inducible transactivation function (AF-2).

For NHR signaling, the general concept is that in the absence of a ligand, NHRs are often associated with NHR corepressor complex, resulting in an inhibition of NHR induced transcription. Following hormone/ligand treatment, NHRs undergo conformational changes that result in the release of the corepressor complex and recruitment of coactivator complex [2,3], leading to transcriptional activation *via* a specific hormone response element (HRE) (Fig. **1B**).

One criterium to classify NHRs is based on the requirement of dimerization partners. Most steroid hormone receptors, such as the glucocorticoid receptor (GR) and estrogen receptors α and β (ERα and ERβ), are homodimeric and do not require a dimerization partner. Another group of NHRs

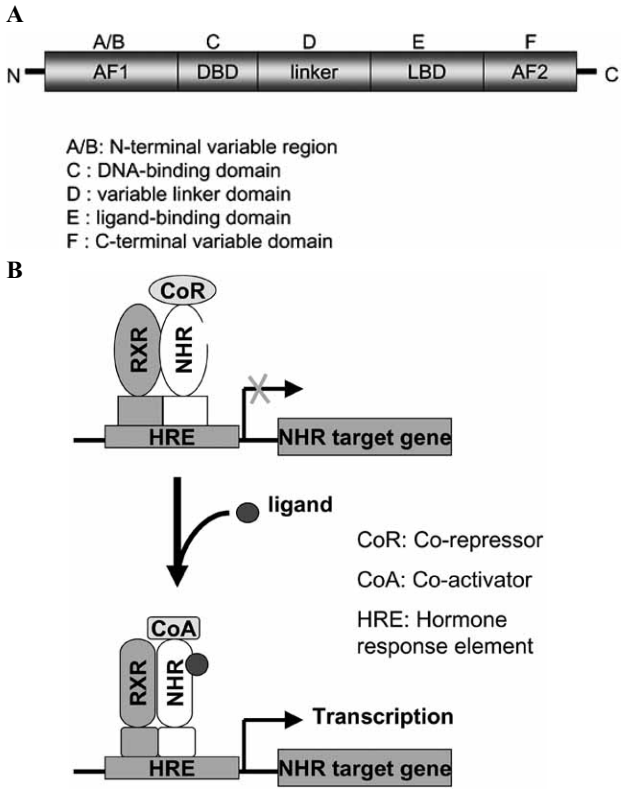

Fig. (1). NHRs are ligand-dependent transcription factors that regulate target gene expression. (A) Schematic domain structure of a typical NHR. (B) Current model of NHR-mediated gene regulation.

*Address correspondence to these authors at the Department of Pharmaceutical Sciences, and Department of Chemistry and Center for Chemical Methodologies and Library Development, University of Pittsburgh, Pittsburgh, PA 15260, USA; E-mail: pwipf@pitt.edu; wex6@pitt.edu

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require the retinoid X receptor (RXR) heterodimerization partner. The RXR heterodimeric receptors include the thyroid hormone receptor (TR), vitamin D receptor (VDR) and many members of the "orphan nuclear receptors", or oNHRs, such as retinoid acid receptor (RAR), peroxisome proliferation activating receptors α, γ, and δ/β (PPARα, γ, and δ/β), farnesoid X receptor (FXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), and liver X receptor α and β (LXRα and β). oNHRs lacked known physiological ligands when they were identified [3,4].

One of the most active research areas in the NHR field is the synthesis and identification of small molecule ligands for NHRs including the oNHRs. This is due to the potential of NHRs as therapeutic targets. The identification of synthetic ligands can not only realize the therapeutic potentials of NHRs but also provide valuable pharmacological tools to dissect the functions of NHRs.

2. SYNTHESIS OF A DISCOVERY LIBRARY OF HOMOALLYLIC AMIDES, ALLYLIC AMIDES AND *C*-CYCLOPROPYLALKYLAMIDES

Wipf *et al*. reported a new method for the synthesis of homoallylic amides, allylic amides and *C*-cyclopropylalkylamides [5,6]. This methodology yielded a 67-member discovery library (Table **1**). The library members were prepared using a novel three-component aldimine addition reaction as outlined in Scheme **1**.

This 67-member discovery library was initially designed to develop novel ER antagonists. Indeed, we have recently reported the identification of *C*-cyclopropylalkylamide **5a** (CK1-183, see Table **1**) as an ERα antagonist [7]. The use of this library in ER antagonist identification is not the focus of this review. Additional screening of the same library yielded novel PXR agonists [8], which will be reviewed in greater detail below.

3. USE OF THE DISCOVERY LIBRARY IN THE IDENTIFICATION OF PXR AGONISTS: A PXR AGONIST WITH UNIQUE SPECIES-DEPENDENT STEREOSELECTIVITY AND ITS IMPLICATIONS IN DRUG DEVELOPMENT

3.1. PXR as a Xenobiotic Receptor

PXR belongs to the oNHR family of NHRs. Upon ligand activation, PXR forms a heterodimer with RXR and induces target gene expression by binding to a PXR response element (PXRE) in the promoter regions of the target genes [9-11]. In this manner, PXR transduces chemical signals into transcriptional responses that govern a network of target genes involved in various physiological processes. Compared to other oNHRs, the PXR ligand binding pocket has a hydrophobic ligand-binding cavity that is composed of a large, smooth surface containing only a small number of polar residues, suggesting that activators need not conform to a restricted conformation or orientation [12-14]. As a result, the PXR LBD can accommodate a structurally diverse array of hydrophobic compounds.

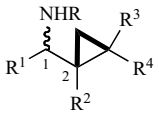
PXR activates the expression of a wide range of xenobiotic target genes, including Phase I cytochrome P450 enzymes [15, 16], Phase II conjugating enzymes [17,18], and "Phase III" drug transporters [19,20]. PXR was first found to be a regulator of *CYP3a* gene expression. PXR regulates *CYP3a* gene expression by binding to DR3 (direct repeat spaced by three nucleotides) in rodent *CYP3a* genes and ER6 (everted repeat spaced by six nucleotides) in the human *CYP3a4* gene promoter [15,16,21,22]. In addition, PXR regulates the expression of other P450 genes from various species, including *Cyp2b6*, *Cyp2b9*, *CYP2c8*, *CYP2c9* and *Cyp2c19* [20,23-26]. Paradoxically, the products from the Phase I reactions are electrophiles or nucleophiles that are still potentially toxic. These electrophiles or nucleophiles need to be further detoxified by Phase II conjugating enzymes and exported outside the cells by the drug transporters. Interestingly, PXR also regulates Phase II conjugating enzymes, such as glutathione *S*-transferases [17], sulfotransferases [27] and UDP-glucuronosyltransferase [18], as well as transporters, such as *MDR1* [20], *MRP2* [28] and the brain P-glycoprotein [19]. The simultaneous induction of Phase I and Phase II enzymes and transporters by PXR is not only an efficient way to eliminate xenobiotics, but also represents an essential mechanism to avoid production of excess harmful metabolic intermediates.

3.2. Activation of PXR

Consistent with the role of PXR as a xenobiotic receptor, PXR is activated by a variety of xenobiotics including drugs known to induce hepatic and intestinal CYP3A activity [15,16,21,22]. These include metyrapone, clotrimazole, phenobarbital, spironolactone and *trans*-nonachlor. Other PXR activators include some natural products, such as vitamin K2 [29], vitamin E [30], carotenoids [31], and drugs such as the herbal remedy St. John's wort (SJW) [32] and the Chinese herbal medicines Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch) [33], the calcium channel blocker nifedipine, the HIV protease inhibitor ritonavir [28], the antineoplastics paclitaxel [20], tamoxifen and 4-hydroxytamoxifen [34], the antidiabetic agent troglitazone [35]; the cholesterol-lowering drugs lovastatin and SR12813 [22,35], the sedative glutethimide, and the endocrine disruptors bisphenol A, diethylhexylphthalate and nonylphenol [for a review, see Ref 9]. Several groups have also identified bile acids [36,37] and their intermediates [38,39] as endogenous PXR activators.

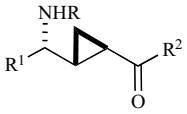
An interesting feature of PXR is its species-specific ligand selectivity. While the synthetic anti-glucocorticoid pregnenolone 16α-carbonitrile (PCN) is capable of activating rodent PXR, it has little activity on human PXR. In contrast, the antibiotic rifampicin (RIF) can serve as an efficient PXR activator in humans but not in rodents [15,40]. The differences in the ligand binding pockets of PXRs from different species have been proposed as the determining factor for the ligand selectivity [14]. Further analysis demonstrated that the Phe305 of mPXR and rPXR and the Leu308 residue in their human counterpart that are located within or are neighboring the flexible loop that forms part of the pore to the ligand-binding cavity are critical for the species-dependent ligand selectivity [41]. To further investigate the difference in PXR pharmacology across species, Xie *et al*. created so-called "humanized" mice in which the mPXR was deleted *via* homologous recombination and hPXR was introduced into the mouse liver through a liver-specific transgene [40]. These

Table 1. Structures of the 67-Member Discovery Library



C-Cyclopropylalkylamides*	C_1-C_2	R	R^1	R^2	R^3	R^4
5a (CK1183)	*anti*	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2N(Ts)CO_2Et$
5b	*anti*	$P(O)Ph_2$	3-OMe-Ph	H	H	C_4H_9
5c	*anti*	$P(O)Ph_2$	2-OMe-Ph	H	H	C_4H_9
5d	*anti*	$P(O)Ph_2$	4-Cl-Ph	H	H	C_4H_9
5e	*anti*	$P(O)Ph_2$	Ph	H	H	CH_2CH_2OH
5f	*anti*	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2CO_2Si(^iPr)_3$
5g	*anti*	$P(O)Ph_2$	Ph	H	H	CO_2Me
5h	*anti*	$P(O)Ph_2$	Ph	H	H	$CH=CH_2$
5i	*anti*	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2OSi(^tBu)Ph_2$
5j	*anti*	$P(O)Ph_2$	Ph	H	H	C_4H_9
5k	*syn*	$P(O)Ph_2$	Ph	H	H	C_4H_9
5l	*anti*	$P(O)Ph_2$	PhCC	H	H	C_4H_9
5m	*anti*	CO_2CH_2Ph	Ph	H	H	CO_2Me
5n	*anti*	CO_2CH_2Ph	Ph	H	H	$C(O)NH^iPr$
5o	*syn*	CO_2CH_2Ph	Ph	H	Me	$C(O)NH^iPr$
5p	*syn*	$P(O)Ph_2$	Ph	H	Me	C_4H_9
5q	*anti*	$P(O)Ph_2$	Ph	Et	H	Et
5r	*anti*	$P(O)Ph_2$	Ph	Me	H	$CH=CH_2$
5s	*anti*	Ts	Ph	H	H	C_4H_9
5t	*syn*	Ts	Ph	H	H	C_4H_9
5u	*anti*	CO_2CH_2Ph	Ph	Me	H	$C(O)NH^iPr$
5v	*anti*	Ts	$PhCH_2CH_2$	H	H	C_4H_9
5w	*anti*	C(O)Ph	Ph	H	H	C_4H_9
5x	*anti*	C(O)Ph-4-NO_2	Ph	H	H	C_4H_9

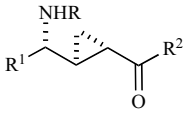
* Diastereomerically pure



C-Cyclopropylalkylamino carboxylates*	R	R^1	R^2
5-1a	CO_2CH_2Ph	Ph	(S)-NHCH(Me)Ph
5-1b	CO_2CH_2Ph	Ph	NHPh-4-Br
5-1c	CO_2CH_2Ph	Ph	L-Phe-OMe
5-1d	C(O)Ph-4-Br	Ph	OMe

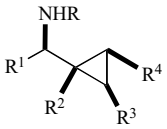
* Enantiomerically pure

(Table 1. Contd….)

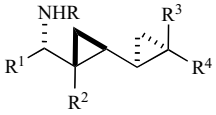


C-Cyclopropylalkylamino acids*	R	R^1	R^2
5-2a	CO_2CH_2Ph	Ph	L-Phe-OMe

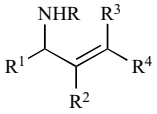
* Enantiomerically pure



C-Cyclopropylalkylamides	R	R^1	R^2	R^3	R^4
5-3a	$P(O)Ph_2$	Ph	H	Me	C_4H_9
5-3b	$C(O)Ph\text{-}3,5\text{-}(NO_2)_2$	Ph	H	Me	C_4H_9
5-3c	C(O)Ph	Ph	H	Me	C_4H_9
5-3d	$P(O)Ph_2$	Ph	Me	H	H



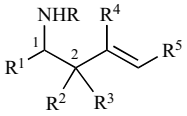
C-Cyclopropylalkylamides	R	R^1	R^2	R^3	R^4
5-4a	$C(O)Ph\text{-}3,5\text{-}(NO_2)_2$	$Ph\text{-}4\text{-}CO_2Me$	H	Me	C_6H_{13}
5-4b	$P(O)Ph_2$	Ph	H	Me	CH_2CH_2OH
5-4c	$P(O)Ph_2$	$Ph\text{-}4\text{-}CO_2Me$	H	Me	C_6H_{13}



Allylic amides	R	R^1	R^2	R^3	R^4
6a	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2N(Ts)CO_2Et$
6b	$P(O)Ph_2$	$(E)\text{-}PhCH{=}C(CH_3)$	H	H	C_4H_9
6c	$P(O)Ph_2$	Ph	H	H	C_4H_9
6d	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2OSi(^tBu)Ph_2$
6e	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2CO_2Si(^iPr)_3$
6f	$P(O)Ph_2$	$(E)\text{-}PhCH{=}CH$	H	H	C_4H_9
6g	$P(O)Ph_2$	$4\text{-}CO_2Me\text{-}Ph$	H	H	C_4H_9
6h	$P(O)Ph_2$	3-OMe-Ph	H	H	C_4H_9
6i	$P(O)Ph_2$	2-OMe-Ph	H	H	C_4H_9
6j	$P(O)Ph_2$	$4\text{-}NO_2\text{-}Ph$	H	H	C_4H_9

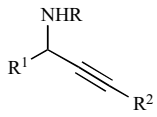
(Table 1. Contd….)

Allylic amides	R	R^1	R^2	R^3	R^4
6k	$P(O)Ph_2$	3-NO_2-Ph	H	H	C_4H_9
6l	$P(O)Ph_2$	4-Cl-Ph	H	H	C_4H_9
6m	$P(O)Ph_2$	PhCC	H	H	C_4H_9
6n	Ts	Ph	H	H	C_4H_9
6o	Ts	$PhCH_2CH_2$	H	H	C_4H_9
6p	$P(O)Ph_2$	Ph	H	Me	C_4H_9
6q	$P(O)Ph_2$	Ph	Me	H	H
6r	$P(O)Ph_2$	Ph	Et	H	Et
6s	$P(O)Ph_2$	4-CO_2Me-Ph	$Si(CH_3)_3$	H	(*E*)-CH=CHC_6H_{13}
6a1	$P(O)Ph_2$	Ph	H	H	$CH_2CH_2N(Ts)CO_2Et$



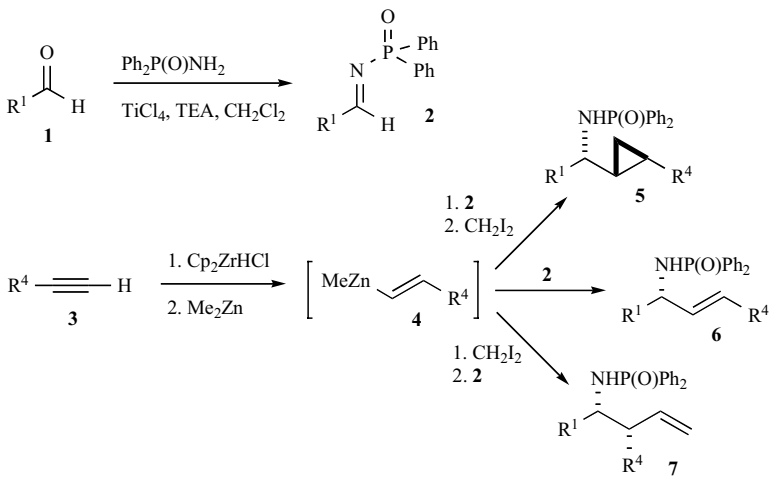
Homoallylic amides*	C1-C2	R	R^1	R^2	R^3	R^4	R^5
7a	*anti*	$P(O)Ph_2$	Ph	$CH_2CH_2OSi(^tBu)Ph_2$	H	H	H
7b		$P(O)Ph_2$	Ph	Me	Me	H	Me
7c	*anti*	$P(O)Ph_2$	4-CO_2Me-Ph	$CH_2CH_2OSi(^tBu)Ph_2$	H	H	H
7d	*syn*	$P(O)Ph_2$	4-OMe-Ph	C_4H_9	H	H	H
7e	*syn*	$P(O)Ph_2$	Ph	C_4H_9	H	H	H
7f	*syn*	Ts	Ph	C_4H_9	H	H	H
7g	*syn*	Ts	Ph	$CH_2CH_2OSi(^tBu)Ph_2$	H	H	H
7h	*syn*	$P(O)Ph_2$	Ph	Et	H	Et	H
7i	*anti*	$P(O)Ph_2$	Ph	Et	H	Et	H
7j		$P(O)Ph_2$	Ph	H	H	Me	H
7k	*syn*	3,5-$(NO_2)_2$-PhC(O)	Ph	C_4H_9	H	H	H

* Diastereomerically pure



Propargylic amides	R	R^1	R^2
7-1a	$P(O)Ph_2$	Ph	C_4H_9

mice exhibited a "humanized" hepatic xenobiotic response-profile, readily responding to the human-specific inducer RIF in a concentration range equivalent to the standard oral dosing regimen in humans, but were not responsive to the rodent-specific ligand PCN [40]. More recently, the hPXR humanization has been extended to the intestine by using the liver- and intestine-specific fatty acid binding protein (FABP) promoter to direct the hPXR transgene expression [42].



Scheme 1. Synthesis of a discovery library of *C*-cyclopropylalkylamides, allylic amides and homoallylic amides. By controlling the solvent and the order of addition of reagents, *C*-cyclopropylalkylamides (**5**), allylic amides (**6**) or homoallylic amides (**7**) were prepared from readily available starting materials, aldehydes (**1**) and alkynes (**3**).

3.3. PXR as a Potential Therapeutic Target

Although PXR was identified as a xenobiotic receptor, many of its target genes are involved in the biotransformation and homeostasis of endogenous and exogenous compounds that may influence physiological and pathological responses in mammals. Accordingly, PXR has been suggested as a therapeutic target to remove endogenous and exogenous toxins. For example, a sustained PXR activation in transgenic mice conferred resistance to LCA-induced cholestatic liver damage [37], which may be attributed to PXR-mediated induction of *Cyp3a11* and *Sult2a9* gene expression [27,37]. Activation of PXR in mice has also been shown to promote bilirubin clearance and prevent hyperbilirubinemia [18,43].

Despite the promise of PXR as a therapeutic target, a major challenge in developing PXR-activating drugs is how to avoid the concern of drug-drug interactions. Drug-induced production of drug metabolizing enzymes and transporters is the molecular basis for many clinical drug-drug interactions [44]. As a xenobiotic receptor, PXR has been implicated in potential drug-drug interactions since the cloning of this receptor [for reviews, see Ref 9,10,45-48]. The side effect of St. John's wort in triggering severe adverse drug-drug interactions with oral contraceptives, the HIV protease inhibitor indinavir, and the immunosuppressant cyclosporin has been proposed to be due to the activation of PXR by this herb and the subsequent induction of the CYP3A system [32]. In a recently published study, we showed that the Chinese herbal medicines Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch.) activated PXR and induced the expression of CYP3A and 2C isoforms. Moreover, administration of the extracts of these two herbs in rats increased the metabolism of the co-administered warfarin, a known substrate for CYP2C9 [33].

Drug-drug interactions are clearly a concern, but we argue that the potential of drug-drug interactions alone should not exclude PXR as a therapeutic target. This notion is supported by the fact that many PXR activating agents, including RIF, have been successfully used as clinical drugs, long before they were recognized as PXR agonists [15,16]. For example, RIF has been shown to relieve pruritus in cholestatic liver disease by stimulating 6α-hydroxylation and elimination of bile acids. Similarly, the therapeutic value of St. John's wort is largely sustained even after the revelation of its potential for PXR activation and drug-drug interaction. The same notion can also be extended to another xenobiotic receptor, CAR. Phenobarbital, later found to be a CAR activator, has long been used clinically to relieve jaundice. Nevertheless, considering the potential for adverse effects, it may be desirable to use a PXR-activating drug as a single agent in order to avoid drug-drug interactions.

3.4. Identification of PXR Agonists from the Discovery Library

Although many PXR agonists have been identified and reported, a continued effort to develop and/or identify novel PXR modulators is necessary. First of all, many PXR agonists have been reported but little is known about PXR antagonists. From a drug metabolism point of view, it is tempting to speculate that co-administration of a PXR antagonist may have the benefit of increasing the therapeutic efficacy of a drug. But a PXR antagonist may also suffer concerns of toxicity due to a compromised drug clearance. On the other hand, when PXR is explored as a therapeutic target, it is necessary to develop potent and ideally human-specific PXR agonists. Novel PXR ligands cannot only be used to realize the therapeutic potential of this receptor, but also provide invaluable pharmacological tools to dissect the PXR functions.

We utilized an *in vitro* cell culture system to screen for PXR activators. A chimeric receptor in which the LBD of PXR was fused to the DBD of the yeast transcription factor Gal4 and a Gal4-responsive reporter tk-UAS-Luc plasmid that contains three copies of the Gal4 binding site were co-transfected into monkey kidney CV1 cells. After 24h incuba-

tion, cells were treated with the library compounds at a concentration of 10 μM for 24 h before the luciferase assay. A CMV-β-galactosidase (β-Gal) plasmid was also transfected for normalization [8]. The PXR activator can bind to the LBD of PXR and trigger the activation of the Gal4 reporter, resulting in a higher expression of luciferase. Fold induction was calculated by dividing the value of normalized luciferase activity of compound-treated cells by that of vehicle-treated cells.

Many compounds from the discovery library of allylic amides, homoallylic amides and *C*-cyclopropylalkylamides showed some degree of PXR activation [8]. **5n** (equivalent to **S20** which stands for Sample 20 in [8]), benzyl-2-(isopropylcarbamoyl)-2-methylcyclopropyl)(phenyl)methylcarbamate, is the most potent compound in this library, causing a 5.4 ± 0.6 fold induction of PXR activity [8]. It has a cyclopropylalkylamide scaffold and three stereogenic carbons, and can be resolved into two enantiomers, **(+)-5n**, benzyl (*R*)-((1*S*, 2*S*)-2-(isopropylcarbamoyl)-2-methylcyclopropyl)(phenyl) methylcarbamate and **(–)-5n**, benzyl (*S*)-((1*R*,2*R*)-2-(isopropylcarbamoyl)-2-methylcyclopropyl)(phenyl)methylcarbamate (Fig. **2**). The carbon scaffold of **5n** is distinct from the chemical structures of known PXR agonists such as RIF and PCN (Fig. **2**). Interestingly, the structurally closely related allylic amide **6h** (**S2** in Ref 8), *N*-[(2*E*)-1-(3-methoxyphenyl)-2-heptenyl]-*P*,*P*-diphenylphosphinoylamide, and *C*-cyclopropylalkylamide **5b (S7** in Ref 8)**,** *N*-[(*R*)-[(1*R*,2*R*)-2-butylcyclopropyl](3-methoxyphenyl)methyl]-*P*,*P*-diphenylphosphinoylamide, are also potent PXR activators, causing fold inductions of 4.0 ± 0.5 and 4.4 ± 0.7, respectively [8]. Treatment with **5n** also induced the expression of drug metabolizing enzymes and transporters in reporter gene assays, in primary human hepatocytes and in "humanized" hPXR transgenic mice [8]. It is important to note that, while combinations of transient transfection, primary hepatocyte cultures and humanized mice represent comprehensive biological readouts of PXR, a direct binding assay between the PXR protein and a candidate agonist is necessary to convincingly establish a *bona fide* PXR ligand. Moreover, in cell- or animal-based assays, we cannot exclude the possibility that the metabolites, rather than the parent compounds, are responsible for the activation. It is known that there are extensive cross-talks among xenobiotic receptors, such as those between PXR and CAR [26]. Although the cell-based assays using receptor naïve cells showed that **5n** did not activate CAR and FXR [8], we cannot exclude the possibility that the effects of **5n** on drug metabolizing enzyme expression in the hepatocytes and in the mouse livers were not mediated by receptors other than PXR. Compound **5n** exhibited good solubility in the cell-based reporter assay in the concentration range that we used (up to 40 μM final concentration). Compound **5n** also showed little cytotoxicity as judged by the co-transfected β-galactosidase activities.

3.5. Species Selectivity of 5n Enantiomers

Interestingly, the two enantiomers of **5n** showed a species-specific activation of PXR activity. hPXR was activated by **(+)-5n** up to 9.5-fold, in contrast to the 2-fold induction by **(–)-5n**. On the other hand, mPXR was more readily activated by **(–)-5n** with a fold induction of 11.5, while the induction by **(+)-5n** was only 4-fold [8]. The stereoselectivity was further confirmed in primary hepatocyte cultures. **(+)-5n** was a more potent inducer of human CYP3A gene expression than (-)-**5n**, but the opposite was true when the mouse hepatocytes were used [8]. The species-dependent stereoselectivity of **5n** 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

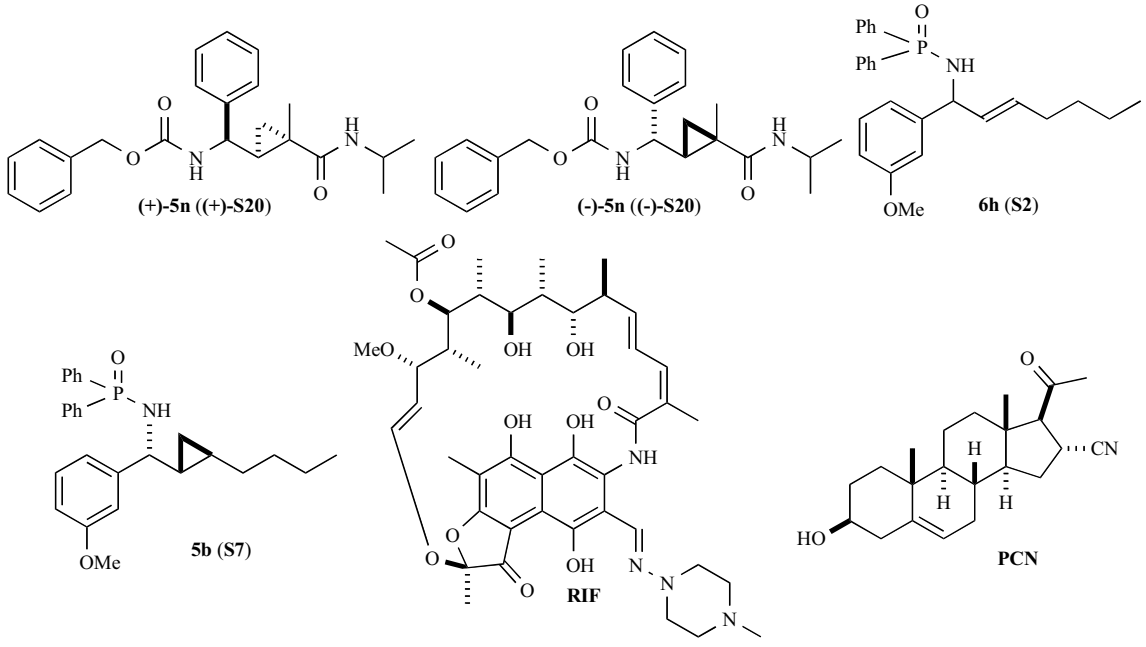


Fig. (2). Structures of PXR activating agents.

receptors [14]. To investigate whether the residues of PXR protein that determine the species-dependent ligand specificity also determine the stereoselectivity of **5n** enantiomers, we used rPXR-F305L and hPXR-L308F mutants [41]. Results showed that the preference of rPXR to **(–)-5n** was completely abolished in the rPXR-F305L mutant. However, the L308F mutation had little effect on the hPXR's preference for **(+)-5n** [8]. These findings suggest that Phe305 of rPXR, which is located in the flexible loop that forms part of the pore to the ligand-binding cavity, is also critical for the preference of rPXR for **(–)-5n**, while amino acids other than the corresponding Leu308 in hPXR determine the preference of hPXR for **(+)-5n**.

3.6. Implications of the Enantiospecificity of PXR Ligand in Drug Development

To our knowledge, **5n** represents the first compound whose enantiomers have opposite species preference in activating a xenobiotic receptor. These findings are of consequence to drug development. There are at least two conceivable benefits of this new information. For pharmaceutical agents whose therapeutic effects do not rely on PXR, the choice of PXR-neutral but therapeutically effective enantiomers may help to avoid unwanted drug-drug interactions. Since the induction of drug-metabolizing enzymes is the underlying mechanism of drug-drug interactions, and many of the PXR target genes are involved in this process, the activation of PXR may cause severe drug-drug interactions and side effects. Therefore, it is desirable to avoid PXR activation activity when designing compounds for therapeutic purposes. In contrast, for drugs whose therapeutic target is PXR, an hPXR-specific enantiomer will be necessary in order to achieve the intended therapeutic effects in humans. 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.

It remains to be determined whether the **5n** enantiomers have the appropriate properties, such as pharmacokinetics and bioavailability, to serve as pharmacological agents in humans. Future work will require the development of more potent PXR activating agents of this new ligand scaffold. Moreover, a crystal structure analysis of **5n**-bound PXR LBD would extend our understanding of the molecular basis for ligand recognition and enantiomer preference by PXR. It is possible that 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 nuclear receptor is being used as a drug target.

4. SUMMARY AND OUTLOOK

Due to the potential of NHRs as therapeutic targets, the pharmaceutical industry has embarked on research programs designed to identify cognate or surrogate ligands for NHRs including oNHRs. By combining a structurally diverse chemical library with functional assays using transient transfection, primary human hepatocyte cultures and "humanized" transgenic mice, a practical approach to identify novel NHR agonist and/or antagonists can be realized. Indeed, a similar strategy has been successfully employed to identify PXR activators from traditional Chinese medicines. We found that the traditional Chinese medicines Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch) activate PXR and increase the clearance of warfarin [33].

Our results have also demonstrated that the same discovery library may be simultaneously applied to multiple NHR targets. Although this discovery library of amino acid analogues was initially designed for an ER modulator screen, the activation on PXR by the library compounds may not be a surprise considering that PXR is the body's designated "xenosensor". The most interesting finding in the application of this library in the PXR screen is the revelation of the unique species-dependent stereoselectivity of certain PXR ligands and the potential implications of this stereoselectivity in drug development.

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