Title: Discovery of a highly active ligand of human Pregnane X Receptor: a case study from pharmacophore modeling and virtual screening to “in vivo” biological activity.


INSERM U824, Montpellier, F-34298, Univ Montpellier 1, Montpellier, F-34060, France (G.L., A.P., A-M.B., P.B.)
INSERM, U554, Montpellier, F-34090, Univ Montpellier 1 and 2, CNRS, UMR5048, Centre de Biochimie Structurale, Montpellier, F-34090, France. (C.B., V.N., J-F.G., G.S., W.B., A.C.)
INSERM, U632, Montpellier, F-34293, Univ Montpellier 1, Montpellier, F-34060, France. (J-M.P.)
Running title: Identification of Synthetic PXR Agonists

Corresponding author: Patrick Balaguer Equipe INSERM SHEC, Signalisation Hormonale, Environnement et Cancer, Centre de Recherche en Cancérologie de Montpellier (CRCM), Parc Euromédecine – CRLC Val d’Aurelle, F-34298 Montpellier, France Cedex 5. Tel.: +33 467 612 409; fax: +33 467 613 787. p.balaguer@valdorel.fnclcc.fr

Number of pages: 34
Number of tables: 3
Figures: 5
References: 38
Abstract: 217 words
Introduction: 668 words
Discussion: 1411 words

Abbreviations: CYP, cytochrome P450; DMEM, Dulbecco’s Modified Eagle’s medium; DCC-FCS, dextran-coated charcoal treated fetal calf serum; DR, direct repeat, EC50, effective concentration for half-maximal activity; ER, everted repeat; FCS, fetal calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; hPXR, human Pregnane X Receptor; LBD, ligand-binding domain; C2BA-4, (1-(2-chlorophenyl)-N-[1-(1-phenylethyl)-1H-benzimidazol-5-yl]methanesulfonamide); RXR, Retinoid X Receptor; RT-PCR, real-time polymerase chain reaction; SDS, sodium dodecyl sulfate; SMRT, silencing mediator for retinoic and thyroid hormone receptor; SRC-1, steroid receptor coactivator-1.
Abstract

The human Pregnane X receptor (hPXR) is a nuclear receptor that regulates the expression of phase I and phase II drug-metabolizing enzymes, as well as that of drug transporters. In addition, this receptor plays a critical role in cholesterol homeostasis and in protecting tissues from potentially toxic endobiotics. hPXR is activated by a broad spectrum of low affinity compounds including xenobiotics and endobiotics such as bile acids and their precursors. Crystallographic studies revealed a ligand binding domain (LBD) with a large and conformable binding pocket that likely contributes to the ability of hPXR to respond to compounds of varying size and shape. Here, we describe an in silico method that allowed the identification of 9 novel hPXR agonists. We further characterize the compound C2BA-4, a methanesulfonamide that activates PXR specifically and more potently than does the reference compound SR12813 in our stable cell line expressing a Gal4-PXR and a GAL4 driven luciferase reporter gene. Furthermore treatment of primary human hepatocytes with C2BA-4 results in a marked induction of the mRNA expression of hPXR target genes such as cytochromes P450 3A4 and 2B6. Finally, C2BA-4 is also able to induce hPXR-mediated in vivo luciferase expression in HGPXR stable bioluminescent cells implanted in mice. The study suggests new directions for the rational design of selective hPXR agonists and antagonists.
Introduction

The Pregnane X receptor (PXR; NR1I2; (Lehmann et al., 1998), also known as SXR (Blumberg et al., 1998) and PAR (Bertilsson et al., 1998), which is a member of the nuclear receptor superfamily, is a ligand-dependent transcription factor that regulates the expression of multiple drug metabolizing enzymes and transporters in the liver and the intestine (Kliewer et al., 2002). PXR interacts with a variety of DNA response elements (direct repeats DR-3, DR-4, and DR-5, and everted repeats ER-6 and ER-8) in the 5'-flanking regions of genes as a heterodimer with the 9-cis retinoic acid receptor (RXR) (Blumberg et al., 1998; Kast et al., 2002; Xie et al., 2000). Upon ligand binding, the DNA-bound activated PXR/RXR heterodimer recruits nuclear receptor coactivators thereby initiating the transcription of target genes (Kliewer et al., 2002; Orans et al., 2005). Recently, Johnson et al. (2006) demonstrated that the corepressor SMRT binds and regulates the transcriptional activity of PXR.

PXR is a key regulator of phase I (cytochrome P450), phase II (conjugating), and phase III (ABC transporters) metabolizing and detoxifying enzymes involved in endobiotic and xenobiotic clearance (Kliewer et al., 2002). Because the PXR pathway is activated by a large number of prescription drugs, PXR is thought to be involved in drug metabolism and efflux as well as in drug-drug interactions. Indeed gene knockout studies confirmed a role for PXR in regulating the metabolism of endogenous steroids and xenobiotics (Staudinger et al., 2001). PXR is also involved in lipid homeostasis by activating genes that facilitate lipogenesis and by suppressing the β-oxidative pathways (Zhou et al., 2006). Even though PXR was identified as a xenobiotic sensor, some evidences suggest that PXR could be a potential therapeutic target for several human diseases. Recent studies indicate that PXR
plays a role in bilirubin clearance, prevents hyperbilirubinemia and also hepatorenal toxicity from cholesterol metabolites (Orans et al., 2005; Xie et al., 2003). Furthermore, it was recently shown that PXR ligands could be putative neuroprotectors in Niemann-Pick disease (Langmade et al, 2006).

Several agonists of hPXR have been described including natural and synthetic steroids such as 5β-pregnane-3,20-dione and estradiol (Jones et al, 2000, Xue et all, 2007a), the cholesterol-lowering drugs lovastatin and SR12813 (Jones et al., 2000; Lehmann et al., 1998), the synthetic oxysterol ligand T0901317 (Xue et all, 2007b), the antibiotic rifampicin (Blumberg et al., 1998; Lehmann et al., 1998) and the active agent of St. John’s wort, hyperforin (Wentworth et al., 2000). Crystal structures of the human PXR ligand-binding domain (hPXR LBD) revealed a typical three-layered α-helical sandwich, commonly found in nuclear receptors (Moras and Gronemeyer, 1998). However, PXR possesses a flexible and conformable ligand-binding pocket that adjusts its shape to accommodate ligands of distinct sizes and structures (Watkins et al., 2003; Watkins et al., 2001). The large and conformable binding pocket likely contributes to its ability to respond to low affinity compounds, including endobiotics (Chrencik et al., 2005, Xue et al, 2007a). Recently, Chrencik et al. (2005) reported the 2.8 Å resolution crystal structure of hPXR LBD in complex with the macrolide antibiotic rifampicin. They showed that rifampicin contacts 18 amino acid side chains in the PXR ligand-binding pocket through hydrogen bonds and hydrophobic interactions.

Because hPXR is a potential therapeutic target for several human pathologies, new generation of hPXR modulators with improved selectivity and affinity for PXR might represent novel therapeutic tools. Furthermore, such ligands could serve as scaffold
for the design of potent PXR antagonists (Synold et al., 2001, Tabb et al., 2004, Zhou et al., 2006).

In this report, we describe a structure-based and high-throughput virtual screening method that allowed discovering highly active PXR agonists. Based on the crystal structure of hPXR LBD in complex with rifampicin (Chrencik et al., 2005), we designed a pharmacophore and used this information to select the compound library which was subsequently used for “in silico” screening. Using this approach, we were able to identify 9 original hPXR agonists and one of them, C2BA-4, a methanesulfonamide, presents a higher activity than SR12813 on hPXR in biological assays.
Materials and Methods

Materials

Materials for cell culture, RNA extraction TRIzol reagent, SuperScript-II First-Strand Synthesis System for RT-PCR and Lipofectamine were from Invitrogen (Cergy-Pontoise, France). Luciferin and G418 were purchased from Promega (Charbonnières-les-Bains, France). SR12813 was purchased from Tebu-bio (Le Perray en Yvelines, France).

Virtual screening

The structure-based design of new PXR agonists was performed using the crystal structure of hPXR LBD in complex with rifampicin (pdb code: 1SKX). Hydrogen atoms were added to the protein structure by using the InsightII/Biopolymer-Discover3 modules (Accelrys Inc., San Diego, CA) at pH 7.0 and their positions were subjected to 1000 iterations of steepest descent minimization followed by 1000 steps of conjugate gradient minimization using the CFF force field. All non-hydrogen atoms of the protein as well as the ligand were held rigid during these minimizations. The X-ray structure of the complex was then heated and equilibrated at 298 K for 3 ps with amino-acid side chains within a 5 Å distance to the ligand unfixed while keeping the rest of the protein fixed. Then at 298 K, a 5 ps molecular dynamic (MD) simulation with implicit water followed by an energy-minimization was repeated 50 times in the same condition of unfixed and fixed atoms, with total energy of interaction between hPXR and rifampicin monitored using the intermolecular command of the Discover3 module (Insight, Accelrys). This energy evaluation was used to select the lowest-energy-minimized LBD conformation among the ten last MD minimized structures. A heavy atom root-mean-square deviation (rmsd) value between selected and starting structures of the MD simulation was 0.39 Å and both structures presented
comparable volumes of LBDs. These two different target structures with the ligand removed were used for the virtual screening process. A 2D database search was performed with the MDL.ISIS/Base software (http://www.mdli.com/) against the ChemBridge database (http://chembridge.com/chembridge/) and 496 molecules with specific pharmacophoric constraints were selected. These molecules retrieved in a multi-SDF file were converted to 3D structures (single conformation) with CORINA (Molecular Networks GmbH), then docked and evaluated by the Surflex v1.27 program (Jain., 2003). For virtual screening calculations, no water molecules were added during the protomol generation. For the 2 structures used, the poses of molecules with a score higher than 6, were visualized and analyzed.

**Plasmids**

The pPM-LBDhPXR expression plasmid was described previously (Ourlin et al., 2003). The yeast Gal4 DNA binding domain fused to the LBD of hPXR (107 to 434 aa) was generated from the pPM-LBD using PCR primers that introduced BamHI sites and subcloned into pSG5-puro (gift from Hinrich Gronemeyer, Strasbourg, France).

The pET15b-hPXR expression plasmid was generated by PCR amplification of cDNA-encoding amino acids 107 to 434 of hPXR using oligonucleotides 5’-CGCGCGCATATGAAGGAGATGATCATG-3’ and 5’-GCGCGCGGATCCTCAGCTACCTGTGATGCCG-3’. All plasmids were fully sequenced.

**Generation of stable reporter cell lines**

HG5LN and HGPXR cells were described previously (Lemaire et al., 2006; Seimandi et al., 2005). Briefly, HG5LN cells were obtained by integration of a GAL4-responsive gene (GAL4RE5-bGlob-Luc-SV-Neo) in HeLa cells (Seimandi et al.,
2005). The HGPXR cell line was obtained by transfecting HG5LN cells with a plasmid (pSG5-GAL4(DBD)-hPXR(LBD)-puro) which enables the expression of the DNA binding domain of the yeast activator GAL4 (met 1 to ser 147) fused to the ligand binding domain of hPXR (met 107 to ser 434) and confers resistance to puromycin. For the strain culture, cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing phenol red and 1 g/l glucose, and supplemented with 5% fetal calf serum (FCS) in a 5% CO2 humidified atmosphere at 37°C. HG5LN cell medium was supplemented with 1 mg/ml geneticin and HGPXR cell medium with 1 mg/ml geneticin and 0.5 µg/ml puromycin.

For tests, cells were grown in DMEM without phenol red, supplemented with 3% dextran-coated charcoal treated fetal calf serum (DCC-FCS).

Living cell luciferase assay

Cells were seeded at a density of 5 x 10^4 cells per well, in 96-well white opaque tissue culture plates (Becton Dickinson) and grown in 200 µl DCC-FCS. Tested compounds were added 24 h later at concentrations between 10 nM and 10 µM and cells were incubated for 16 h with compounds. At the end of incubation, compound containing medium was removed and replaced by 0.3 mM luciferin containing culture medium. Luciferase activity was measured in a MicroBeta Wallac luminometer (EG&G Wallac, Turku, Finland) and luminescence was measured in intact cells for 2 s per well.

In vivo bioluminescent imaging of HGPXR implanted cells

The implantation technique of luminescent reporter cells was described earlier (Lemaire et al., 2006; Pillon et al., 2005). Briefly, female athymic nude mice, about 50 days old and weighing 18 g to 20 g, were obtained from Harlan France (Gannat, France) and acclimatized for a week before the experiment started. Mice were housed
in self-contained filter-top plastic cages (4 mice/cage) maintained under the following standard conditions: 22°C ± 2°C, 45% ± 10% relative humidity, 12 hr light/12 hr dark cycle each day. Mice were given a standard diet (UAR, Epinay-sur-Orge, France) and water *ad libitum*.

About 8 \(10^5\) cells, prepared in serum-free DMEM, were subcutaneously (sc) grafted onto the mouse right dorsal flank (HGPXR cells) and left dorsal flank (HG5LN cells, as an internal control), and a week later tumor size was considered sufficient for performing the *in vivo* experiments.

The PXR activities of different compounds were measured as follows. Mice were injected intraperitonealy with 100 µl of C2BA-4, rifampicin or SR12813 dissolved in DMSO at 25, 15, 5 mg/kg body weight. Mice were imaged before injection and 8 h after injection. For bioluminescence imaging procedure, mice were first sedated using the isoflurane gas anesthesia system from T.E.M. (Bordeaux, France), with 4% isoflurane in air in an anesthesia induction box and then with 1.5% isoflurane in air/O2 (80/20) continuously delivered via a nose cone system in the dark box of the NightOWL LB 983 NC100 CCD camera (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was then measured after the mice had been intraperitoneally (ip) injected with luciferin saline solution, 125 mg/kg body weight, which gave rise to a luminescent signal that was maximal 10 min later and remained stable for 20 min. The photons emitted from luciferase were integrated for 2 min, and the pseudocolor luminescent image was generated using WinLight software (Berthold Technologies). A gray-scale body-surface reference image was also collected. The overlay of the body image and the luminescence representation allowed the localization of the xenografts. The luminescent signal intensities from the regions of interest (ROI) were obtained and the data were expressed as photon flux...
(photons/s). Background photon flux was defined from a ROI of the same size placed in a non-luminescent area near the animal, and then subtracted from the measured luminescent signal intensity.

Mean values ± SD were calculated from at least four independent experiments. Statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) with turkey’s post-hoc test to evaluate the effect of SR12813 or C2BA-4. Six mice were necessary to accomplish the present work. All experiments were performed in compliance with the French guidelines for experimental animal studies (Agreement No. B-34-172-27).

**Limited proteolytic digestion**

LBD of hPXR in pET-15b expression plasmid (1 µg) was generated by coupled transcription/translation in rabbit reticulocyte lysate using the TNT system according to the manufacturer’s instructions (Promega, Madison WI). [35S]Methionine was included in the transcription/translation mix to generate [35S]PXR. Briefly, after a 30 min incubation with ligand at room temperature, hPXR protein was digested at 25°C with 250 µg/ml trypsin for 10 min. Proteolysis was terminated by adding sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min.

The proteolytic fragments were separated on a 10% SDS-polyacrylamide gel. Gels were dried and radio-labeled digestion products were visualized by autoradiography using a bio-imaging analyzer (Fujix BAS1000 Phosphorimager). Autoradiograms were analyzed using an image analyzing software. Experiments were performed 3 times. To measure the relative levels of the bands protected by a test compound on a gel, the intensity of the band of PXR-LBD treated with ethanol, around 30 kDa, was taken as 1.
Liver samples, hepatocyte cultures and treatment

Hepatocytes were prepared from lobectomy segments resected from adult patients for medical purposes unrelated to our research program. The use of these human hepatic specimens for scientific purposes has been approved by the French National Ethics Committee.

Human hepatocytes were prepared and cultured as previously described (Pascussi et al., 2000). Cells were plated into collagen-coated P12 dishes at 0.17 x 10^-6 cells/cm^2 in a hormonally and chemically defined medium consisting of a mixture of Williams’ E and Ham’s F-12 (1:1 v/v). Treatment with 10^-6 M and 10^-5 M effector solutions or solvent (DMSO 0.1%) started 48 h after plating and lasted 24h.

Total RNA purification and CYP3A4 quantitative PCR

Total RNA was isolated with TRIzol reagent. cDNA was synthetised from 1 µg of total RNA using the SuperScript-II First-Strand Synthesis System for RT-PCR at 42°C for 60 min in the presence of random hexamers (Invitrogen), and then 10-fold diluted in water. Two microliters were used in duplicate for quantitative PCR amplification of CYP2B6, CYP3A4 and β-actin, as internal control, using the Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The following program was used: denaturation step at 95°C for 10 min, 45 cycles PCR (denaturation at 95°C for 10 s, annealing at 65°C for 8 s, elongation at 65°C for 15 s). Sense and reverse primers, respectively, were as follows: β-actin: 5’-tgggcatgttgcagaaggt and 5’-ttcatcagatgcagttg; CYP3A4: 5’-cacaaaccggaggcttttg and 5’-ttccatcagtctaggccccaa; CYP2B6: 5’-ggccatcggggcttttg and 5’-agggcctttggatttccg. The curves of amplification were read with the Roche’s Light Cycler Software using the comparative cycle threshold (Ct) method. Relative quantifications of the target mRNAs were calculated after
normalization of CtS with respect to the β-actin levels. Values are expressed as fold induction compared to untreated cells (DMSO 0.1%) +/- S.D.. For each donor, statistical analysis of the results was carried out using a two sample, two-tailed Student’s t test to evaluate the effect of RIF, SR12813 or C2BA-4 treatments on the control. Results were considered statistically significant at p<0.05.

**Data analysis and statistics**

In the transactivation assay, each compound was tested at various concentrations in at least three separate experiments in triplicate wells and data are presented as the mean ± S.D.

Individual agonist dose-response curves were fitted using the sigmoidal dose-response function of a graphics and statistics software (Graph-Pad Prism, version 4.0, 2003, Graphpad Software Incorporated, San Diego. CA). EC$_{50}$, effective concentration for half-maximal luciferase activity was calculated via non-linear regression. This equation was used to fit the data in the graphic software. Transactivation data are presented as EC$_{50}$ values for each compound tested.

One-way analysis of variance (ANOVA) was used to demonstrate statistical difference between the activity percentage of control and tested compounds with the help of GraphPad Prism software (GraphPad, San Diego, CA). Calculation of statistical significance (P values < 0.05) between treated and controlled groups were performed using Tukey’s post-hoc test.
Results

Structure-based design and virtual screening.

In order to rationalize the identification of original and highly active hPXR ligands, a structure-based approach was used. Crystal structures of the ligand-binding pocket of hPXR in complex with a variety of agonists (PDB codes: 1SKX, 1NRL and 1ILH) were analyzed to derive a pharmacophore model for potential ligands (Chrencik et al., 2005; Watkins et al., 2003; Watkins et al., 2001). As previously mentioned, the crystal structure of hPXR LBD in complex with rifampicin reveals a canonical fold consisting of a three layered-helical sandwich with however a unique five-stranded antiparallel β-sheet as well as a highly hydrophobic and flexible ligand binding pocket. Rifampicin, one of the largest PXR ligands identified so far, interacts with 18 amino acid side chains of hPXR which are not all in contact with the smaller ligands. Indeed, comparison of all hPXR LBD structures reveals that only six residues are consistently involved in ligand binding (Supplementary data I). Met243, Trp299 and Phe420 are involved in hydrophobic interactions whereas Ser247, Gln285 and His407 form hydrogen bonds with the ligands (Orans et al., 2005). Based on these crucial interactions and in agreement with previously reported pharmacophore models for PXR activators (Ekins and Erickson, 2002; Schuster and Langer, 2005), we built a 2D pharmacophore made up from 2 to 4 hydrophobic phenyl groups and at least one hydrogen-bond acceptor that could form a hydrogen bond with polar residues. Then, on the basis of the chemical structure of known PXR ligands, we also selected compounds presenting i) up to three hydrogen-bond donors, ii) a Cl heteroatom, which is present in several PXR agonists like the pesticides chlordane or chlordecone (Kretschmer and Baldwin., 2005; Lemaire et al., 2004) and iii) 2 nitrogen
atoms allowing the formation of additional hydrogen bonds and the design of original molecules when compared to reference ligands SR12813 and hyperforin. Finally, several molecular features were added to our pharmacophoric constraints such as a molecular weight up to 500 Daltons and a cLog P between 5 and 6 to favour membrane crossing. A subset of 496 molecules was so retrieved from the ChemBridge Database (February 2004) and docked using the Surflex program (Jain, 2003). The virtual screening was performed using the recently described structure of the rifampicin-bound hPXR LBD (Chrencik et al., 2005) with or without further molecular dynamic (MD) optimization. After virtual screening (see Materials and Methods), docking poses of top ranked molecules (score higher than 6) were visualized and analyzed for their associations with hydrophobic residues and their capabilities to form at least one hydrogen bond with Ser247, Gln285 or His407. At the final stage, 6 molecules were retrieved from the screening using the X-ray structure (C2BA-3, C2BA-7, C2BA-10, C2BA-11, C2BA-12 and C2BA-248), 6 from the MD structure (C2BA-4, C2BA-5, C2BA-6, C2BA-8, C2BA-13, and C2BA-251) and 1 (C2BA-9) obtained from both target structures. Table A in supplementary section provides Chembridge IDs and docking scores for the 13 compounds (set I). These molecules that showed a promising docking mode were purchased for further biological evaluation.

C2BA-4 is a potent PXR activator in HGPXR cells.

HGPXR cells obtained as described in Materials and Methods were designed to detect hPXR agonists, and the intermediary HG6LN cell line allowed us to evaluate non-PXR-mediated luciferase gene expression. We first evaluated the 13 compounds identified by virtual screening for PXR activation using the HGPXR stable cell line. In a previous work
(Lemaire et al, 2006), we studied the activities of the most potent known PXR ligands, rifampicin, hyperforin and SR12813 on the HGPXR cell line. These ligands displayed full agonist activities with different EC$_{50}$ values (720±30, 110±15 and 137±45 nM, respectively). Due to its highest activity, the human and rabbit PXR activator SR12813 (Jones et al., 2000) was chosen as our reference molecule for this study, and the activation values obtained with all the tested compounds were expressed relative to the luciferase activity observed in the presence of 1 µM SR12813 and taken as 100. The baseline activity of the HGPXR cells was 21.3±3.1 %. The structures and EC$_{50}$ values of original and reference ligands are displayed in Table 1 (active compounds) and Table 2 (inactive compounds) (see also Set I, Nine of the thirteen compounds showed agonistic activity (Fig 1A and 1B). They were categorized into three potency groups, weak (EC$_{50}$ > 10 µM), moderate (1 µM < EC$_{50}$ < 10 µM) (Fig 1B) and strong (EC$_{50}$ < 1 µM) (Fig 1A).

Four of them (C2BA-4, C2BA-7, C2BA-10 and C2BA-13) were found strong inducers of luciferase expression. EC$_{50}$ values were 49, 108, 163 and 191 nM, respectively. C2BA-8, C2BA-6 and C2BA-5 were moderate inducers. Their EC$_{50}$ values were 1306, 1893 and 2856 nM, respectively. Finally, C2BA-248 and C2BA-251 produced noticeable transactivation but even at high concentration, luciferase expression did not reach 100%. The structures of inactive compounds (C2BA-3, C2BA-9, C2BA-11 and C2BA-12) are displayed in Table 2.

To evaluate the strength of our docking studies, we selected an additional set of 9 compounds (Set II, see Table B in supplementary section) from the same subset of 496 molecules bearing the pharmacophoric constraints. In Set II, we retrieved compounds that form fewer contacts with critical residues (cut-off distance 3Å) but presenting a binding score higher or around 6. When tested for their activity, only one molecule was found to be a strong inducer (EC$_{50}$ <1µM) whereas 4 compounds had
an EC$_{50}$ ranking from 1 to 5 µM and 4 molecules were inactive. Thus, this additional set of compounds led to less potent molecules when compared to the 13 compounds of Set I.

To assess the PXR-independent luciferase expression as well as the toxicity of the new compounds, all ligands were systematically tested for their ability to activate parent HG$_{5}$LN cells. Among molecules of Set I, C2BA-12 and C2BA-3 showed luciferase activity in HG$_{5}$LN cells (Fig 1C), demonstrating that the activity observed in HGPXR cells was non-PXR specific. In addition, C2BA-7, C2BA-8, C2BA-10, C2BA-13, C2BA-248 and C2BA-7, C2BA-13 were toxic at 10 µM) and 5 µM respectively (Fig 1C). The toxicity of these compounds was also observed in HGPXR cells. At 1 µM, C2BA-7, C2BA-13 and C2BA-248 reached a plateau at 100% maximal HGPXR activity. At higher concentrations, a decrease in HGPXR luciferase expression was observed. For C2BA-8 and C2BA-248, the plateau was observed at 3 µM. Because C2BA-4 presented the lowest EC$_{50}$ on HGPXR cells as well as an absence of toxicity, this compound was selected for additional studies. No PXR-independent toxicity was observed for the 9 compounds of Set II (data not shown).

**Limited proteolytic digestion**

To determine whether C2BA-4 interacts directly with PXR and induces conformational changes, we analyzed the resistance of C2BA-4 hPXR to limited proteolysis (Fig 2). hPXR LBD labeled with [$^{35}$S]methionine was pre-incubated with ethanol (vehicle) or increasing concentrations (10.0 µM, 3.0 µM, 1.0 µM, 0.3 µM) of C2BA-4 or SR12813, prior to digestion with 250 µg/ml trypsin. The input lane represents undigested PXR (47kDa). Incubation of the receptor with 250 µg/ml trypsin in the absence of ligand (ETOH) led to complete digestion of hPXR-LBD. In
contrast, SR12813 produced a 30 kDa major protease resistant fragment, following partial protease digestion. The protease protection pattern induced by C2BA-4 was similar to that obtained with SR12813 pattern. C2BA-4 was slightly more potent at 0.3 µM but a higher degree of protection was obtained with SR12813 at high concentration (10 µM) (Fig. 2). These results indicate that the two ligands interact directly with hPXR LBD and induce similar conformational changes.

**C2BA-4 induces CYP3A4 and CYP2B6 expression in human hepatocytes**

The effect of C2BA-4 on PXR target gene expression was further evaluated in human hepatocytes. Cultured human hepatocytes from 4 different donors were treated with the control solvent (DMSO 0.1%), rifampicin (5 µM or 10 µM), SR12813 (1 µM) or C2BA-4 (5 µM) for 24 h. Total mRNA was isolated and QRT-PCR was performed to detect the expression level of CYP3A4 and CYP2B6 mRNAs. As expected, rifampicin (RIF) and SR-12813 strongly and significantly increased CYP3A4 and CYP2B6 mRNA expressions in these cells (Table 3). More interestingly, C2BA-4 induced the expression of CYP3A4 (from 8- to 74- fold induction compared to untreated cells) and CYP2B6 (from 3- to 46- fold induction compared to untreated cells) mRNAs in all culture preparations tested. In addition, we observed that the increase of CYP3A4 and CYP2B6 by C2BA-4 was dose-dependent in FT259 (Fig. 3) and very close to that obtained with rifampicin. These results demonstrate that, in agreement with our *in vitro* studies, C2BA-4 is able to activate PXR in human hepatocytes, leading to an increase of transcription of its main target genes such as CYP3A4 and CYP2B6.
In vivo response of xenografts to hPXR ligands

To test C2BA-4 activity in the context of the whole organism, HGPXR cells were implanted in nude mice (Fig. 4) as previously described (Lemaire et al, 2006). Mice were subcutaneously grafted with HGPXR cells onto the right dorsal flank and with HG5LN cells as an internal control onto the left dorsal flank, as described in Materials and Methods. The luminescent signal was detected before (Fig. 4A and 4C) and 8 h after (Fig. 4B and 4D) i.p. injection of 25 mg/kg SR12813 or C2BA-4 using a CCD camera. The specific responses in the HGPXR tumor were normalized by taking into account the basal response obtained in the HG5LN tumor before calculation of the fold induction. Detailed photon counting (photon/s) and induction factor calculation are reported in Fig. 4E. Although signal intensities could be different from one mouse to another due to a difference in tumor size, induction factors were reproducible. HG5LN internal control was used to detect non-specific activation of luciferase promoter and to detect any reproducibility problems due to substrate i.p. injection that may cause variations in luciferin bioavailability and perturb signal intensity. Injection of 5, 15, and 25 mg/kg SR12813 or C2BA-4 caused a dose dependant increase in the induction factor (Fig. 4F). At 25 mg/kg, induction factor averages of SR12813 and C2BA-4 were 2.5 ± 1 and 2.2 ± 0.8, respectively.
Discussion

PXR has a central role in human detoxification biology, regulating the expression of a critical set of gene products involved in xenobiotic and endobiotic metabolism. Thus agonists of hPXR enhancing the removal of endogenous and exogenous toxins are potential pharmacological tools and therapeutic agents. Recent studies suggest that treatment with PXR activators may delay the progressive degeneration of Niemann-Pick C1 disease (Langmade et al., 2006). Furthermore, such ligands could be used as scaffold for the design of compounds with antagonistic activity (Bourguet et al., 2000; Shiau et al., 2002; Steinmetz et al., 2001, Xue et al., 2007b). Development of PXR antagonists is desirable as a potential way to control the up-regulation of drug metabolism pathways during some therapeutic treatments.

Using a computational approach, the aim of our study was to identify original agonistic ligands. A structure-based approach to design ligands for hPXR requires a consideration of three characteristics of hPXR LBD: i) a large size, ii) a high flexibility with dynamic accommodations and iii) the hydrophobic nature of key residues implicated in the interaction with ligands. Indeed, by contrast with other nuclear receptors, hPXR contains a 60 amino acid insertion located in the binding site which seems to account for the first two characteristics. Moreover, the absence of salient features of the LBD in terms of shape and electrostatic potential was reported as unfavorable for the efficiency of a virtual screening on hPXR (Schapira et al., 2003). These particular characteristics of PXR limit the effectiveness of a structure-based design approach. However, as mentioned by Orans et al. (2005), six amino acid side chains were found to be involved in ligand binding in all the LBD structures of hPXR determined to date. Thus, among the Surflex docking results with a score higher than
6, we selected a set of 13 molecules interacting with these residues via a combination of hydrophobic contacts and hydrogen bonds. From these 13 compounds, 9 were found to exhibit an agonistic activity (Table 1), 3 came from the virtual screening performed with the X-ray hPXR-rifampicin complex structure whereas 6 agonistic compounds were retrieved from docking on an MD-optimized complex conformation. The two LBD conformations led to the identification of highly active compounds with EC50 below 200 nM (Table A, Set I, supplementary data).

Using an additional set of 9 compounds that presented a less favorable mode of binding as judged by proximity with the 6 critical residues, we also identified 5 additional ligands of hPXR but with less potent activity (Table B, Set II, supplementary data). Taken together, these results clearly validate the pharmacophoric constraints employed and underline the potential of virtual screening in PXR agonist discovery. Furthermore, they suggest that the criterion of proximity with the 6 critical residues appears to be a good filter to identify highly active compounds even in the case of hPXR which can adapt its LBD to many molecules.

One of the new hPXR ligands, C2BA-4 (1-(2-chlorophenyl)-N-[1-(1-phenylethyl)-1H-benzimidazol-5-yl]methanesulfonamide) was found without any toxic effect and able to activate hPXR with a better EC50 than SR12813 (Table 1) which led us to consider C2BA-4 as a potential hPXR ligand. Indeed, in a partial trypsin digestion assay C2BA-4 effectively protected hPXR from trypsin digestion (Figure 2), demonstrating a direct receptor-ligand interaction. C2BA-4 altered the protease sensitivity of hPXR and generated ligand-protection patterns similar to that observed for the SR12813-bound receptor. Moreover, our results show that C2BA-4 can alter the conformation of hPXR at a lower concentration than does SR12813 (Figure 2),
suggesting that C2BA-4 displays a higher affinity. To further characterize C2BA-4 as a potent PXR ligand, we studied its effect on CYP3A4 and CYP2B6 transcription (Xie et al., 2000) in primary culture of human hepatocytes. The current study shows that C2BA-4, induces an increase of CYP3A4 and CYP2B6 mRNA levels in human hepatocytes from 4 different donors and after 24 hours of exposure with an efficacy comparable to that of rifampicin or SR12813. Of note, the observation that the response levels were different between donors is due to the fact that CYP3A4 and CYP2B6 are inducible enzymes and that their induction varies markedly (up to 40-fold) across the population due to drug-mediated variation in CYP transcription (Lamba et al., 2005).

Activation of hPXR and induction of drug-metabolizing enzymes in vitro may not imply that there is relevant induction of PXR-mediated gene in rodent or in human. To test whether C2BA-4 induces PXR activity in vivo, we implanted the HGPXR reporter cells in nude mice. This assay allowed an in vivo detection of C2BA-4 response, the advantage of our model being that we were able to measure human and not mouse PXR activation. Despite the better in vitro effectiveness of C2BA-4 when compared to SR12813, our data obtained in primary human hepatocytes and in grafted nude mice show that C2BA-4 and SR12813 similarly activated hPXR. Differences in the bioavailability or metabolism of the two compounds might account for this apparent discrepancy.

Taken together, these results indicate that C2BA-4 represent a new potent agonist ligand of hPXR and could serve as novel lead for further chemical optimizations and pharmacological investigations. The putative most relevant mode of binding of this compound presented in Figure 5, docked either in X-ray and MD-derived structures, was found to be very close to that of the rifampicin (Chrencik et al., 2005,
supplementary data 1). Indeed, the sulfonamide moiety of C2BA-4 could be hydrogen-bonded with Ser 247 and His 407 in the central part of the cavity as does rifampicin which forms one hydrogen bond with Ser 247, one with His 407 and two with Gln 285. The 1-phenylethyl moiety of C2BA-4 could interact with Trp 299 and the 2-chloro-benzyl group with Phe 420 (Figure 5). These two residues are systematically implicated in the agonist binding to hPXR. In addition, C2BA-4 seems to be at interacting distance with Val 211 and Leu 239 which participate in the binding of rifampicin but not SR12813 nor hyperforin (Chrencik et al, 2005). Then, it is interesting to note the proximity of the 2-chloro-benzyl group with residues forming Helix-12 of the hPXR LBD, in particular Met 425 (Figure 5). In several nuclear receptors, Helix-12 plays an important role as its position and its mobility, which vary according to the agonistic or antagonistic nature of the ligand, determine the interacting interface and the type of co-factor proteins that could bind to nuclear receptors. Thus, in several studies (Bourguet et al, 2000) it has been observed that bulky antagonistic ligands prevent Helix-12 from adopting the active conformation of LBD.

This putative mode of binding of C2BA-4 will be useful for the rational design of analogues able to present additional contacts with the hPXR LBD. Indeed, the chemical structure of C2BA-4 opens the way to the synthesis of a large number of chemical analogues which could be investigated to develop agonistic compounds with higher activity. Moreover, co-crystallization studies of C2BA-4 in complex with hPXR are under progress to validate the mode of binding.

Recently, one paper describing a new commercially available LXR and PXR ligand (T0901317) was published (Xue et al, 2007b). Using transient PXR and LXR transfection, this new ligand showed EC$_{50}$ of 12.6 nM and 100 nM for PXR and LXR.
respectively. Interestingly, T0901317 derivatives designated to obtain PXR antagonists were found to be potent agonists with an enhanced selectivity and affinity for PXR (EC$_{50}$ of 3 nM and > 10 µM, for PXR and LXR respectively). The promiscuity and the structural conformability of the PXR ligand binding pocket make antagonist design particularly difficult. However, based on the putative mode of binding of C2BA-4, several C2BA-4 analogues with bulky substituent groups on the chlorophenyl moiety could be designed in an attempt to reverse the agonistic activity towards an original antagonistic action by preventing helix 12 from adopting an active conformation. If successful, the finding of hPXR antagonists could provide a unique tool to control drug metabolism and to reduce the activation of xenobiotic metabolism pathways during therapeutic treatment of disease. Finally, the virtual screening approach described in this report could represent a framework to develop relevant in silico tests to predict the ability of environmental compounds to bind PXR.

**Conclusion**

The current study represents a strategy for the identification of hPXR ligands by combining a structure-based design with a functional approach using both cell culture and whole animals. The original agonists described here, in particular the most active, will be further chemically optimized to design novel selective hPXR modulators with hopefully improved therapeutic indexes and great medical benefit.
References


Lamba J, Lamba V and Schuetz E (2005) Genetic variants of PXR (NR1I2) and CAR (NR1I3) and their implications in drug metabolism and pharmacogenetics. Curr Drug Metab 6(4):369-383.


Footnotes
This study has been partly supported by the European Union Network CASCADE (FOOD-CT-2003-506319) and ANR JCJC-05-47810 (J-M.P). CB is a recipient of the MENRT from the French Government. VN is a recipient of the French Young Researcher Program (INSERM).

Reprint request: Patrick Balaguer Equipe INSERM U824, Signalisation Hormonale, Environnement et Cancer, Centre de Recherche en Cancérologie de Montpellier (CRCM), Parc Euromédecine – CRLC Val d’Aurelle, F-34298 Montpellier, France Cedex 5. Tel.: +33 467 612 409; fax: +33 467 613 787. p.balaguer@valdorel.fnclcc.fr

&: These authors contributed equally to this work.
Legends of figures

Fig. 1 C2BA-4 is an efficient hPXR agonist. The activity of HGPXR cells was measured as a function of the concentration of (A) potent inducers: SR12813, C2BA-4, C2BA-7, C2BA-10, C2BA-13, and (B) SR12813 and moderate and weak inducers: C2BA-5, C2BA-6, C2BA-8, C2BA-248, C2BA-251. Activity was expressed as a percentage of 1 µM SR12813-induced activity. (C) The activity of HG5LN control cells was measured at 0.3, 1, 3 and 10 µM of C2BA-3, C2BA-7, C2BA-8, C2BA-10, C2BA-12, C2BA-13 and C2BA-248. Means ± SD were calculated from three independent experiments performed in triplicate.

Fig. 2 Binding of C2BA-4 to PXR. PXR protein was synthesized and labeled with [35S]methionine in reticulocyte lysate and incubated with C2BA-4 and SR12813 or vehicle alone before trypsin digestion. The reaction was terminated by boiling in SDS-containing protein sample buffer. PXR proteolytic pattern was analyzed by SDS-PAGE and fixed/dried gel were visualized by PhosphorImager. (A) C2BA-4 and SR12813 treatments protect from trypsin digestion in dose-dependent manner. A trypsin resistant fragment (30 kDa) was generated in presence C2BA-4 and SR12813. Figure shown is representative of three experiments. (B) Autoradiograms were analyzed using an image analyzing software. To measure the relative levels of the 30 kDa band protected by C2BA-4 or SR12813 on SDS-PAGE gel, the intensity of the band of PXR-LBD treated with ethanol was taken as 1.

Fig. 3 Dose-dependent effect of C2BA-4 on CYP3A4 and CYP2B6 gene expression in cultured human hepatocytes. Human hepatocytes from FT259 were treated for 24 h with solvent (DMSO 0.1%), rifampicin, C2BA-4 at different concentrations ranging from 0.1 µM to 5 µM as indicated. Twenty hours later, cells were harvested in TRIzol,
total RNA was extracted and CYP3A4 and CYP2B6 levels were measured via quantitative real-time PCR using the Light Cycler apparatus. As an internal control, the β-actin mRNA level was measured similarly to normalize data. Data are represented as fold induction compared to non treated cells +/- SD.

Fig. 4 Effect of C2BA-4 on in vivo induction of hPXR in nude mice. (4A-4D) Bioluminescent imaging of nude mouse xenografts: HG5LN (left flank) and HGPXR (right flank) xenograft were imaged before (4A) and 8 h after (4B) 25 mg/kg SR12813 ip injection or before (4C) and 8 h after (4D) 25 mg/kg C2BA-4 ip injection. (E) Photon counting and induction factors of HG5LN and HGPXR xenografts before and after stimulation by 25 mg/kg SR12813 or C2BA-4. Light intensities (photons/s) were calculated using WinLight software (Berthold Technologies) as described by Pillon et al. (2005). Specific signal was background subtracted and ratio of HGPXR specific values relative to HG5LN gives the induction factor for SR12813 or C2BA-4. (4F) SR12813 and C2BA-4 were ip injected in nude mice at the indicated concentrations (mg/kg) to establish in vivo dose response curves. Mean values ± were calculated from 4 independent experiments and six mice were necessary to perform in vivo experiments.

Fig. 5 Putative mode of binding for C2BA-4 in the ligand binding pocket of hPXR. C2BA-4 could form hydrogen bonds with at least ser 247, his 407 and hydrophobic contacts with met 243, phe 420, trp 299. Hydrogens are not represented for clarity. The crystallographic structure of hPXR (PDB code: 1SKX) is represented in yellow, the MD structure of hPXR in blue.
Table 1: Chemical structures and EC<sub>50</sub> values of active PXR ligands obtained with HGPXR reporter cell lines. C2BA-4 is used as a racemic solution.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Structure</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Name</th>
<th>Chemical Structure</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR12813</td>
<td><img src="image" alt="Structure" /></td>
<td>137 ± 45</td>
<td>C2BA-8</td>
<td><img src="image" alt="Structure" /></td>
<td>1308 ± 675</td>
</tr>
<tr>
<td>C2BA-4</td>
<td><img src="image" alt="Structure" /></td>
<td>49 ± 15</td>
<td>C2BA-10</td>
<td><img src="image" alt="Structure" /></td>
<td>163 ± 76</td>
</tr>
<tr>
<td>C2BA-5</td>
<td><img src="image" alt="Structure" /></td>
<td>2856 ± 1558</td>
<td>C2BA-13</td>
<td><img src="image" alt="Structure" /></td>
<td>191 ± 81</td>
</tr>
<tr>
<td>C2BA-6</td>
<td><img src="image" alt="Structure" /></td>
<td>1893 ± 790</td>
<td>C2BA-248</td>
<td><img src="image" alt="Structure" /></td>
<td>1893 ± 790</td>
</tr>
<tr>
<td>C2BA-7</td>
<td><img src="image" alt="Structure" /></td>
<td>108 ± 52</td>
<td>C2BA-251</td>
<td><img src="image" alt="Structure" /></td>
<td>108 ± 52</td>
</tr>
</tbody>
</table>
Table 2: Chemical structures of inactive PXR ligands.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2BA-3</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>C2BA-9</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>C2BA-11</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>C2BA-12</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
Table 3: Effect of C2BA-4 on the expression of PXR target genes in cultured human hepatocytes. Human hepatocytes from four different donors (FT259, FT261, FT267 and FT268) were treated for 24 h with solvent (DMSO 0.1%), rifampicin (RIF 5 µM or 10 µM), SR12813 (SR 1 µM), C2BA-4 (5 µM). Twenty hours later, cells were harvested in TRIzol, total RNA was extracted and cDNA was synthesized. CYP3A4 and CYP2B6 levels were measured via quantitative real-time PCR using the Light Cycler apparatus. As an internal control, the β-actin mRNA level was measured similarly to normalize data. Data are expressed as fold induction compared to non treated cells ± SD. nd: none determined. *, P<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Donor #1 (FT259)</th>
<th>Donor #2 (FT261)</th>
<th>Donor #3 (FT267)</th>
<th>Donor #4 (FT268)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP3A4</td>
<td>CYP2B6</td>
<td>CYP3A4</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>RIF</td>
<td>49.92 *</td>
<td>10.22 *</td>
<td>35.79 *</td>
<td>11.53 *</td>
</tr>
<tr>
<td></td>
<td>± 6.95</td>
<td>± 1.43</td>
<td>± 0.53</td>
<td>± 0.4</td>
</tr>
<tr>
<td>SR</td>
<td>nd</td>
<td>nd</td>
<td>21.08 *</td>
<td>23.39 *</td>
</tr>
<tr>
<td></td>
<td>± 2.48</td>
<td>± 1.69</td>
<td>± 1.57</td>
<td>± 1.01</td>
</tr>
<tr>
<td>C2BA-4</td>
<td>74.07 *</td>
<td>46.06 *</td>
<td>23.38 *</td>
<td>31.26 *</td>
</tr>
<tr>
<td></td>
<td>± 8.71</td>
<td>± 5.58</td>
<td>± 0.45</td>
<td>± 2.27</td>
</tr>
</tbody>
</table>
### E

<table>
<thead>
<tr>
<th></th>
<th>Light emitted from xenograft</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HG5LN (photons/s)</td>
<td>HGPX (photons/s)</td>
<td>Relative to HG5LN</td>
</tr>
<tr>
<td>SR12813</td>
<td>9676</td>
<td>25583</td>
<td>2.64</td>
</tr>
<tr>
<td>0 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>20506</td>
<td>143948</td>
<td>7.02</td>
</tr>
<tr>
<td>C2BA-4</td>
<td>41360</td>
<td>12151</td>
<td>0.29</td>
</tr>
<tr>
<td>0 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>137719</td>
<td>131018</td>
<td>0.95</td>
</tr>
</tbody>
</table>

### F

![Graph showing fold change for SR12813 and C2BA-4 with different drug doses (25 mg/kg, 15 mg/kg, and 5 mg/kg).](image)