Identification of Clinically Utilized Drugs that Activate Pregnan X Receptors

Sunita J. Shukla, Srilatha Sakamuru, Ruili Huang, Timothy A. Moeller, Paul Shinn, Danielle VanLeer, Douglas S. Auld, Christopher P. Austin and Menghang Xia

NIH Chemical Genomics Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD (S.J.S., S.S., R.H., P.S., D.V.L., D.S.A., C.P.A., M.X.); Celsis In Vitro Technologies, Halethorpe, MD (T.A.M.)
Running Title: Clinically utilized drugs that activate PXR

Correspondence should be addressed to:

Menghang Xia, PhD
National Institutes of Health
9800 Medical Center Drive
Rockville, MD 20850
phone: 301-217-5718
fax: 301-217-5736
email: mxia@mail.nih.gov

Text pages: 37
Tables: 3
Figures: 5
References: 40

Words in
Abstract: 231
Introduction: 681 (not including word count for references throughout introduction)
Discussion: 1,288 (includes word count for references in discussion)
Nonstandard Abbreviations:

PXR, pregnane X receptor; NR, nuclear receptor; CYP, cytochrome P450; GSTs, glutathione-S-transferases; MDR1, multidrug resistance protein 1; LBD, ligand binding domain; CYP3A4, cytochrome P450 3A4; DBD, DNA binding domain; hPXR, human pregnane X receptor; qHTS, quantitative high-throughput screening; rPXR, rat pregnane X receptor; PXRE, PXR response element; RXR, retinoid X receptor; NPC, NIH Chemical Genomics Center Pharmaceutical Collection; FDA, Food and Drug Administration; DMSO, dimethyl sulfoxide; FRD, flying reagent dispenser; TR-FRET, time-resolved fluorescence resonance energy transfer; CRCs, concentration response curves; MOA, mechanism of action; SAR, structure activity relationship; SOM, self-organizing map; LC/MS, liquid chromatography/mass spectrometry; TFA, trifluoroacetic acid; TOF, time-of-flight mass spectrometer; CDK, cyclin-dependent kinase; SHP, small heterodimer partner; SMRT, silencing mediator of retinoid and thyroid hormone receptors; SRC-1, steroid receptor coactivator-1; HNF4α, hepatocyte nuclear factor α; PGC-1α, peroxisome proliferators-activated receptor γ co-activator-1α; RXR, retinoid X receptor; XREM, xenobiotic responsive enhancer molecule/PXRE; ER6, everted repeat with a six-nucleotide spacer; DR3, direct repeat with a three-nucleotide spacer; Ab, Tb3+-labeled antibody; SD, standard deviation
Abstract

The pregnane X receptor (PXR) binds xenobiotics and regulates the expression of several drug metabolizing enzymes and transporters. Human PXR (hPXR) activation and CYP3A4 induction can be involved in drug-drug interactions resulting in reduced efficacy or increased toxicity. However, there are known species-specific differences with regard to PXR activation that should be taken into account when extrapolating animal PXR data to humans. We profiled 2,816 clinically used drugs from the National Institutes of Health Chemical Genomics Center Pharmaceutical Collection (NPC) for their ability to activate hPXR and rat PXR (rPXR) at the cellular level, induce human CYP3A4 at the cellular level, and bind human PXR at the protein level. From 6-11% of drugs were identified as active across the 4 assays, which included assay-specific and pan-active compounds. The lowest concordance was observed between the hPXR and rPXR assays, and many compounds active in both assays nonetheless demonstrated significant potency differences between species. Analysis based on clustering potency values demonstrated the greatest activity correlation between the hPXR activation and CYP3A4 induction assays. SAR analysis identified chemical scaffolds which were pan-active (e.g., dihydropyridine calcium channel blockers) and others which were uniquely active in individual assays (e.g., steroids and fatty acids). These results provide important information on PXR activation by clinically utilized drugs, highlight the species-specificity of PXR activation by xenobiotics, and provide a means of prioritizing compounds for follow-up studies and optimization efforts.
Introduction

The pregnane X receptor (PXR) is a member of the nuclear receptor (NR) family, based on its sequence homology to other NRs (Kliewer et al., 1998). PXR is expressed in the intestine and liver across species (Kliewer, 2003) and plays a critical role in the regulation of genes involved in drug metabolism and efflux (LeCluyse, 2001; Ekins and Schuetz, 2002; Ekins et al., 2008), most notably cytochrome P450 (CYP) enzymes, glutathione-S-transferases (GSTs) and multidrug resistance protein 1 (MDR1) (Kliewer, 2003). Unlike other nuclear receptors, the PXR ligand binding domain (LBD) is largely hydrophobic and extremely flexible, thus allowing for conformational changes to accommodate structurally diverse molecules (Watkins et al., 2001). Structurally diverse PXR ligands include antibiotics, calcium channel blockers, steroids, dietary supplements, environmental pollutants, antimycotics, protease inhibitors and statins (Blumberg and Evans, 1998; Kliewer et al., 1998; Jones et al., 2000; Goodwin et al., 2002; Zhang et al., 2008).

Several studies have identified PXR as an important regulator of cytochrome P450 3A4 (CYP3A4), involved in the metabolism of more than 50% of all prescribed drugs in humans with the aid of various co-activators (Figure 1A) (Lehmann et al., 1998; Jones et al., 2000; Sinz et al., 2008). The ability of PXR to bind a broad range of substrates has been shown to correlate with the induction of CYP3A4 expression (Timsit and Negishi, 2007). PXR activation and CYP3A4 induction are involved in approximately 60% of all drug-drug interactions (Evans, 2005), in which an administered drug modulates the metabolism of a co-administered drug, leading to decreased efficacy or increased toxicity.
Most DNA binding domains (DBDs) of mammalian PXR receptors are highly conserved (95% identity), however the PXR LBD is highly divergent across species, sharing roughly 70% sequence identity (Jones et al., 2000; Moore et al., 2000). This divergence, noted by specific amino acid differences in the crystal structure of the PXR LBD (Ekins and Schuetz, 2002), plays a role in species-specific induction of PXR and CYP3A4 (Jones et al., 2000). Pharmacologic studies in primary hepatocytes also suggest that species-specific variation in CYP3A4 induction is not a consequence of CYP3A4 promoter structure, but rather NRs such as PXR (Xie et al., 2000). Previous studies that have profiled compound-dependent PXR activation using species-specific hepatocytes found marked differences in ligand binding (Kocarek et al., 1993; Kocarek et al., 1995; Barwick et al., 1996; Jones et al., 2000). For example, rabbit and human PXR are activated by rifampicin, however there are differences in PXR activation by other ligands such as dexamethasone (Jones et al., 2000). Thus, the validity of using animal models or nonhuman hepatocytes to predict CYP3A induction in humans has been questioned (Jones et al., 2000). Furthermore, the use of primary human hepatocytes is not feasible due to unavailability and poor quality of human liver donors.

Despite knowledge regarding drug-drug interactions, little is known about the mechanism by which these agents activate PXR and induce CYP3A4 expression. Thus far, the majority of PXR and CYP3A4 activity profiling has come from computational prediction; however, there is a paucity of published experimental data on PXR activation and CYP3A4 induction by clinically utilized drugs, particularly those approved more than 10 years ago, when PXR was discovered. Furthermore, only a small fraction of the published data report EC_{50} values (Khandelwal et al., 2008). Thus, new in vitro methods
to assess human PXR (hPXR) activation are warranted due to the aforementioned reasons regarding species differences and availability and quality of human liver donors.

Due to its importance in xenobiotic metabolism and clinical pharmacology, a comprehensive publicly available database of the PXR activity found in clinically used drugs would be tremendously valuable to both guide the use of currently prescribed pharmaceuticals, and aid in the development of new drugs. Furthermore, rats are the preferred models for drug metabolism and pharmacokinetic (DMPK) studies, hence rat-human PXR activation differences are important to identify and quantify. We have previously reported the profiling of a structurally diverse collection of up to 17,000 compounds for PXR binding and CYP activity (Shukla et al., 2009; Veith et al., 2009) using quantitative high-throughput screening (qHTS) (Shukla et al., 2009). In this study, we utilized qHTS to profile over 2,800 clinically used drugs and bioactive compounds for their ability to activate hPXR and rat PXR (rPXR) and induce human CYP3A4 using cell-based \textit{in vitro} assays. The activities of these drugs at the cellular level (human) were further compared to their ability to bind the human PXR LBD at the protein level (Shukla et al., 2009) to identify compounds which directly bind to the LBD and those which potentially outside this region along the full-length PXR. Finally, we identified compounds that induced human CYP3A4 with an additional cell-based assay. The results obtained here will be clinically beneficial and can be used to prioritize compounds for further testing in \textit{in vitro} and \textit{in vivo} systems.
Materials and Methods

Cell Lines and Cell Culture Conditions

DPX-2 (utilized for hPXR activation and CYP3A4 induction studies) and rPXR cell lines were purchased from Puracyp (Carlsbad, CA). The DPX-2 cell line construction has been previously described (Raucy et al., 2002). Briefly, HepG2 cells derived from human heptocellular carcinoma cells were co-transfected with an expression vector containing the full-length coding region of hPXR, a PXR response element (PXRE), and a luciferase construct containing the CYP3A4 enhancer harboring distal and proximal promoters (Yueh et al., 2005). The PXRE is required for binding of the PXR and retinoid X receptor (RXR) heterodimer in the regulatory regions of target genes prior to activation. The luciferase construct containing the CYP3A4 enhancer (upstream of the luciferase reporter gene) is necessary for detection of CYP3A4 transcription, as measured by luminescence. The rPXR cell line was generated through co-transfection with an expression vector containing the full-length rPXR and a luciferase construct into a rodent hepatoma cell line (Sinz et al., 2007). Cell culture and assay medium were purchased from Puracyp (Carlsbad, CA) and the cells were maintained at 37ºC under a humidified atmosphere and 5% CO₂.

NCGC Pharmaceutical Collection (NPC)

The NPC collection (R. Huang et al, in preparation) currently contains 2,816 clinically utilized and bioactive small molecules, 52% of which are Food and Drug Administration (FDA) approved for human or animal use in the United States. The remaining drugs are either approved for use in other countries, such as Europe, Canada or
Japan, or are compounds that have been tested in clinical trials. The NPC library was prepared as fifteen inter-plate titrations which were serially diluted 1:2.236 in dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA) in 384 well plates. The stock concentrations of the test compounds ranged from 10 mM to 0.13 µM. Transfer of the diluted compounds from 384 well plates to 1,536 well plates was performed using an Evolution P³ system (Perkin Elmer, Wellesley, MA). Each treatment plate included concurrent DMSO and positive control wells and concentration-response titrations of controls, all occupying columns 1-4 (described in the respective assay methods sections). During screening, the compound plates were sealed and kept at room temperature, while other copies were maintained at -80 °C for storage.

**hPXR and rPXR Activation**

Summarized protocols for the hPXR and rPXR assays (Figure 1Bi) can be found in Supplementary Tables 1 and 2, respectively. To measure PXR activation by a luciferase reporter readout, DPX-2 and rPXR cell lines were dispensed in white solid bottom 1,536 well plates (Greiner Bio-One North America, Monroe, NC) at 2,000 cells/5 µL/well in assay medium using a Flying Reagent Dispenser (FRD, Aurora Discovery, Calsbad, CA). Plates were incubated at 37°C for 5 hours before transferring 23 nL of each compound from the NPC via pin tool (Kalypsys, San Diego, CA), with final compound concentrations ranging from 7.5 nM to 46 µM. The final concentration of DMSO in the culture medium was <0.5%. Positive controls used to assess maximal response were rifampicin and dexamethasone for the hPXR and rPXR assays, respectively. All positive control compounds in the primary screens were also used as positive controls in the
respective follow-up studies. The plates were incubated with compound for 24 hours. Five microliters of One-Glo™ luciferase (Promega, Madison, WI) reagent was added to the plates and incubated at room temperature for 30 minutes prior to reading on a ViewLux plate reader (PerkinElmer; Shelton, CT).

**CYP3A4 Induction Assay**

A summarized protocol for Figure 1Bii can be found in Supplementary Table 3. CYP3A4 induction, a luciferase reporter readout, was measured separately from PXR activation in DPX-2 cells using P450-Glo™ Screening Systems (Promega). Briefly, DPX-2 cells were dispensed in white solid bottom 1,536 well plates at 2,000 cells/5 μL/well in assay medium using an FRD. Next, the plates were incubated for 3.5 hours prior to addition of 23 nL of each compound in the NPC. Additionally, 1 μl of luciferin assay substrate was added to the cells and incubated at 37°C for 3 hours. Five microliters of P450-Glo™ Reagent was subsequently added to plates and incubated at room temperature for 30 minutes prior to reading on the ViewLux plate reader. This particular reagent is robust and has been previously used for CYP3A4 profiling (Veith et al., 2009) with little to no fluorescence interference. Rifampicin was the positive control used to assess maximal response in the CYP3A4 induction assay. The positive control compound in the primary screens was also used as positive controls in the respective follow-up studies.

**Determining Induction of CYP3A4 in Cryopreserved Human Hepatocytes**
The procedure for thawing and culturing plateable cryopreserved human hepatocytes was performed as described in instructions for use from Celsis In Vitro Technologies product information. Briefly, cryopreserved human hepatocytes were thawed at 37°C for approximately two minutes and transferred into 5 mL of InVitroGRO™ CP medium at 37°C. The hepatocyte suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to a final concentration of 0.5 x 10^6 viable cells per mL. Cells were dispensed into 96-well BD BioCoat™ collagen I coated culture plate (Becton Dickenson, Massachusetts) at a final density of 50,000 viable hepatocytes per well. On day 2 of culture, BD Matrigel™ (Becton Dickenson, Massachusetts) was added to the culture at 0.25 mg per mL of protein in InVitroGRO™ CP medium. On days 3 and 4, cells were incubated with the positive control rifampicin [25 µM] in InVitroGRO™ HI medium to induce CYP3A4 protein levels or with test compound in 7-point dosing range from 0.033-33 µM for rimexolone, oxatomide, colforsin, nilvadipine and famprofazone, and 0.01-100 µM for bumecainum. Vehicle control wells were incubated with 0.5% DMSO in InVitroGRO™ HI medium. On day 5, media was removed and metabolism was determined using P450-Glo™ CYP3A4 with Luciferin-IPA (Promega, Wisconsin) according to manufacturer’s instructions for use. After 30 minute incubation with luciferin-IPA substrate, medium was transferred to opaque 96-well microtiter plate containing an equal volume of P450-Glo™ reaction buffer. The cell culture plate was washed with InVitroGRO™ KHB buffer and viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Wisconsin) according to manufacturer’s instructions for use.
PXR Ligand Binding Domain Assay

The hPXR LBD assay (Figure 1Biii) was performed with LanthaScreen™ time-resolved fluorescence resonance energy transfer (TR-FRET)-based technology (Invitrogen, Carlsbad, CA) and is described in detail elsewhere (Shukla et al., 2009). Briefly, the assay was performed using the LanthaScreen™ TR-FRET PXR Competitive Binding Assay Kit which contains the TR-FRET PXR Assay Buffer, Fluormone™ PXR Green (fluorescein-labeled PXR ligand), human PXR-LBD (GST) (amino acids 111-434), and LanthaScreen™ Tb-anti-GST antibody. PXR LBD was diluted in the assay buffer to 1.5X final recommended concentration to maintain optimal protein stability during the screening process in the 1,536 well plate format. An FRD was used for all reagent dispensing. SR12813, a potent PXR agonist, was used as a positive control to assess maximal response in the assay. The control columns were arranged as previously described (Shukla et al., 2009).

Cytotoxicity Assay in DPX-2 Cells

Cell viability was measured using a luciferase-coupled ATP quantization assay of metabolically active cells (ATPlite™ 1step Luminescence Assay System, Perkin Elmer, Boston, MA). Cells were dispensed at 2,000 cells/5 μL/well in 1536-well white solid bottom assay plates using an FRD. The assay plates were incubated at 37°C for 5 hours to allow for cell attachment, followed by addition of compounds via pin tool. After compound addition, plates were incubated for 24 hours at 37°C. At the end of the incubation period, 5 μL of ATPlite reagent was added, plates were incubated at room temperature for 20-30 minutes, and luminescence intensity was determined using a
ViewLux plate reader. Positive control columns were arranged as the following: Column 1, concentration-response titration of tetraoctyl ammonium bromide from 2.8 nM to 92 μM; Column 2, 92 μM tetraoctyl ammonium bromide; Column 3, DMSO only; and Column 4, 23 μM tetraoctyl ammonium bromide. Compounds with curve classes 1.1, 1.2, 2.1 or 2.2 were considered cytotoxic, while class 4 curves were considered inactive and all other curve classes considered inconclusive (See Data Analysis section).

Data Analysis and Follow-up Studies

Data normalization and curve fitting were performed as previously described (Shukla et al., 2009). Raw plate reads for each titration point were normalized relative to the following controls, which represented 100% response: 23 μM rifampicin (hPXR activation and CYP3A4 induction assays), 46 μM dexamethasone (rPXR), or 92 μM tetra-n-octylammonium bromide (cytotoxicity in DPX-2 cells) and DMSO only wells (0%). The concentration response curves (CRCs) for every compound were fit to the Hill equation, yielding half-maximal inhibition (IC₅₀) or activation (EC₅₀) and maximal response (efficacy) values. Fitting of experimental data to the Hill equation was amended for bell shaped concentration response curves, which showed an initial increase in activity at low compound concentrations followed by a sharp decline at higher compound concentrations: concentrations greater than the concentration of maximal response where masked for regression purposes to determine the AC₅₀ of the maximal response. There were a total number of 34 plates in the primary qHTS screen, which included 15 plates corresponding to each concentration per NPC library set and one DMSO-only plate at the beginning and end of each NPC library plate stack (final DMSO concentration of 0.45%).
Compounds from the primary qHTS screen were classified into 4 major curve classes according to quality of curve fit and efficacy as previously described (Shukla et al., 2009). Compounds with class 1.1, 1.2, 2.1, and 2.2 curves were associated with high confidence and quality CRCs. The aforementioned curve classes were considered as active, while compounds with class 4 curves were deemed inactive since they are not associated with any activity across the concentrations tested. All other curve classes (including those curves with single point activity) were deemed inconclusive.

Hierarchical clustering of compound activity patterns was performed with Spotfire DecisionSite 8.2. (Cambridge, MA) using correlation of the log EC$_{50}$ values as the similarity metric. Compounds that were associated with CRC classes 1-3 in at least 3 assays were included for clustering.

Follow-up studies were performed on a new aliquot of sample to confirm sample integrity and assay reproducibility. Compounds that were class 4 in one test and class 1.1, 1.2, 2.1 or 2.2 (efficacy>50%) in another test were not considered as confirmed. All other cases were considered confirmed. In the follow-up studies, 72 compounds were cherry picked from the original solutions and chosen based on activities and structural diversity across the 4 assays. The compounds also had efficacy >50% and potency <10 µM across at least one assay with class 1.1, 1.2, 2.1, and 2.2 curves (See Data Analysis section). Compounds were prepared in duplicate 24-point two-fold dilution titrations in DMSO, with final concentrations ranging from 0.11 pM to 92 µM. These 72 compounds (Supplementary Table 4) were re-tested in the hPXR, rPXR, CYP3A4 induction and PXR binding assays. For more extensive investigation and confirmation of previous results, 26 out of 72 compounds were ordered from commercial vendors and re-tested in the
respective assays in duplicate 24-point two-fold dilution titrations. These compounds were chosen based on potency <10 μM in at least one out of four assays with curve classes 1.1, 1.2, 2.1, and 2.2, novel mechanism of action (MOA) with regard to hPXR activation or CYP3A4 induction assays, and no apparent cytotoxicity.

Of the 26 compounds re-tested, 8 were purchased from Sigma-Aldrich (St. Louis, MO): ciglitizone, colforsin, diclazuril, felodipine, oxatomide, tacrolimus, troglitazone and zearalanol. Nine compounds were purchased from Prestwick Chemical (Washington, DC): famprofazone, fenbendazole, fluorometholone, hydralazine hydrochloride, methacycline hydrochloride, methylprednisolone 6-alpha, rimexolone, terconazole, and thonzonium bromide. Hetacillin potassium and thiamylal sodium were purchased from Microsource (Gaylordsville, CT). Plicamycin and sirolimus were provided by the National Cancer Institute (Bethesda, MD). Other compounds purchased were bumecainum (Asinex, Winston-Salem, NC), nilvadipine (Bosche Scientific, New Brunswick, NY), riodipine (Enamine, Kiev, Ukraine); suramin (Biomol, Plymouth Meeting, PA), and zafirlukast (Toronto Research, Ontario, Canada).

All compounds were considered confirmed between solution cherry-picked and re-ordered samples unless high confidence curve classes became inactive or vice-versa between the follow-up assays.

**Structure Activity Relationship (SAR) Analysis**

*Clustering of compounds by activity patterns*

Compounds were clustered hierarchically based on their log EC_{50} patterns from the primary screen across the four PXR assays within Spotfire DecisionSite 8.2 (Spotfire
Inc., Cambridge, MA) using the correlation of log EC$_{50}$ values as the similarity metric (Figure 5A). To facilitate SAR analysis, a simplified clustering of compound activity profiles was performed as follows: each compound was converted into a [0,1] bit string with length four that represents its activity profile with each bit corresponding to activity in a PXR assay. A bit was set to 1 if the compound was class 1-3 in the corresponding PXR assay and 0 if the compound was class 4. Eight different activity profiles (or bit patterns) were observed from the data and the compounds were categorized according to their activity pattern (Figure 5B).

**Clustering of compounds by structure**

The NPC compounds were clustered using the Self-Organizing Map (SOM) algorithm based on similarity in their Daylight® structural fingerprints yielding 351 clusters (data not shown).

**Compounds that co-cluster by structure and activity**

Compounds that belong to the same activity cluster may fall into several structure clusters and the same is true for compounds that belong to the same structure cluster. Combining the two sets of clustering results, the NPC compounds were further segregated into 695 structure-activity clusters, such that each of these clusters contains structurally similar compounds that also share similar activity patterns. Over 70% of these structure-activity clusters contain only one compound. We further examined the clusters that contain at least four compounds and found 16 such clusters (data not shown).
Chemical analysis for compound purity

Initial purity and identity determination was performed on a Waters Acquity liquid chromatography/mass spectrometry (LC/MS) (Waters Corporation, Milford, MA). A 2.2 minute gradient of 5 to 100% acetonitrile (containing 0.025% trifluoroacetic acid (TFA)) in water (containing 0.05% TFA) was used with a 3 minute run time at a flow rate of 0.5 mL/min. The column, a Phenomenex Luna, 2.0x 100mm, with a 2.5 µm particle size, was used at a temperature of 45° C. Purity determination was performed using a Photo Diode Array Detector and an Evaporative Light Scattering Detector. Mass determination was performed using a Waters Micromass ZQ mass spectrometer with electrospray. Data was analyzed using the Waters OpenLynx software. Samples with inconclusive data, which required additional purity and identity determination, were processed on an Agilent 1200 LC/MS. This system utilized a 6.8 minute gradient of 4 to 100% acetonitrile in water, with similar TFA proportions to the Acquity run in both solvents, over an 8.5 minute run time. The column used was a Phenomenex Luna 3.0x100mm, with a 3.0 µm particle size, at a temperature of 50° C. Purity determination was performed using an Agilent Diode Array Detector. Mass determination was performed using an Agilent Quadrupole LC/MS. Samples which could not be identified due to poor ionization were further analyzed using an Agilent Time-Of-Flight Mass Spectrometer (TOF, Agilent Technologies, Santa Clara, CA). Mobile phase conditions were similar with the exception of 0.1% formic acid replacing TFA. Confirmation of molecular formula was confirmed using electrospray ionization with the Agilent Masshunter software version B.02.
Results

*Profiling clinically used drugs against human PXR.* In this study, we profiled over 2,800 compounds in the NPC for hPXR activity in a cell-based hPXR activation assay and an hPXR LBD assay (Shukla et al., 2009) (Figure 1) in qHTS format. The hPXR activation assay was carried out with rifampicin, a well known hPXR agonist, as a positive control. The assay performed well across thirty-four 1,536-well plates with an average rifampicin EC$_{50}$ of 3.5 μM and signal to background ratio of 5.7 (Supplementary Table 4). A total of 310 compounds from the primary screen were classified as activators of hPXR on the basis of high confidence curve classes (see Methods) and EC$_{50}$ values ≤ 30 μM, yielding a hit rate of 11% (Table 1). There were 8 active compounds with potency values below 1 μM; however, the majority of the compounds had potency values above 10 μM (Table 1). To confirm compound activity, 72 compounds with no cytotoxicity or luciferase inhibition (data not shown) were cherry-picked from their original solutions and re-tested in the hPXR assay (Supplementary Table 5). Activity was confirmed in 66 out of the 72 re-tested compounds, yielding a confirmation rate of 92% (Table 2). Based on potency and novelty of compound mechanism with respect to PXR activity from the primary screen, 26 of the 72 compounds were chosen for further follow up in the hPXR assay and ordered from commercial vendors. All compound structures were confirmed by LC/MS and the purity of all compounds was 100% with the exception of fenbendazole (92%). Ten of the 26 compounds had EC$_{50}$ values <15 μM (Supplementary Table 6), three of which were dihydropyridine calcium channel antagonists, nilvadipine, felodipine, and riodipine. Seven out of the 26 compounds activated hPXR only at higher concentrations (ie, EC$_{50}$ values above 23 μM). In contrast,
9 compounds showed no activity in hPXR up to the highest concentration tested (46 μM), which is also true for the cherry-pick solution activities.

We have previously screened the entire NPC for PXR ligand binding, which is a complementary dataset to identify drugs which activate hPXR via binding to the LBD (Shukla et al., 2009). We sought to compare the results of these two assays to obtain more information on the mechanism of PXR activation, especially because both studies were performed in a dose-response format. The hPXR LBD assay identifies PXR activation by direct LBD binding whereas the hPXR cell-based assay can identify compounds which activate hPXR either through direct ligand binding or through modulation of hPXR activation by signaling pathways (Lin et al., 2008). The primary qHTS of the hPXR LBD assay performed well, with a signal to background ratio of 3.1 and average EC$_{50}$ of 0.44 μM for the positive control SR12813 (Supplementary Table 4). Of the 2,816 NPC compounds, 194 (7%) produced high quality CRCs (Table 1). The activities of 71 compounds out of 72 re-tested from the original solution were confirmed, yielding a 99% confirmation rate between the primary and follow-up assays (Table 2). Twenty-four of the 26 selected compounds confirmed, with suramin and zafirlukast being most potent (Supplementary Table 6).

We wanted to assess the concordance rate among active compounds in the hPXR and PXR LBD assays in order to evaluate how many compounds from the hPXR assay may bind directly to the PXR LBD. To be more inclusive when calculating concordance rate, we assessed all compounds (non curve class 4) that showed activity. Among the 2,814 compounds screened from the NPC, 603 compounds demonstrated concordant activity in both assays, while 846 compounds demonstrated activity in either assay,
indicating a concordance rate of 71.3% (603/846). There were 7 high efficacy compounds active in both assays upon re-testing, including ciglitazone and zafirlukast (Supplementary Table 6).

**Species selectivity of drugs against hPXR and rPXR.** Although compound mediated hPXR activation is most important clinically, rPXR activation is equally important in drug development since drug metabolism and pharmacokinetic studies are generally first performed in the rat prior to data extrapolation to humans. The rPXR cell-based primary screen performed well, with a signal to background ratio of 4.7 and an average EC$_{50}$ of 2.6 μM across the screen for the dexamethasone positive control (Supplementary Table 4). Of the 2,816 compounds profiled, 175 (6%) produced high confidence CRCs, approximately half of which had potency values <10 μM (Table 1). Seventy-two compounds were cherry-picked and activities of all 72 were confirmed (Table 2).

Given the species-specific differences in hPXR and rPXR, we were interested in the concordance rate among active compounds in the two assays. There were 512 compounds which demonstrated concordant activity in both assays, while 750 compounds demonstrated activity in either assay, indicating a concordance rate of 68.3%. Among the 26 compounds re-tested in the follow-up study, plicamycin was the most potent compound that was only active in the rPXR assay (Supplementary Table 6, Figure 2A), where the bell-shaped CRC observed for plicamycin was not due to cytotoxicity. Other compounds active only in the rPXR assay included hydralazine hydrochloride and 6-α-methylprednisolone (Figure 2A). In contrast, ciglitizone and troglitazone only showed activity in the hPXR specific assay (Supplementary Table 6, Figure 2B).
Rimexolone, fluorometholone, fenbendazole, and diclazuril were more potent in the rPXR assay (Figure 3). These results indicate greater selectivity for rPXR in the follow-up assays, while compounds such as fluorometholone were uniquely active and potent in the primary assay (Table 3). In contrast, several compounds including thiamylal sodium, oxatomide, bumecaimum, nilvadipine, riodipine, and felodipine displayed greater potency in the hPXR assay, indicating a greater selectivity for hPXR (Figure 4, Supplementary Table 6).

**Effect of drugs on CYP3A4 induction.** Ligand binding to PXR frequently activates transcription of CYP3A4 and several other CYP genes (Ekins and Schuetz, 2002). To investigate the downstream mechanism of drug action, we screened the NPC with regard to human CYP3A4 induction. The primary qHTS measuring CYP3A4 induction performed well (Supplementary Table 4) and revealed 212 active compounds (8%) with high quality CRCs. Less than fifty percent of compounds displayed potency values <10 μM (Table 1). The activities of 70 compounds out of 72 re-tested were confirmed, yielding a 97% confirmation rate (Table 2).

There were 564 concordant compounds with regard to activity in the hPXR and CYP3A4 assays, and 761 compounds that were active in either assay, resulting in a concordance rate of 74.1% (564/761). Of the 26 compounds re-tested in the CYP3A4 assay, 3 dihydropyridine calcium channel antagonists, riodipine, nilvadipine, and felodipine showed the greatest potency (Supplementary Table 6). As expected, cluster analysis of EC₅₀ patterns revealed the closest similarity between the hPXR activation and CYP3A4 induction assays with regard to compound activity profiles (Figure 5A).
Furthermore, linear regression, performed using the log_{10} transformed EC_{50} values derived from the 26 re-tested compounds, revealed a statistically significant correlation (r^2=0.75, P<0.0001) between the hPXR activation and CYP3A4 induction assays. Taken together, these data suggest that a majority of compounds that activate hPXR also induce CYP3A4 for drug metabolism.

In order to test the effects of novel CYP3A4 DPX-2 inducers in cryopreserved primary hepatocytes, we tested 6 compounds, bumecainum, colforsin, famprofazone, nilvadipine, oxatomide, and rimexolone. All 6 compounds showed potent CYP3A4 induction in the cryopreserved primary human hepatocytes, confirming the DPX-2 results (Supplementary Table S6).

**Identification of structure-activity relationships based on activity profiling.** The NPC drugs were clustered both by similarity in structure and activity (EC_{50}) patterns (see Methods section for details). While most drugs showing similar activity patterns were structurally diverse, consistent with the promiscuous nature of PXR, several activity clusters contained drugs with common structural scaffolds (Figure 5B). Figure 5B shows eight different activity profiles and structural classes associated with each activity profile.

The most prevalent profile was characterized by activity in the hPXR activation and CYP3A4 induction assays. There were 47 drugs having this profile (cluster 1, Figure 5B), comprising four major structural classes: phenothiazines, dibenzazepines, thioxanthenes and benztropines. Representative compounds from this profile are shown in Table 3. The second most prevalent number of drugs included those which displayed activity only in the hPXR LBD assay, comprising 30 drugs in three structural classes:
unsaturated fatty acids, tetracyclines, and cephalosporins (cluster 2 Figure 5B). Cluster 3 drugs demonstrated only CYP3A4 activity, typified by the hydroxyquinolines and cardiac glycosides. In contrast, 16 compounds in two major structural series, the steroid hormones and 4-anilinoquinazolines, comprised cluster 4, correlating with hPXR activation. Corticosteroids were associated with species-specific rPXR activity and included dexamethasone (Table 3), a well-known rPXR activator (LeCluyse, 2001), among the 15 drugs included in cluster 5. Conversely, nine drugs were active across all three human assays and included the imidazole antifungals (cluster 6). Seven compounds (dihydropyridine calcium channel blockers), including nilvadipine, nifedipine and riodipine, were pan-active across all 4 assays (cluster 7) and have been previously shown to activate hPXR (Drocourt et al., 2001). Interestingly, a second scaffold, which was part of the dihydropyridine calcium channel blockers, was not associated with CYP3A4 activity (cluster 6). Variations in the substitution groups at the R2 and R4 positions seem to differentiate activity between clusters 6 and 7 and result in compound-specific behavior with regard to CYP3A4 induction. The corticosteroid/glucocorticoid scaffold was associated with cluster 8, where activity is seen across all of the cell-based assays. Although this cluster shares a common backbone with the steroid and corticosteroid clusters associated with activity clusters 4 and 5, respectively, small changes in the ring structure and substituent groups have changed its activity profile across assays.

Discussion

In the present study, we have profiled a diverse array of clinically utilized drugs for species-specific PXR activation and CYP3A4 induction. Compounds identified and
validated from the individual assays represent a range of known and novel PXR structurally divergent ligands. We observed that many drugs induced CYP3A4 through PXR activation and that the utilization of different assay formats and species-specific cell lines allowed for the construction of pharmacological profiles for PXR activity across each group.

To our knowledge, this is the first study which examines a large set of clinically approved drugs in a qHTS format to identify inducers of PXR and CYP3A4. The assays utilized were highly robust and allowed us to directly compare hPXR and rPXR activation at the cellular level. The use of stably transfected cell lines containing the PXR coding region and CYP3A4 promoter and enhancer elements to identify CYP3A4 inducers has been previously validated (Raucy et al., 2002; Yueh et al., 2005; Sinz et al., 2007). Recently, the use of in vitro cell-based methods which measure nuclear receptor activation has been demonstrated as a reliable method of identifying CYP inducers in a species-specific manner (Mueller et al., 2009).

It is worth noting that comparisons between different assay formats may produce discrepant results and warrant interrogation when interpreting the data. For example, compounds which were active in the hPXR assay and negative in the hPXR LBD assay may not have been able to displace the tracer molecule, thus appearing inactive. There are well-known examples, such as docetaxel and paclitaxel, where compound binding in one assay does not correlate to PXR binding in cell-based transactivation assays (Harmsen et al., 2007; Sinz et al., 2008), thus many research groups have replaced PXR radioligand binding assays with cell-based assays (Sinz et al., 2006). In any case, active compounds from the hPXR LBD assay which do not reproduce in a cell-based assay could act as
antagonists, may not enter the cell or be degraded in the cellular milieu. Finally, there has been recent evidence regarding the modulation of hPXR through ligand binding and/or cell signaling pathways such as protein kinase pathways (Lin et al., 2008). One recent study (Lin et al., 2008) identified 2 cyclin-dependent kinase (CDK) inhibitors which strongly activated hPXR in a cell-based transactivation assay but only weakly activated PXR in the same binding assay format used in our study. Thus, this indicates that direct hPXR binding is not completely responsible for hPXR activation due to CDK pathway inhibition, which otherwise negatively regulates hPXR activity (Lin et al., 2008).

Although we do not have many CDK inhibitors represented in our NPC collection, compounds which are only active in the hPXR assay or have different potencies between the hPXR and hPXR LBD assays will warrant further functional investigation with regard to hPXR modulation via other pathways.

Active compounds in the hPXR cell-based assay which did not induce CYP3A4 could indicate a true effect or a CYP3A4 inhibitor which first binds PXR. Additional factors, such as negative feed back of CYP3A4 expression and other biochemical limitations with regard to the degree of CYP3A4 expression elicited by PXR activation (Luo et al., 2002) may have contributed to inactivity in the CYP3A4 induction assay. There were a few compounds which did not show PXR activation but demonstrated CYP3A4 induction (Supplementary Table 5), however the majority of these demonstrated low efficacy and potency. Upon re-testing from commercial vendors (Supplementary Table 6), there were no compounds which showed such an effect.

Our primary screening confirmed several compounds known to activate PXR, such as rifampicin, an antibiotic used in the treatment of tuberculosis. These results are
consistent with a previous finding that rifampicin strongly activates PXR and induces CYP3A4 in HepG2 cells (Yasuda et al., 2008). Ritonavir, a protease inhibitor known to activate hPXR (Luo et al., 2002), was active in the hPXR assay with an EC$_{50}$ value of 4 μM. Among the 26 compounds re-tested in our study, the majority of these compounds are novel with regard to prior knowledge concerning species-specific and common activators of PXR. Rimexolone is a corticosteroid and anti-inflammatory agent used in eye drops to reduce intraocular pressure (Kavuncu et al., 2008). This compound showed activity in both cell-based hPXR and rPXR assays and is a novel inducer of PXR activity. Although the relationship between hPXR, rPXR and rimexolone is novel, human and rodent PXR are generally hormonally regulated (Goodwin et al., 2002).

This study confirms that nilvadipine, a calcium channel blocker used in the treatment of hypertension, activated hPXR and induced CYP3A4 (Niwa et al., 2004). Two other calcium channel blockers, felodipine and riodipine showed similar activity (Supplementary Table 5). Felodipine is a known CYP3A4 substrate and is a commonly used in vivo probe for clinical studies involving CYP3A4 and 3A5 induction (Sinz et al., 2008). We also found that these compounds were 3-6 fold more potent in the hPXR assay compared to the rPXR assay, which provides useful information for species selectivity.

The anti-tumor compound plicamycin, was one of the most potent compounds in the rPXR follow-up assay (Figure 2, Supplementary Table 6). Plicamycin, also referred to as mithramycin, is a naturally occurring antibiotic used in the treatment of testicular carcinomas and acute mylogenous leukemia (Lahiri et al., 2008). This is a novel finding regarding species-specific induction of PXR by plicamycin and further analogs can be studied with regard to drug development and co-administration with other drugs. The
bell-shaped CRC of plicamycin could be explained by titration of co-regulators interacting with rPXR in the HepG2 cell line, which would warrant further investigation. Ciglitazone, a PPAR-γ agonist used in the treatment of diabetes (Pershadsingh et al., 1993), demonstrated hPXR-specific induction (Figure 2). This is consistent with a previous study showing several fold (>5) ciglitazone-mediated hPXR activation compared to mouse PXR (Vignati et al., 2004). Troglitazone, a PPAR-γ agonist and ciglitazone analog, is used in the treatment of type 2 diabetes (Jones et al., 2000) and showed hPXR specific activation (Figure 2). Previous studies have noted that these types of thiazolidinedione anti-diabetic drugs assert their effects through activation of the PPAR-γ/RXR heterodimer (Lehmann et al., 1995). Additionally, troglitazone activates hPXR at concentrations similar to those required to activate PPAR-γ, which provides additional evidence for interactions with other drugs such as oral contraceptives (Jones et al., 2000).

In addition to primary screening and follow-up assays, SAR analysis is a useful tool in order to potentially eliminate problematic chemotypes early in drug discovery (Sinz et al., 2008). SAR analysis revealed that most of the active compounds across the 4 assays are structurally diverse with multiple structural classes found within each activity group. Nevertheless, several classes of compounds that share the same chemical scaffolds were associated with different activity groups (Figure 5B). Overall, our method was validated by the association of activity cluster 5 (activity in the rPXR assay only) with corticosteroids, which included the control compound dexamethasone. This approach was further validated by the association of hPXR activity (cluster 4) with steroid hormones. The structural classes associated with activity cluster 1 (benztropines, dibenzazepines,
phenothiazines, and thioxanthenes) are commonly used as antipsychotic drugs and have limited data regarding their association with hPXR activation and CYP3A4 induction, thus this may be a novel finding of the study. Terconazole, an azole-containing topical antifungal drug with anti-inflammatory properties (Liebel et al., 2006), demonstrated activity in the hPXR assay but not the CYP3A4 induction assay, suggesting regulation of CYP3A4 through other mechanisms.

As with the lack of a CYP isozyme diverse data set prior to a recent study (Veith et al., 2009), until now there has not been a publically available comprehensive data set of hPXR and rPXR activation and CYP3A4 induction, by clinically utilized drugs to facilitate early drug discovery and avoid drug-drug interactions.

**Acknowledgement:** We thank Darryl Leja for illustrations, Bill Leister for quality control measurements of the compounds, and Dr. Judy Raucy for critical review of this manuscript.
Authorship Contributions

*Participated in research design:* Shukla, Xia
*Conducted experiments:* Shukla, Sakamuru, Shinn, VanLeer, Moeller, Xia
*Performed data analysis:* Shukla, Huang, Xia
*Wrote or contributed to the writing of the manuscript:* Shukla, Huang, Moeller, Auld, Austin, Xia
References


Footnotes

This research was supported in part by the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health.
Figure Legends

Figure 1: Ligand mediated PXR activation and CYP3A4 induction and Assay Schematics. (A) Upon ligand binding, PXR translocates into the nucleus. In the absence of PXR bound to ligand, an orphan nuclear receptor such as small heterodimer partner (SHP) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) are transcribed by co-activators such as steroid receptor coactivator-1 (SRC-1), hepatocyte nuclear factor α (HNF4α) and peroxisome proliferators-activated receptor γ co-activator-1α (PGC-1α), resulting in the inhibition of CYP3A4 transcription. SHP/SMRT transcription is blocked by ligand-activated PXR and the ligand-PXR complex forms a heterodimer with RXRα prior to stabilization by SRC-1 and other co-activators. The co-activator-PXR-RXRα complex binds the xenobiotic responsive enhancer molecule/PXRE (XREM/PXRE) region containing an everted repeat with a spacer of 6 nucleotides (ER6) in the proximal promoter and direct repeats with a 3 nucleotide spacing (DR3) in the distal XREM upstream of the CYP3A4 transcription start site (Stanley et al., 2006; Sinz et al., 2007). (Bi) The hPXR/rPXR activation assay relies on a luciferase reporter gene (green), which is downstream of the PXRE (grey). Upon ligand binding to the PXRE, the amount of luciferase enzyme (green dots) is quantified upon the addition of luciferin substrate and detection after cell lysis. (Bii) The hPXR CYP3A4 induction assay relies on a luciferase-linked CYP3A4 promoter construct. Upon ligand binding to the PXRE, the amount of CYP3A4 enzyme expression (blue dots) is measured upon the addition of CYP3A4 substrate and detection after cell lysis. (Biii) The hPXR LBD assay relies on the proximity of two fluorophores, where excitation of a Tb³⁺-labeled antibody (Ab) which binds to the GST tagged portion of the nuclear receptor (blue triangle). Excitation at 340
nm results in fluorescent emission at 495 nm and energy transfer to a fluorescein labeled acceptor ligand capable of binding to the PXR LBD. The transfer of energy to the acceptor molecule results in fluorescent emission at 520 nm and a high TR-FRET ratio (520 nm/495 nm). In the presence of a PXR ligand (competitor), the displacement of the tracer molecule from the nuclear receptor is observed, resulting in a disruption of the energy transfer and loss of the TR-FRET signal.

Figure 2: Species-specific compounds involved in PXR activation. Compounds shown are taken from the set of 26 which were re-tested in each assay. Each CRC represents the average response of triplicates with error bars demonstrating the standard deviation (SD) in the rPXR (A) and hPXR (B) assays.

Figure 3: Compounds selectively potent in the rPXR assay. Compounds shown are taken from the set of 26 which were re-tested in each assay. Each CRC represents the average response of triplicates with error bars demonstrating the SD in the rPXR and hPXR assays for rimexolone (A), fluorometholone (B), fenbendazole (C) and diclazuril (D). ■ represents hPXR data and ▲ represents rPXR data.

Figure 4: Compounds which are selectively potent in the hPXR assay. Compounds shown are taken from the set of 26 which were re-tested in each assay. Each CRC represents the average response of triplicates with error bars demonstrating the SD in the rPXR and hPXR assays for thiamylal sodium (A), oxatomide (B) and bemecainum (C). ■ represents hPXR data and ▲ represents rPXR data.
Figure 5: Hierarchical clustering and SAR analysis of all cell-based and hPXR LBD assays. (A) Clustering was based on non-class 4 log₁₀ transformed compound EC₅₀ values across 3 or more assays. The cell-based assays generally cluster more closely together, followed by the hPXR LBD assay. (B) Each rectangle represents a cluster of structurally similar compounds. Eight activity clusters based on compound activity and potency across the 4 assays from the primary qHTS data. Each cluster was associated with 1-4 structure classes. The white boxes indicate generally less potent/inactive compounds and the red boxes indicate compounds which were generally more potent/active.
Table 1: Potencies for Active* Compounds from Primary qHTS Across Assays

<table>
<thead>
<tr>
<th>Potency (μM)</th>
<th>hPXR</th>
<th>hPXR LBD</th>
<th>CYP3A4 Induction</th>
<th>rPXR</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>1-10</td>
<td>124</td>
<td>98</td>
<td>86</td>
<td>79</td>
</tr>
<tr>
<td>10 -32</td>
<td>178</td>
<td>88</td>
<td>113</td>
<td>87</td>
</tr>
</tbody>
</table>

*Curve classes 1.1, 1.2, 2.1 and 2.2
Table 2: Confirmation of Follow-Up Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primary qHTS vs. Cherry-Pick (%)*</th>
<th>Cherry-Pick vs. Re-acquired Samples (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPXR</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>hPXR LBD</td>
<td>99</td>
<td>92</td>
</tr>
<tr>
<td>rPXR</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>97</td>
<td>100</td>
</tr>
</tbody>
</table>

*Out of 72 compounds re-tested
**Out of 26 compounds re-tested
# Table 3: Representative Species-Specific Compounds Across Activity Groups

<table>
<thead>
<tr>
<th>Cluster # (Cluster Name)</th>
<th>Compound</th>
<th>hPXR Potency (μM)*</th>
<th>CYP3A4 Induction Potency (μM)*</th>
<th>rPXR Potency (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Phenothiazines)</td>
<td>Tropanyl 3,5-Dimethylbenzoate</td>
<td>3.5</td>
<td>15.8</td>
<td>Inactive</td>
</tr>
<tr>
<td>1 (Thioxanthenes)</td>
<td>Thiothixene Hydrochloride</td>
<td>12.6</td>
<td>7.1</td>
<td>Inactive</td>
</tr>
<tr>
<td>1 (Benztropines)</td>
<td>t-Butylhydroquinone</td>
<td>12.6</td>
<td>2.0</td>
<td>Inactive</td>
</tr>
<tr>
<td>5 (Corticosteroids)</td>
<td>Carminomycin</td>
<td>Inactive</td>
<td>Inactive</td>
<td>2.8</td>
</tr>
<tr>
<td>5 (Corticosteroids)</td>
<td>Dexamethasone acetate</td>
<td>Inactive</td>
<td>Inactive</td>
<td>4.5</td>
</tr>
<tr>
<td>5 (Corticosteroids)</td>
<td>Fluorometholone</td>
<td>Inactive</td>
<td>Inactive</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Taken from the primary qHTS screen
Figure 3
Figure 5B