Identification and Validation of Novel hPXR Activators Amongst Prescribed Drugs via Ligand-Based Virtual Screening

Yongmei Pan, Linhao Li, Gregory Kim, Sean Ekins, Hongbing Wang and Peter W. Swaan

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Penn Street, Baltimore, MD 21201, USA; Collaborations in Chemistry, Jenkintown, PA 19046, USA (SE); Department of Pharmacology, University of Medicine & Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA (SE).
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b) Corresponding Author:

Peter W. Swaan, Ph.D., Department of Pharmaceutical Sciences, University of Maryland,
20 Penn Street, HSF2-621, Baltimore, MD 21201 USA
Tel: (410) 706-0103
Fax: (410) 706-5017
Email: pswaan@rx.umaryland.edu

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hPXR, Human pregnane X receptor; LBD, ligand binding domain; XV ROC AUC, Leave-one-out cross-validation based ‘receiver operator curve’ area under the curve.
Abstract

Human pregnane X receptor (hPXR) plays a key role in regulating metabolism and clearance of endogenous and exogenous substances. Identification of novel hPXR activators among commercial drugs may aid in avoiding drug-drug interactions during coadministration. We applied ligand-based computational approaches for virtual screening of a commonly prescribed drug database (SCUT). Bayesian classification models were generated with a training set comprising 177 compounds using Fingerprints and 117 structural descriptors. A cell-based luciferase reporter assay was used for evaluation of chemical-mediated hPXR activation in HepG2 cells. All compounds were tested at 10μM concentration with rifampicin and DMSO as positive and negative controls, respectively. The Bayesian models showed specificity and overall prediction accuracy up to 0.92 and 0.69 for test set compounds. Screening the SCUT database with this model retrieved 105 hits and 17 compounds from the top 25 were chosen for in vitro testing. The reporter assay confirmed that nine drugs, i.e., fluticasone, nimodipine, nisoldipine, beclomethasone, finasteride, flunisolide, megestrol, secobarbital, and aminogluthethimide were previously unidentified hPXR activators. Thus, the present study demonstrates that novel hPXR activators can be efficiently identified amongst FDA approved and commonly prescribed drugs, which should lead to detection and prevention of potential drug-drug interactions.
Introduction

Nuclear receptors (NRs) are a class of transcription factors that control gene expression and play a key role in the development, homeostasis and metabolism of living organisms (di Masi et al., 2009). The Pregnane X receptor (PXR) belongs to the NR1I family and regulates enzymes and transporters involved in xenobiotic detoxification as well as maintaining homeostatic balance of endobiotics, including bile acids, cholesterols and steroid hormones (Jyrkkarinne et al., 2008). PXR mediates activation of gene sets pertinent to xenobiotic metabolism, such as cytochrome 450 (CYP) superfamily members CYP1, CYP2B, CYP2C, and CYP3A4 in rodents and humans (Maglich et al., 2002; Plant, 2007; di Masi et al., 2009). A very broad range of substances have been identified as hPXR activators in vitro, including commercial drugs, pesticides, environmental contaminants, and natural products (Timsit and Negishi, 2007).

Due to its vital role in drug metabolism, it is not surprising that human (h)PXR has been found responsible for decreased drug efficacy and increased drug toxicity (Ma et al., 2008; di Masi et al., 2009). For example, co-administration of rifampicin, a hPXR activator used for treatment of tuberculosis (Chrencik et al., 2005) with a variety of drugs (including oral contraceptives (Ma et al., 2008), the anesthetic midazolam (Backman et al., 1996), and HIV protease inhibitors (Ivanovic et al., 2008)), resulted in decreased drug efficacy mainly due to hPXR-mediated increased expression of CYP3A4 (Ivanovic et al., 2008; Ma et al., 2008; di Masi et al., 2009). Thus, identification of novel hPXR activators amongst commercial drugs is important in predicting hPXR mediated drug-drug interactions.

Crystal structures of hPXR ligand binding domain (hPXR-LBD) indicate that its
binding cavity is much larger than that of other NR members (Xu et al., 2004; Chrencik et al., 2005; di Masi et al., 2009). Several key amino acid residues are responsible for the high flexibility of its binding site that is critical for recognizing promiscuous ligands of various dimensions and chemical properties (Ekins et al., 2009). Probably due to the flexibility of the hPXR-LBD and the limitation of docking algorithms, docking structurally diverse molecules is a challenge (Ekins et al., 2008; Khandelwal et al., 2008; Yasuda et al., 2008; Ekins et al., 2009). Therefore, docking methods have been suggested for use in combination with other computational methods to improve prediction (Khandelwal et al., 2008; Yasuda et al., 2008; Ekins et al., 2009). The flexibility and large size of the hPXR-LBD necessitates development of multiple pharmacophores for consensus prediction by considering interactions between a ligand various binding sites (Yasuda et al., 2008). Recently, ligand-based Structure-Activity Relationship (SAR) approaches, such as machine learning methods (Khandelwal et al., 2008) and Bayesian statistics (Ekins et al., 2009; Zientek et al., 2010) have been applied to generate models by using just binary classification of ligands (e.g. activator and nonactivator) instead of quantitative data while using 2D instead of 3D descriptors.

In the current study, we applied Bayesian models to identify novel hPXR activators by virtual screening of an in house database of frequently prescribed FDA approved drugs (SCUT) (Chang et al., 2006). We confirmed nine novel hPXR activators out of seventeen predicted hPXR activators by luciferase reporter assay; this indicates that ligand-based virtual screening combined with experimental validation assays is a valuable tool for efficient retrieval of novel ligands that interact with hPXR.
Methods

**Principal Component Analysis (PCA) of SCUT Database Molecules and Training and Test Set Compounds.** Datasets consisting of 177 (Ung et al., 2007; Khandelwal et al., 2008) and 145 (Khandelwal et al., 2008) previously published hPXR activators/nonactivators were used as training and test sets, respectively. In the training set, 98 compounds with EC$_{50}$ < 100 μM were classified as hPXR activators while 79 compounds with EC$_{50}$ > 100 μM were classified as nonactivators. The current test set consisted of previously reported 82 activators and 63 nonactivators. 104 independent variables representing molecular size, solubility, flexibility, polarity, charge, surface area, and hydrogen bond features were calculated with “Calculate Molecular Properties” protocol of Discovery Studio 2.1 (DS 2.1, Accelrys, San Diego, CA). The PCA plot is a useful tool to assess similarity among training and test set compounds so as to understand potential outlier prediction (Khandelwal et al., 2007). A PCA plot of training, test and SCUT database compounds was performed with the protocol “Calculate Principal Components” DS2.1 by using 3 as the minimum number of components and 0.65 minimum variance explained.

**Building and Validation of Bayesian Models.** Bayesian statistics is a classification approach based on a learn-by-example protocol. A Bayesian model is created by estimating the frequencies of features when a hypothesis is true (Xia et al., 2004). To apply the model to a sample, a weight is calculated for each feature using a Laplacian-corrected estimator. The prediction of the likelihood of a sample is made by summing up the weights associated with each feature (Xia et al., 2004; Rogers et al., 2005). Extended-connectivity fingerprints maximum diameter 6 (ECFP$_6$), functional-
class fingerprints maximum diameter 6 (FCFP_6) (Rogers et al., 2005) and another 117 structural descriptors were calculated with DS2.1. Laplacian-corrected Bayesian classification models (Xia et al., 2004; Rogers et al., 2005) were generated using the “Create Bayesian Model” protocol. Leave-one-out cross-validation based ‘receiver operator curve’ area under the curve (XV ROC AUC) (Zweig and Campbell, 1993) was calculated for the training set compounds as an assessment of predictive capacity of Bayesian models. The Bayesian models were validated with the test set as well. The activities of the test set compounds were predicted by the “Calculate Molecular Properties” protocol with the Bayesian models.

**Virtual Screening of SCUT Database Drugs with Bayesian Models.** Drugs from the SCUT database were virtually screened for prediction of their hPXR activities through the same protocol as for test set compounds by a Bayesian model with the highest-ranking predictive performance (see results).

**Docking of Test Set and SCUT Drugs to hPXR Ligand Binding Domain with FlexX and Surflex.** Docking programs were applied to evaluate binding between ligands and LBD of hPXR. In the FlexX docking algorithm (BioSolveIT, Sankt Augustin, Germany), the protein is kept rigid while the ligand is treated flexibly. The overall strategy of this docking method is the incremental construction algorithm (Rarey et al., 1996), in which a ligand is first split up into fragments. The selection of the base fragment is centered on a recognition technique called pose clustering; subsequently, the ligand is built up incrementally by adding other fragments with a simple greedy strategy. The ranking of fragment placements and estimation of binding energy use terms of the Böhm scoring function with minor changes (Rarey et al., 1996).
Surflex (Tripos, St. Louis, MO) is similar to FlexX in that a molecule is first fragmented and the conformations of each piece are further explored. Here, a “protomol”, or idealized active site ligand comprising a cluster of molecular fragments featuring the binding pocket surface (Ruppert et al., 1997), is generated and serves as a target for alignment of fragment conformations based on the molecular similarity method (Jain, 2003). An entire molecule is then assembled by the incremental construction approach (Welch et al., 1996) or a genetic algorithm called the ‘Whole Molecule Algorithm’ (Jones et al., 1997). The Hammerhead empirical function is used as scoring function of putative poses as well as objective function of local optimization (Jain, 2003).

Previous docking studies indicated that the crystal structure of hPXR-LBD (PDB ID: 1NRL) (Watkins et al., 2003) performed better in predicting hPXR activators/nonactivators than three other hPXR-LBD crystal structures when using FlexX (Khandelwal et al., 2008). Accordingly, the crystal structure 1NRL was selected as the docking target. In FlexX, residues within 6.5Å of a ligand were defined as the active site. The maximum number of solutions per iteration and fragmentation was 500. The single top docked orientation with the best score (in kcal/mol (Stahl, 2000)) of each ligand was generated. In Surflex docking, the protomol was generated based on active site residues, with proto_thresh of 0.5 and proto_bloat of 0. The maximum number of fragment conformations was 20 and the maximum number of poses per ligand was 20.

Selected retrieved compounds from Bayesian modeling were tested in vitro to validate their activity against hPXR.

**Chemicals and Reagents.** Rifampicin (RIF) was purchased from Sigma-Aldrich (St. Louis, MO). The Dual-Luciferase Reporter Assay System was purchased from
Promega (Madison, WI). Drugs butorphanol (+)-tartrate salt, megestrol 17-acetate vetroanal, cefoxitin sodium, estramustine sodium phosphate, flunisolide, fludrocortisone acetate, fluticasone propionate, sulfasalazine, secobarbital methanol solution, triamcinolone were purchased from Sigma-Aldrich. Finasteride, tobramycin, amikacin disulfate, nimodipine were purchased from Alexis Biochemicals (San Diego, CA). DL-aminogluthethimide was purchased from LKT Laboratories (Gardena, CA). Oridonin was from Chromadex (Irvine, CA). Nisoldipine and beclomethasone dipropionate were obtained from Dr. James Polli and Dr. Richard N. Dalby’s laboratories, University of Maryland, Baltimore.

**Plasmid Constructs.** The CYP2B6 reporter construct, containing both PBREM and the distal XREM [CYP2B6-2.2 kilobases (kb)], were generated as described previously (Wang et al., 2003; Tolson et al., 2009). The pSG5-hPXR expression plasmid was acquired from Dr. Steve Kliewer (University of Texas, Southwestern Medical Center, Dallas, TX). The pRL-TK Renilla luciferase plasmid used to normalize firefly luciferase activities was purchased from Promega.

**Transient Transfection in HepG2 Cells.** HepG2 cells seeded in 24-well plates were transfected with CYP2B6-2.2 kb reporter construct in the presence of hPXR expression vector using FuGENE 6 Transfection Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were treated with solvent [0.1% dimethyl sulfoxide (DMSO)] or test compounds (including positive control RIF and drugs) at the concentrations of 10 μM for another 24 h. In parallel experiments, the highly cytotoxic chemotherapeutic agent, mytomycin was tested at 0.1, 1 and 10 μM concentrations. Subsequently, all cell lysates
were assayed for firefly activities normalized against the activities of *Renilla* luciferase using the Dual-Luciferase Kit (Promega). Data are represented as mean ± S.D. of three individual transfections.

**Dose-Dependent Assay.** Based on preliminary screening results, four drugs highly responsive to hPXR activation or deactivation at 10 µM were further tested at the concentrations of 0.1, 1 and 10 μM in HepG2 cells transfected with hPXR expression and CYP2B6 reporter constructs. The dual-luciferase activities of different treatments were measured and calculated as described above.

**Cytotoxicity Assay.** To ensure that the observed hPXR activation of mitomycin in HepG2 cells was not confounded by potential cytotoxic effects, an MTT assay was carried out in parallel. The cells were dispensed in a 96-well plate at a density of 1.5 × 10⁴ cells / well. After 24-h incubation, cells were treated with oridonin at 20, 40 μM (positive control) or mitomycin at various concentrations for 24 hr. A 20-μl aliquot of MTT solution (5.0 mg/ml) was added to each well followed by 4-h incubation, and the resulting crystals were dissolved in 150 μl DMSO. Absorbance (A) was measured with a microplate reader (BIO-RAD, Hercules, CA).
Results

Principal Component Analysis. PCA is useful as an estimation of whether molecules occupy different descriptor spaces so as to understand potential outlier predictions of the test set and database compounds. PCA was performed on the SCUT database and the test and training set compounds based on 104 descriptors. The first two and three principal components explained 62 and 68.1% of the variance, respectively, indicating the plot of the first two components roughly represented the descriptor space occupied by the molecules. Figure 1A demonstrates that the test set accommodated a similar space as the training set molecules; the training set drugs covered most of the descriptor space of that occupied by the SCUT database drugs (Fig. 1B).

Bayesian Model Building and Validation. The human PXR data for model development (training set \( n = 177 \)) and validation (test set \( n = 145 \)) were taken from recent publications (Ung et al., 2007; Khandelwal et al., 2008). 117 structural descriptors including topological variables as well as structural fingerprints ECFP_6 and FCFP_6 (calculated with DS2.1) were applied for model development. Four Bayesian models were generated with combinations of parameters. The first two models ECFP-1 and FCFP-1 (Table 1) were obtained with 117 descriptors together with ECFP_6 or FCFP_6, respectively. The other two models ECFP-2 and FCFP-2 were developed by 74 descriptors without topological features as well as ECFP_6 or FCFP_6. The predictive performance of Bayesian models were evaluated by XV ROC AUC based on leave-one-out cross-validation of training set compounds. XV ROC AUC reflects the relationship between sensitivity and specificity, ranging from 0 to 1 with a higher the number indicating a better model (Zweig and Campbell, 1993). The models were also validated
with an external test set consisting of 145 molecules. The predicted performance of the models was demonstrated by their sensitivity (SE), specificity (SP), overall prediction accuracy (Q) and Matthew’s correlation coefficient (C values) calculated from the empirical true positive (TP), true negative (TN), false positive (FP), and false negative (FN) (Table 1).

The XV ROC AUC values of ECFP-1, FCFP-1, ECFP-2 and FCFP-2 models revealed good internal prediction in terms of leave-one-out cross-validation of training set compounds (Table 1). Model validation with the external test set (Table 1) revealed a good specificity (SP) for all models, but a lower sensitivity (SE). This indicated that the models identified a low ratio of false positives but high ratio of false negatives.

The effects of using topological descriptors and different fingerprints, i.e., ECFP_6 and FCFP_6 on the predictive performance of the Bayesian models are discussed in Supplemental Information (Supplemental Results 1, Figure S1). Compared to previous models developed in our laboratory using machine learning methods (Khandelwal et al., 2008), the Bayesian models performed better in terms of higher accuracy and Matthew’s correlation values. Amongst the four Bayesian models, the ECFP-2 model was deemed superior: although ECFP-2 has lower Q and C than ECFP-1 and FCFP-1, the advantage of this model is its high specificity (92.1%) indicating a lower occurrence of false positives. To possibly increase the chance of a hit during experimental tests, the ECFP-2 model was selected for virtual screening to identify novel hPXR inhibitors.

**Virtual Screening of SCUT Database with ECFP-2 Bayesian Model and Docking Programs** SCUT database compounds were screened with the ECFP-2 model,
retrieving 113 hits according to their Bayesian scores. The results of docking test set and
SCUT compounds into hPXR are included in Supplemental Information (Supplemental
Results 2 & Table S1&S2). Among the 113 hits obtained by virtual screening with
Bayesian model ECFP-2, 8 compounds were removed from the list due to failed docking
by FlexX and disfavored binding energies indicated by Surflex. Among the left 105 hits,
28 compounds belonging to the training or test set were discarded. The top 50 hits were
used to search against PubMed for previously documented hPXR affinity. Four
compounds with recorded studies on their hPXR activity were removed. The top 25 hits
without any previously documented hPXR study were checked for feasibility in using
them in experimental assays. Ultimately, 17 compounds were selected for in vitro testing
based on their commercial availability (Table 2). Interestingly, four predicted hPXR
activators, i.e., beclometasone, triamcinolone, fludrocortisones, and fluticasone were
docked unsuccessfully by FlexX but had favorable Surflex scores. This divergent result
could be attributed to the different docking algorithms between FlexX and Surflex and
the lack of protein flexibility in docking.

hPXR Activation by Predicted Drugs. hPXR governs the transactivation of
multiple drug-metabolizing genes such as CYP3A4, CYP2B6, UGT1A1, as well as drug
transporters such as MDR1. The CYP2B6 reporter construct containing both the proximal
PBREM and the distal XREM is highly responsive to chemical-mediated hPXR
activations (Tolson et al., 2009). Here, we investigated the ability of seventeen drugs to
activate hPXR-mediated CYP2B6 reporter expression in HepG2 cells. As shown in
Figure 2 and Table 2, compared with vehicle control, nine drugs, i.e., beclometasone,
finasteride, flunisolide, fluticasone propionate, megestrol acetate, nimodipine,
nisoldipine, secobarbital and aminoglutethimide showed noticeable increases of hPXR-mediated CYP2B6 reporter activities at 10 μM. Rifampicin (RIF) is an established hPXR activator (Cheng et al., 2009; Messina et al., 2009). Among the active molecules, fluticasone propionate and nimodipine exhibited robust hPXR activation to the level which challenge that of RIF, while nisoldipine was not as potent as RIF but still caused a significant increase of hPXR-mediated CYP2B6 reporter activities. Megestrol, beclometasone, finasteride and flunisolide caused increased activity by 2.7 to 5.6 folds. Secobarbital and aminoglutethimide demonstrated 1.57 and 1.42 fold higher Luc activity than control vehicle, indicating their marginal effects on hPXR activation. One interesting observation was, instead of being an hPXR activator, mitomycin had dramatically decreased the CYP2B6 luciferase activity to just 12% of basal level. The drugs amikacin, cefoxitin, estramustine, fludrocortisone, sulfasalazine, tobramycin and triamcinolone did not have observed enhanced Luc activity (Table 2; Fig. 2).

To further confirm their effects on hPXR-mediated Luc activity, a dose-dependent assay of mitomycin and the three compounds with the most potent Luc activity, i.e., fluticasone propionate, nimodipine, and nisoldipine were performed (Fig. 3). Fluticasone propionate, nimodipine, and nisoldipine significantly enhanced the PXR-mediated CYP2B6 reporter expression in a dose-dependent manner, with the highest activation at the concentration of 10 μM. By contrast, mytomycin (0.1 uM) only moderately decreased CYP2B6 Luc activity compared to the vehicle control. Notably, at the concentration of 1 and 10 μM, the Luc activity decreased by approximately 90% compared with control. Mitomycin is a chemotherapeutic agent associated with high cytotoxicity (Crowston et al., 2006). To further confirm whether the decreased Luc response with mytomycin was
due to the deactivation of hPXR or to its nonspecific cytotoxicity, cell viability testing was performed on mitomycin in HepG2 cells as measured by MTT assay. At concentrations of 0.1, 0.5, 1, and 2 μM, mitomycin demonstrated minor cytotoxicity, with cell viability between around 90 and 80% (Fig. 4). However, at concentrations above 5 μM, mitomycin showed clear cytotoxicity with cell viability dropping to approximately 50%. Given that mitomycin decreases PXR-mediated CYP2B6 Luc activity by 90% at concentrations that only cause minor cytotoxicity, mitomycin may represent a novel hPXR deactivator.

Overall, based on the in vitro tests, with Luc activity 12-fold higher than control, fluticasone propionate, nimodipine, and nisoldipine can be regarded as potent hPXR activators. Megestrol, beclometasone, finasteride, flunisolide, secobarbital and aminoglutethimide demonstrated increased Luc activity between 1.57 to 5.6 fold; these compounds can be categorized as weak to moderate hPXR activators (Fig. 5).

**Binding of Newly Identified hPXR Activators with the hPXR-LBD.** Surflex was employed to demonstrate the interactions between the binding pocket of hPXR-LBD and some of the newly identified hPXR activators, i.e., fluticasone, nimodipine, nisoldipine, and megestrol (Fig. 6). Fluticasone (Fig. 6A) and nimodipine (Fig. 6B) have hydrogen bond interactions with the side chains of H407 and S247, whereas Nisoldipine (Fig. 6C) has hydrogen bonds with these residues and T408. Conversely, hPXR-LBD docking of beclomethasone reveals hydrogen bonding with residues N285 and H407 (Fig. 6D). Docking of the other newly identified hPXR activators is shown in Supporting Information (Supplemental Figure S2). In general, we observed that, due to the relatively large size of the binding pocket, compounds may move around and interact with different
sites within the binding pocket. Similar to the three binding modes of SR12813 in hPXR-LBD (Watkins et al., 2001), the side chains of residues H407, S247, N285 were predicted to be involved in hydrogen-bonding interaction with the docked molecules. Compounds megestrol, flunisolide, secobarbital, and aminoglutethimide (Figures 6D and S2F, S2H and S2I in Supporting Information) have hydrogen-bonding with side chain of N285. The results are consistent with previous docking study (Khandelwal et al., 2008) indicating that the side chain NH of N285 forms hydrogen-bonding interactions with hPXR activators. In addition, the backbone of A209 was found to form hydrogen bonds with two drugs, i.e., aminoglutethimide and mitomycin, as shown in Figures S2I and S2J in Supporting Information. The predicted involvement of S247 and N285 in the interaction between an activator and hPXR is supported by mutagenesis in which the mutation of these two residues is responsible for less promiscuity of mPXR (di Masi et al., 2009).
Discussion

The Need for Integrated Application of Computational and Experimental Approaches. The integrated usage of docking and SAR models to determine ligand, substrate or inhibitor specificity has greatly advanced our understanding of the mechanism of receptors and drug transporters (Khandelwal et al., 2008; Krueger et al., 2009). To date, several combinations of structure- and/or ligand-based methods have been reported to characterize hPXR and activator interactions (Gao et al., 2007; Ekins et al., 2008; Khandelwal et al., 2008; Yasuda et al., 2008), but application of this strategy to identify novel hPXR activators among commercial FDA approved drugs has not been explored on a large scale.

Previous docking studies on hPXR indicated that directly using the ‘cutoff score’ from docking programs was limited for prediction and classification of hPXR activators and nonactivators (Ekins et al., 2008; Khandelwal et al., 2008; Yasuda et al., 2008; Ekins et al., 2009). To overcome this limitation, our present work applied Bayesian models for identification of hPXR activators. The models were generated with known hPXR activators and nonactivators by using different combinations of structural descriptors. The sensitivity, specificity, and overall accuracy evaluated based on external test set compounds served as a means of model validation and their values indicated that the Bayesian models were able to successfully identify hPXR activators from nonactivators. The Bayesian model with highest specificity was selected for virtual screening of the SCUT database. Although the model has lower sensitivity than other models, the high specificity indicated that this model could result in less false positives, thus increasing the efficiency of experimental tests. The hits were ranked and selected according to their
Bayesian scores. Only those with the highest scores and favorable binding energies obtained with docking programs were considered for in vitro testing. The combined computational approach used in this study with experimental assays could be applied in identification of ligands for other proteins.

**Newly Identified hPXR Activators, Antagonist and Nonactivators.** Among the seventeen tested drugs, nine were confirmed as hPXR activators. Mitomycin, although predicted as a hPXR activator, turned out to be a newly identified hPXR antagonist that significantly decreased luciferase activity by 88%. Five of the novel hPXR activators (Table 2), i.e., beclometasone, finasteride, flunisolide, fluticasone, and megestrol belong to the corticosteroid/glucocorticoid family, two drugs, i.e., nimodipine, and nisoldipine are dihydropyridine analogs and secobarbital is a phenobarbital analog. Although there are no published interactions between any of the nine drugs and PXR, there are quite a few corticosteroid analogs, including dexamethasone, progesterone, 17α-hydroxy progesterone, 5β-Pregnane-3,20-dione and budesonide (Bhadhprasit et al., 2007; Zimmermann et al., 2009) that are hPXR activators. Dihydropyridines such as nifedipine, nicardipine, and isradipine (Drocourt et al., 2001) have also been reported as PXR activators, while a secobarbital analog, phenobarbital has been identified as a moderate hPXR activator (Lemaire et al., 2004). No aminoglutethimide analog has been shown previously to have an interaction with hPXR. Therefore, the Bayesian model successfully identified nine novel hPXR activators with no previously recorded hPXR interaction, one of which belongs to a novel therapeutic class not previously known to impact PXR activity.
Two tested corticosteroids, triamcinolone and fludrocortisone did not improve hPXR-mediated CYP2B expression indicated by luciferase reporter assay. The structures and LogPs of these two drugs are shown in Figure 5 and Table 3. It can be seen that triamcinolone and fludrocortisone have lower LogPs than other corticosteroids which activate hPXR. It is widely known that PXR activators are very hydrophobic (Ekins et al., 2009). The more hydroxyl groups on triamcinolone contribute to its higher hydrophilicity than other corticosteroid analogs therefore possibly compromising its activity to hPXR. The hydroxyl groups may also represent unfavorable hydrogen bonding interactions. Estramustine is an estradiol derivative and is indicated as a hPXR non-activator in the current study. 17ß-estradiol is a well established moderate hPXR activator (Xue et al., 2007). As shown in Figure 3, hPXR activation of estramustine is likely lost due to the attachment of a nitrogen mustard-carbamate ester to the phenyl hydroxyl of estradiol. Previous studies showed that the carbamic ester in estramustine is hydrolyzed in vivo by liver, prostate and intestine (Gunnarsson et al., 1983). As a result, one would expect that estramustine could have in vivo hPXR activity obtained from its metabolite estradiol.

Clinical Pharmacokinetic Implications for hPXR Mediated Drug-drug Interaction. Due to the critical role of hPXR in regulation of genes involved in metabolism of xenobiotics, identification of novel hPXR activators/non-activators among commercial drugs could benefit a priori identification of drug-drug interactions. The empirical or predicted physicochemical properties of newly identified hPXR activators, a suggested antagonist and non-activators are included in Table 3. Megestrol acetate can be regarded as a moderate hPXR activator with 5.61 increased fold of Luciferase activity than vehicle. It has been used for treatment of weight loss in patients with cancer and
HIV/AIDS (Mulligan et al., 2007). A previous clinical study demonstrated when the anti-
HIV drug indinavir was coadministered with megestrol acetate, its efficacy decreased
significantly in terms of pharmacokinetic parameters (~36% for \(C_{\text{max}}\) and ~28% for AUC)
(all clinical PK data in this section were retrieved from www.rxlist.com). Indinavir is
metabolized mainly by CYP3A in liver (Chiba et al., 1997). Considering hPXR is a
regulator of the cytochrome P450 superfamily and megestrol is an activator of hPXR, one
would expect that the drug-drug interaction between megestrol estate and indinavir could
be hPXR mediated.

Nimodipine and nisoldipine are moderate hPXR activators according to the
current study. The oral bioavailability of both drugs is relatively low (13% and 5%,
respectively), due to high first-pass metabolism in the liver and gut wall. Drug-drug
interactions have been observed between cimetidine and nimodipine or nisoldipine. But
we are not aware of recorded decreased drug efficacy due to coadministration with
nimodipine or nisoldipine. Since the available studies of nimodipine/nisoldipine-
mediated drug interactions are quite limited, more clinical tests are required. Fluticasone
is a potent hPXR activator according to the current study. However, significant
involvement of fluticasone in drug interactions are not expected due to its low plasma
concentration following extensive first-class metabolism after inhaled dosing.

Beclometasone, finasteride, and flunisolide were identified as moderate hPXR
activators. Beclometasone dipropionate is metabolized rapidly by high capacity esterases
widely distributed in tissues. Possibly due to the fast clearance of beclometasone and its
major metabolite B-17-MP, no beclometasone mediated drug interaction has been
observed. Finasteride has been tested with a few clinical drugs, including antipyrine,
digoxin, propranolol, and theophylline. Possibly because of its low affinity to hPXR, it did not seem to influence the P450 system and no meaningful drug interactions have been identified. Flunisolide can be used as nasal spray and there has been no drug interaction identified.

Secobarbital is a barbiturate derivative. Although there has been few drug interactions observed with secobarbital, its analog phenobarbital (a well recognized hPXR activator) was found to decrease drug efficacy when it was administered concurrently with drugs including anticoagulants, corticosteroids, doxycycline and estradiol. The Phenobarbital-mediated decrease of drug efficacy is attributed to increased metabolism induced by P450 enzymes in liver. Since secobarbital is suggested as a weak hPXR activator, its involvement in drug-drug interactions is yet to be determined. Aminoglutethimide was reported to accelerate dexamethasone metabolism and abolish the effects of coumarin and warfarin possibly due to aminoglutethimide promoted induction of hepatic microsomal enzymes. The confirmation of aminoglutethimide being a weak hPXR activator is consistent with these previously observed drug interactions.

Mitomycin is used as a chemotherapy agent and was identified as a novel hPXR antagonist. Due to serious side effects caused by its cytotoxicity (Crowston et al., 2006), the application of this drug is tightly restricted. Although a hPXR antagonist could be used with other drugs to increase efficacy, the low basal activity of hPXR and the high toxicity of mitomycin to normal tissues prevent it to from being a practical co-administrated drug for therapeutic purposes (Pagano et al., 2001). However, it may suggest we look at other less cytotoxic analogs in order to understand the activity.
In conclusion, the combined ligand based screening and experimental assays outlined in this study could present a method to identify potential therapeutic hPXR activators and non-activators confirmed by *in vitro* testing. We have shown in this study that Bayesian models generated with available hPXR activators and non-activators can be used to suggest new potential drug candidates for experimental testing, as well as identify other known activators that are not included for model generation. Identification of hPXR inhibitors provides insights for understanding hPXR mediated drug-drug interactions. Clinical pharmacokinetics and drug interactions pertinent to newly recognized hPXR activators have been investigated and possible hPXR mediated drug interactions were discussed. Thus, the application of ligand based virtual screening in combination with *in vitro* testing represents a means to rationally identify and subsequently validate commercial drugs as activators for a protein of interest.
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Authorship Contribution

Participated in research design: Pan, Ekins, Wang, Swaan

Conducted experiments: Pan, Li, Kim

Contributed new reagents or analytic tools: Wang

Performed data analysis: Pan, Swaan

Wrote or contributed to the writing of the manuscript: Pan, Ekins, Swaan, Wang
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Computational models to assign biopharmaceutics drug disposition 

Machine learning methods and docking for predicting human pregnane X 

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DMD #3580


antibiotics that activate pregnane X receptor and induce CYP3A4 in liver and


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Figure Legends

**Figure 1.** PCA plots among test set and training set drugs (A), and among training set and SCUT database drugs (B). The first two principal components explained 62% of variance.

**Figure 2** Effects of drugs on hPXR-mediated CYP2B6 reporter gene activation. HepG2 cells were transfected with hPXR expression vectors in the presence of CYP2B6-2.2 kb reporter construct. Transfected cells were then treated with vehicle or drugs (10 μM) for 24 h. RIF (10 μM) was used as positive control for hPXR. Luciferase activities were determined and expressed relative to vehicle control. Data represent the mean ± S.D. (n = 3).

**Figure 3** Dose dependent assay of fluticasone, nimodipine, nisoldipine and fluticasone. The compounds were tested their Luciferase activity in HepG2 cells at the concentrations of 0.1, 1, and 10 μM. CT indicates control (0.1% DMSO) and RIF indicates rifampcin. Data represent the mean ± S.D. (n = 3).

**Figure 4** Cytotoxicity of mitomycin in HepG2 cells measured by MTT assay. The cells were dispensed in 96-well plate at a density of $1.5 \times 10^4$ cells / well. After 24-h incubation, they were treated with oridonin at 20, 40 μM (positive control) and mitomycin at various concentrations for 24 h. A 20-μl aliquot of MTT solution (5.0 mg/ml) was added to each well followed by 4-h incubation, and the resulting crystals
were dissolved in 150 μl DMSO. CT indicates control with 0.1% DMSO. Data represent the mean ± S.D. (n = 3).

**Figure 5** Chemical structures and therapeutic classifications of newly identified hPXR activators, nonactivators and a deactivator.

**Figure 6** The Surflex docked (1NRL) conformations of newly identified PXR activators fluticasone (A), nimoldipine (B), nisoldipine (C), beclomethasone (D). The hydrogen bonding interaction is shown as black dotted lines. The protein backbone is shown as ribbon (orange), amino acid residues are shown in the stick mode whereas the PXR activators are shown in the ball and stick mode. The images were created using Pymol (v. 1.3).
Table 1 Predictive performance of FlexX docking and Bayesian models with training set \((n = 177, \text{leave-one-out cross-validation})\) and test set \((n = 145)\).

<table>
<thead>
<tr>
<th>name</th>
<th>ECFP-1</th>
<th>FCFP-1</th>
<th>ECFP-2</th>
<th>FCFP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-fingerprints</td>
<td>ECFP_6</td>
<td>FCEP_6</td>
<td>ECFP_6</td>
<td>FCFP_6</td>
</tr>
<tr>
<td>No. of descriptors</td>
<td>117</td>
<td>117</td>
<td>74</td>
<td>74</td>
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<tr>
<td>XV ROC AUC (%)(^a)</td>
<td>87.0</td>
<td>85.2</td>
<td>86.4</td>
<td>87.7</td>
</tr>
<tr>
<td>TP/FP/TN/FN(^b)</td>
<td>37/8/55/45</td>
<td>48/11/52/34</td>
<td>29/5/58/53</td>
<td>33/8/55/49</td>
</tr>
<tr>
<td>SE (%)(^b)</td>
<td>45.1</td>
<td>58.5</td>
<td>35.4</td>
<td>40.2</td>
</tr>
<tr>
<td>SP (%)(^b)</td>
<td>87.3</td>
<td>82.5</td>
<td>92.1</td>
<td>87.3</td>
</tr>
<tr>
<td>Q (%)(^b)</td>
<td>63.4</td>
<td>69.0</td>
<td>60.0</td>
<td>60.7</td>
</tr>
<tr>
<td>C (%)(^b)</td>
<td>34.7</td>
<td>41.4</td>
<td>32.1</td>
<td>30.3</td>
</tr>
</tbody>
</table>

\(^a\) Based on training set compounds.

\(^b\) Predictive performance validation by test set compounds. True positive (TP), true negative (TN), false positive (FP), false negative (FN), sensitivity (SE), specificity (SP), overall prediction accuracy (Q), and Matthew’s correlation coefficient (C)(Ung et al., 2007; Khandelwal et al., 2008). SE = TP/(TP + FN), SP = TN/(TN + FP), Q = (TP + TN)/(TP + TN + FP + FN), C = [(TP * TN) – (FN * FP)]/[(TP + FN) (TP + FN) (TN + FN) (TN + FP)]\(^{1/2}\).
Table 2 Molecules predicted following SCUT database search with Bayesian model, FlexX and SurfleX.

<table>
<thead>
<tr>
<th>No.</th>
<th>name</th>
<th>Bayesian score</th>
<th>FlexX score (kcal/mol)</th>
<th>SurfleX score (-LogKD)</th>
<th>category</th>
<th>PXR Luc activity (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>beclometasone</td>
<td>21.201</td>
<td>x</td>
<td>4.47</td>
<td>corticosteroid</td>
<td>3.95±0.25</td>
</tr>
<tr>
<td>2</td>
<td>triamcinolone</td>
<td>19.05</td>
<td>x</td>
<td>4.33</td>
<td>corticosteroid</td>
<td>0.8±0.07</td>
</tr>
<tr>
<td>3</td>
<td>megestrol acetate</td>
<td>18.56</td>
<td>-10.2</td>
<td>5.91</td>
<td>corticosteroid</td>
<td>5.61±0.35</td>
</tr>
<tr>
<td>4</td>
<td>fludrocortisone</td>
<td>16.43</td>
<td>x</td>
<td>4.05</td>
<td>corticosteroid</td>
<td>0.9±0.08</td>
</tr>
<tr>
<td>5</td>
<td>fluticasone propionate</td>
<td>15.71</td>
<td>x</td>
<td>6.25</td>
<td>corticosteroid</td>
<td>18.5±1.46</td>
</tr>
<tr>
<td>6</td>
<td>estramustine</td>
<td>11.65</td>
<td>-5.8</td>
<td>4.53</td>
<td>17-beta-estradiol</td>
<td>1.04±0.08</td>
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<tr>
<td>7</td>
<td>finasteride</td>
<td>10.49</td>
<td>-15.7</td>
<td>6.47</td>
<td>antiandrogen</td>
<td>2.97±0.31</td>
</tr>
<tr>
<td>8</td>
<td>nisoldipine</td>
<td>10.14</td>
<td>-24.3</td>
<td>5.19</td>
<td>dihydropyridine</td>
<td>11.7±2.69</td>
</tr>
<tr>
<td>9</td>
<td>flunisolide</td>
<td>9.85</td>
<td>-16.8</td>
<td>4.8</td>
<td>corticosteroid</td>
<td>2.7±0.14</td>
</tr>
<tr>
<td>10</td>
<td>mitomycin</td>
<td>8.09</td>
<td>-13.5</td>
<td>4.1</td>
<td></td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>11</td>
<td>nimodipine</td>
<td>7.99</td>
<td>-23.2</td>
<td>7.63</td>
<td>dihydropyridine</td>
<td>16.5±0.44</td>
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<tr>
<td>12</td>
<td>cefoxitin</td>
<td>7.51</td>
<td>-17</td>
<td>5.11</td>
<td>Cephalosporin</td>
<td>1.07±0.04</td>
</tr>
<tr>
<td>13</td>
<td>amikacin</td>
<td>7.22</td>
<td>-15.2</td>
<td>7.73</td>
<td></td>
<td>0.92±0.03</td>
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<tr>
<td>14</td>
<td>aminoglutethimide</td>
<td>7.12</td>
<td>-20</td>
<td>6.63</td>
<td></td>
<td>1.42±0.07</td>
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<tr>
<td>15</td>
<td>tobramycin</td>
<td>6.43</td>
<td>-9.6</td>
<td>7.78</td>
<td></td>
<td>1.13±0.07</td>
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<tr>
<td>16</td>
<td>sulfasalazine</td>
<td>6.31</td>
<td></td>
<td>6.81</td>
<td>Mesalazine</td>
<td>0.8±0.08</td>
</tr>
<tr>
<td>17</td>
<td>secobarbital</td>
<td>6.1</td>
<td>-12</td>
<td>5.53</td>
<td>Phenobarbital</td>
<td>1.57±0.06</td>
</tr>
</tbody>
</table>

*a “x” indicates unsuccessful dock by FlexX.
Table 3 Empirical or predicted physicochemical properties of newly identified hPXR activators, an antagonist and non-activators.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular weight</th>
<th>LogP</th>
<th>Solubility</th>
<th>Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluticasone propionate</td>
<td>500.6</td>
<td>3.4</td>
<td>insoluble</td>
<td>0.51 (intranasal)</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>418.4</td>
<td>2.7</td>
<td>1.20e-02mg/mL</td>
<td>100 (intravenous)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13 (oral)</td>
</tr>
<tr>
<td>Nisoldipine</td>
<td>388.4</td>
<td>3.1</td>
<td>5.7e-03mg/mL</td>
<td>5</td>
</tr>
<tr>
<td>Beclomethasone dipropionate</td>
<td>408.9</td>
<td>1.3</td>
<td>49.39mg/L</td>
<td>2</td>
</tr>
<tr>
<td>Finasteride</td>
<td>372.5</td>
<td>4.7</td>
<td>11.7mg/L</td>
<td>63</td>
</tr>
<tr>
<td>Flunisolide</td>
<td>434.5</td>
<td>1.1</td>
<td>insoluble</td>
<td>6.7</td>
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<tr>
<td>Megestrol</td>
<td>342.5</td>
<td>3.2</td>
<td>2µg/mL</td>
<td>Well absorbed</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>334.3</td>
<td>-1.6</td>
<td>soluble</td>
<td>N.A.</td>
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<tr>
<td>Triamcinolone</td>
<td>394.4</td>
<td>0.2</td>
<td>80mg/L</td>
<td>N.A. *</td>
</tr>
<tr>
<td>secobarbital</td>
<td>238.3</td>
<td>2.3</td>
<td>550mg/mL</td>
<td>N.A.</td>
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<tr>
<td>aminoglutethimide</td>
<td>232.3</td>
<td>1.3</td>
<td>Practically insoluble</td>
<td>&gt;95</td>
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<tr>
<td>Fludrocortisone</td>
<td>380.5</td>
<td>0.3</td>
<td>140mg/L</td>
<td>N.A.</td>
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<td>estramustine</td>
<td>440.4</td>
<td>5.7</td>
<td>3.85e-04mg/mL</td>
<td>N.A.</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>427.5</td>
<td>-0.02</td>
<td>1.95e-01mg/mL</td>
<td>5</td>
</tr>
<tr>
<td>amikacin</td>
<td>585.6</td>
<td>-7.4</td>
<td>1.85e+005mg/mL</td>
<td>53</td>
</tr>
<tr>
<td>tobramycin</td>
<td>467.3</td>
<td>-5.8</td>
<td>1e+003mg/mL</td>
<td>11.7</td>
</tr>
<tr>
<td>sulfasalazine</td>
<td>398.4</td>
<td>2.5</td>
<td>1.65e-02mg/mL</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

* N.A., not available

b Predicted solubility by DrugBank.
Figure 1

(A) and (B) illustrate the distribution of different sets in the first two components. (A) shows the test set (blue diamonds) and the training set (pink diamonds). (B) displays the SCUT set (green diamonds) and the training set (pink diamonds).
Figure 3

Cell viability %

DMSO  RIF  flu 0.1  Flu 1  Flu 10  Mitomycin 0.1  Mitomycin 1  nimodipine 0.1  nimodipine 1  nimodipine 10  nisoldipine 0.1  nisoldipine 1  nisoldipine 10