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Piperine activates human pregnane X receptor to induce the expression of cytochrome P450 3A4 and multidrug resistance protein 1

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Abstract

Activation of the pregnane X receptor (PXR) and subsequently its target genes, including those encoding drug transporters and metabolizing enzymes, while playing substantial roles in xenobiotics detoxification, might cause undesired drug-drug interactions. Recently, an increased awareness has been given to dietary components for potential induction of diet-drug interactions through activation of PXR. Here, we studied, whether piperine (PIP), a major component extracted from the widely-used daily spice black pepper, could induce PXR-mediated expression of cytochrome P450 3A4 (*CYP3A4*) and multidrug resistance protein 1 (*MDR1*). Our results showed that PIP activated human PXR (hPXR)-mediated *CYP3A4* and *MDR1* expression in human hepatocytes, intestine cells, and a mouse model; PIP activated hPXR by recruiting its coactivator SRC-1 in both cellular and cell-free systems; PIP bound to the hPXR ligand binding domain in a competitive ligand binding assay *in vitro*. The dichotomous effects of PIP on induction of *CYP3A4* and *MDR1* expression observed here and inhibition of their activity reported elsewhere challenges the potential use of PIP as a bioavailability enhancer and suggests that cautions should be taken for PIP consumption during drug treatment in patients, particularly those who favor daily pepper spice or rely on certain pepper remedies.

Keywords

Piperine; pregnane X receptor; cytochrome P450 3A4; multidrug resistance protein 1; drug-drug interaction

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Introduction

The pregnane X receptor (PXR; NR1I2), which belongs to the nuclear hormone receptor superfamily, is predominantly expressed in the liver and gastrointestinal tract and has been well-characterized as a xenobiotic sensor that binds to structurally diverse chemicals, including numerous clinical drugs, phytochemicals, dietary constituents, and endogenous substances (Willson and Kliewer, 2002). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor and binds to the promoter of PXR target genes to control their expression (Chen, 2008). Ligand binding to PXR recruits coactivators to induce the expression of the target genes, which encode proteins involved in xenobiotic detoxification and endobiotic metabolism, such as drug-metabolizing enzymes and transporters (Thunah et al., 2011). Abnormal activation of PXR by xenobiotics may lead to undesirable adverse drug-drug or food-drug interactions, a possible liability concern in drug development and clinical therapy. While the effect of clinical drugs on PXR activity has been heavily investigated, considerably less is known about the effect of dietary food and pure herbal constituents (Chang and Waxman, 2006; Chang, 2009).

Cytochrome P450 3A4 (CYP3A4) and multidrug resistance protein 1 (MDR1, also known as P-glycoprotein or P-gp), both expressed in the liver and intestine, are major PXR target genes. CYP3A4, a monooxygenase, mainly functions in catalyzing the first step of detoxification of xenobiotics by a hydroxylation reaction (Poulton et al., 2013). MDR1, a member of the ATP-binding cassette transporter family, acts as an efflux pump to limit the absorption of xenobiotics and contributes to the extrusion of many drugs in the intestine (Crowe and Tan, 2012). Because CYP3A4 and MDR1 together contribute to the metabolism and transportation of >50% of clinically used drugs and a great number of xenobiotics (Guengerich, 1999; Veith et al., 2009), induction or inhibition of CYP3A4 and MDR1 may cause drug-drug and dietary-drug interactions.

It has been recognized that many functional inhibitors of CYP3A4 and MDR1 also regulate their gene expression by modulating PXR activity. For example, CYP3A4 inhibitors clotrimazole (Zhang et al., 2002) and tamoxifen (Zhao et al., 2002), the MDR1 inhibitor nifedipine (Viale et al., 2009), and the dual CYP3A4 and MDR1 inhibitor ritonavir (Bierman et al., 2010; Kumar et al., 1996) increase *CYP3A4* and *MDR1* gene expression by activating human PXR (hPXR) (Drocourt et al., 2001; Luo et al., 2002; Moore et al., 2000b; Sane et al., 2008), while the dual CYP3A4 and MDR1 inhibitor ketoconazole (KET) (Gibbs et al., 1999; Rautio et al., 2006) decreases *CYP3A4* and *MDR1* gene expression by inhibiting hPXR activity (Venkatesh et al., 2011). Dietary constituents and phytochemicals have also been shown to affect both gene expression and protein function of CYP3A4 and MDR1. For example, St. John's wort extract is well known to not only strongly inhibit the activity of CYP3A4 and MDR1, but also induce their gene expression by activating hPXR (Dresser et al., 2003; Hafner et al., 2010; Moore et al., 2000a; Obach, 2000).

Piperine (PIP, 1-piperoylpiperidine), a major component extracted from black pepper used as a daily spice or a traditional medicine, was reported to be an inhibitor of the activity of human CYP3A4 and MDR1 (Bhardwaj et al., 2002), resulting in increased bioavailability of orally co-administrated drugs that were CYP3A4 and MDR1 substrates. Therefore, PIP has been proposed as a potential bioavailability enhancer for such drugs (reviewed in Han, 2011). However, it remains unknown whether PIP can also regulate *CYP3A4* and *MDR1* gene expression through modulating hPXR activity. In this study, we used a number of *in vitro* and *in vivo* measurements to show that PIP could activate the transcriptional activity of hPXR and subsequently induce the expression of *CYP3A4* and *MDR1* in human intestine cells and hepatocytes. Together with the reported results that PIP inhibits the activity of CYP3A4 and MDR1, our findings revealed a dichotomous effect of PIP.

Materials and Methods

Chemicals and Plasmids

PIP, rifampicin (RIF), paclitaxel, KET, SR12813, T0901317 (TO), clotrimazole, and pregnenolone-16 β -carbonitrile (PCN) were purchased from Sigma (St. Louis, MO). pcDNA3-hPXR and pGL3-*CYP3A4*-luc plasmids were described previously (Lin et al., 2008). pcCMV6-entry and pCMV6-mPXR plasmids were obtained from OriGene (Rockville, MD). MDR1 full-length promoter construct containing 8 kb of the *MDR1* promoter region (p-8055MDR) was kindly provided by Dr. Oliver Burk (Geick et al., 2001) (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Germany) and used to construct pGL4-*MDR1*-luc in pGL4.14 (Promega, Madison, WI) following a method described previously (Lin et al., 2008).

Cell Culture

The human intestinal epithelial cell lines LS174T and LS180, the human liver carcinoma cell line HepG2, HEK293T cell line, and mouse liver cell line Hepa-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in modified Eagle's minimal essential medium (MEM) from ATCC with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. According to the product information from ATCC, LS180 is derived from Dukes' type B colorectal adenocarcinoma, and LS174T is a variant of LS180 that has been maintained by using trypsin in the subculture protocol (ATCC) (Rutzky et al., 1980). HepG2-hPXR-*CYP3A4*-Luc and LS180-hPXR-*CYP3A4*-Luc cells stably expressing hPXR and *CYP3A4* promoter reporter gene (*CYP3A4*-Luc) were maintained under the double selection of 400 μ g/ml G418 and 200 μ g/ml Hygromycin B (Invitrogen, Carlsbad, CA). Human primary hepatocytes were obtained through the Liver Tissue Cell Distribution System (Pittsburgh, PA), which was funded by NIH Contract #N01-DK-7-0004 / HHSN267200700004C, and cultured in hepatocyte medium (Sigma).

PXR Transactivation Assay

The cells were transfected with pcDNA3, pcDNA3-hPXR, *CYP3A4*-luc, and/or pGL4-*MDR1*-luc plasmids using FuGENE 6 (Roche Diagnostics). Twenty-four hours after transfection in growth media, ~10,000 live cells were plated in each well of a 96-well culture plate (PerkinElmer, Waltham, MA) and grown for an additional 24 h in phenol red-free MEM (Invitrogen) supplemented with 1% charcoal/dextran-treated FBS (HyClone) and other additives, as described in the cell culture section. Forty-eight hours after transfection, a luciferase assay was performed to measure luminescence using the Dual-Glo luciferase assay system (Promega) and EnVision microplate reader (PerkinElmer).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis (qPCR)

Total RNA was isolated from LS174T cells, LS180 cells, and human primary hepatocytes using Maxwell 16 LEV simplyRNA purification kits (Promega). qPCR was performed using Taqman gene expression assays (Applied Biosystems, Carlsbad, CA) specific for *CYP3A4*, *MDR1*, and *hPXR*, and *GAPDH* was used as the reference gene according to the manufacturer's protocol in an ABI 7900HT system (Applied Biosystems). The comparative Ct method was used for relative quantification for gene expression with the following formula: $Ct = Ct(\text{test gene}) - Ct(GAPDH)$; $Ct(\text{test gene}) = Ct(\text{test gene in treatment group}) - Ct(\text{test gene in vehicle control group})$; the fold changes of mRNA = 2^{-Ct} , which indicated the relative mRNA level of the corresponding transcript to the control samples.

Western Blotting Analysis

The cells were rinsed once with cold phosphate-buffered saline and then lysed in RIPA lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS). Whole cell lysates containing ~25 µg of total protein lysates were loaded into Nupage 4% to 12% bis-Tris gels (Invitrogen) with Nupage MES SDS running buffer (Invitrogen). The proteins were then transferred to a nitrocellulose membrane using the iBlot gel transfer system (Invitrogen) and iBlot gel transfer stacks (Invitrogen). For Western blotting, the membrane was blocked for 1 h with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE), probed with mouse monoclonal antibodies against CYP3A4 (K03; Schuetz et al., 1996b), MDR1, and hPXR (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with secondary goat anti-mouse antibody labeled with infrared dye (LI-COR Biosciences). Antigen-antibody interactions were visualized, and the intensity of the protein band was quantified using an Odyssey infrared imager (LI-COR Biosciences). The intensity of each protein band was normalized to that of actin to generate the relative intensity, with the relative intensity of the DMSO treated sample set as “1.0”. A representative immunoblot from at least two independent experiments is shown.

Small Interfering RNA Transfection

hPXR was knocked down in cells by transient transfection of small interfering RNA (siRNA). Briefly, cells cultured in 6-well plates were treated with 200 nM ON-TARGETplus SMARTpool siRNA targeting PXR (L-003415, Thermo Fisher Scientific, Waltham, MA) or Nontargeting Pool (D-001810, Thermo Scientific) together with 7.5 µl/well Lipofectamine RNAiMAX Transfection (Invitrogen) diluted in 1 ml of medium with serum and without antibiotics. After 48 h transfection, the cells were treated with indicated compounds for another 24 h and then collected for the PXR transactivation assay and qPCR analysis.

Mammalian Two-Hybrid Assay

The CheckMate mammalian two-hybrid system (Promega) was used. pACT-hPXR, pBIND-SRC-1, and pG5-luc (a GAL4 luciferase reporter construct) were cotransfected into HEK293T cells. The pBIND-SRC-1 plasmids also constitutively expressed Renilla luciferase, which was used as an internal transfection control. Dual-Glo Luciferase Assay (Promega) was used to measure luciferase activity. Expression of hPXR and SRC-1 was confirmed by Western blotting analysis (data not shown). The relative luciferase activity for pG5-luc was determined by normalizing firefly luciferase activity with Renilla luciferase activity.

SRC-1 Coactivator Recruitment Assay

A Lanthascreen time-resolved fluorescence resonance energy transfer (TR-FRET) assay was performed to examine SRC-1 recruitment by activated hPXR according to the manufacturer's instructions (Invitrogen). Briefly, purified GST-hPXR ligand-binding domain (LBD) and a mixture of Tb-anti-GST antibody/Fl-SRC1-4 peptide was added to each well containing test compound or DMSO solvent control for final concentrations of 5 nM hPXR-LBD, 5 nM Tb-anti-GST, and 500 nM Fl-SRC1-4 peptide (total volume: 20 µl per well; 384-well solid black plates). Assay plates were then incubated at room temperature for 1 h. An excitation filter centered at 340 nm was used to excite the terbium-labeled antibody, and emission filters centered at 490 nm and 520 nm were used to detect the terbium and fluorescein emission intensities, respectively. A post-excitation lag time of 100 µs followed by an integration time of 200 µs was used to collect the time-resolved emission signals on a PHERAStar plate reader (BMG Labtech, Durham, NC) to calculate the 520/490 TR-FRET ratio.

Competitive Ligand-Binding Assay

A Lanthascreen TR-FRET PXR competitive binding assay was conducted according to the manufacturer's protocol as described previously (Lin et al., 2008). Briefly, assays were performed in a volume of 20 μ l in 384-well solid black plates with 5 nM GST-hPXR ligand-binding domain, 40 nM fluorescent-labeled hPXR agonist (Fluomore PXR Green, Invitrogen), 5 nM terbium-labeled anti-GST antibody, and test compound at different concentrations. The reaction mixture was incubated at 25°C for 20 min, and then fluorescent emissions of each well were measured. Net TR-FRET ratio was calculated by subtracting the background TR-FRET ratio obtained from 10 μ M SR12813 TR-FRET ratio.

Molecular Modeling and Docking Study

The structure of PIP was obtained from the ZINC database (code 1529772) (Irwin et al., 2012). The hPXR-LBD protein crystal structure was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>) in PDB format (PDB code 209I). All water molecules and ligands from the protein structures were removed using the molecular graphics system Pymol (<http://www.pymol.org>). The PDBQT files for docking were generated in AutoDockTools (ADT) version 1.5.6 (<http://mglttools.scripps.edu>), which was used to add polar hydrogens and Kollman charges to the protein structures. The search space with XYZ dimensions 24 Å $\times$ 24 Å $\times$ 24 Å was centered at coordinates 8.934 (x), 30.182 (y), 25.546 (z). Docking of PIP to hPXR-LBD was carried out with the program AutoDock Vina version 1.1.1 (Trott and Olson, 2010) with the exhaustiveness value set to 300. The docking results were analyzed and the figures created in Pymol with the assistance of Ligplot+ (Laskowski and Swindells, 2011). The Molecular Lipophilicity Potential Interaction Score (MLPInS) using Fermi's type distance function (Vistoli et al., 2010) was calculated using the package VEGA (Pedretti et al., 2004).

Hydrodynamic Injections, Treatment, and *In Vivo* Imaging

We performed all animal experiments in accordance with a protocol approved by St. Jude Children's Research Hospital Institutional Animal Care and Use Committee. Male C57BL/6 mice (8–15 weeks old, Charles River Laboratories, Wilmington, MA) were housed at 22–23°C with a 12 h light/dark cycle and free access to food and water in the St. Jude Animal Resources Center certified by the American Association for Accreditation of Laboratory Animal Care. All animals within an experiment were matched for age and body weight. *In vivo* delivery of *CYP3A4*-luc reporter gene and hPXR gene into mouse liver was performed using the hydrodynamic injection method as described (Liu et al., 1999; Schuetz et al., 2002). Briefly, mice were given a rapid (5–10 s) tail vein injection of 25 μ g of linearized *CYP3A4*-luc plasmid DNA with or without linearized hPXR plasmid DNA in sterile saline in a volume equal to 10% of body weight. Imaging for luciferase activity was performed 2–8 weeks after somatic gene transfer, during which the bioluminescence was stable in the mice. Cohorts of 5 mice were given injections of VivoGlo luciferin (150 mg/kg of body weight, i.p., Promega), anesthetized under 2.5% isoflurane, and imaged 10 min later in the Xenogen IVIS 200 system (Xenogen) to obtain a basal image. Five mice in each group were then treated intraperitoneally with vehicle control, 10 mg/kg RIF, or 50 mg/kg PIP every 24 h for 2 days. Twelve hours after the last treatment, the mice were imaged as described above. Uniform regions of interest were drawn around the liver, and total photon flux was analyzed with Living Image 3.2 software (Xenogen). The induction of *CYP3A4*-luc reporter activity was calculated using the imaging from the same mouse as follows: induction rate = total photon flux (after treatment) / total photon flux (before treatment).

Statistical Analysis and Curve Fitting

The Student's *t* test was used to determine statistical significance of unpaired samples. Differences were considered significant for $p < 0.05$, 0.01, or 0.001 and not significant (N.S.) for $p > 0.05$. Dose-response data were fitted to estimate EC₅₀ (half-maximal effective concentration) with the following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X)})$, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Results

PIP induced *CYP3A4* and *MDR1* expression in human hepatocytes and intestine cells

Because liver and intestine (duodenum, jejunum and colon) (Greiner et al., 1999; Kolars et al., 1992; van de Kerkhof et al., 2007; van de Kerkhof et al., 2008) are the major organs with highly inducible expression of *CYP3A4* and/or *MDR1* in response to uptake of xenobiotics, we first examined the effects of PIP on endogenous *CYP3A4* and *MDR1* expression in human hepatocytes and intestine cell lines. First, the primary human hepatocytes from 3 different donors (HH1956, 1962, and 1890) were treated for 72 h with PIP or with RIF or paclitaxel (both known hPXR agonists) (Figure 1). PIP induced *CYP3A4* mRNA in a dose-dependent manner (6- and 10-fold at 50 μM , comparable to the levels induced by 5 μM RIF) (Figure 1A). Under the same conditions, PIP and RIF only slightly increased the *MDR1* mRNA levels (<2-fold) (Figure 1B). The extents of increase of *CYP3A4* and *MDR1* protein levels by the drug treatment were consistent with those of their mRNA levels (Figure 1C). Together, the results showed that PIP induced *CYP3A4* and *MDR1* expression in human primary hepatocytes.

Next, we tested the effects of PIP in human intestine cells LS174T and LS180, which reportedly express endogenous hPXR with inducible activity and have been used as intestinal models to study the function of hPXR (Geick et al., 2001; Gupta et al., 2008; Harmsen et al., 2008; Kota et al., 2010; Schuetz et al., 1996a), and LS180-hPXR, which stably expresses ectopic hPXR (Figure 2). Similarly as in the human hepatocytes, in all of the intestinal cell lines, PIP increased the levels of *CYP3A4* (Figure 2A) and *MDR1* mRNA (Figure 2B) in a dose-dependent manner, with much greater magnitude of *CYP3A4* mRNA levels than of *MDR1* (~20-fold vs. ~5-fold at the highest concentration of PIP); the mRNA induction levels at the highest concentration of PIP were comparable to those induced by RIF. The extents of increase of *MDR1* protein levels by PIP treatment were consistent with those of their mRNA levels in a dose-dependent manner (Figure 2C). Although PIP induced a great magnitude of *CYP3A4* mRNA level, the protein level of endogenous *CYP3A4* was not detectable (Figure 2C), possibly due to extremely low levels of *CYP3A4* protein in these intestine cell lines, while the elevated *CYP3A4* gene transcription has been shown to be functionally correlated to its increased enzymatic activity (Gupta et al., 2008). In LS180-hPXR cells, both PIP and RIF induced much greater *CYP3A4* and *MDR1* expression at both mRNA and protein levels than in its parental cell line, LS180 (Figure 2), indicating that the elevated hPXR level enhanced the effect of PIP and RIF in inducing *CYP3A4* and *MDR1* expression and suggesting that PIP acts through hPXR.

PIP activated hPXR-regulated *CYP3A4* and *MDR1* promoters

The greater *CYP3A4* and *MDR1* induction by PIP in LS180-hPXR prompted us to determine whether PIP acts through hPXR. We first tested the effect of PIP on two hPXR-regulated promoter reporter constructs, *CYP3A4-luc* and *MDR1-luc*, by transiently transfecting each reporter construct with or without hPXR into human intestinal (LS174T and LS180) and liver (HepG2) cell lines and then treating the cells with RIF or PIP, approximately at its physiologically relevant level (25 μM) (Han et al., 2008) (Figure 3). Similar to treatment with RIF, treatment with PIP induced significantly higher levels of

activity of *CYP3A4*-luc (Figure 3A) and *MDR1*-luc (Figure 3B) in an hPXR-dependent manner (Figure 3). Therefore, PIP, functionally like RIF, activated hPXR-regulated *CYP3A4*-luc and *MDR1*-luc reporter activity in all three cell lines.

To confirm the effect of PIP on *CYP3A4* and *MDR1* promoters, a dose-response experiment was conducted, and the half-maximal effective concentration (EC_{50}) was determined using reporter assays (Table 1). With overexpressed hPXR, PIP induced hPXR-mediated reporter activity at EC_{50} values ranging from 2–4 μ M in all three cell lines, and the maximal induction occurred at varied concentrations depending on the reporters and cell lines. Without overexpression of hPXR, PIP induced the activity of *CYP3A4*-luc and *MDR1*-luc at slightly lower EC_{50} values (Table 1), consistent with the results showing that PIP induced endogenous *CYP3A4* and *MDR1* gene expression (Figures 1 and 2), and suggesting that the effect of PIP was at least partially mediated by endogenous hPXR activation. The ranges of EC_{50} values of PIP were similar to those of RIF under the same conditions (Table 1).

We further determined the potency of PIP on activating hPXR's transcriptional activity on the *CYP3A4* promoter in two stable reporter cell lines: human liver cell line (HepG2-hPXR-*CYP3A4*-luc) and intestinal cell line (LS180-hPXR-*CYP3A4*-luc), both stably transfected with hPXR and the *CYP3A4*-luc promoter reporter gene. PIP induced the hPXR transactivation activity in a dose-dependent manner with an estimated EC_{50} values of 6.4 μ M in HepG2-hPXR-*CYP3A4*-luc cells and 2.2 μ M in LS180-hPXR-*CYP3A4*-luc cells (Figure 4 and Table 1), comparable with the EC_{50} values obtained from their parental cells with the transiently overexpressed hPXR. The range of EC_{50} values suggested that PIP had high potency to activate hPXR transcriptional activity, comparable to that of RIF in general.

Downregulation of hPXR diminished the effect of PIP on hPXR target gene expression

To confirm that PIP induced hPXR activity, we tested the effect of PIP on the expression of hPXR target genes when hPXR expression was knocked down or its activity was pharmacologically inhibited. In LS180-hPXR-*CYP3A4*-luc cells, when hPXR expression was genetically knocked down using siRNA, resulting in ~80–90% ablation of hPXR expression (data not shown), the induction of *CYP3A4* and *MDR1* mRNA levels by both PIP and RIF were significantly abrogated compared with non-targeting control siRNA (Figures 5A and B). Consistent with their mRNA levels, the induction of hPXR-mediated *CYP3A4* promoter activity by PIP and RIF was also significantly lower (Figure 5C). Similarly, when we used KET (a known hPXR inhibitor) to pharmacologically inhibit hPXR activity, the induction of *CYP3A4* promoter activity by PIP or RIF in LS180-hPXR-*CYP3A4*-luc cells was significantly diminished (Figure 6A); the induction of *CYP3A4* by PIP and RIF was significantly reduced at both the mRNA (Figure 6B) and protein levels (Figure 6C) in human hepatocytes; the induction of *MDR1* by PIP and RIF was significantly reduced at protein levels in LS174T and LS180 intestinal cells (Figure 6D). Therefore, hPXR was required for PIP to induce the expression of hPXR target genes *CYP3A4* and *MDR1*.

Interaction between PIP and hPXR

We then sought the mechanism responsible for PIP to activate hPXR. Because previous studies have shown that steroid receptor coactivator-1 (SRC-1) contributes to the ligand-induced activation of hPXR (Sui et al., 2012), we first tested whether PIP treatment could result in recruitment of SRC-1 to hPXR. In a mammalian two-hybrid system in which the interaction between hPXR and SRC-1 induced by PXR agonist leads to a specific reporter activity (Pondugula et al., 2009), 25 μ M PIP significantly increased the reporter signal (Figure 7A) to a similar extent as RIF did, indicating that PIP induced the interaction between hPXR and SRC-1 in the cells (Figure 7A). Furthermore, in a cell-free SRC-1

recruitment assay, 25 μ M PIP directly promoted the binding of SRC-1 to the hPXR ligand binding domain (hPXR-LBD), comparable to the activity of 0.13 μ M TO (a potent hPXR agonist). Importantly, hPXR inhibitor KET inhibited the interaction between SRC-1 and hPXR (Figure 7B). Together, these results indicated that PIP activated hPXR function by recruiting co-activator SRC-1.

The recruitment of SRC-1 by PIP in the cell-free system also suggested that PIP, like RIF and other PXR agonists, might directly bind to the hPXR-LBD. To test this, a cell-free competitive hPXR-LBD binding assay using a fluorescein-labeled PXR ligand as a tracer was performed. At 2.5 μ M (its EC_{50} value of hPXR promoter activity), PIP decreased ~20% of the tracer binding to hPXR-LBD compared with vehicle control (Figure 7C), whereas the three hPXR agonists, TO, SR12813 (known as potent hPXR agonists), and clotrimazole (known as a moderate hPXR agonist) at their EC_{50} values of hPXR promoter activity decreased ~70%, ~40%, and ~35% of the tracer binding to hPXR-LBD, respectively (Figure 7C). Furthermore, a molecular docking study using AutoDock Vina predicted the mode of PIP binding to hPXR-LBD. PIP was positioned in the binding pocket (Figure 7D, *left*). The top pose was selected based on the AutoDock score and the MLPInS. In this model, the interactions involved the residues Leu206, Leu209, Lys210, Val211, Pro227, Pro228, Glu235, Ile236, Phe237, Ser238, Leu240, Met243, Phe288, Trp299, and Tyr306, with a hydrogen bond between the amide NH-group of Leu239 and one of the oxygen atoms in the five-member ring of PIP (Figure 7D, *right*). Together, these results indicated that PIP could directly interact with hPXR to promote co-activator recruitment, thus activating hPXR as an agonist.

PIP induced hPXR activation *in vivo*

We next tested PIP activation of hPXR *in vivo* using a mouse model. Due to the divergence of PXR-LBD across different species, most PXR ligands show species-specific PXR activity. To establish an appropriate mouse model to investigate the effect of PIP on hPXR activity *in vivo*, we first evaluated the effect of PIP on mouse PXR (mPXR) activity using Hepa-1 mouse liver cells and HepG2 human liver cells transiently transfected with *CYP3A4*-luc reporter plasmid with or without *hPXR* or *mPXR* constructs, respectively (Figure 8). In both cell lines with hPXR overexpression, only PIP and RIF, but not PCN (a known specific mPXR agonist), significantly induced hPXR-regulated *CYP3A4* promoter activity, compared with vehicle control; in contrast, with mPXR overexpression, only PCN, but not PIP or RIF, significantly induced mPXR-regulated *CYP3A4* promoter activity (Figures 8A and B), consistent with their species specificity on the activity of PXR. These results suggested that PIP was specific for hPXR (Figure 8A and B). Taking the advantage of its hPXR specificity, we generated transient hPXR-transgenic reporter mice to evaluate the effect of PIP on hPXR activity *in vivo*, in which wild-type C57/B6 mice were given injections of *CYP3A4*-luc reporter plasmid with or without *hPXR* plasmid by hydrodynamic injection (Liu et al., 1999; Schuetz et al., 2002). The change of *CYP3A4*-luc reporter activity after drug treatment was used to indicate its induction by the drug. In the mice without hPXR expression, neither RIF nor PIP treatment significantly increased *CYP3A4*-luc reporter activity compared with vehicle control (Figures 8C). In the mice with hPXR expression, both PIP and RIF significantly increased levels of *CYP3A4*-luc reporter activity compared with vehicle controls (Figure 8D and E). Therefore, PIP, similar to RIF, specifically activated hPXR-mediated *CYP3A4* transactivation *in vivo*.

Discussion

To our knowledge, our study is the first report that PIP, at a physiologically relevant level, could induce hPXR-mediated *CYP3A4* and *MDR1* expression in human hepatocytes and intestine cells, at least partly by directly interacting with hPXR.

There were previously no or very limited experimental data on the effects of PIP on hPXR target genes in human cells. One recent report showed that PIP induced rat *CYP3A23* reporter gene activity in human HepG2 cells (Qiang et al., 2012), but the effect of PIP on neither endogenous CYP expression nor hPXR activity in human cells was known. Several studies showed that PIP could induce MDR1 in rat intestinal tissue (Han et al., 2008; Qiang et al., 2012), but whether the induction depends on PXR was unclear. Interestingly, a recent study revealed PIP-induced MDR1 expression in Caco-2, an hPXR-deficient human intestinal cell line, indicating PXR-independent MDR1 induction (Han et al., 2008). Our findings suggested that at least an hPXR-dependent mechanism was partially responsible for the effect of PIP on MDR1 induction in the intestinal LS174 and LS180 cells. It is, however, possible that other PXR-independent mechanisms also contribute to the induction of MDR1 by PIP, since multiple mechanisms, including those mediated by hypoxia-inducible factor-1, p53, constitutive androstane receptor and even chromosomal rearrangement as well as epigenetic mechanisms have been reported to regulate the expression of MDR1 (reviewed in Chen, 2010).

Our findings of the effects of PIP on induction of CYP3A4 and MDR1 pose a challenge for a current proposal using PIP as a potential bioavailability enhancer for drugs that are CYP3A4 and MDR1 substrates. PIP was found to directly inhibit the activity of CYP3A4 and MDR1 proteins (Bhardwaj et al., 2002) and therefore was proposed as a potential bioavailability enhancer for co-administered drugs that are CYP3A4 and MDR1 substrates (reviewed in Han, 2011). Since PIP can induce expression but also inhibit the activities of CYP3A4 and MDR1, its ultimate net effect on drug metabolism and bioavailability is dependent on the balance of these two opposing effects. The dichotomous effects of PIP could help explain the observations from several clinical studies attempting to use PIP as a bioavailability enhancer for drugs that are CYP3A4 substrates. A recent human study showed that an herbal extract containing PIP (24 mg) failed to enhance the bioavailability of midazolam (a CYP3A4-selective substrate); the plasma pharmacokinetics of midazolam remained unchanged in the presence of PIP (Volak et al., 2013). Similarly, in two other human studies, co-administration of 20 mg PIP showed no significant inhibition on the rate of metabolism of two CYP3A4 substrates, nevirapine (200 mg) and carbamazepine (500 mg), as indicated by the unchanged elimination constant rate (K_{el}) (Kasibhatta and Naidu, 2007; Pattanaik et al., 2009), suggesting that PIP at this dose probably had no significant effect on inhibiting CYP3A4 activity in human. The increased bioavailability of the two CYP3A4 substrates (nevirapine and carbamazepine) by PIP co-treatment, as indicated by the increased AUC, might be mediated through other mechanisms, such as decreased hydrochloric acid secretion and delay in gastric emptying (Pattanaik et al., 2006). The failure of PIP to inhibit the activity of CYP3A4 in these clinical studies could be explained by the dichotomous effects of PIP: the induction of CYP3A4 and MDR1 expression by PIP may offset the inhibitory effects of PIP on the activity of these two proteins. Therefore, the proposal to use PIP as a bioavailability enhancer for CYP3A4 and MDR1 substrates warrants further clinical evaluation.

The physiologically relevant level of PIP was estimated based on information from the literature. As the major active component of black pepper, PIP accounts for 5–9% of its content (Bhardwaj et al., 2002). As a widely daily-used spice, an average daily consumption of black pepper is around 359 mg (~60–110 μ mol of PIP) in the United States (Kindell SS, 1984; Bhardwaj et al., 2002). Given that the plasma volume is ~2.5–3 L in the average adults (Crispell et al., 1950; FRANK and GRAY, 1953; Anderson and Anderson, 2002), when the absorption efficacy or first-pass effect of intestine and liver are not taken into account, a theoretical range of plasma concentration of PIP should be less than 20–44 μ M (60 μ mol/3L – 110 μ mol/2.5L). Considering the fact that black pepper consumption is normally a minute amount but repeated multiple times in the daily diet, PIP might actually

sustain in the circulatory and digestion system at concentrations close to the theoretical range (~20–44 μM). This estimation is in line with a recent clinical study showing that the maximum plasma concentration of PIP was around 6 μM after a single dose of orally administrated PIP (24 mg) in healthy volunteers (Volak et al., 2013). Thus, when 359 mg of daily consumption of black pepper was accounted, we estimated that 25 μM , as used in the present study, would be in the range of a physiologically relevant level of PIP in average adults.

The net effects of PIP on CYP3A4 and MDR1 will depend on the level of PIP used. In one study, PIP inhibited CYP3A4-catalyzed formation of verapamil metabolites D-617 and norverapamil *in vitro* with IC_{50} values of 53.8 and 64.4 μM , respectively, and inhibited the efflux of MDR1 substrates digoxin and cyclosporine A in Caco-2 cells with IC_{50} values of 15.5 and 74.1 μM , respectively (Bhardwaj et al., 2002). In another study, PIP inhibited digoxin efflux in a similar Caco-2 cell system at a minimal concentration of 50 μM (Han et al., 2008). These results all suggested that the dietary level of PIP may not be high enough to exert 50% inhibitory effects on the activity of CYP3A4 and MDR1 proteins. On the other hand, our study showed PIP induced hPXR-mediated transactivation of CYP3A4 and MDR1 at relatively low concentrations (EC_{50} at ~2.5 μM), and 25 μM PIP is high enough to increase *CYP3A4* and *MDR1* expression in intestine cells, suggesting that induction of *CYP3A4* and *MDR1* expression by PIP may offset its weak inhibitory effects on these two proteins at such low concentrations. Another study showed that treatment with 50 μM PIP for 48 h functionally increased, but not inhibited, the efflux of digoxin by MDR1 in a Caco-2 cell monolayer model (Han et al., 2008), suggesting that the stimulatory effect of PIP on MDR1 expression offsets its inhibitory effect on the activity of MDR1.

PIP's net effects on CYP3A4 and MDR1 activity could have important clinical implications in food-drug and herb-drug interactions. The altered activity of CYP3A4 and MDR1 by PIP could lead to undesired pharmacokinetic interactions during drug therapy. Such undesired effects by dietary food and pure herbal constituents have got attention in clinical and preclinical studies, such as hyperforin in St. John's wort and ginkgolides in Ginkgo biloba (Lau et al., 2012). The active components in St. John's wort are identified with strong PXR agonism (Moore et al., 2000a). In some preclinical/clinical studies, co-treatment of St. John's wort with different drugs, such as cyclosporine A, indinavir, oral contraceptives, tacrolimus, warfarin, verapamil, and fexofenadine, led to undertreatment and failure of the therapeutic drugs, because the plasma concentrations of co-administrated drugs were significantly reduced by St. John's wort due to PXR-mediated CYP3A4 induction (Izzo and Ernst, 2009). Our study suggested that the physiologically relevant level of PIP was sufficient to change *CYP3A4* and *MDR1* expression in human liver and intestine and could potentially affect the absorption and metabolism and subsequently the efficacy of other therapeutic drugs, as seen in the case of St. John's wort. Recently, one study showed that long-term and low-dose treatment of PIP in rats significantly decreased the bioavailability of oral diltiazem, an MDR1 substrate, due to the significant induction of MDR1 in rat small intestine (Qiang et al., 2012), consistent with our observation in human cells. However, due to the dichotomous effects of PIP on CYP3A4 and MDR1, the extent to which PIP can affect food-drug interactions during therapy warrants further study.

In our study, PIP's agonism of hPXR activity was demonstrated unambiguously in the cell-free and cell-based assays as well as in a humanized PXR reporter mouse model. The direct binding of PIP to hPXR-LBD was only evidenced at one concentration (2.5 μM , ~ EC_{50} of the reporter activity) in the TR-FRET competitive assay. Because higher concentrations of PIP quenched the fluorescence signal and compromised the TR-FRET assay results, the binding of PIP to hPXR-LBD cannot be determined at higher concentrations. Nevertheless, the results indicated that the IC_{50} of PIP binding to hPXR-LBD would be higher than 2.5

μM, which was much higher than those of known hPXR agonists TO and SR12813 (23 and 87 nM, respectively) (Lin et al., 2008). In addition, xenobiotics have been shown to activate hPXR indirectly (Wang et al., 2012), such as by modifying the phosphorylation status and activity of hPXR (Dong et al., 2010; Lin et al., 2008; Pondugula et al., 2009; Pondugula et al., 2010). PIP has been shown to inhibit the activity of several kinases, including mitogen-activated protein kinases, IkappaB, and protein kinase B (Akt) (Doucette et al., 2013; Kumar et al., 2007; Lin et al., 1999). In addition to its direct binding on activation of hPXR, whether and how PIP could indirectly activate hPXR warrants further investigation.

In summary, PIP induced *CYP3A4* and *MDR1* expression by activating hPXR in human hepatocytes and intestine cells. Given that black pepper is one of the most extensively-used daily spices and its active component PIP is also commercially available as a health supplement, our findings indicated that there may be an increased chance of undesired food-drug interactions caused by the uptake of black pepper or piperine-containing food, and caution should be taken for use of black pepper while undergoing other drug therapeutics.

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Abbreviations

CYP	cytochrome P450
KET	ketoconazole
MDR1	multidrug resistance protein 1
PCN	pregnenolone-16 -carbonitrile
PIP	piperine
PXR	pregnane X receptor
RIF	rifampicin
SRC-1	steroid receptor coactivator-1
TO	T0901317

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Highlights

- Piperine induces PXR-mediated *CYP3A4* and *MDR1* expression.
- Piperine activates PXR by binding to PXR and recruiting coactivator SRC-1.
- Piperine induces PXR activation *in vivo*.
- Cautions should be taken for piperine consumption during drug treatment.

Figure 1. PIP induced *CYP3A4* and *MDR1* expression in human hepatocytes at both mRNA and protein levels

A) Human *CYP3A4* and B) *MDR1* mRNA expression was analyzed by real-time PCR in primary human hepatocytes (HH1956 and HH1962) after treatment with vehicle control (0.1% DMSO), RIF, or increasing concentrations of PIP as indicated for 72 h. Results are presented as fold increase over the DMSO control. Data represent mean $\pm$ SEM from three independent experiments: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the vehicle control by t test. C) *CYP3A4* and *MDR1* protein levels were determined by Western blotting in the hepatocytes (HH1890) after treatment with vehicle control (0.1% DMSO), RIF, paclitaxel (Taxol), or PIP as indicated for 72 h. The numbers at the bottom indicate the

relative intensity of the protein bands, with the DMSO treated sample set as “1.0”. D)
Chemical structure of PIP.

Figure 2. PIP induced CYP3A4 and MDR1 expression in human intestinal cells at both mRNA and protein levels

A) Human *CYP3A4* and B) *MDR1* mRNA expression was analyzed by real-time PCR in the intestinal cells LS174T, LS180, and LS180-hPXR (with overexpression of hPXR) after treatment with vehicle control (0.1% DMSO), RIF, or increasing concentrations of PIP as indicated for 72 h. Results are presented as fold increase over the DMSO control. Data represent mean $\pm$ SEM from three independent experiments: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the vehicle control by *t* test. C) CYP3A4 and MDR1 protein levels in the same cells were determined by Western blotting. The numbers at the bottom indicate the relative intensity of the protein bands, with the DMSO treated sample set as "1.0".

Figure 3. PIP induced hPXR transactivation of *CYP3A4* and *MDR1* promoter

A) *CYP3A4* and B) *MDR1* promoter activity in LS174T, LS180, and HepG2 cells (from top to bottom panel) after treatment were determined: the cells were transiently co-transfected with pGL3-*CYP3A4*-luc reporter or pGL4-*MDR1*-luc reporter plasmids with either empty vector pcDNA3 (EV) or pcDNA3-hPXR plasmids. pRL-TK Renilla luciferase (Rluc) control reporter vectors were used as a transfection control. After 24 h of transfection, cells were treated with vehicle control (0.1% DMSO), RIF, or PIP as indicated for another 24 h. *CYP3A4* and *MDR1* promoter activity is presented as relative luciferase units (RLU) after being normalized to the Renilla luciferase internal control. Data represent mean $\pm$ SEM from

three independent experiments. p values are indicated between the two groups compared by t test.

Figure 4. PIP induced dose-dependent hPXR-mediated *CYP3A4* promoter activity
CYP3A4 promoter activities were determined in A) HepG2-hPXR-*CYP3A4*-Luc cells (liver cell line with stably overexpressed hPXR and *CYP3A4*-luc reporter gene) and B) LS180-hPXR-*CYP3A4*-luc cells (intestinal cell line with stably overexpressed hPXR and *CYP3A4*-luc reporter gene) after treatment with increasing concentrations of RIF or PIP as indicated for 24 h. *CYP3A4* promoter activity is expressed as RLU, and drug concentration is expressed in a log scale. Data at each point represent mean $\pm$ SEM from quadruplicate measurements.

Figure 5. Knockdown of *hPXR* expression abolished PIP-induced *hPXR* transcriptional activity in LS180-*hPXR-CYP3A4*-luc cells

A) *CYP3A4* and B) *MDR1* mRNA expression and C) *CYP3A4* promoter activity were determined in LS180-*hPXR-CYP3A4*-luc cells after treatment: the cells were transiently transfected with *hPXR* siRNA (*hPXR* KD) or non-target control siRNA (NT), and 48 h after transfection, the cells were treated for another 24 h with vehicle control (0.1% DMSO), RIF, or PIP, as indicated. The induction of each gene mRNA level or *CYP3A4* promoter activity by the treatment was normalized as fold increase over the DMSO control. Data represent mean $\pm$ SEM from three independent experiments. p values are indicated between the two groups compared by t test.

Figure 6. Pharmacologic inhibition of hPXR activity abolished PIP-induced *CYP3A4* and *MDR1* expression in human primary hepatocytes and intestinal cells at promoter activation, mRNA or protein levels

A) *CYP3A4* promoter activity was determined in LS180-hPXR-*CYP3A4*-luc cells after treatment with RIF or PIP with or without 25 μ M ketoconazole (KET, a known hPXR inhibitor) as indicated for 24 h. The induction of *CYP3A4* promoter activity by the treatment was normalized as percentage increase over the DMSO control. B) *CYP3A4* mRNA (determined by real-time PCR) and C) *CYP3A4* protein levels (by Western blotting) in primary human hepatocytes (HH1890) after treatment with vehicle control (0.1% DMSO), RIF, PIP, or PIP plus 25 μ M KET as indicated for 48 h. Results are presented as fold increase over the DMSO control. In all graphs, data represent mean $\pm$ SEM from three independent experiments. p values are indicated between the two groups compared by t test. D) *MDR1* protein levels by Western blotting in LS174T and LS180 human intestinal cells treated as described in (C). For the Western blotting analysis, the numbers at the bottom indicate the relative intensity of the protein bands, with the DMSO treated sample set as "1.0".

Figure 7. Determination of direct interaction between PIP and hPXR

A) PIP's effects on recruitment of coactivator SRC-1 to hPXR in a mammalian two-hybrid assay. In this assay, two fusion proteins, VP16-hPXR (full-length hPXR fused to *Herpes simplex* virus VP16 activation domain) and GAL4DBD-SRC-1 (GAL4-DNA binding domain fused to SRC-1), were transiently expressed in HEK293T cells, and the interaction between hPXR and SRC-1 induced by PXR agonist was indicated by specific *GAL4*-luc reporter activity. The cells were treated with vehicle control (0.1% DMSO), RIF, or PIP as indicated. The *GAL4*-luc reporter activity is presented as RLU after being normalized to the Renilla luciferase internal control. B) PIP's effects on recruitment of SRC-1 to hPXR-LBD in an *in vitro* time-resolved fluorescence resonance transfer (TR-FRET) SRC-1 coactivator

recruitment assay. hPXR-LBD, Tb-anti-GST antibody, and fluorescein-labeled SRC-1–4 peptide were incubated together with or without test compounds, including TO (a known hPXR agonist), KET (a known hPXR inhibitor), and PIP. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm (emission from acceptor fluorophore) by the emission at 490 nm (emission from donor terbium) and used to indicate SRC-1's binding to hPXR-LBD. C) PIP bound to hPXR-LBD in a TR-FRET competitive binding assay. hPXR-LBD, Tb-anti-GST antibody, and a fluorescein-labeled hPXR ligand (referred to as a “tracer”) were incubated together in the presence of vehicle control (DMSO) or test compounds as indicated. The known hPXR agonists TO, SR12813 (SR), and clotrimazole (CLO) were used as positive controls. The TR-FRET ratio indicated the binding of tracer to hPXR-LBD, and a decrease of the TR-FRET ratio suggested the test compounds' binding to hPXR-LBD by outcompeting the tracer's binding. In all graphs, data represent mean $\pm$ SEM from three independent determinations: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the DMSO control by *t* test. D) Molecular docking of PIP bound to hPXR-LBD. *Left*, hPXR-LBD (PDB code 209I) is represented as cartoon and PIP as stick. *Right*, PXR-LBD residues (carbon atoms in yellow) potentially involved in the interaction with PIP (carbon atoms in white). Oxygen and nitrogen atoms are represented in red and blue, respectively. A hydrogen bond (pink dot line) is indicated between the amide NH group of Leu239 and one of the oxygen atoms in the five-member ring of PIP.

Figure 8. PIP induced hPXR transactivation of *CYP3A4* promoter activity *in vivo*

PIP's effects on the activity of mouse PXR (mPXR) and human PXR (hPXR) in A) Hepa-1 mouse liver cells and B) HepG2 human liver cells. The cells were transfected for 24 h with pGL3-*CYP3A4*-luc reporter plasmids and pRL-TK Renilla luciferase plasmids with pcDNA3-hPXR or pCMV6-mPXR plasmids as indicated. The transfected cells were treated for 24 h with vehicle control (0.1% DMSO), RIF, pregnenolone-16 α -carbonitrile (PCN), or PIP as indicated. CYP3A4 promoter reporter activity is presented as relative luciferase units (RLUs) normalized to the Renilla luciferase internal control. C, D, and E) PIP's effects on the human CYP3A4 promoter activity *in vivo*. C) The *CYP3A4*-luc reporter mice (n=5) or D) hPXR-*CYP3A4*-luc reporter mice (n=10) were generated by the hydrodynamic injection

of the pGL3-*CYP3A4*-luc reporter gene alone or in combination with pcDNA3-hPXR. The reporter mice were treated with vehicle control (1% DMSO), RIF, or PIP as indicated every 24 h for 2 days. The luciferase activity in these reporter mice was recorded by bioluminescence imaging 12 h before and 12 h after treatment, and the induction of *CYP3A4* promoter reporter activity was calculated as described in “Materials and Methods.” Each data point represents the induction of *CYP3A4* promoter reporter activity by the treatment in an individual mouse, and lines indicate the mean of induction in each treatment group. *p* values are indicated between the two groups compared by *t* test. E) The bioluminescence images of luciferase activity from 5 representative hPXR-*CYP3A4*-luc reporter mice before and after treatment are shown. Rainbow scale represents relative light units (photon flux per second and square centimeter), which positively reflects the luciferase activity.

Table 1

Potency of PIP on *CYP3A4* and *MDR1* promoter activity in human liver and intestinal cells with and without hPXR overexpression (half-maximal effective concentration, EC₅₀, μ M)

Cells were transiently co-transfected with pGL3-*CYP3A4*-luc reporter or pGL4-*MDR1*-luc reporter plasmids with pcDNA3 (empty vector) or pcDNA3-hPXR plasmids, pRL-TK Renilla luciferase vectors were used as a transfection control. After 24 h of transfection, cells were treated for 24 h with vehicle control (0.1% DMSO) or 11 increasing concentrations of RIF or PIP. Cells stably expressing hPXR and *CYP3A4*-Luc received the same treatment.

	Tissue type	<i>CYP3A4</i> promoter reporter				<i>MDR1</i> promoter reporter			
		without PXR transfection		with PXR transfection		without PXR transfection		with PXR transfection	
		RIF	PIP	RIF	PIP	RIF	PIP	RIF	PIP
LS174T	intestine	1.64	0.67	4.7	3.9	2.21	1.7	3.26	2.2
LS180	intestine	0.89	2.45	1.6	3.6	0.67	2.07	0.72	3.09
HepG2	liver	1.92	0.78	1.2	2.5	N.D. ^b	0.98	1.23	2.55
Stable cell line									
LS180-hPXR- <i>CYP3A4</i> -Luc	intestine	N.A. ^a	N.A.	0.59	2.2	N.A.	N.A.	N.A.	N.A.
HepG2-hPXR- <i>CYP3A4</i> -Luc	liver	N.A.	N.A.	1.6	~6.4 ^c	N.A.	N.A.	N.A.	N.A.

^a N.A., not available

^b N.D., not detectable

^c Estimated EC₅₀ value (half-maximal effective concentration) with the 95% confidence interval: 3.6–11.1 μ M