



Agonism of human pregnane X receptor by rilpivirine and etravirine: Comparison with first generation non-nucleoside reverse transcriptase inhibitors



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ABSTRACT

Rilpivirine and etravirine are second generation non-nucleoside reverse transcriptase inhibitors approved recently by the United States Food and Drug Administration for the treatment of human immunodeficiency virus-1 infection. Pregnane X receptor (PXR) is a member of the superfamily of nuclear receptors that regulate the expression of various genes controlling diverse biological functions. The present study investigated the effects of rilpivirine and etravirine on the activity of human PXR (hPXR), including the mode of activation, and compared them to those of efavirenz, nevirapine, and delavirdine, which are first generation non-nucleoside reverse transcriptase inhibitors. In transiently transfected HepG2 cells, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated human, mouse, and rat PXR. Results from mechanistic studies indicated that rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, bound to the ligand-binding domain of hPXR, as assessed by a transactivation assay and by a competitive ligand-binding assay using time-resolved fluorescence resonance energy transfer; triggered nuclear translocation of a green fluorescence protein-tagged hPXR, as visualized by confocal imaging; and recruited steroid receptor coactivator-1 (SRC-1), SRC-2, and SRC-3 to hPXR, as demonstrated by mammalian two-hybrid assays. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, increased hPXR target gene (*CYP3A4*) expression in primary cultures of human hepatocytes. In summary, select non-nucleoside reverse transcriptase inhibitors activated human and rodent PXR. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, were identified as agonists of hPXR, as assessed in mechanistic experiments, and inducers of *CYP3A4*, as determined in primary cultures of human hepatocytes.

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1. Introduction

Human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) still remains one of the leading causes of mortality in humans, claiming an estimated 1.8 million lives annually [1]. Unfortunately, a cure for this disease has not been found yet; however, the current therapeutic options are able to suppress viral load and increase Cluster of Differentiation 4 (CD4) counts in HIV/AIDS patients [2]. An important class of drugs that is routinely used for the treatment of HIV-1 infection, along with other anti-retroviral drugs, is the non-nucleoside reverse transcriptase inhibitors [3]. Efavirenz, nevirapine, and delavirdine

(Fig. 1) are first generation non-nucleoside reverse transcriptase inhibitors approved for clinical use by the United States Food and Drug Administration (U.S. FDA). The most recent additions to this drug class are rilpivirine and etravirine (Fig. 1), which were approved for clinical use by the U.S. FDA in May 2011 [4] and January 2008 [5], respectively. Rilpivirine and etravirine, like other non-nucleoside reverse transcriptase inhibitors, inhibit HIV-1 reverse transcriptase enzyme allosterically [5]. They are potent second generation non-nucleoside reverse transcriptase inhibitors. A therapeutic option is to use rilpivirine in combination with other anti-retrovirals. Recently, the U.S. FDA approved a rilpivirine-based triple drug combination with the brand names of Complera[®] (containing 25 mg rilpivirine, 300 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the U.S.A.) and Eviplera[®] (containing 25 mg rilpivirine, 245 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the European Union) for the treatment of HIV-1 infection [4]. Like rilpivirine, etravirine is

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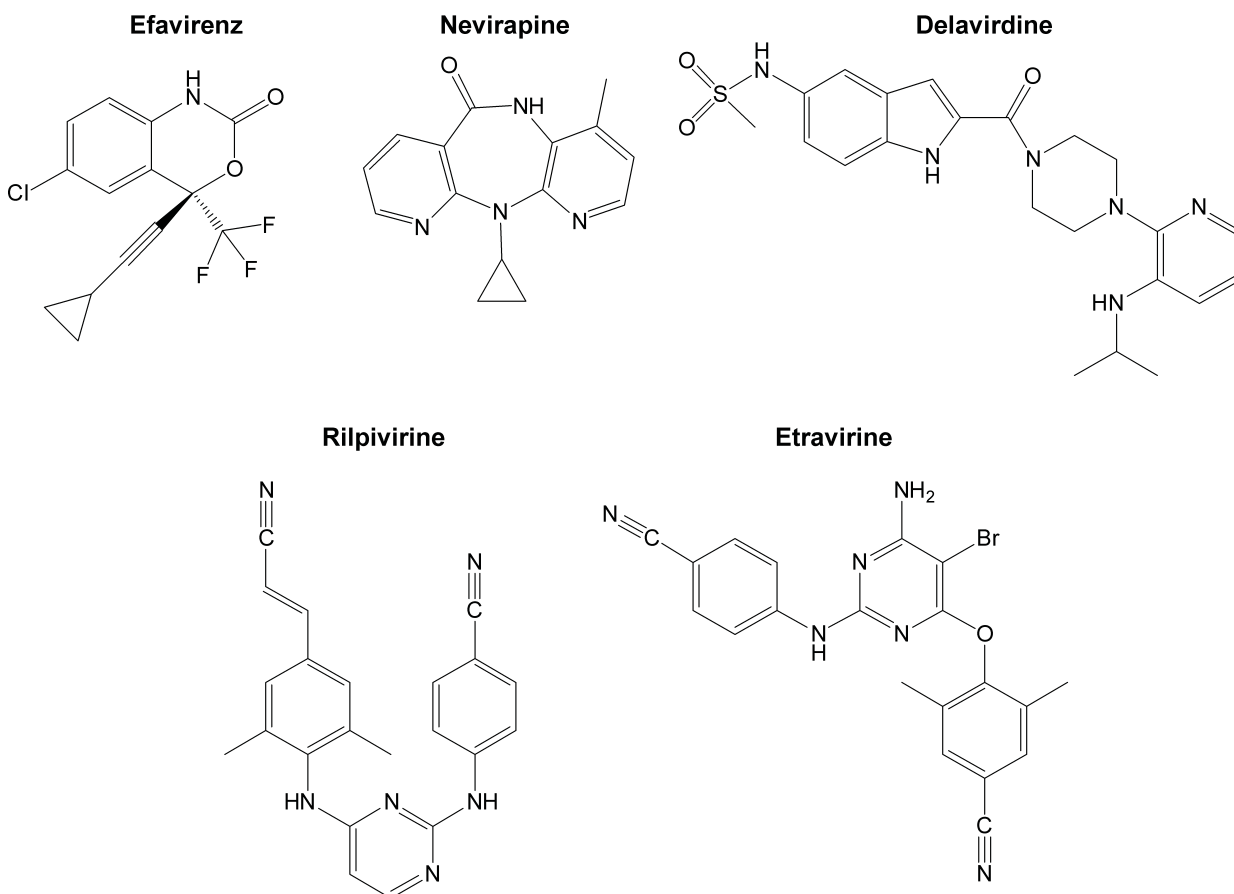


Fig. 1. Chemical structures of efavirenz, nevirapine, delavirdine, rilpivirine, and etravirine.

reported to be effective in both non-nucleoside reverse transcriptase inhibitor-naïve and non-nucleoside reverse transcriptase inhibitor-resistant strains of HIV-1, and it is used in combination with other anti-retrovirals [6].

Nuclear receptors represent a broad group of ligand-activated transcriptional factors and are categorized as endocrine receptors, adopted orphan receptors, and orphan receptors [7]. They play a key regulatory role in various biological processes, ranging from development and differentiation to metabolic homeostasis. Pregnane X receptor (PXR; designated as NR1I2 according to the nomenclature of nuclear receptors) is a member of the superfamily of nuclear receptors [8]. It acts as xenobiotic sensors and regulates expression of genes involved in drug transport, such as *ABCB1* (P-glycoprotein) [9] and *ABCC2* (MRP2) [10], and those involved in drug metabolism, such as *CYP3A4* [11–13]. Other than playing a major role in many of the pharmacokinetic drug interactions [14], PXR has been linked to the development of bile acid toxicity [15], drug-induced hepatotoxicity [16], and anticancer drug resistance [17]. It has also been proposed as a therapeutic target for various disease states, including cholestatic liver disease [18], inflammatory bowel disease [19], and dyslipidemia [20].

Among the first generation non-nucleoside reverse transcriptase inhibitors, efavirenz has been reported to activate human PXR (hPXR) [21–23]. By comparison, nevirapine only weakly activates hPXR, and this occurs not at a therapeutic concentration (7.5 μM) [23], but at a suprapharmacological concentration (50 μM) [22]. However, the mechanism on how these drugs activate hPXR is still not known. In a recent study, treatment of primary cultures of human hepatocytes with etravirine (10 μM) increased *CYP3A4* mRNA expression [24]. This increase in *CYP3A4* mRNA expression by etravirine was attenuated by pretreatment with sulforaphane

[24], which is an in vitro antagonist of hPXR [25]. However, there is no direct experimental evidence as to whether the second generation non-nucleoside reverse transcriptase inhibitors (i.e. rilpivirine and etravirine) affect the transcriptional activity of PXR or how they may activate hPXR.

In the present study, we systematically evaluated the effects of rilpivirine and etravirine on the activity of hPXR, including their mode of activation, and compared them to those of first generation non-nucleoside reverse transcriptase inhibitors; i.e., efavirenz, nevirapine, and delavirdine. Given that PXR is known to show pronounced species-dependent activation by drugs and other chemicals [26], we determined whether rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors activate mouse PXR (mPXR) and rat PXR (rPXR). Our experimental approaches involved cell-based luciferase reporter gene assays, competitive ligand binding assay by time-resolved fluorescence resonance energy transfer (TR-FRET), in vitro nuclear translocation analysis by confocal microscopy, mammalian two-hybrid assay, and hPXR target gene (*CYP3A4*) expression analysis in human hepatocytes in culture. The results are discussed in the context of drug-dependent activation of human and rodent PXR by select non-nucleoside reverse transcriptase inhibitors and their mode of activation.

2. Materials and methods

2.1. Chemicals and reagents

Rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Dextran, Triton X-100 (Union Carbide Corporation,

Houston, TX, U.S.A.), rifampin, pregnenolone 16 α -carbonitrile (PCN), 4-pregnane-20 β -ol-3-one, sodium phenobarbital, and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, MO, U.S.A.). Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl) ethenyl-1,1-bisphosphonate (SR12813) was obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, U.S.A.). Testosterone and 6 β -hydroxytestosterone were purchased from Steraloids (Newport, RI, U.S.A.). Cytotoxicity Detection Kit (LDH) and FuGENE 6 transfection reagent were purchased from Roche Diagnostics (Laval, QC, Canada). Matrigel basement membrane matrix was obtained from BD Biosciences (Mississauga, ON, Canada). Hanks' balanced salt solution (HBSS), PureLink RNA Mini Kit, PicoGreen Double-Stranded DNA Quantification Kit, LanthaScreen TR-FRET PXR Competitive Binding Assay, and ProLong[®] Gold antifade reagent with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were procured from Life Technologies (Burlington, ON, Canada), and the Dual-Luciferase Reporter Assay System was from Promega (Madison, WI, U.S.A.). Charcoal-stripped fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.) was bought from Thermo Fisher Scientific (Nepean, ON, Canada), and all other reagents to culture HepG2 cells were obtained from Life Technologies (Burlington, ON, Canada). Cryopreserved hepatocyte thawing medium, hepatocyte plating medium, hepatocyte maintenance medium, and various medium supplements were provided by Triangle Research Labs, LLC (Research Triangle Park, NC, U.S.A.).

2.2. Plasmids

pCMV6-XL4-hPXR, pCMV6-entry-mPXR, pCMV6-AC-rPXR, pCMV6-AC-GFP-hPXR, pCMV6-XL4, pCMV6-AC, and pCMV6-entry were obtained from OriGene Technologies (Rockville, MD, U.S.A.). pFR-luc reporter were purchased from Agilent Technologies (Santa Clara, CA, U.S.A.). The internal control *Renilla reniformis* luciferase pGL4.74 [hRluc/TK] plasmid was procured from Promega (Madison, WI, U.S.A.). The pGL3-basic-CYP3A4-XREM-luc reporter construct was prepared according to a published method [27]. pVP16-hPXR-LBD, pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID were constructed as detailed previously [28,29]. The pVP16 and pM empty vectors were purchased from Clontech (Mountain View, CA, U.S.A.). The plasmid constructs were sequenced by the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC, Canada) and their sequence identity was confirmed by comparing with published sequence.

2.3. HepG2 cells

HepG2 human hepatocellular carcinoma cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in T-75 culture flasks in minimum essential medium supplemented with heat-inactivated fetal bovine serum (10%, v/v), penicillin G (100 U/ml), streptomycin (100 μ g/ml), and L-glutamine (2 mM). Cells were maintained at 37 °C in a humidified incubator with 95% air and 5% CO₂. Culture medium was changed every 3 days, and cells were sub-cultured weekly.

2.4. Determination of cytotoxicity of non-nucleoside reverse transcriptase inhibitors

Cultured HepG2 cells were plated in 24-well plates at a density of 100,000 cells/well. At 48 h post-plating, the cells were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 0.1–25 μ M), dextran (1%, w/v; negative control), or Triton X-100 (0.1%, v/v; positive control) in a volume of 0.5 ml culture medium. At the end of the 24 h treatment period,

culture supernatant was collected and cells were lysed in 0.5 ml lysis buffer containing 2%, v/v Triton X-100 and 20 mM EDTA in phosphate-buffered saline (pH 7.4). LDH levels were quantified in the culture supernatant and cell lysate using the Roche Cytotoxicity Detection Kit (LDH, Roche Diagnostics, Laval, QC, Canada), as described earlier [28]. Results are expressed as the amount of LDH in the culture supernatant as a percentage of the total cellular LDH; i.e., sum of LDH in the culture medium and cell lysate. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

2.5. Transient transfection and reporter gene assays

To investigate the effect of rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors on the activity of hPXR, mPXR, and rPXR, HepG2 cells were plated onto 24-well microplates at a density of 100,000 cells/well in a volume of 0.5 ml of culture medium. At 24 h post-plating, cultured cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 μ l/ μ g of DNA), serum-free Opti-MEM (20 μ l/well), pGL4.74 [hRluc/TK] internal control vector (5 ng/well), a reporter construct (50 ng/well), and either a receptor expression plasmid or the corresponding empty vector (50 ng/well) for 24 h, as detailed in the appropriate figure legend. Transfected cells were treated with 0.5 ml of supplemented culture medium containing DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), a negative control, or a positive control at the concentrations indicated in the appropriate figure legend. At the end of the 24 h treatment period, HepG2 cells were lysed. Firefly luciferase and *R. reniformis* luciferase activities were quantified using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.). Luminescence was measured in a GloMax 96 microplate luminometer (Promega, Madison, WI, U.S.A.). Normalized luciferase activity was determined by calculating the ratio of firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

2.6. hPXR-LBD transactivation assay

The hPXR-LBD transactivation assay was performed as described previously [30]. Briefly, cultured HepG2 cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 μ l/ μ g of DNA), serum-free Opti-MEM (20 μ l/well), pGL4.74 [hRluc/TK] internal control vector (5 ng/well), pFR-luc reporter (100 ng/well), and either the pM-hPXR-LBD (Met-107 to Ser-434) expression plasmid or the pM empty vector (100 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented culture medium containing DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 μ M), PCN (10 μ M; negative control for hPXR [26]), rifampin (10 μ M; positive control for hPXR [26]), or SR12813 (10 μ M; positive control for hPXR [26]) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were quantified as described above. Each experiment was performed in triplicate and a total of five independent experiments were conducted.

2.7. Competitive ligand-binding assay

A LanthaScreen hPXR competitive binding assay based on the principle of time-resolved fluorescence resonance energy transfer

(TR-FRET) was conducted as described previously [30]. A non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 μ M), PCN (10 μ M; negative control), phenobarbital (1000 μ M; positive control), SR12813 (10 μ M; positive control), or DMSO (1%, v/v; vehicle control) was incubated with hPXR ligand-binding domain (5 nM), Fluoromone PXR Green (40 nM), terbium-labeled anti-glutathione transferase (10 nM), and dithiothreitol (0.05 mM) at ambient temperature for 1 h in the dark. TR-FRET was quantified using a PHERAstar FS microplate reader (BMG Labtech GmbH, Allmendgruen, Germany) with the following settings: excitation wavelength, 337 nm; emission wavelengths 490 nm (terbium emission) and 520 nm (fluorescein emission); delay time, 100 μ s; and integration time, 200 μ s. TR-FRET ratio, background TR-FRET ratio, and net TR-FRET ratio were determined as detailed previously [30]. Data are expressed as percentage of the net TR-FRET ratio in the DMSO-treated control groups. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

2.8. *In vitro* nuclear translocation of green fluorescence protein-tagged hPXR

HepG2 cells were cultured on poly-d-lysine-coated glass cover slips in 24-well plates at a density of 25,000 cells/well. At 24 h post-plating, cultured HepG2 cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 μ l/ μ g of DNA), serum-free Opti-MEM (20 μ l/well), and pCMV6-AC-GFP-hPXR (green fluorescence protein-tagged hPXR; 50 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented medium containing DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 μ M), PCN (10 μ M; negative control for hPXR) [26], or rifampin (10 μ M; positive control for hPXR [26]) for 24 h. Subsequently, the cells were fixed with *p*-formaldehyde (4%, v/v) for 20 min on ice. After three washes in phosphate-buffered saline (pH 7.4), the cover slips containing cells were mounted on glass slides using ProLong[®] Gold Antifade Reagent with DAPI. The cells were visualized under an Olympus FV10i confocal microscope interfaced with the FluoView 10i software (FV10i version 1.2c) and analyzed using the NIH, ImageJ software (National Institutes of Health, U.S.A.).

2.9. Mammalian two-hybrid assay

Recruitment of steroid receptor coactivator-1 (SRC-1), steroid receptor coactivator-2 (SRC-2), and steroid receptor coactivator-3 (SRC-3) to hPXR was assessed by a mammalian two-hybrid assay [29]. Briefly, at 24 h post-plating, cultured HepG2 cells were transfected with pGL4.74 [*hRluc*/TK] internal control vector (10 ng/well), pFR-luc reporter plasmid (100 ng/well), a coactivator expression plasmid (10 ng/well), and either pVP16-hPXR-LBD expression plasmid or the corresponding pVP16 empty vector (40 ng/well). The coactivator expression plasmids were pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID. At 24 h post-transfection, cells were treated with supplemented culture medium containing DMSO (0.1%, v/v; vehicle control),

a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 μ M), PCN (10 μ M; negative control for hPXR), rifampin (10 μ M; positive control for hPXR), or SR12813 (10 μ M; positive control for hPXR) for 24 h. Luciferase activity was measured and normalized as described under *Transient Transfection and Reporter Gene Assays*. Each experiment was performed in triplicate and a total of five independent experiments were conducted.

2.10. Culture and treatment of human hepatocytes

The demographics of the donors (GC4008 and HUM4021) are listed in Table 1. Cryopreserved human hepatocytes (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) were thawed and plated according to protocols listed at <http://triangleresearchlabs.net/products/cryopreserved-hepatocytes/>. Cell viability was 88% and 90% for hepatocyte samples GC4008 and HUM4021, respectively, as assessed by trypan blue exclusion [31]. Hepatocytes were plated and cultured at a density of 3.5×10^5 cells per well in a BioCoat 24-well plate at 37 °C in a humidified incubator with 95% air and 5% CO₂. They were allowed to attach for 4 to 6 h. Subsequently, the medium was aspirated and 0.5 ml of fresh hepatocyte maintenance medium (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) and Matrigel (0.25 mg/ml) were added to each well. Plates were placed in the incubator, and this was followed by a change in the culture medium on Day 2 and drug treatment was initiated on Day 3. Cultured hepatocytes were treated with hepatocyte maintenance medium containing DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 μ M), or rifampin (10 μ M; positive control for CYP3A4 expression [32]), as described in the figure legend. The drug-containing culture medium was replaced every 24 h and the treatment was continued for 48 h for hepatocyte sample GC4008 and 72 h for hepatocyte sample HUM4021.

2.11. Isolation of total RNA, reverse transcription, and real-time PCR analysis

RNA isolation and reverse transcription were conducted as described previously [28]. Briefly, cultured human hepatocytes were lysed and total cellular RNA was isolated using PureLink RNA Mini Kit (Life Technologies, Burlington, ON, Canada). Total RNA was reverse-transcribed using Superscript II reverse transcriptase, and total cDNA was quantified using PicoGreen Double-Stranded DNA Quantitation Kit (Life Technologies, Burlington, ON, Canada).

The sequences of the primers used to amplify CYP3A4 cDNA [33] and HPRT1 cDNA [34] were specified in our previous publication [28]. The sequence of the forward primer to amplify the cDNA of 18s rRNA was 5'-CTT-TGG-TCG-CTC-GCT-CCT-C-3', whereas the sequence of the reverse primer was 5'-CTG-ACC-GGG-TTG-GTT-TTG-AT-3'. The primers were synthesized and their specificity was verified by sequencing the purified amplicons (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.).

CYP3A4 cDNA and HPRT1 cDNA were amplified by real-time PCR in a LightCycler (Roche Diagnostics, Laval, QC, Canada) according to conditions described previously [28]. To amplify the cDNA of 18s rRNA, each PCR reaction contained 1 ng total

Table 1
Demographics of human hepatocytes donors.

Donor identification	Race	Sex	Age (years)	Reported BMI ^a	Smoking status	Alcohol use	Drug use	Serological data
GC4008	Caucasian	Male	69	24.7	Stopped 24 years ago	No	No	All negative
HUM4021	Caucasian	Male	13	18.0	Non-smoker	No	No	All negative

^a Body Mass Index.

cDNA, 1 U Platinum *Taq* DNA polymerase in 1 × PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 3 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate, 0.25 mg/ml bovine serum albumin, 0.2 μM forward and reverse primers, and 1:30,000 SYBR Green I. The PCR cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 s, 62 °C for 6 s, and 72 °C for 10 s. To construct calibration curves (cross point versus log cDNA copies), CYP3A4, HPRT1, and 18s rRNA amplicons were generated from human liver QUICK-Clone cDNA (Clontech, Mountain View, CA, U.S.A.) and then purified and quantified as described previously [35]. CYP3A4 mRNA expression was normalized to that of housekeeping genes (HPRT1 and 18s rRNA).

2.12. Testosterone 6β-hydroxylation assay

Testosterone 6β-hydroxylation assay was conducted in cultured human hepatocytes as described previously [28]. Briefly, cultured human hepatocytes were first washed twice with HBSS and subsequently incubated with testosterone (200 μM final concentration) for 15 min at 37 °C. The incubation samples were then transferred to a 24-well plate and stored at -80 °C until analysis. High-performance liquid chromatographic (HPLC) analyses of 6β-hydroxytestosterone was performed as described previously [35], but with several modifications. In the present study, 4-pregnen-20β-ol-3-one (in acetonitrile) was selected as the internal standard based on its structural similarity to the

analyte (6β-hydroxytestosterone), and 10 μl of the internal standard (2000 pmol) was added to 150 μl of the hepatocytes incubation sample. A 50 μl of that sample was injected onto the HPLC. The detector response of the internal standard was used to normalize the detector response of 6β-hydroxytestosterone. The HPLC system (Waters, Milford, MA, U.S.A.) was equipped with a Waters model 1525 binary pump, Waters model 717 plus autosampler, and Waters model 2487 dual wavelength absorbance detector. The chromatographic separation was carried out on a Waters SymmetryShield C18 column (150 mm × 4.6 mm; i.d., 5 μm) linked to a Phenomenex (Torrance, CA, U.S.A.) SecurityGuard cartridge (4.0 mm × 3.0 mm; i.d., 5 μm). The mobile phases consisted of water containing 0.5% (v/v) acetic acid (A) and methanol containing 0.5% (v/v) acetic acid (B), and the optimized flow rate was 0.8 ml/min. A gradient method was used to separate 6β-hydroxytestosterone from the internal standard and the matrix components with the following solvent conditions: linear gradient from 55 to 90% B (0–12 min), isocratic at 90% B (12–15 min), linear gradient from 90 to 55% B (15–16 min), and isocratic at 55% B (16–25 min). The detector response for 6β-hydroxytestosterone and internal standard was recorded at a wavelength of 242 nm. Data acquisition and processing were conducted using the Waters Breeze software (version 3.20). A calibration curve was constructed with the authentic 6β-hydroxytestosterone standard (100–12,500 pmol; diluted in HBSS) for each experiment.

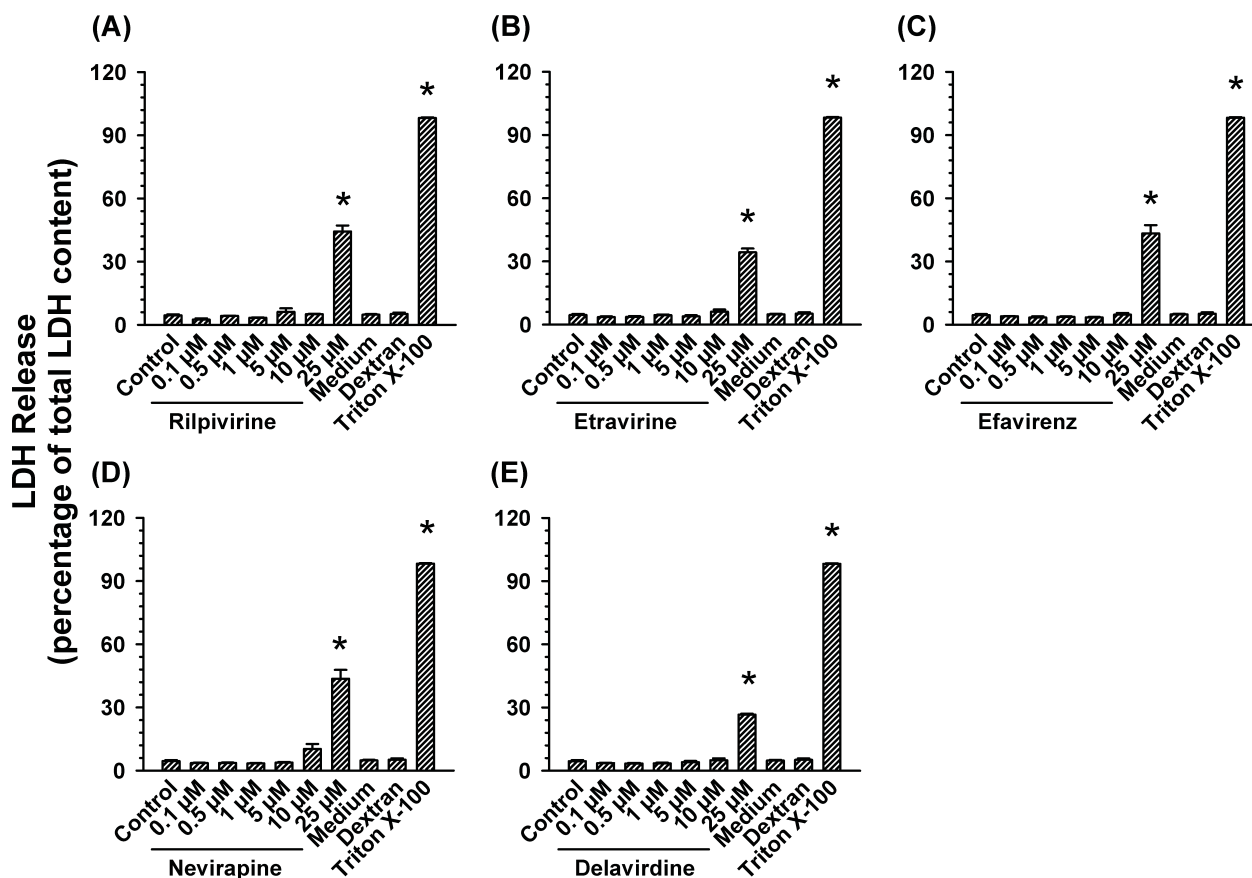


Fig. 2. Effect of non-nucleoside reverse transcriptase inhibitors on LDH release in HepG2 cells. Cultured cells were treated with varying concentrations (0.1–25 μM) of (A) rilpivirine, (B) etravirine, (C) efavirenz, (D) nevirapine, or (E) delavirdine for 24 h. The control treatments were DMSO (0.1%, v/v; vehicle for the non-nucleoside reverse transcriptase inhibitors), culture medium (vehicle for dextran and Triton-X), dextran (1%, w/v; negative control), and Triton X-100 (0.1%, v/v; positive control). LDH levels were measured in the culture medium and cell lysates, and the amount of LDH in the culture medium is expressed as a percentage of the total cellular LDH. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M percentage of total LDH content for three independent experiments. *, significantly different from the control group treated with DMSO ($P < 0.05$).

2.13. Data analyses

The half-maximal effective concentration (EC_{50}) and maximal response (E_{max}) for hPXR activation were calculated using the following equation in GraphPad Prism[®] 5.00 (GraphPad Software, Inc., La Jolla, CA, U.S.A.): $E = E_0 + (E_{max} - E_0)/(1 + 10^{(\log EC_{50} - C)})$, where E is effect (i.e. fold increase over vehicle-treated control group), E_0 is basal effect, and C is drug concentration. Data analyses were performed by one-way or two-way analysis of variance. Where appropriate, two-way analysis of variance was followed by the Student–Newman–Keuls multiple comparison test (SigmaPlot 11.0; Systat Software, Inc., San Jose, CA, U.S.A.). The level of statistical significance was set a priori at $P < 0.05$.

3. Results

3.1. Cytotoxicity assessment of rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors

To determine non-cytotoxic concentrations of the selected non-nucleoside reverse transcriptase inhibitors for investigation in the subsequent in vitro cell-based reporter gene assays, release of intracellular LDH into the culture medium was employed as a cytotoxicity marker [31]. LDH release was quantified in cultured HepG2 cells treated with increasing concentrations (0.1–25 μ M) of rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine. LDH release did not increase at drug concentrations of up to 10 μ M for rilpivirine (Fig. 2A), etravirine (Fig. 2B), efavirenz (Fig. 2C), nevirapine (Fig. 2D), or delavirdine (Fig. 2E). Expected results were obtained for the positive control (0.1%, v/v Triton X-100; Fig. 2A–E) and the negative control (1%, w/v dextran; Fig. 2A–E). In all subsequent experiments, the concentration of the non-nucleoside reverse transcriptase inhibitors did not exceed 10 μ M.

3.2. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activate hPXR

The effect of rilpivirine and etravirine on the activity of hPXR was assessed in cell-based reporter gene assays and compared with an equimolar concentration (5 μ M) of efavirenz, nevirapine, and delavirdine in hPXR-transfected HepG2 cells. As shown in Fig. 3A, rilpivirine and etravirine increased hPXR activity by 11- and 17-fold, respectively, over the vehicle-treated control group. Among the other non-nucleoside reverse transcriptase inhibitors tested, efavirenz (7-fold), but not nevirapine or delavirdine, activated hPXR. Rifampin (10 μ M), a positive control for hPXR activation [13], produced a 20-fold increase over the vehicle-treated control group, whereas PCN (10 μ M), a negative control [26], did not have an effect.

3.3. Concentration-response relationship in the effect of non-nucleoside reverse transcriptase inhibitors on hPXR activity

Concentration-response experiments were performed to further characterize the activation of hPXR by rilpivirine, etravirine, and efavirenz. As shown in Fig. 3B, rilpivirine, at concentrations of 0.05 and 0.1 μ M, had no effect on the activity of hPXR, whereas it activated hPXR at greater concentrations (0.5–10 μ M), with a maximal response of 11-fold produced at 5 μ M. Etravirine had a profile similar to rilpivirine as 0.05 and 0.1 μ M had no effect on hPXR activity, whereas greater concentrations (0.5–10 μ M) of this drug activated hPXR, with maximal response (17-fold) produced at 5 μ M. Efavirenz at a concentration of 0.5 or 1 μ M had no effect on hPXR activity, whereas at 5 and 10 μ M, it increased the activity by 7- and 10-fold, respectively. In contrast to rilpivirine, etravirine and efavirenz, neither nevirapine nor

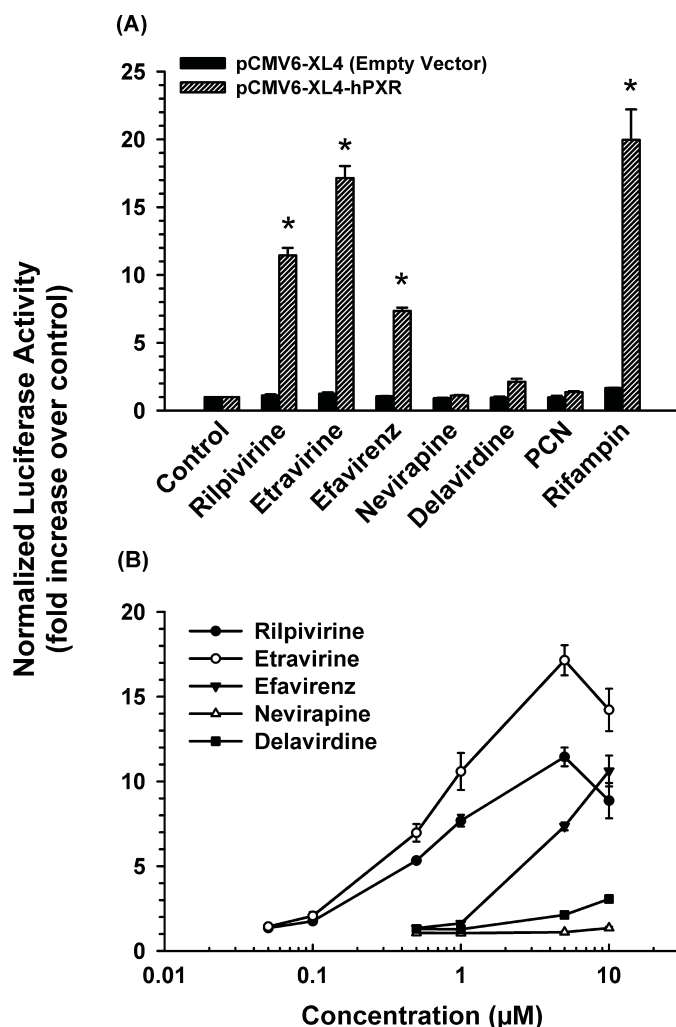


Fig. 3. Comparative effect of rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine on the activity of hPXR. Cultured HepG2 cells were transfected with pGL3-basic-CYP3A4-XREM-luc, pGL4.74 [hRtluc/TK], and either pCMV6-XL4-hPXR or pCMV6-XL4 (empty vector) for 24 h. (A) For the single concentration experiment, transfected cells were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), PCN (10 μ M; negative control), or rifampin (10 μ M; positive control) for 24 h. (B) Concentration-response curves for hPXR activation by rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors were generated by treating transfected cells with DMSO (0.1%, v/v; vehicle control) or a non-nucleoside reverse transcriptase inhibitor at 0.05–10 μ M for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean \pm S.E.M. for three independent experiments. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the hPXR expression plasmid ($P < 0.05$).

delavirdine activated hPXR at concentrations up to and including 10 μ M (Fig. 3B).

The calculated EC_{50} and E_{max} values (mean \pm S.E.M.) for rilpivirine-mediated hPXR activation were 0.4 ± 0.2 μ M and 11 ± 1 fold, respectively, whereas those for hPXR activation by etravirine were 0.6 ± 0.1 μ M and 17 ± 1 fold, respectively. These values are comparable with the literature EC_{50} and E_{max} values for hPXR activation by rifampin [36], which is a prototypic hPXR agonist [13]. It was not possible to calculate the EC_{50} and E_{max} values for efavirenz because maximal effect was not achieved in our dose-response experiment (Fig. 3B).

3.4. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activate mPXR and rPXR

Pronounced species-dependent chemical activation of PXR has been reported [26]. Therefore, we determined the effect of the non-nucleoside reverse transcriptase inhibitors (Fig. 3A and B) on the activity of mPXR and rPXR. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated mPXR (Fig. 4A) and rPXR (Fig. 4B). This pattern of response is similar to the profile shown for hPXR (Fig. 3A). PCN (10 μ M), which is a positive control for ligand activation of mPXR and rPXR [37], activated both mPXR and rPXR, whereas rifampin (10 μ M), which is a negative control [37], had no effect (Fig. 4A and B).

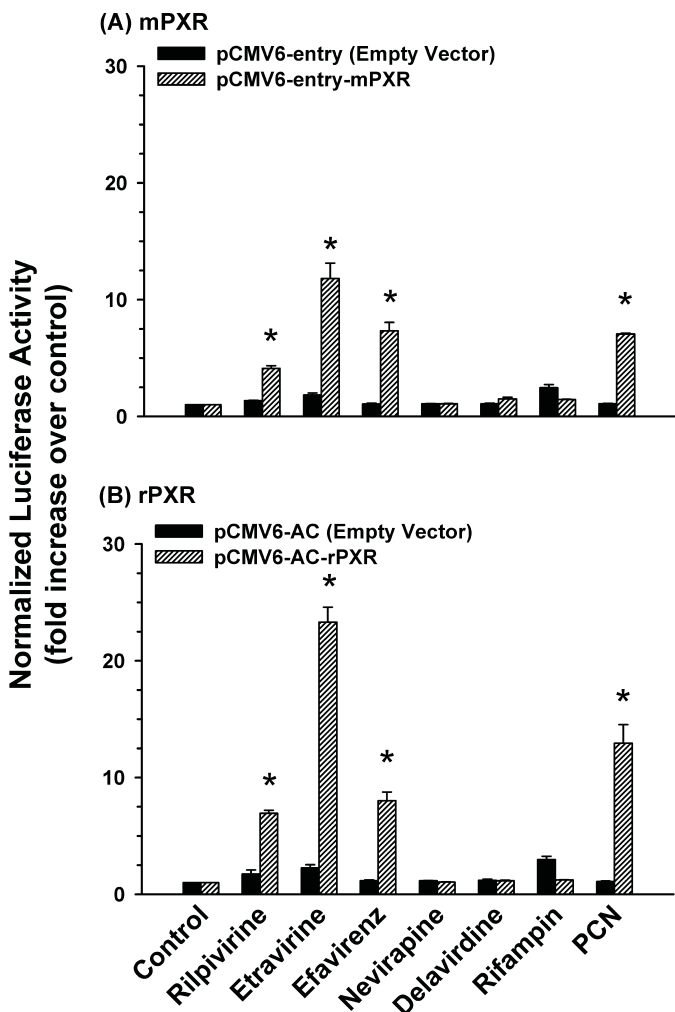


Fig. 4. Activation of mPXR and rPXR by rilpivirine, etravirine, and efavirenz. Cultured HepG2 cells were transfected with (A) pGL3-basic-CYP3A4-XREM-luc, pGL4.74 [hRluc/TK], and either pCMV6-entry-mPXR, or pCMV6-entry (empty vector); or (B) pGL3-basic-CYP3A4-XREM-luc, pGL4.74 [hRluc/TK], and either pCMV6-AC-rPXR or pCMV6-AC (empty vector) for 24 h. Transfected cells were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), rifampin (10 μ M; negative control), or PCN (10 μ M; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean \pm S.E.M. for three independent experiments. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the mPXR or rPXR expression plasmid ($P < 0.05$).

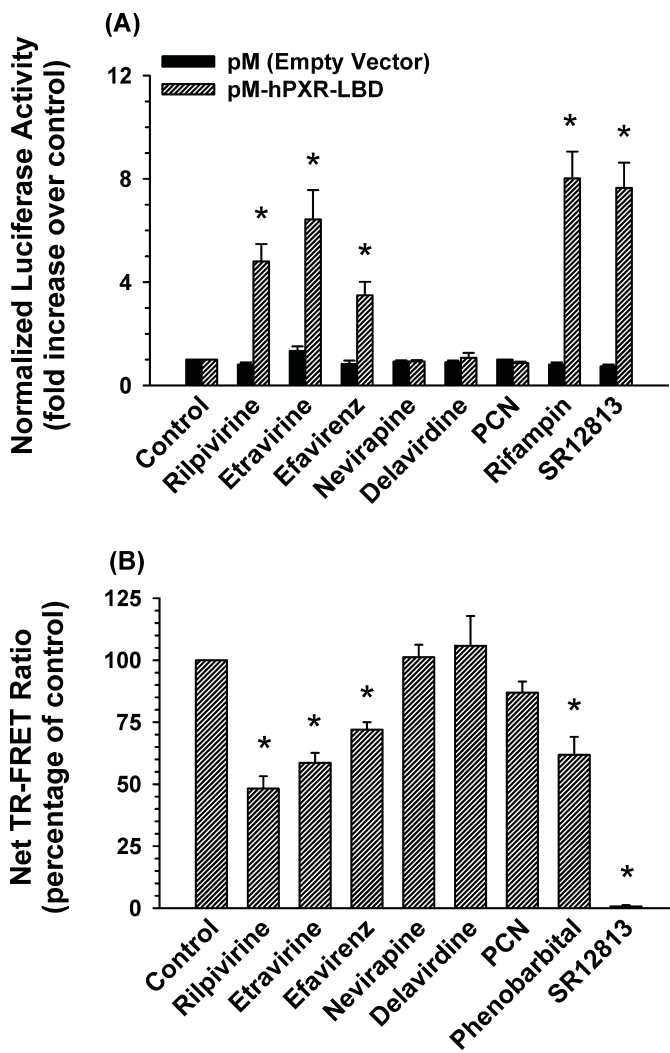


Fig. 5. (A) Effect of rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine on transactivation of the ligand-binding domain of hPXR. Cultured HepG2 cells were transfected with pGL4.74 [hRluc/TK], pFR-luc, and either pM-hPXR-LBD or the pM empty vector for 24 h. Transfected cells were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), PCN (10 μ M; negative control), rifampin (10 μ M; positive control), or SR12813 (10 μ M; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean \pm S.E.M. for five independent experiments. *, significantly different from the same treatment group transfected with the pM empty vector and from the vehicle-treated control cells transfected with pM-hPXR-LBD ($P < 0.05$). (B) TR-FRET analysis of the binding of rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors to the ligand-binding domain of hPXR. A non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), PCN (10 μ M; negative control), phenobarbital (1000 μ M; positive control), SR12813 (10 μ M; positive control), or DMSO (0.1%, v/v; vehicle control) was incubated with human PXR ligand-binding domain (5 nM), Fluormone PXR Green (40 nM), terbium-labeled anti-glutathione transferase (10 nM), and dithiothreitol (0.05 mM). Net TR-FRET ratio was determined by subtracting the background TR-FRET ratio from the TR-FRET ratio. Results are expressed as a percentage of net TR-FRET ratio in the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean \pm S.E.M. for three independent experiments. *, significantly different from the vehicle-treated control group ($P < 0.05$).

3.5. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, transactivate the ligand-binding domain of hPXR

To investigate whether rilpivirine, etravirine, and efavirenz act as agonists of hPXR, a series of mechanistic experiments were performed, starting with a cell-based reporter gene assay whereby HepG2 cells were transfected with a plasmid (i.e. pM-hPXR-LBD; Met-107 to Ser-434) expressing the ligand-binding domain of hPXR. As shown in Fig. 5A, rilpivirine, etravirine, and efavirenz produced a 4.8-, 6.4-, and 3.5-fold increase in the luciferase activity, respectively. As expected, nevirapine and delavirdine had no effect on the transactivation of hPXR-LBD, consistent with data shown in a reporter gene assay using the full-length hPXR (Fig. 3A and B). The positive controls (10 μ M rifampin and 10 μ M SR12813) and the negative control (10 μ M PCN) produced the expected results (Fig. 5A).

3.6. TR-FRET analyses indicate binding of rilpivirine, etravirine, and efavirenz to the ligand-binding domain of hPXR

To corroborate the hPXR-LBD transactivation data (Fig. 5A), a TR-FRET competitive ligand-binding assay was conducted. As shown in Fig. 5B, rilpivirine, etravirine, and efavirenz (each at 5 μ M) decreased the net TR-FRET emission ratio to 48%, 58%, and 72% of the control level, respectively, whereas nevirapine and delavirdine had no effect. Analyses with positive controls indicate that phenobarbital (1000 μ M) produced an effect (61% of the control level) comparable to that of rilpivirine, etravirine, and efavirenz, whereas SR12813 (10 μ M) was the most effective (<1% of the control level). The negative control (10 μ M PCN) yielded the expected result.

3.7. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, stimulate nuclear translocation of GFP-tagged hPXR

In its inactive form, hPXR resides mainly in the cytoplasmic compartment of the cells, and upon activation by an agonist, the

agonist-activated receptor complex translocates from the cytoplasm into the nucleus [38]. According to confocal microscopy (Fig. 6), the GFP-tagged hPXR translocated to the nucleus in HepG2 cells treated with rilpivirine, etravirine, or efavirenz, as evident by the predominant localization of green fluorescence in the nucleus. A similar finding was obtained in cells treated with the positive control rifampin, in agreement with published data [39]. In contrast, the fluorescence was predominantly in the cytoplasm in GFP-hPXR-transfected cells treated with nevirapine, delavirdine, or PCN (negative control).

3.8. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, promote recruitment of coactivators to hPXR

To assess whether rilpivirine, etravirine, and efavirenz recruit coactivators to hPXR, mammalian two-hybrid assays were conducted. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, increased the luciferase reporter activity in HepG2 cells transfected with pVP16-hPXR-LBD and a coactivator expression plasmid for SRC-1 (Fig. 7A), SRC-2 (Fig. 7B), or SRC-3 (Fig. 7C). Experiments with the positive controls (10 μ M rifampin and 10 μ M SR12813) and negative control (10 μ M PCN) yielded the expected results (Fig. 7A–C).

3.9. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, increase the expression of a hPXR target gene (CYP3A4) in cultured human hepatocytes

Primary cultures of human hepatocytes were used to compare the effect of first and second generation non-nucleoside reverse transcriptase inhibitors on the induction of CYP3A4, which is a known target gene of hPXR [11–13]. In sample GC4008 (Fig. 8A), rilpivirine, etravirine, and efavirenz increased CYP3A4 mRNA expression by 5.7-, 4.3-, and 1.8-fold, respectively, whereas nevirapine and delavirdine had no effect. A similar trend was observed in sample HUM4021 where rilpivirine, etravirine, and efavirenz increased CYP3A4 mRNA expression by 3.1-, 6.3-, and

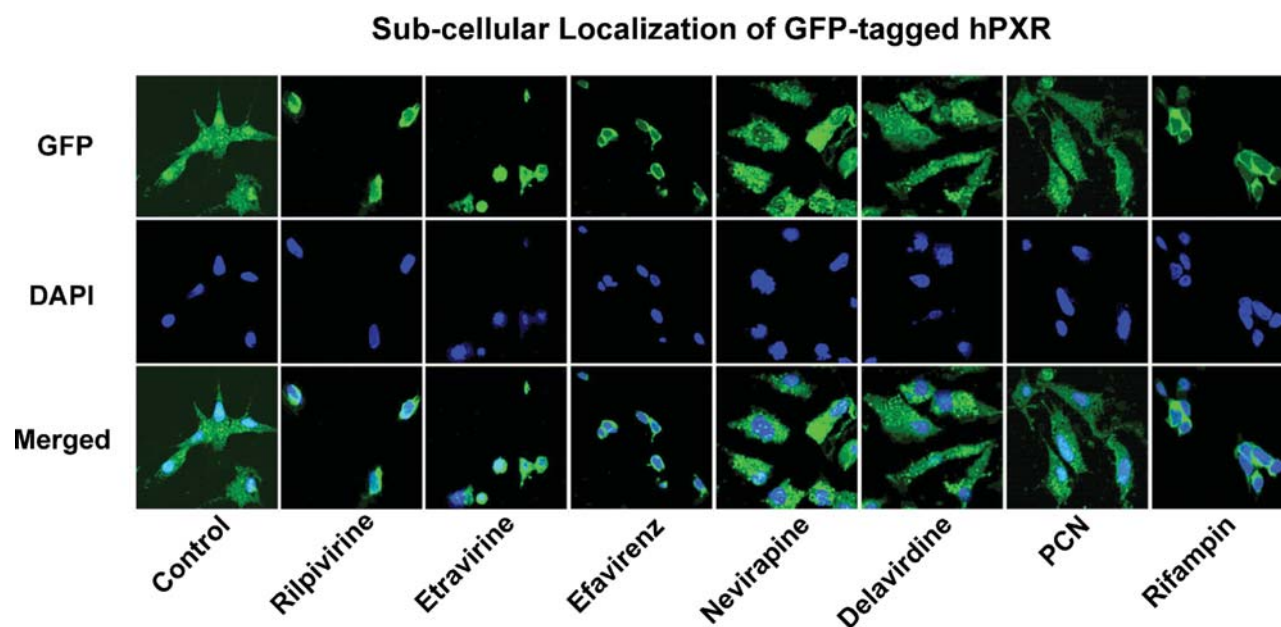


Fig. 6. Localization of GFP-tagged hPXR in HepG2 cells treated with rilpivirine, etravirine, or a first generation non-nucleoside reverse transcriptase inhibitor. Cultured HepG2 cells were transfected with pCMV6-AC-GFP-hPXR for 24 h and treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), PCN (10 μ M; negative control), or rifampin (10 μ M; positive control) for another 24 h. Subsequently, the cells were fixed with *p*-formaldehyde (4%, v/v) and mounted on glass slides using ProLong[®] Gold Antifade Reagent with DAPI for confocal microscopy. Shown are representative photomicrographs illustrating the localization of GFP-tagged hPXR, DAPI-stained nuclei, and merged images for each treatment group.

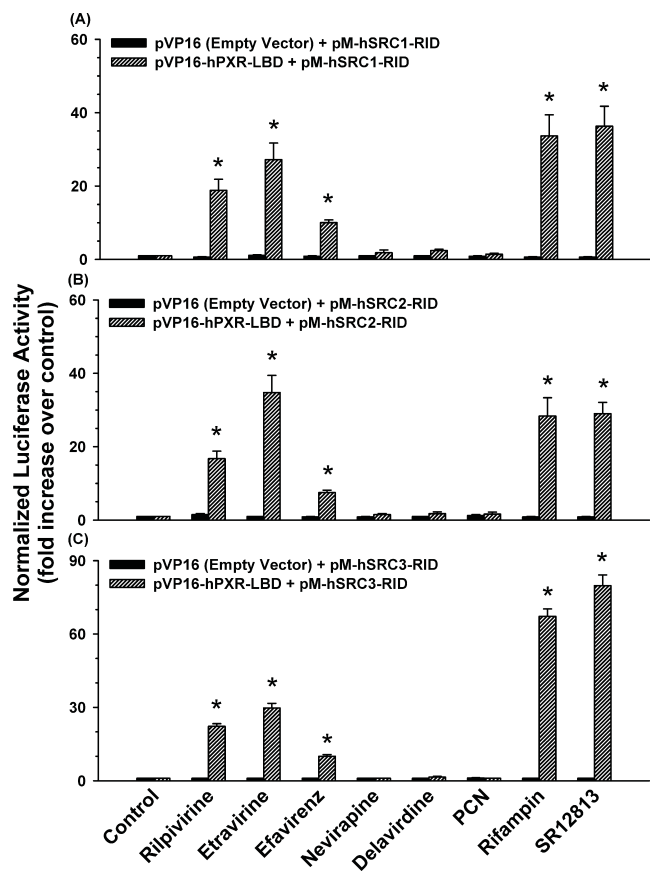


Fig. 7. Mammalian two-hybrid assay to evaluate the recruitment of steroid receptor coactivators to hPXR by rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors. Cultured HepG2 cells were transfected with pGL4.74 [*hRluc*/TK], pFR-luc, a coactivator expression plasmid, and either pVP16-hPXR-LBD or pVP16 (empty vector) for 24 h. The coactivator plasmids were (A) pM-hSRC1-RID, (B) pM-hSRC2-RID, and (C) pM-hSRC3-RID. Transfected cells were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), PCN (10 μ M; negative control), rifampin (10 μ M; positive control), or SR12813 (10 μ M; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean \pm S.E.M. for five independent experiments. *, significantly different from the same treatment group transfected with corresponding empty vector and from the vehicle-treated control cells transfected with pVP16-hPXR-LBD expression plasmid ($P < 0.05$).

1.7-fold, respectively, and nevirapine and delavirdine had no effect (Fig. 8B). By comparison, rifampin (10 μ M; positive control) increased the expression of CYP3A4 mRNA by 3.8-fold in sample GC4008 and 4.8-fold in sample HUM4021. The same overall pattern of response was obtained regardless of whether HPRT1 (Fig. 8A and B) or 18s rRNA (data not shown) was used as the reference gene to normalize the CYP3A4 mRNA data. CYP3A catalytic activity, as determined by the metabolic conversion of testosterone to 6 β -hydroxytestosterone [40], was also measured. In sample GC4008, etravirine and efavirenz increased CYP3A catalytic activity by 2.5- and 2.8-fold, respectively (Fig. 8C), whereas rilpivirine and delavirdine decreased it and nevirapine had no effect. By comparison, in sample HUM4021, rilpivirine, etravirine, and efavirenz increased CYP3A catalytic activity by 3.1-, 3.9-, and 3.2-fold, respectively (Fig. 8D), whereas delavirdine decreased it and nevirapine had no effect. In the same experiment, rifampin increased CYP3A activity by 8.7- and 4.1-fold in samples GC4008 and HUM4021, respectively.

4. Discussion

PXR is a ligand-activated transcriptional factor belonging to the superfamily of nuclear receptors [8] and it is known to regulate the expression of biologically important genes [20]. We report for the first time that the second generation non-nucleoside reverse transcriptase inhibitors rilpivirine and etravirine act as orthosteric agonists of hPXR. This conclusion is based on the experimental evidence indicating that both rilpivirine and etravirine 1) bound to the ligand-binding domain of hPXR, as demonstrated by hPXR-LBD transactivation and TR-FRET competitive ligand-binding assay; 2) stimulated nuclear translocation of GFP-tagged hPXR into the nuclear compartment, as shown by confocal microscopy; and 3) recruited steroid receptor coactivators SRC-1, SRC-2, and SRC-3 to hPXR, as indicated by mammalian two-hybrid assays. Our concentration-response data indicated that rilpivirine and etravirine activated hPXR at a concentration range of 0.5–10 μ M, with maximal effect observed at 5 μ M. As a comparison, the steady-state maximum plasma concentrations of rilpivirine, etravirine, and efavirenz have been reported to be $0.30 \pm 0.08 \mu$ M (mean \pm S.D.) [41], $1.81 \pm 0.66 \mu$ M (mean \pm S.D.) [42], and 12.98μ M (95% confidence interval, 7.95–18.27 μ M) [43], respectively, in specific studies conducted in HIV patients. Overall, our data show that rilpivirine and etravirine activate hPXR at low micromolar concentrations and by a mechanism that involves orthosteric agonism of the receptor.

Comparison of rilpivirine and etravirine with the first generation non-nucleoside reverse transcriptase inhibitors, such as efavirenz, nevirapine and delavirdine, revealed drug-specific agonism of hPXR by this class of anti-HIV drugs, as demonstrated by the mechanistic experiments in the present study. Among the five drugs investigated, only rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, were identified as agonists of hPXR. At an equimolar concentration (5 μ M), the rank order in the activation of hPXR was etravirine > rilpivirine > efavirenz, based on data from our cell-based reporter gene assay. By comparison, as shown in the present study, nevirapine did not activate hPXR at $\leq 10 \mu$ M, in agreement with a previous study reporting a lack of an effect at 7.5 μ M [23]. This drug weakly activates hPXR at a concentration of 50 μ M [22]. Overall, it is interesting that despite the chemical diversity within the first and second generation non-nucleoside reverse transcriptase inhibitors [5], three out of the five drugs investigated are capable of activating hPXR. This not only points to the well-known broad substrate specificity of hPXR [44], but it also provides an opportunity to utilize this information to deduce the structure-activity relationship for newer non-nucleoside reverse transcriptase inhibitors that may be valuable in designing newer drugs in this class.

PXR has been reported to show remarkable species-dependent differences in the ligand-binding domain [26]. Illustrating this fact are the examples of rifampin and PCN. Rifampin is a well-known activator of hPXR, but it has no activity on mPXR or rPXR [37]. In contrast, PCN activates mPXR and rPXR, but not hPXR [37]. As assessed at an equimolar concentration (5 μ M) in the present study, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated mPXR and rPXR, a profile that is qualitatively similar to that obtained in our hPXR activation experiment. According to site-directed mutagenesis studies, Leu-308 [45] and Gln-285 [46] in the ligand-binding domain of hPXR, Ile-282 in the ligand-binding domain of mPXR [46], and Phe-305 and Asp-318 in the ligand-binding domain of rPXR [47] were identified as amino acids imparting ligand-dependent species-specific activation of PXR. Future in silico docking studies, including those that target these specific amino acid residues, may provide insights into the binding pattern of non-nucleoside reverse transcriptase inhibitors in the ligand-binding domain of hPXR, mPXR, and rPXR, and the

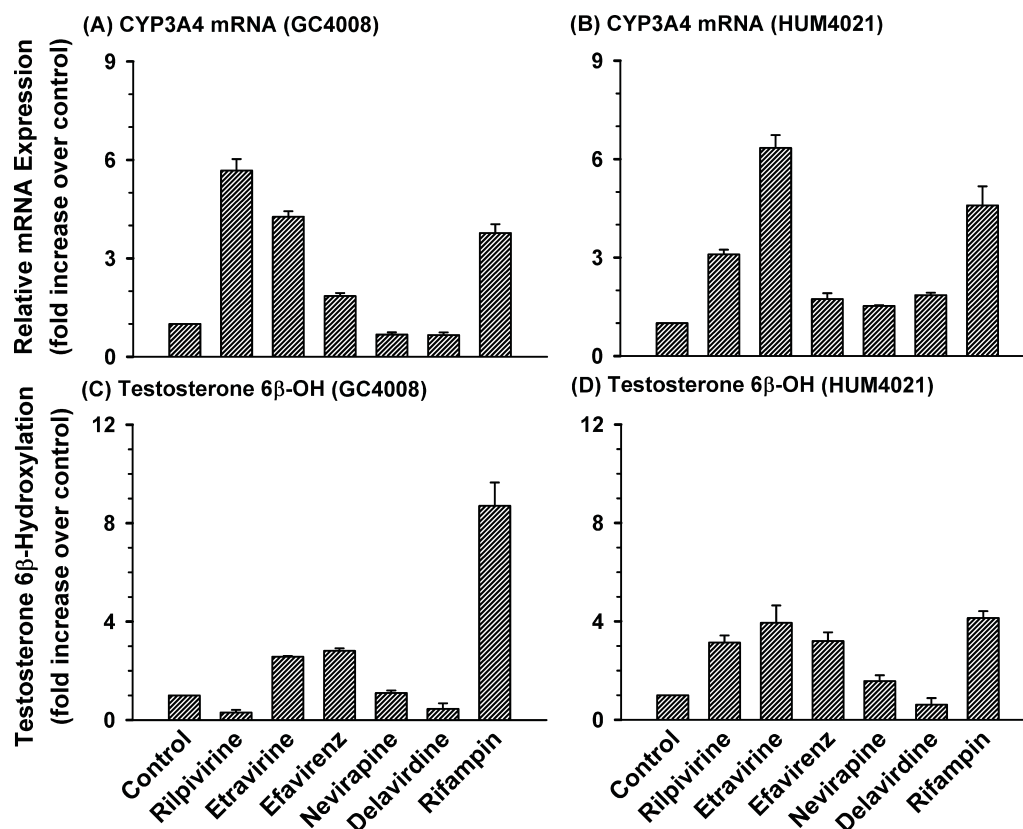


Fig. 8. Induction of CYP3A4 in primary cultures of human hepatocytes treated with rilpivirine, etravirine, or a first generation non-nucleoside reverse transcriptase inhibitor. Cultured hepatocytes were treated with DMSO (0.1%, v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 μ M), or rifampin (10 μ M; positive control) for (A and C) 48 or (B and D) 72 h. Hepatocytes were lysed, total RNA was isolated from pooled cell lysates (three wells per treatment group), and CYP3A4 and HPRT1 mRNA levels were analyzed by real-time PCR. CYP3A4 mRNA level was normalized to HPRT1 mRNA level. Data are shown as mean \pm S.D. of triplicate PCR analyses for hepatocyte samples (A) GC4008 and (B) HUM4021. To determine CYP3A4 catalytic activity, testosterone 6 β -hydroxylation assay was performed using HPLC analyses. Data are expressed as mean \pm S.D. of three wells for hepatocyte samples (C) GC4008 and (D) HUM4021.

lack of species-dependent PXR activation by this class of anti-HIV drugs. Overall, our data suggest that it may be appropriate to utilize mouse and rat models to assess the biological interactions between hPXR and the non-nucleoside reverse transcriptase inhibitors rilpivirine, etravirine, and efavirenz.

As shown in our experiment with human hepatocytes in culture, agonism of hPXR by rilpivirine was associated with an increase in the mRNA expression of CYP3A4, which is a target gene of hPXR [11–13]. The increase was accompanied by an elevation in CYP3A-mediated testosterone 6 β -hydroxylation activity in hepatocyte sample HUM4021 (Fig. 8D), but a decrease in sample GC4008 (Fig. 8C). The reason for this differential finding in testosterone 6 β -hydroxylation activity is not clear, but perhaps rilpivirine elicits a non-genomic effect (e.g. mechanism-based inactivation) that dominates over the genomic effect (i.e. transcriptional increase in CYP3A4 gene expression) and accounts for the net decrease in testosterone 6 β -hydroxylation that occurs after 2 days of drug treatment (Fig. 8C). Studies are planned to explore this possibility. The reported induction of CYP3A enzymes by etravirine [24] and present study) and efavirenz [21–23,48,49] in cultured human hepatocytes also occurs in vivo in humans, as inferred from pharmacokinetic studies. The administration of etravirine decreases the area under the plasma concentration-time profile for maraviroc [50], which is metabolized almost exclusively by CYP3A enzymes [51]. Similarly, human pharmacokinetic studies have shown that efavirenz increases the elimination of CYP3A substrates, as assessed by the erythromycin breath test [52], the metabolic formation of omeprazole sulfone from omeprazole [53], and the metabolic formation of 4 β -hydroxycholesterol from

cholesterol [54]. The lack of CYP3A4 induction by nevirapine at ≤ 10 μ M in cultured human hepatocytes ([23] and present study) is consistent with a lack of an effect of therapeutic doses of this drug on CYP3A activity in vivo, as determined by the erythromycin breath test [55]. In the case of delavirdine at a concentration of ≤ 10 μ M, this drug did not activate hPXR or increase CYP3A4 gene expression; rather, in our cultured human hepatocyte experiment, it decreased CYP3A-mediated testosterone 6 β -hydroxylation, consistent with the identification of delavirdine as a CYP3A inhibitor in vivo in human subjects [56] and a CYP3A mechanism-based inactivator in vitro in microsomal incubations [57]. Overall, given that PXR plays a major role in regulating the expression of genes involved not only in drug transport and metabolism, but also in other biological processes [20], it will be important to assess the effects of rilpivirine and etravirine on the expression of hPXR target genes other than CYP3A4. Studies are currently underway to investigate in detail the functional consequences of hPXR activation by the second generation non-nucleoside reverse transcriptase inhibitors.

In summary, among the five non-nucleoside reverse transcriptase inhibitors investigated in the present study, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, are activators of hPXR, mPXR, and rPXR. Agonism of hPXR by rilpivirine, etravirine, and efavirenz was demonstrated by the ability of these drugs to bind to the ligand binding domain of hPXR, promote nuclear translocation of GFP-tagged hPXR, and recruit receptor coactivators (SRC-1, SRC-2, and SRC-3). hPXR activation by rilpivirine, etravirine, and efavirenz was associated with induction of a hPXR target gene (CYP3A4). Overall, our novel

findings indicate ligand-dependent regulation of PXR function by select non-nucleoside reverse transcriptase inhibitors and provide insight into the molecular mechanism of hPXR activation by rilpivirine, etravirine, and efavirenz. As activation of PXR and induction of target gene expression form the molecular basis of drug-drug interactions [14] and other biological actions [20], the results from the present study provide a rational basis for investigating novel actions of the second generation non-nucleoside reverse transcriptase inhibitors rilpivirine and etravirine.

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References

- [1] Flexner C. HIV drug development: the next 25 years. *Nat Rev Drug Discov* 2007;6:959–66.
- [2] Oguntibeju OO. Quality of life of people living with HIV and AIDS and antiretroviral therapy. *HIV AIDS* 2012;4:117–24.
- [3] Jayaweera D, Dilanchian P. New therapeutic landscape of NNRTIs for treatment of HIV: a look at recent data. *Expert Opin Pharmacother* 2012;13:2601–12.
- [4] De Clercq E. When rilpivirine meets with tenofovir, the start of a new anti-HIV drug combination era. *Biochem Pharmacol* 2012;84:241–8.
- [5] De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents* 2009;33:307–20.
- [6] Croxtall JD. Etravirine: a review of its use in the management of treatment-experienced patients with HIV-1 infection. *Drugs* 2012;72:847–69.
- [7] Sonoda J, Pei L, Evans RM. Nuclear receptors: decoding metabolic disease. *FEBS Lett* 2008;582:2–9.
- [8] Germain P, Staels B, Dacquet C, Spedding M, Laudet V. Overview of nomenclature of nuclear receptors. *Pharmacol Rev* 2006;58:685–704.
- [9] Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal *MDR1* by rifampin. *J Biol Chem* 2001;276:14581–87.
- [10] Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, et al. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002;277:2908–15.
- [11] Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeborg L, Sydow-Backman M, et al. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA* 1998;95:12208–13.
- [12] Blumberg B, Sabbagh Jr W, Juguillon H, Bolado Jr J, van Meter CM, Ong ES, et al. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 1998;12:3195–205.
- [13] Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 1998;102:1016–23.
- [14] Wang YM, Ong SS, Chai SC, Chen T. Role of CAR and PXR in xenobiotic sensing and metabolism. *Expert Opin Drug Metab Toxicol* 2012;8:803–17.
- [15] Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, et al. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* 2001;98:3369–74.
- [16] Guo GL, Moffit JS, Nicol CJ, Ward JM, Aleksunes LA, Slitt AL, et al. Enhanced acetaminophen toxicity by activation of the pregnane X receptor. *Toxicol Sci* 2004;82:374–80.
- [17] Chen Y, Tang Y, Guo C, Wang J, Boral D, Nie D. Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochem Pharmacol* 2012;83:1112–26.
- [18] Kakizaki S, Takizawa D, Tojima H, Horiguchi N, Yamazaki Y, Mori M. Nuclear receptors CAR and PXR: therapeutic targets for cholestatic liver disease. *Front Biosci* 2011;17:2988–3005.
- [19] Shah YM, Ma X, Morimura K, Kim I, Pregnane Gonzalez FJ. X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF- κ B target gene expression. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G1114–22.
- [20] Gao J, Xie W. Targeting xenobiotic receptors PXR and CAR for metabolic diseases. *Trends Pharmacol Sci* 2012;33:552–8.
- [21] Hariparsad N, Nallani SC, Sane RS, Buckley DJ, Buckley AR, Desai PB. Induction of CYP3A4 by efavirenz in primary human hepatocytes: Comparison with rifampin and phenobarbital. *J Clin Pharmacol* 2004;44:1273–81.
- [22] Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, et al. Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J Pharmacol Exp Ther* 2007;320:72–80.
- [23] Svard J, Spiers JP, Mulcahy F, Hennessy M. Nuclear receptor-mediated induction of CYP450 by antiretrovirals: Functional consequences of *NR1I2* (PXR) polymorphisms and differential prevalence in whites and sub-Saharan Africans. *J Acquir Immune Defic Syndr* 2010;55:536–49.
- [24] Yanakakis LJ, Bumpus NN. Biotransformation of the antiretroviral drug etravirine: Metabolite identification, reaction phenotyping, and characterization of autoinduction of cytochrome P450-dependent metabolism. *Drug Metab Dispos* 2012;40:803–14.
- [25] Zhou C, Poulton EJ, Grun F, Bammler TK, Blumberg B, Thummel KE, et al. The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Mol Pharmacol* 2007;71:220–9.
- [26] Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, et al. The Pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* 2000;14:27–39.
- [27] Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 1999;56:1329–39.
- [28] Lau AJ, Yang G, Rajaraman G, Baucom CC, Chang TKH. Human pregnane X receptor agonism by *Ginkgo biloba* extract: Assessment of the role of individual ginkgolides. *J Pharmacol Exp Ther* 2010;335:771–80.
- [29] Lau AJ, Yang G, Chang TKH. Isoform-selective activation of human constitutive androstane receptor by *Ginkgo biloba* extract: functional analysis of the SV23, SV24, and SV25 splice variants. *J Pharmacol Exp Ther* 2011;339:704–15.
- [30] Lau AJ, Yang G, Yap CW, Chang TKH. Selective agonism of human pregnane X receptor by individual ginkgolides. *Drug Metab Dispos* 2012;40:1113–21.
- [31] Jauregui HO, Hayner NT, Driscoll JL, Williams-Holland R, Lipsky MH, Galletti PM. Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes - freshly isolated cells, cell suspensions, and primary monolayer cultures. *In Vitro* 1981;17:1100–10.
- [32] Pichard L, Fabre I, Fabre G, Domergue J, Saint Aubert B, Mourad G, et al. Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cytochrome P450 oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metab Dispos* 1990;18:595–606.
- [33] Schuetz EG, Beck WT, Schuetz JD. Modulators and substrates of P-glycoprotein and cytochrome P450 3A coordinately up-regulate these proteins in human colon carcinoma cells. *Mol Pharmacol* 1996;49:311–8.
- [34] Qiu H, Durand K, Rabinovitch-Charle H, Rigaud M, Gazaille V, Clavere P, et al. Gene expression of HIF-1 α and XRCC4 measured in human samples by real-time RT-PCR using the sigmoidal curve-fitting method. *Biotechniques* 2007;42:355–62.
- [35] Chang TKH, Chen J, Teng XW. Distinct role of bilobalide and ginkgolide A in the modulation of rat CYP2B1 and CYP3A23 gene expression by *Ginkgo biloba* extract in cultured hepatocytes. *Drug Metab Dispos* 2006;34:234–42.
- [36] Chang TKH. Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by herbal medicines. *AAPS J* 2009;11:590–601.
- [37] Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, et al. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 2000;275:15122–27.
- [38] Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K, Kawajiri K. Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR. *Mol Pharmacol* 2003;63:524–31.
- [39] Lichti-Kaiser K, Brobst D, Xu C, Staudinger JL. A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* 2009;331:65–76.
- [40] Yuan R, Madani S, Wei XX, Reynolds K, Huang SM. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 2002;30:1311–9.
- [41] Goebel F, Yakovlev A, Pozniak AL, Vinogradova E, Boogaerts G, Hoetelmans R, et al. Short-term antiviral activity of TMC278—a novel NNRTI—in treatment-naïve HIV1-infected subjects. *AIDS* 2006;20:1721–6.
- [42] DeJesus E, Lalezari JP, Osiyemi OO, Ruane PJ, Ryan R, Kakuda TN, et al. Pharmacokinetics of once-daily etravirine without and with once-daily darunavir/ritonavir in antiretroviral-naïve HIV type-1-infected adults. *Antivir Ther* 2010;15:711–20.
- [43] Nanzigu S, Eriksen J, Makumbi F, Lanke S, Mahindi M, Kiguba R, et al. Pharmacokinetics of the nonnucleoside reverse transcriptase inhibitor efavirenz among HIV-treated Ugandans. *HIV Med* 2012;13:193–201.
- [44] Chang TKH, Waxman DJ. Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev* 2006;38:51–73.
- [45] Tirona RG, Leake BF, Podust LM, Kim RB. Identification of amino acids in rat pregnane X receptor that determine species-specific activation. *Mol Pharmacol* 2004;65:36–44.
- [46] Ostberg T, Bertilsson G, Jendeborg L, Berkenstam A, Uppenberg J. Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. *Eur J Biochem* 2002;269:4896–904.
- [47] Song X, Li Y, Liu J, Mukundan M, Yan B. Simultaneous substitution of phenylalanine-305 and aspartate-318 of rat pregnane X receptor with the corresponding human residues abolishes the ability to transactivate the CYP3A23 promoter. *J Pharmacol Exp Ther* 2005;312:571–82.

- [48] Kamiguchi N, Aoyama E, Okuda T, Moriwaki T. A 96-well plate assay for CYP4503A induction using cryopreserved human hepatocytes. *Drug Metab Dispos* 2010;38:1912–6.
- [49] Mugundu GM, Hariparsad N, Desai PB. Impact of ritonavir, atazanavir and their combination on the CYP3A4 induction potential of efavirenz in primary human hepatocytes. *Drug Metab Lett* 2010;4:45–50.
- [50] Kakuda TN, Abel S, Davis J, Hamlin J, Scholler-Gyure M, Mack R, et al. Pharmacokinetic interactions of maraviroc with darunavir-ritonavir, etravirine, and etravirine-darunavir-ritonavir in healthy volunteers: results of two drug interaction trials. *Antimicrob Agents Chemother* 2011;55:2290–6.
- [51] Lu Y, Hendrix CW, Bumpus NN. Cytochrome P450 3A5 plays a prominent role in the oxidative metabolism of the anti-human immunodeficiency virus drug maraviroc. *Drug Metab Dispos* 2012;40:2221–30.
- [52] Mouly S, Lown KS, Kornhauser D, Joseph JL, Fiske WD, Benedek IH, et al. Hepatic but not intestinal CYP3A displays dose-dependent induction by efavirenz in humans. *Clin Pharmacol Ther* 2002;72:1–9.
- [53] Michaud V, Ogburn E, Thong N, Aregbe AO, Quigg TC, Flockhart DA, et al. Induction of CYP2C19 and CYP3A activity following repeated administration of efavirenz in healthy volunteers. *Clin Pharmacol Ther* 2012;91:475–82.
- [54] Josephson F, Bertilsson L, Bottiger Y, Flamholz L, Gisslen M, Ormaasen V, et al. CYP3A induction and inhibition by different antiretroviral regimens reflected by changes in plasma 4-beta-hydroxycholesterol levels. *Eur J Clin Pharmacol* 2008;64:775–81.
- [55] Mouly S, Rizzo-Padoin N, Simoneau G, Verstuft C, Aymard G, Salvat C, et al. Effect of widely used combinations of antiretroviral therapy on liver CYP3A4 activity in HIV-infected patients. *Br J Clin Pharmacol* 2006;62:200.
- [56] Cheng CL, Smith DE, Carver PL, Cox SR, Watkins PB, Blake DS, et al. Steady-state pharmacokinetics of delavirdine in HIV-positive patients: effect on erythromycin breath test. *Clin Pharmacol Ther* 1997;61:531–43.
- [57] Voorman RL, Maio SM, Payne NA, Zhao Z, Koepfingler KA, Wang X. Microsomal metabolism of delavirdine: evidence for mechanism-based inactivation of human cytochrome P450 3A. *J Pharmacol Exp Ther* 1998;287:381–8.