Title: Camptothecin Attenuates Cytochrome P450 3A4 Induction by Blocking the Activation of Human Pregnane X Receptor

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Running Title: CPT attenuates CYP3A4 induction by Blocking hPXR activation

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Abbreviations: CPT, camptothecin; DME, drug metabolizing enzyme; CYP3A4, cytochrome P450 3A4; hPXR, human pregnane X receptor; DDI, drug-drug interaction; SRC-1, steroid receptor cofactor-1; 1,25-(OH)2VD3, 1α, 25-Dihydroxyvitamin D3; CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime; PCN, pregnenolone-16α-carbonitrile; hVDR, human vitamin D receptor; hCAR3, human constitutive androstane receptor 3; RXRα, retinoid X receptor α; MCS, multiple cloning sites; GST, glutathione S-transferase; LBD, ligand-binding domain; FRET, fluorescence resonance energy transfer; HPLC, high performance liquid chromatography; LOQ, limit of quantification; EMSA, electrophoretic
mobility shift assays; MDR-1, multi-drug resistance gene-1; MRP-1, multiple drug resistance protein 1; SRC186, 186 amino acid residues of the human SRC-1 protein.

**Recommended section assignment:** Metabolism, Transport, and Pharmacogenomics
Abstract

Differential regulation of drug metabolizing enzymes (DMEs) is a common cause of adverse drug effects in cancer therapy. Due to the extremely important role of cytochrome P450 3A4 (CYP3A4) in drug metabolism and the dominant regulation of human pregnane X receptor (hPXR) on CYP3A4, finding inhibitors for hPXR could provide a unique tool to control drug efficacies in cancer therapy. Camptothecin (CPT), was demonstrated as a novel and potent inhibitor (IC$_{50}$ = 0.58 µM) of an hPXR-mediated transcriptional regulation on CYP3A4 in this study. In contrast, one of its analogues, irinotecan (CPT-11), was found to be an hPXR agonist in the same tests. CPT disrupted the interaction of hPXR with steroid receptor coactivator-1 (SRC-1) but had effects on neither the competition of ligand binding, nor the formation of the hPXR and retinoid X receptor α (RXRα) heterodimer, nor the interaction between the regulatory complex and DNA responsive elements. CPT treatment resulted in delayed metabolism of nifedipine in human hepatocytes treated with rifampicin, suggesting a potential prevention of drug-drug interactions (DDIs) between CYP3A4 inducers and CYP3A4-metabolized drugs. Since CPT is the leading compound of topoisomerase I inhibitors, which comprise a quickly developing class of anti-cancer agents, the finding indicates the potential of a new class of compounds to modify hPXR activity as agonists/inhibitors and is important in the development of CPT analogues.
Introduction

Topoisomerase I inhibitors are a quickly developing class of anti-cancer agents. Clinical trials with the leading compound of this class of drugs, camptothecin (CPT), showed remarkable anti-cancer activity by interrupting DNA replication in cancer cells (Chen and Liu, 1994). Several semi-synthetic derivatives of CPT, like topotecan and irinotecan (CPT-11), have been used in cancer chemotherapy (Creemers et al., 1994). Though the main mechanism by which topoisomerase I inhibitors produce their antitumor effects has been widely investigated, both the mechanism of inter-individual variability in the plasma disposition and the potential to regulate the efficacy of other compounds when used in combination are not fully understood (van Warmerdam et al., 1996).

Upregulation/downregulation of drug metabolizing enzymes (DMEs) is a common cause of unpredictable drug effects during cancer therapy (Rochat, 2005), especially the alteration in the expression of cytochrome P450 3A4 (CYP3A4), an enzyme involved in the metabolism of 60% of drugs (Guengerich, 1999), including many anti-cancer drugs. Human pregnane X receptor (hPXR, also called steroid and xenobiotic receptor), a ligand-activated nuclear receptor, has been characterized as a dominant transcriptional factor of CYP3A4 (Chen and Nie, 2009). Evidence has shown that hPXR translocates from cytoplasm to the nucleus to increase the transcription of CYP3A4 as well as other DMEs and transporters after treatment with its agonists (Kawana et al., 2003; Chen et al., 2007). In contrast, the hPXR inhibitors block the biological actions of hPXR. Since many anti-cancer drugs have narrow therapeutic indexes, the modulation on hPXR-CYP3A4 pathway may cause significant toxicity or unpredictable ineffective therapy. At the present, although many hPXR agonists have been reported, few hPXR compounds antagonizing hPXR-CYP3A4 pathway have been identified.
Since the CYP3A4-hPXR pathway is extremely important in drug efficacy (Bertilsson et al., 1998; Chen et al., 2009), establishing an interaction between CYP3A4-hPXR pathway and CPT analogues is important for reducing the drug-drug interactions (DDIs) and toxicity during drug development and clinical applications. In this study the effects of CPT and its analogue, irinotecan, on hPXR-CYP3A4 pathway, and the underlying mechanisms were characterized. CPT potently attenuated CYP3A4 induction via blocking the activation of nuclear receptors, especially hPXR. The clinical relevance of CYP3A4 inhibition was proven by attenuated nifedipine metabolism in CPT-pretreated primary cultures of human hepatocytes. Further mechanistic studies revealed that CPT inhibited hPXR by interrupting the binding of steroid receptor cofactor-1 (SRC-1) to hPXR. Conversely, irinotecan was a moderate agonist of hPXR, with no additive or inhibitive effects on rifampicin-induced hPXR activations.

Here, we established CPT as a novel and potent inhibitor of hPXR. On one hand, CPT and its analogues can be useful tools for modulating DME expression and drug efficacies. On the other hand, since CPT is the leading compound of a class of quickly developing anti-cancer drugs, the potential effects of CPT analogues on DMEs levels, especially CYP3A4 level, should be noticed in drug development.
Methods

**Reagents and cells.** CPT, irinotecan (supplemental fig. 1), 1α, 25-Dihydroxyvitamin D3 (1,25-(OH)2VD3), 6-(4-Chlorophenyl) imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO), pregnenolone-16α-carbonitrile (PCN), proteinase inhibitors, nifidipine, oxidized nifedipine and nitrendipine were purchased from Sigma-Aldrich (St. Louis, MO). Rifampicin, SR12813 and ketoconazole were from Biomol International L.P. (Plymouth Meeting, PA). Dual-luciferase assay kit, β-galactosidase assay kit, MTS assay kit and TNT® T7 quick coupled transcription/translation system were purchased from Promega (Madison, WI). *Escherichia coli* Rosetta (DE3) and his-bind quick 300 cartridges were acquired from EMD Chemicals Inc. (Gibbstown, NJ). Zeba™ desalt spin column, EZ-Link® Sulfo-NHS-LC-biotin and proFound™ Pull-Down biotinylated protein:protein interaction kit were obtained from Thermo Fisher Scientific (Rockford, IL). RNeasy mini kit was purchased from Qiagen (Valencia, CA). Taqman realtime PCR reagents and primers were obtained from Applied Biosystems (Foster City, CA). EMSA buffer kit for the Odyssey was from Li-COR Biosciences (Lincoln, NE). HepG2, 293T and HCT116 cells were obtained from ATCC (Manassas, VA). Fresh primary cultures of human hepatocytes from 3 donors and hepatocyte culture media kit were obtained from BD biosciences (Woburn, MA). Donor demographics are shown in supplemental table 1. CBS cells were obtained from Dr. Subhas Chakrabarty, SimmonsCooper Cancer institute, Southern Illinois University. All HPLC grade reagents were from ACROS Organics (Morris Plains, NJ).

**Plasmids.** pGL3 vectors were purchased from Promega (Madison, WI). PetDuet-1 and pet28a were from EMD Chemicals Inc. (Gibbstown, NJ). pGEM3 - human vitamin D receptor (hVDR) was produced by ATCC (Manassas, VA). The full-length hVDR cDNA was isolated from the
pGEM3-hVDR plasmid and subcloned into a pcDNA3.1 vector. A pGL3-CYP3A4-XREM-luc plasmid was constructed by inserting two CYP3A4 regulatory elements, a distal enhancer (-7849 to -7219) and a proximate promoter (-369 to +37), into a pGL3 basic vector (Chen et al., 2007). Gal4DB-SRC-1-RID, TK-MH100×4-LUC, pSG5-mouse pregnane X receptor (mPXR), pVP-hPXR, pTracer-CMV2- human constitutive androstane receptor 3 (hCAR3), pTracer-CMV2 and pCMX- retinoid X receptor α (RXRα) were kind gifts from other labs (see Acknowledgements).

Cell culture and drug treatments. HepG2 cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% charcoal-stripped FBS (Gibco, Carlsbad, CA). Only cells of passage 4 were used for transient transfections and drug treatments. Fresh human hepatocytes were available in 24-well collagen I plates. Cells were cultured in Hepato- STIM media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, and 10 ng/ml epidermal growth factor and incubated in a humidified 37°C incubator with 95% air / 5% CO2 atmosphere. Drugs were prepared in vehicle freshly on the day of use. Fresh dosing solutions were added every 24 h so that cells have a total of three exposures to the treatments over a 72 hours time period.

Cell toxicity assessment. Cells were plated into a 96-well plate and treated with drugs at different concentrations or vehicle controls for a certain time period. Cell viabilities after treatments were evaluated by a MTS assay according to the manufacturer’s instructions. Cytotoxicity for human hepatocytes was determined by a Vi-Cell™ XR cell viability analyzer (Beckman Coulter, Fullerton, CA).

Quantitative RT-PCR. Total RNA was prepared using an RNeasy mini kit with on-column DNase treatment. RNA quantitation and quality were determined by an Eppendorf biophotometer. TaqMan one-step RT-PCR assays were performed with 100 ng of RNA sample.
using an ABI Prism 7500 Fast Real-Time PCR System. An initial RT step occurred for 15 min at 48°C and was subsequently followed by heating to 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Primers for CYP3A4 and hPXR are as follows: CYP3A4 forward 5′-TCAGCCTG GTGCTCCTATCTAT-3′, CYP3A4 reverse 5′-AAGCCCTTATGGGTAGGACAAATATTT-3′, CYP3A4 TaqMan probe 5′-FAM-TCCAGGGCC CACACCTGCCT-TAMRA-3′; hPXR forward 5′-GCAGGAGCAATTTCGCCATT-3′, hPXR reverse 5′- TCAGGCAGCATAGCCATGATC -3′, hPXR TaqMan probe 5′-FAM-CCAGCCTGCTC ATAGGTTCTTTGTTCGAA-TAMRA-3′.

The sets of primers or probes for CYP3A4 and hPXR were designed to span exon junctions to prevent detection of any possible contamination of genomic DNA. Each assay contained 0.9 μM each of forward and reverse primer and 0.25 μM TaqMan probe. SYBRgreen amplification of human 28S RNA was used as an internal control (28S Forward 5′-GGTATGGGCCCGACGCT -3′, 28S Reverse 5′- CCGATGCCGCCGACGCTCAT -3′). Standard curves were constructed by serial 10-fold dilutions, ranging from 30 copies to 300000 copies, of interest genes. Quantitations of gene expression levels were determined by interpolation of threshold cycle values to a standard curve. Gene-specific mRNA was subsequently normalized to 28S.

**Western-blot.** Cells were lysed and sonicated in SDS running buffer. The lysate was centrifuged and the supernatant was separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were then electroblotted onto a polyvinylidene fluoride membrane. After blocking, the membrane was incubated with a primary antibody for 1 hour at room temperature followed by an incubation with a secondary antibody for another hour. The antibodies used were listed in supplemental table 2. β-actin was used as a loading control. The proteins were visualized by an
Odyssey Infrared Imaging System and the intensities of protein bands were determined on an Odyssey 2.0 software (Li-COR biosciences).

**Luciferase assay.** Cells were seeded into a 12-well plate. After attachment, cells at 80% confluence were cotransfected with 0.15 µg of pcDNA3.1-hPXR or pTracer-CMV2-CAR3 or pSG5-mPXR or pcDNA3.1-hVDR or vector control, 0.15 µg of pGL3-CYP3A4-XREM and 10 ng of pGL3-CMV-Renilla (each well). Twenty-four hours after transfection, the cells were treated with drugs for 2 hours followed by a fresh medium treatment for another 22 hours. Then cells were harvested and lysed. The luciferase activity was measured and normalized for transfection efficiency with renilla activity.

**Mammalian two-hybrid assays.** HepG2 cells were seeded into a 12-well plate. After attachment, cells at 80% confluence were cotransfected with 500 ng of Gal4DB-SRC-1-RID, 100 ng of pVP-hPXR, 500 ng of TK-MH100×4-LUC and 200 ng of pCMV-LacZ (each well). 24 hours after transfection, the cells were treated with drugs. Then the cells were harvested and lysed. The luciferase activity was measured and normalized for transfection efficiency with β-gal activity.

**Cofactor binding assay.** The residues 130-434 of PXRLBD were engineered as a C-terminal his-tagged fusion protein and subcloned into multiple cloning sites (MCS) 2 of petDuet-1. Residues 623-710 of the human SRC-1 protein were subcloned into MCS1 of petDuet-1 to improve the stability and solubility of PXRLBD. The soluble PXRLBD fragment was obtained in Rosetta (DE3) with the induction of 0.05 mM IPTG in shaker at 22 °C. The protein was purified by his-bind quick 300 cartridges and changed into TBS buffer by a Zeba™ desalt spin column for pull-down assays. The 186 amino acid fragment of human SRC-1 protein with a C-terminal his-tag was amplified from a SRC-1 containing plasmid, subcloned into pet28a plasmid.
and transformed into Rosetta (DE3). The expression was induced by IPTG overnight at 18 °C. The SRC186 fragment was purified by his-bind quick 300 cartridges and changed buffered into PBS. The purified SRC186 fragment was labeled with EZ-Link Sulfo-NHS-LC-Biotin reagent using a 3-fold molar excess of biotin reagent. Then the peptide was purified and changed into TBS buffer. The pull-down study was performed using a proFound™ pull-down biotinylated protein:protein interaction kit according to the manufacturer’s instructions. The biotinylated SRC186 fragment was immobilized on streptavidin-agarose beads. Then the purified PXRLBD peptide and tested compounds were added to the beads. Following the prey capture, the beads were washed and eluted. The elutes were used for SDS-PAGE analysis.

**hPXR competitive binding assay.** This assay was performed by Invitrogen biochemical nuclear receptor profiling service. In brief, the assay was performed in 96-well non-coated polystyrene assay plate. Each well contained 20 µl of reaction mixture (n = 4): tested compounds/control compound (SR12813), 40 nM Fluormone™ hPXR Green (a green fluorescent hPXR ligand), 50 µM DTT, 10 nM purified glutathione S-transferase (GST)-tagged hPXR ligand-binding domain (LBD), 10 nM terbium-labeled anti-GST tag antibody and 1% DMSO. Plates were incubated at room temperature without light and evaporation for 1 to 2 hours. Binding of Fluormone™ tracer is then measured by monitoring fluorescence resonance energy transfer (FRET) from the terbium-labeled antibody to the Fluormone™ tracer, resulting in a high TR-FRET ratio (520 nm fluorescent emission of Fluormone : 490 nm fluorescent emission of terbium). Competitors will displace the Fluormone™ tracer from the receptor and disrupt FRET, resulting in a lower TR-FRET ratio. To read a Lanthascreen TR-FRET assay, the instrument is configured to excite the terbium donor around 340 nm (30 nm bandwidth), and to separately read the terbium emission peak that is centered at approximately 490 nm (10 nm
bandwidth), and the fluorescein emission that is centered at approximately 520 nm (25 nm bandwidth). The 520/490 TR-FRET ratio was measured using a PerkinElmer EnVision® fluorescent plate reader. A 100 µs delay followed by a 200 µs integration time was used.

**Metabolism of nifedipine by human hepatocytes.** Nifedipine was dissolved in 400 µl of medium to give a final concentration of 50 µM. Preliminary experiments were performed to determine the nifedipine concentration and incubation time in the linear ranges. The hepatocytes were incubated in a 37°C incubator (with CO2) for 30 min. At the end of the incubation period, the assay was stopped by adding 400 µl of acetonitrile and 1 µl of 40 uM nitrendipine to each well. The mixtures were centrifuged at 13,000 rpm for 5 min. A 20 µl aliquot of supernatants was used for high performance liquid chromatography (HPLC).

**HPLC analyses.** The HPLC analyses of nifedipine, oxidized nifedipine (CYP3A4 metabolite) and nitrendipine (internal control) were performed on a Shimadzu LC-20AT HPLC system equipped with a Shimadzu SPD 20AV UV detector. A Thermo scientific hypersil gold column (4.6 x 150 mm, 3 µm), preceded by a C18 precolumn cartridge, was used in all assays. The solvent system at a flow rate of 0.5 ml/min comprised 58% of solvent A (0.1 M sodium phosphate, pH 3.0) and 42% of solvent B (100% acetonitrile). The compounds were detected by monitoring of UV absorbance at 230 nm and compared with authentic standard compounds. The concentration was quantified by comparing the ratio of peak area of oxidized nifedipine to that of nitrendipine. A standard curve was constructed with the range of oxidized nifedipine used being from 500 pg to 200 ng. The acceptance standard for the calibration curves was a regression coefficient > 0.99. The limit of quantification (LOQ) was defined as a signal to noise ratio of 10:1, with an acceptable level of variation (<10%).
In vitro translation and electrophoretic mobility shift assays (EMSA). EMSAs were performed using full-length hPXR and human RXRα synthesized in vitro. A pCMX-hRXRα plasmid and a pcDNA3.1-hPXR plasmid were used in a TNT® T7 quick coupled transcription/translation system. Single-strand DNA oligonucleotides were labeled with IRDye™ 800 CM phosphoramidite at 5’end by IDT DNA technologies (Coralville, IA) and then annealed to form double-stranded DNA fragments. The following IRDye-labeled oligonucleotides were used as probes (only the sense strand is shown, with consensus sequence in boldface): CYP3A4-pER6, 5’ IRDye™ 800-TAG AATA TGAACT CAAAGG AGGTCA GTGAGT - 3’; CYP3A4-dER6, 5’ IRDye™ 800-CCCTGAAAT CATGTC GGTTCA AGCA-3’. Proteins were incubated for 20 min at room temperature in darkness with 2.5 nM IRDye-labeled oligos in 10 mM Tris (pH 7.5), 50 mM KCl, 0.25% Tween20, 5% glycerol, 3.5 mM dithiothreitol, 50 ng/µl poly(dI-dC) and 1×proteinase inhibitor cocktail. The mixture was then subjected to electrophoresis on a native Tris-glycine-EDTA polyacrylamide gel in a buffer containing 25 mM Tris base, 190 mM glycine and 1 mM EDTA. Images were generated by scanning the plates directly in an Odyssey infrared scanner at the channel of 800 CM. The quantification was performed by Odyssey 2.0 software. For competition bindings, unlabeled oligonucleotides at a 100-fold molar excess were co-incubated. All shift assays were performed in dark.

Statistical analysis. Student’s t-test (two-tails) was used to analyze the difference between two groups. For comparisons among three or more groups, ANOVA test was used. A P value between groups, if smaller than 0.05, is considered statistically significant.
Results

Attenuation of CYP3A4 induction in primary cultures of human hepatocytes. To assess the regulation of CPT on CYP3A4, primary cultures of human hepatocytes from three donors (supplemental table 1) were treated with CPT alone or in the combination with compounds that had previously been identified as positive (rifampicin(Goodwin et al., 1999)) or negative (ketoconazole(Huang et al., 2007)) for CYP3A4 induction for 72 hours. Hepatocytes had no visible morphological changes or cell deaths after CPT treatment alone or in combination with rifampicin at the concentrations used. During treatment with single drugs, rifampicin significantly induced CYP3A4 in all three donors with a high inter-individual variability (Figs. 1A & 1B). CPT had no significant effects on basal CYP3A4 mRNA level (Fig. 1A). However, the induction of CYP3A4 by rifampicin was decreased by the coadministration with CPT by 60% to 90% (Fig. 1A). A CPT analogue, irinotecan, was tested in parallel in experiments. It showed a moderate induction of CYP3A4 and had no synergetic or inhibitory effects on rifampicin-induced CYP3A4 levels in donor 1 and donor 2 (Fig. 1B). As a dominant regulator of CYP3A4, The hPXR mRNA levels were also evaluated in the same samples. Real-time PCR results showed hPXR levels were unchanged in all treatments when compared with significantly changed CYP3A4 effects (Figs. 1C & 1D).

Western-blots were also performed to evaluate protein levels. The results agreed with mRNA data. CPT reduced the ability of rifampicin to increase CYP3A4 protein levels in hepatocytes markedly in donors 1 and 2 (Fig. 2 & Table 1) and moderately in donor 3 (Table 1). However, its analogue, irinotecan, alone induced CYP3A4 protein levels in donor 1(Table 1). No further CYP3A4 inductions were observed in rifampicin-treated hepatocytes from donors 1 and 2 when rifampicin was co-administered with irinotecan (Table 1 & Fig. 2). The unusual down-regulation
of CYP3A4 induction by CPT led us to investigate whether CPT could attenuate the inductions of other hPXR target genes caused by known inducers. However, a western-blot performed on hepatocytes treated with CPT/irinotecan alone or in combination with rifampicin did not detect significant induction/inhibition of hPXR, multi-drug resistance gene-1 (MDR-1) or multiple drug resistance protein 1 (MRP-1) levels (Fig. 2). The induction/inhibition folds of CYP3A4 protein in all three donors after drug treatments are shown in Table 1. CPT also caused a remarkable decrease in the induction of CYP3A4 mRNA and protein levels in HepG2 cells, a hepatocarcinoma cell line, and CBS cells, a colon cancer cell line. Other CYP3A4 inducers, like SR12813 and taxol, were also tested. The results showed it was not a rifampicin-specific effect (Data not shown).

**Inhibition of nuclear receptors-mediated CYP3A4 inductions.** Previous reports have revealed that hPXR is a dominant regulator of CYP3A4 expression. Although the hPXR levels remained constant during CPT treatments (Figs. 1 & 2), the inhibition of hPXR transactivation activity on CYP3A4 promoter could also suppress CYP3A4 induction. An hPXR-based reporter assay was used to test this hypothesis. Cells were cotransfected with a renilla expression vector, a reporter gene construct containing a responsive element for hPXR in CYP3A4 promoter and an hPXR expression plasmid / vector control. Cells were treated with drugs at different concentrations (Fig. 3A), and the induction potential was then measured by the ability of a test compound to activate transcription of the reporter gene. The results are shown in Fig. 3. CPT strongly attenuated the transactivation of CYP3A4 promoter induced by rifampicin in a concentration-dependent manner (Fig. 3A). The IC$_{50}$ was 0.58 µM. In contrast, irinotecan alone induced hPXR transactivation in a dose-dependent manner and showed no synergetic effects on rifampicin-induced hPXR activity (Fig. 3A). These effects were observed only in cells
cotransfected with the hPXR expression vector, suggesting an hPXR-dependent mechanism of regulation. Similar experiments were then carried out in different cell lines to exclude cell line-specific effects (Fig. 3B). Another hPXR agonist, SR12813, was used in evaluations for the purpose of excluding rifampicin-specific effects. The presence of CPT resulted in 60-80% inhibitions of transcriptional activities induced by SR12813 or rifampicin in other cell lines (Fig. 3B).

The CYP3A4 expression is also regulated by the nuclear receptors, hCAR (Tirona et al., 2003) and hVDR (Thompson et al., 2002). Similar reporter assays were performed in HepG2 cells to evaluate the CPT effects on hCAR- and hVDR- mediated CYP3A4 transactivations. In contrast to hPXR, hCAR has a constitutive activation nature in immortalized cells. To avoid problems in in vitro assessment, a splicing variant hCAR3 was used in assay, which displays a low basal activity and still exhibits chemical-mediated activation in several published studies (Faucette et al., 2007). CPT blocked 50% of CITCO-induced CYP3A4 transactivation through hCAR, whereas irinotecan only slightly affected the induction in both HepG2 cells and 293T cells (Fig. 4A). Interestingly, CPT and irinotecan showed opposite results in hVDR assays. CPT itself worked as an agonist in hVDR-mediated CYP3A4 transactivation and produced a synergetic induction with a strong hVDR agonist, 1,25-(OH)₂VD₃. However, the irinotecan showed inhibitory effects to 1,25-(OH)₂VD₃ - induced activation in this case (Fig. 4B). In control experiments without overexpressions of nuclear receptors, neither hVDR nor hCAR activity was increased/inhibited by drug treatments. In sum, these results show that CPT inhibits both hPXR- and hCAR- mediated transactivations of the CYP3A4 promoter but induced hVDR activity. The total effect is the inhibition on CYP3A4 levels because of the dominant modulation of hPXR on CYP3A4 expression in the context of cooperative modulations of all three nuclear receptors.
Finally, previous studies have reported the notable species-variance in the LBD of hPXR in different species. The role of CPT in mouse PXR activation was also examined. The results in Fig. 4C indicate that CPT blocks the activation of mPXR by PCN, a mPXR agonist, with an IC$_{50}$ of 0.87 µM, suggesting CPT is also an inhibitor of mPXR.

Cytotoxicity assays showed cell viabilities greater than 80% for all cell lines in the testing conditions (data not shown).

**Effects on the formation of hPXR:hRXR$\alpha$: hPXR-responsive elements (hPXRE) complex.** To elucidate the underlying mechanisms of the observed inhibitory effects on hPXR activation, we examined effects of CPT on key steps of hPXR activation. Upon activation, hPXR forms a heterodimer with hRXR$\alpha$, and then binds to the hPXRE present in the regulatory region of target genes (Chen and Nie, 2009). The hPXRE that have been identified in the regulatory regions of CYP3A genes consist of direct repeats of the consensus sequence (A/G)G(T/G)TCA separated by 3 nucleotides (DR3) (Kliewer et al., 1998), everted repeats separated by six bases (ER6) (Liu et al., 2008), and inverted repeats with a six-base spacer (IR6). An EMSA was performed to determine whether CPT interferes with the binding of hPXR:hRXR$\alpha$ complex to CYP3A4 hPXRE. Two important ER6 oligonucleotides were used in assays. One ER6 located at the proximal CYP3A4 promoter is hereafter referred to as CYP3A4 pER6, while the other ER6 located at distal CYP3A4 promoter is hereafter referred to as CYP3A4 dER6 (Fig. 5). A single retarded band was observed when both full-length hPXR and hRXR$\alpha$ were incubated with IRDye™ 800 CM phosphoramidite - labeled CYP3A4 pER6 or CYP3A4 dER6, but not when hPXR or hRXR$\alpha$ was incubated alone. An 100-molar excess of unlabeled CYP3A4 pER6 or CYP3A4 dER6 suppressed the specific hPXR:hRXR$\alpha$:hPXRE complex. The treatments with CPT / irinotecan alone or in the combination with rifampicin had no significant effects on either
hPXR:hRXRα heterodimer formation or the binding of the heterodimer to both ER6 hPXRE (Fig. 5).

**Inhibition on the binding of cofactor to hPXR.** The interaction of hPXR with coactivator is a critical step of hPXR signaling (Rosenfeld et al., 2006). Recently, a hPXR inhibitor, ketoconazole, has been shown to disrupt the interaction of hPXR with SRC-1 (Huang et al., 2007). In this study, we first used the mammalian two-hybrid assay to evaluate whether CPT individually or in combination with rifampicin could affect the interaction of hPXR with SRC-1 in cells. Two fusion plasmids, Gal4DB-SRC-1-RID and pVP-hPXR, were used in assay. Gal4DB-SRC-1-RID was constructed by fusing GAL4-DBD (yeast DNA-binding domain) to SRC-1 receptor interacting domain. pVP-hPXR was a plasmid containing full-length hPXR fused to *Herpes simplex* virus VP16 activation domain. The reporter vector, TK-MH100×4-LUC, was constructed by inserting four copies of the yeast upstream activating sequences UASG enhancer (MH100) into the luciferase reporter plasmid TK-LUC. As shown in Fig. 6A, rifampicin significantly promoted the specific interaction of SRC-1 with hPXR in HepG2 cells. Both CPT and ketoconazole individually inhibited rifampicin-induced SRC-1 recruitment to hPXR. As expected, irinotecan alone increased the interaction between SRC-1 and hPXR but had no interference on rifampicin-induced SRC-1: hPXR binding.

To confirm the results of mammalian two-hybrid system, we further performed an *in vitro* coactivator binding assay. Soluble SRC186 (186 amino acid residues of the human SRC-1 protein, which contains all 3 motifs interacting with hPXR) was labeled by biotin, immobilized on streptavidin-agarose beads to pull down the soluble fragment of PXRLBD in the absence or presence of drugs (Fig. 6B). Consistent with mammalian two-hybrid system, there was a basal interaction between SRC186 and PXRLBD in the absence of drugs. The addition of rifampicin
induced a moderate but significant augment on the pulling-down of PXRLBD. Cotreatment of CPT or ketoconazole with rifampicin suppressed the recruitment of SRC-1 to hPXR (Fig. 6C).

**Competitive ligand binding assay.** We then determined whether CPT exerted its antagonism effects through the competition of LBD binding with agonists. Test compounds were screened using the LanthaScreen® TR-FRET hPXR competitive binding assay. In this assay, ligands are identified by their ability to compete with and displace a green fluorescent hPXR ligand, Fluormone™ PXR Green, from hPXR. When the positive control, SR12813, was tested, an IC$_{50}$ of 0.14 µM was observed. In contrast, at the testing concentrations ranging from 57 pM to 10 µM, CPT did not compete with Fluormone™ PXR Green (Fig. 7). These results suggest that CPT at the concentrations used for the transcription assays, might act outside the pocket to prevent coactivator recruitments.

**Suppression on CYP3A4-mediated nifedipine metabolism in primary cultures of human hepatocytes.** The above data demonstrate that CPT can interfere with the induction of CYP3A4 expression in humans by limiting the activation of nuclear receptors. To determine whether such inhibitions can interfere with the metabolism of coadministered drugs, especially of those metabolized by CYP3A4, and lead to potential DDIs, we performed metabolism assays of nifedipine in CPT-treated primary cultures of human hepatocytes from three donors. Nifedipine is a commonly used CYP3A4 probe substrate and has been successfully used both an in vivo and in vitro to measure CYP3A4 metabolic activity(Zhou et al., 2005). The authentic compounds were used in HPLC-UV system for substrate and metabolites determination. The peak area ratio of oxidized nifedipine (metabolite from CYP3A4) to nitrendipine (internal control) was used for quantitation.
The preliminary chromatographic data showed that the retention times for oxidized nifedipine, nifedipine and nitredipine were 13.2 min, 14.1 min and 26.4 min, respectively. The LOQ of oxidized nifedipine was 45 pg. Rifampicin effectively increased nifedipine metabolism in all three donors (Table 2). In donor 2, codosing CPT with rifampicin almost completely attenuated the metabolism of nifedipine (Fig. 8). A moderate suppression was also observed in donors 1 and 3 (Table 2). Irinotecan coadministration had no effect on rifampicin-induced production of oxidized nifedipine in donor 2. Assays treated with CPT or irinotecan alone did not show notable changes in nifedipine metabolism (Fig. 8 and Table 2). As previously demonstrated in primary cultures of human hepatocytes, CPT also inhibited the induction of nifedipine metabolism by rifampicin in HepG2 cells (data not shown).
Discussion

In this study, we demonstrated by quantitative gene expression analyses that CPT, the leading compound of a class of topoisomerase I inhibitors, could antagonize the induction of CYP3A4 in primary cultures of human hepatocytes. CPT was later identified as an inhibitor of hPXR and mPXR in nuclear receptor transactivation assays. Additional receptor transactivation assays showed that CPT could also suppress hCAR activations but had inductive effects on hVDR activation. Furthermore, the underlying mechanisms of hPXR inhibition were explored. CPT could inhibit the recruitment of its coactivator, SRC-1, to hPXR but had no effects on the ligand binding and hPXR:hRXRα:hPXRE complex formation. The inductive effects of rifampicin on nifedipine metabolism could be attenuated by coadministration with CPT in primary cultures of human hepatocytes, suggesting a potential prevention on DDIs between CYP3A4 inducers and drugs metabolized by the CYP3A4 in human body. A CPT analogue, irinotecan, was tested in the study in parallel. No suppression on rifampicin-induced CYP3A4 expression or function was observed. Although numerous hPXR activators have been identified, to date there are few reports of potent inhibitors of hPXR. Here, we establish CPT as a novel functional inhibitor of hPXR, which can be a useful chemical tool for modulating PXR-regulated gene expression in vitro or in vivo.

CPT is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I. CPT showed remarkable anti-cancer activity but also low solubility and severe adverse drug effects. Numerous derivatives of CPT have been developed to increase the benefits of the chemical (Wall et al., 1993). In this study, one of the CPT analogues, irinotecan, showed itself as a moderate hPXR inducer and had no inhibitory effects on the induction of CYP3A4 by rifampicin. Topotecan (supplemental fig. 1), another semi-synthetic analogue of CPT used for the treatment
of cisplatin refractory ovarian carcinoma, was reported as a CYP3A4 inducer (Schuetz et al., 2002). It suggests the potential of CPT analogues, which have similar structures, to modify hPXR activity, though they might be in opposite directions – agonism or antagonism. At present, whereas the induction of CYP3A4 level via hPXR is fully understood, the suppression of CYP3A4 level via hPXR is less well characterized. The fact that the receptor has conformational flexibility makes studies somewhat inefficient in recognizing key structures in inhibitors (Xue et al., 2007). Computational approaches combined with experimental data starting from a class of compounds have achieved some fruits in revealing the key pharmacophores and binding regions for inhibitor activities of ketoconazole and its derivatives, azoles (Ekins et al., 2007). Different from other reports on single hPXR inhibitors, our study provides a new class of compounds with similar structures but opposite activities. Strategies starting from a class of compounds with similar structures but opposite activities have significant advantages over other studies, since it is easier to detect the key structure and after comparison. Further structure-activity study is needed to understand the molecular determinants of receptor - compound interactions with the intent of creating inhibitors.

In this study, more than one hPXR agonist was tested with CPT. The inductive effects of both rifampicin and SR12813 were attenuated by the coadministration with CPT (Fig. 3B), indicating it was not a drug - specific effect. In the study, hPXR and several other hPXR target genes were also tested (Figs. 1 & 2). It has been suggested that hPXR ligands enhanced hPXR-mediated transcription in a ligand- and promoter-dependent fashion, which in turn differentially regulated the expression of individual hPXR targets, especially CYP3A4 and MDR1 (Masuyama et al., 2005). Although MDR1 and MRP-1 have been reported to be upregulated by rifampicin in some studies (Geick et al., 2001), the treatment of rifampicin failed to induce MDR-1 and MRP-1
levels in hepatocytes after 72 hour treatment in the current study. So far, we can’t determine whether the antagonism on agonist-induced hPXR activation is CYP3A4-specific.

Extensive cross-talks exist among the nuclear receptors. CYP3A4 expression was also regulated by hCAR and hVDR, aside from hPXR(Thompson et al., 2002; Tirona et al., 2003). Results from cell-based nuclear receptor transactivation assays indicate that CPT selectively antagonized hPXR and hCAR but agonized hVDR. Our study showed that CPT exerted its inhibitory effects on hPXR by inhibiting cofactor, SRC-1, recruitment. hCAR works in a mechanism similar to hPXR by recruiting SRC-1 in CYP3A4 regulation(Auerbach et al., 2005). Both receptors exhibit promiscuous, low affinity ligand binding characteristics(Kliewer and Willson, 2002). It is reasonable to hypothesize that the binding of CPT on hCAR is unable to allosterically conform the liganded hCAR complex into one that attracts transcriptional coactivators as is the case in hPXR. Of all receptors, hVDR is the receptor with the highest sequence identity to hPXR(Kliewer et al., 1998). hPXR and hVDR both possess unusually large ligand binding pockets(Watkins et al., 2001) and recognize similar response element motifs in CYP3A4 promoter(Thompson et al., 2002). However, hVDR has been thought of as a high affinity nuclear receptor with different agonist/inhibitor selection mechanism from that of hPXR(Schmiedlin-Ren et al., 2001). Moreover, ligand-induced hVDR transactivation on CYP3A4 in its classical target tissue, the intestine, differs from hPXR-mediated CYP3A4 regulation in hepatocytes(Schmiedlin-Ren et al., 2001; Pavek et al., 2009). Studies are ongoing in our laboratory to clarify the differential regulation mechanisms of different nuclear receptors.

The regulation of DMEs/drug transporters is a common cause of adverse drug effects/drug resistance. As cancer patients are generally co-administered multiple drugs and supplements, it is understandable that unpredictable therapeutic outcomes emerge due to the hPXR mediated
transcriptional effects on drug pharmacokinetics and pharmacodynamics. CPT was discovered in 1966 by its remarkable anticancer activity but also low solubility. In preliminary trails, the mean concentration of CPT in plasma was 0.1 µM (Natelson et al., 1996). Multiple new drug delivery techniques have been used to improve the concentration of CPT in vivo (Schluep et al., 2006), which is comparable to the effective CPT concentration in the current study. In addition, some findings in human studies are consistent with our conclusion that CPT is an hPXR inhibitor. Lockhart et al. showed that CPT coadministration attenuated drug-induced hPXR activation (Lockhart et al., 2004). Chen et al. found the use of CPT could overcome MDR1-mediated drug resistance in colon cancer cells (Chen et al., 1991). In 2004, Yoshikawa et al. revealed the ability of camptothecin analogs to combat drug resistance mediated by the drug resistance gene ABCG2 (Yoshikawa et al., 2004). The references mentioned above generally establish that CPT may function as a hPXR inhibitor. Considering the extremely important role of DMEs/transporters in drug metabolism and the dominant regulation of hPXR on DMEs/transporters, finding inhibitors for hPXR could provide a unique tool for controlling drug metabolism in cancer therapy.

Aside from its important role in xenobiotic defense, hPXR involves in many physiological and pathophysiological processes, such as the homeostasis of bone (Tabb et al., 2003), lipid (Zhou et al., 2006), mineralocorticoid (Zhai et al., 2007), glucocorticoid (Zhai et al., 2007) and thyroid hormone (Wong et al., 2005), inflammatory responses (Gu et al., 2006), cell growth and apoptosis (Zucchini et al., 2005) and uterine contractility (Mitchell et al., 2005). Hence, hPXR inhibitors may have utility as chemical modulators to interrogate the physiological roles of hPXR. Studies have shown that the antineoplastic agent ET-743 (Synold et al., 2001), the antifungal agent ketoconazole (azoles) (Huang et al., 2007), the dietary isothiocyanate
sulforaphane (Zhou et al., 2007), the human immunodeficiency virus protease inhibitor A-792611 (Healan-Greenberg et al., 2008) and the phytoestrogen coumestrol (Wang et al., 2008) are all functional inhibitors of hPXR. Here, we describe a novel, effective hPXR inhibitor, among the topoisomerase I class of drugs, that is a potent hPXR inhibitors described, with an IC₅₀ of 0.58 µM toward rifampin-mediated receptor activation. Although the CPT has innate toxic action as an anti-neoplastic drug, its usage as a hPXR modulator will not be limited since it is less toxic to normal cells. Further, since CPT is a leading compound of a class of topoisomerase I inhibitors, chemists are working on developing more potent compounds. CPT analogues present the promising prospect of finding an hPXR inhibitor with higher efficiency but lower toxicity.

In conclusion, we have identified a novel and potent inhibitor of hPXR in this study. We have found that CPT attenuates the induction of CYP3A4 by inhibiting agonists - activated hPXR and hCAR. CPT disrupts the interaction of liganded - hPXR with coactivator, but exerts effects on neither the ligand binding nor the formation of hPXR:hRXRα:hPXRE complex. In addition, our study and previous reports have shown that two CPT analogues, irinotecan and topotecan (Schuetz et al., 2002), are hPXR agonists, which suggests the potential of CPT analogues to modify hPXR activities in opposite directions - agonism or antagonism. Such information may have utility in the development of new CPT derivatives with minimal propensity for causing induction/inhibition - type drug interactions. Finally, since CPT is the leading compound in a group of quickly developing small molecules, the finding offers a promise to obtain better hPXR modulators which may be used to tune the efficacy of therapeutics that serve as hPXR agonists in clinic.
Acknowledgements

We acknowledge and thank the following people/affiliations for providing plasmids:

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<th>Provider/Affiliations</th>
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<td>Gal4DB-SRC-1-RID and</td>
<td>Dr. Sridhar Mani / Albert Einstein College of Medicine</td>
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<td>TK-MH100×4-LUC</td>
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<td>pSG5-mPXR and pVP-hPXR</td>
<td>Dr. Jeff L. Staudinger / Department of Pharmacology and</td>
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<td>Toxicology, University of Kansas</td>
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<td>pTracer-CMV2-CAR3 and</td>
<td>Curtis J. Omiecinski / Center for Molecular Toxicology and</td>
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<td>Dr. David John Mangelsdorf / Howard Hughes Medical</td>
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References


Footnotes

Footnote to the title: This work was supported by the Fiscal Year 2009 Penny Severn Postdoctoral Fellowship from Offices of Women’s Health, Illinois Department of Public Health; the U.S. Department of Defense Postdoctoral Prostate Cancer Training Award [Grant PC081012]; and the National Institutes of Health/ National Cancer Institute [Grants R15CA133776, R01CA13345].

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Legends for figures

Fig. 1. CYP3A4/hPXR mRNA levels in primary cultures of human hepatocytes after drug treatments. Columns: mean induction folds (±SD) when compared with mRNA levels in DMSO-treated group from four repeats. Bars: SD. *, $P < 0.05$; **, $P < 0.01$, when comparing groups treated with CPT or irinotecan alone with DMSO-treated groups; or when comparing groups treated with CPT or irinotecan in combination with rifampicin with rifampicin-treated groups. A. CYP3A4 mRNA levels in hepatocytes treated with CPT alone or in combination with rifampicin. B. CYP3A4 mRNA levels in hepatocytes treated with irinotecan alone or in combination with rifampicin. C. hPXR mRNA levels in hepatocytes treated with CPT alone or in combination with rifampicin. D. hPXR mRNA levels in hepatocytes treated with irinotecan alone or in combination with rifampicin.

Fig. 2. Western-blot of proteins in primary cultures of human hepatocytes (Donor 2) after drug treatments. β-actin was used as a loading control. CYP3A4 protein levels were normalized to the vehicle control and expressed as fold-induction.

Fig. 3. Transactivation of the CYP3A4 promoter by the hPXR. The luciferase activities were normalized to the renilla signals. The induction potential was then indicated as induction folds when compared with vehicle control group (DMSO, 0.1%). Column: the mean hPXR induction potential determined in triplicate independent experiments; *, $P < 0.05$; **, $P < 0.01$, when comparing groups treated with CPT or irinotecan or ketoconazole alone with DMSO-treated groups; or when comparing groups treated with CPT or irinotecan or ketoconazole in
combination with rifampicin with rifampicin-treated groups. A. assays in HepG2 cells. Rifampicin, a classic hPXR agonist, was used as a positive control. Ketoconazole, a known hPXR inhibitor, was used as an inhibition control. B. Assays in other cell lines. Both rifampicin and SR12813 (another hPXR agonist) were used to induce hPXR transactivation.

Fig. 4. Transactivations of the CYP3A4 promoter by the hCAR, hVDR and mPXR. The luciferase activities were normalized to the renilla signals. The induction potential was then indicated as induction folds when compared with vehicle control group (DMSO, 0.1%). Column: the mean induction potentials determined in triplicate independent experiments; Bar: S.D. *, $P < 0.05$; **, $P < 0.01$, when comparing groups treated with CPT or irinotecan alone with DMSO-treated groups; or when comparing groups treated with CPT or irinotecan in combination with agonist with agonist-treated groups. A. transactivation of the CYP3A4 promoter by hCAR3 after drug treatments. CITCO, a selective agonist for the human CAR, was used as positive control. B. transactivation of the CYP3A4 promoter by the hVDR after drug treatments. 1, 25-(OH)$_2$VD$_3$, a strong agonist of hVDR, was used as a positive control. C. transactivation of the CYP3A4 promoter by the mPXR after drug treatments. PCN, a strong activator of mPXR, was used as positive control.

Fig. 5. Analyses of the formation of hPXR:hRXR$\alpha$:hPXRE complex by EMSA. Images were generated by scanning the plates directly in an Odyssey infrared scanner at the channel of 800 CM. The quantification was performed by Odyssey 2.0 software.
Fig. 6. Recruitment of Coactivator, SRC-1, to hPXR. A. Analyses of the recruitment of SRC-1 to hPXR by a mammalian two-hybrid system. Rifampicin treatment and ketoconazole treatment were used as controls. The luciferase activity was measured and normalized for transfection efficiency with $\beta$-gal activity. The interaction potentials were indicated as luciferase induction folds when compared with vehicle control group (DMSO, 0.1%). Column: the mean induction folds determined in triplicate independent experiments; Bar: S.D. *, $P < 0.05$; **, $P < 0.01$, when comparing groups treated with CPT or irinotecan in combination with rifampicin with rifampicin-treated groups. B. SDS-PAGE analyses of purified proteins obtained from *E. Coli* expression. SRC186 was overexpressed in *E.Coli* Rosetta (DE3). The expected protein size is 28 kd. The residues 130-434 of PXRLBD were obtained from *E.Coli* Rosetta (DE3), with a coexpression with residues 623-710 of the human SRC-1. The expected size of PXRLBD is 32 kd. C. Analysis of hPXR and SRC-1 binding *in vitro* by a pull-down assay. Preyed PXRLBD was normalized by inputted SRC186. Rifampicin treatment and ketoconazole treatment were used as controls. The interaction potential was indicated as induction fold when compared with the vehicle control group (DMSO, 0.1%).

Fig. 7. The competition of binding to PXR-LBD in a LanthraScreen® TR-FRET competitive binding assay. The assay was performed in 96-well non-coated polystyrene assay plate. The 520/490 TR-FRET ratio was measured using a PerkinElmer EnVision fluorescent plate reader with laser excitation and emission filters. Negative control without competitor (1% DMSO) and positive control with SR12813 were performed in parallel.
Fig. 8. HPLC analyses of the metabolism of nifedipine in primary cultures of human hepatocytes from Donor 2. The oxidized nifedipine, nifedipine and nitrendipine were eluted at 13.2 min, 14.1 min and 26.4 min, respectively. The metabolisms of nifedipine were quantified by comparing the ratio of peak area of oxidized nifedipine to that of nitrendipine. Rif: rifampicin; Iri: irinotecan.
Tables

TABLE 1

Induction of CYP3A4 protein levels in primary cultures of human hepatocytes.

The data are indicated as folds of CYP3A4 levels when compared with DMSO-treated group in each donor.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Drug treatments</th>
<th>DMSO</th>
<th>CPT&lt;sup&gt;a&lt;/sup&gt; 1µM</th>
<th>Iri&lt;sup&gt;b&lt;/sup&gt; 1µM</th>
<th>Rif&lt;sup&gt;c&lt;/sup&gt; 10µM</th>
<th>Rif 10µM + CPT 1µM</th>
<th>Rif 10µM +Iri 1µM</th>
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<tbody>
<tr>
<td>1</td>
<td>DMSO</td>
<td>1</td>
<td>1.5</td>
<td>4.1</td>
<td>8.8</td>
<td>1.9</td>
<td>10</td>
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<tr>
<td>2</td>
<td>1 µM CPT</td>
<td>1</td>
<td>1.8</td>
<td>-&lt;sup&gt;d&lt;/sup&gt;</td>
<td>53</td>
<td>4.7</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>1 µM Rif 10µM</td>
<td>1</td>
<td>1.2</td>
<td>-</td>
<td>31</td>
<td>12</td>
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</tr>
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<sup>a</sup> CPT: camptothecin;

<sup>b</sup> Iri: irinotecan;

<sup>c</sup> Rif: rifampicin;

<sup>d</sup> "-", not determined.
TABLE 2

Metabolisms of nifedipine in primary cultures of human hepatocytes.

The metabolisms of nifedipine were quantified by comparing the ratio of peak area of oxidized nifedipine to that of nitrendipine in HPLC analyses. The data are indicated as induction folds of the production of oxidized nifedipine (CYP3A4 metabolite) when compared with DMSO-treated group in each donor.

<table>
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<tr>
<th>Donor</th>
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<th>CPT&lt;sup&gt;a&lt;/sup&gt; 1µM</th>
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<sup>a</sup> CPT: camptothecin;

<sup>b</sup> Iri: irinotecan;

<sup>c</sup> Rif: rifampicin;

<sup>d</sup> "-", not determined.
Figure 2

Human Hepatocytes - donor 2

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<table>
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Induction folds (CYP3A4 protein)
Figure 5

<table>
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<tr>
<td>hPXR</td>
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</tr>
<tr>
<td>hRXRa</td>
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<td>-</td>
</tr>
<tr>
<td>Rifampicin (10 μM)</td>
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<td>-</td>
</tr>
<tr>
<td>CPT (1 μM)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>irinotecan (1 μM)</td>
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</tr>
<tr>
<td>5IRD800 CYP3A4 dER6</td>
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<td>+</td>
</tr>
<tr>
<td>CYP3A4 dER6 competitor</td>
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![Image of gel electrophoresis](image_url)

-7800 CYP3A4 dER6 -7600 +1
cctTGAAATcagtGTTCAagca

-200 CYP3A4 pER6 +1
tagaattTGAACTcaaggAGGTCAGtgagt

CYP3A4
Figure 8