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Intestinal human colon adenocarcinoma cell line, LS180, is an excellent model to study PXR- but not CAR-mediated CYP3A4 and MDR1 induction: studies with Anti-HIV Protease Inhibitors

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Running Title: LS180 cells - an excellent model to study PXR-mediated induction

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Abbreviations:

PI, anti-HIV Protease inhibitors; CYP, cytochrome P450; MDR1, Multi-drug resistance transporter; P-gp, P-glycoprotein; AUC, area under the plasma concentration-time curve; PXR, pregnane X receptor; RXR, retinoid X receptor-α; AhR, aryl hydrocarbon receptor; CAR1, constitutive androstane receptor; CAR3, natural splice variant of CAR1; OATP, organic anion transporter; BCRP, breast cancer resistance protein; PCR, polymerase chain reaction; RIF, rifampin; RTV, ritonavir; NFV, nelfinavir; APV, amprenavir; SQV, saquinavir; IDV, indianvir; LPV, lopinavir; TPV, tipranavir; ATV, atazanavir.

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Abstract

Lack of an established cell line model to study induction of cytochrome P450s (CYPs) and drug transporters poses a challenge in predicting *in vivo* drug-drug interactions. Although not well characterized, LS180 cells could be an excellent cell line to study induction of CYPs and transporters because they express PXR. Therefore, as part of a larger study of *in vitro* to *in vivo* prediction of inductive drug interactions, we determined induction of various CYPs and drug transporters by the anti-HIV protease inhibitors (PIs) and the prototypic inducer, rifampin, in LS180 cells. Amongst these proteins, the various PIs significantly induced (n=3-5) only CYP3A4 and MDR1 transcripts (2-50 fold). CYP3A4 activity (1'-OH midazolam formation) was increased (2-fold) by rifampin (10 μM) but was reduced by the PIs (1.5-7 fold). Surprisingly, CAR1 was not found to be expressed in these cells. Additionally, using a reporter assay, we found that PIs did not activate CAR3 but significantly activated PXR (2-24 fold), which correlated well with induction of CYP3A4 and MDR1 transcripts (~ r = 0.9). Furthermore, in a PXR-knockdown stable LS180 cell line, induction of CYP3A4 and MDR1 mRNA, following treatment with PIs and rifampin, was significantly reduced (1.4-5 fold) when compared with that in PXR non-silenced cells. Based on these data, we conclude that LS180 cells could be used as a readily available, high throughput cell line to screen for PXR-mediated induction of CYP3A4 and MDR1 transcripts. These data also indicate that the majority of the PIs are likely to produce intestinal drug-drug interactions by inactivating or inhibiting CYP3A enzymes even though they induce CYP3A4 and MDR1 transcripts via PXR.

To date, much of the focus on clinically significant drug interactions has been on metabolic-based inhibitory drug interactions. However, there is increasing appreciation that inductive drug interactions are more common than previously thought and such interactions can also extend to transporters. For this reason, FDA has recently issued draft guidance on models to study inductive drug interactions. This draft guidance states that "*At this time, the most reliable method to study a drug's induction potential is to quantify the enzyme activity of primary hepatocyte cultures following treatments including the potential inducer drug, a positive control inducer drug (rifampin for CYP3A4), and vehicle-treated hepatocytes (negative control), respectively*" (http://www.fda.gov/cder/guidance/6695dft.pdf). These guidelines are notable for their absence of models to study inductive intestinal drugs interactions.

The intestine is a site of expression of CYPs (especially CYP3A4/5) and multiple transporters (e.g. P-glycoprotein). As oral administration of drugs results in relatively high concentrations of the drugs in the intestinal lumen, the potential for clinically significant drug interactions (both inhibitory and inductive) at this site is high. While a lot of attention has been paid towards CYP-based intestinal drug interactions there is increasing evidence that transporter-based drug interactions can also occur at this site. Therefore, there is a need to identify models (e.g. cell-lines) or systems that can be used to rapidly determine intestinal drug interactions.

Anti-HIV protease inhibitors (PIs) are some of the most potent drugs available on the market with respect to their propensity to produce drug interactions (Barry et al., 1999; Malaty and Kuper, 1999; Back, 2006). While a plethora of studies have shown their ability to produce clinically significant CYP-based inhibitory drug interactions, their

ability to produce inductive drug interactions is less well appreciated (Malaty and Kuper, 1999; Unadkat JD and Wang Y, 2000). In addition, there is evidence to suggest that PIs can inhibit and induce P-gp (encoded by MDR1) (Profit et al., 1999; Perloff et al., 2000; Huang et al., 2001; Perloff et al., 2004; Storch et al., 2007). Therefore, as part of a larger study of *in vitro* to *in vivo* prediction of inductive drug interactions of the PIs, we quantified the induction of CYPs and transporters, by the PIs, in an intestinal cell line, LS180 (this study), in human hepatocytes (Dixit et al., 2007) and *in vivo* (Kirby et al., 2006a).

Materials and Methods

Chemicals: Protease Inhibitors (ritonavir, nelfinavir, amprenavir, saquinavir, indinavir, lopinavir, tipranavir, atazanavir) were obtained from NIH AIDS reagent program. Rifampin was purchased from Bedford laboratories, Bedford OH. Midazolam and 1'-OH midazolam were purchased from Cerilliant (Round Rock, TX). CITCO was purchased from Biomol International LP (Plymouth Meeting, PA). HPLC grade solvents (methanol, acetonitrile and water) and DMSO were purchased from Fisher Scientific. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburg, PA), and were of highest purity available.

Cell culture and molecular biology reagents: LS180 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). All the cell culture reagents, as recommended for growth and maintenance of LS180 cells (e.g. culture medium, trypsin etc.) were purchased from ATCC. Fetal bovine serum was obtained from Hyclone

(Logan, UT). Lipofectamine, plus reagent and transfection medium (Opti-MEM) were obtained from Invitrogen technologies (Carlsbad, CA). PCR master mix, reverse transcription reagents and TaqMan probes were purchased from Applied Biosystems (Foster city CA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNA and RNA extraction kits were purchased from Qiagen (Valencia, CA). The Luciferase Reporter Assay System and the β-galactosidase assay kit were purchased from Promega (Madison, WI). Other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted.

Plasmid constructs: The expression plasmid for human PXR (pSG5-hPXRΔATG) and the reporter plasmid, (XREM-CYP3A4)-tk-luc reporter were kindly provided by Dr Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC). Details of the plasmid have been published previously (Goodwin et al., 1999). An expression vector containing β-galactosidase cDNA under T7 promoter, pCH110 was purchased from Amersham Biosciences (Piscataway, NJ). The expression plasmids for human CAR1, CAR3 and RXR and their respective controls were generously provided by Dr. Curtis Omiecinski (Pennsylvania State University, University Park, Pennsylvania) (Auerbach et al., 2005). The CYP2B6-PBREM/XREM reporter plasmid was a kind gift from Dr. Hongbing Wang (University of Maryland, Baltimore, MD) (Wang et al., 2003).

Cell Culture: LS180 cells were grown as suggested by ATCC except that they were sub-cultured using 5mM EDTA to minimize clumping. Briefly, the cells were maintained in

Dulbecco's modified Eagle medium with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin G, and 50 µg/ml streptomycin.

Creating PXR knock-down cell line in LS180 cells (PXRsi): PXR knock-down cell line was created by transfecting lentivirus containing shRNA for PXR. The primer pair designed for PXR shRNA was as follows-

5' - CCG GCG GCA TGA AGA AGG AGA TGA TCT CGA GAT CAT CTC CTT CTT CAT GCC GTT TTT G - 3'

5' - AAT TCA AAA ACG GCA TGA AGA AGG AGA TGA TCT CGA GAT CAT CTC CTT CTT CAT GCC G - 3'

Briefly, lentivirus was produced by co-transfection of lentiviral plasmid (pKLO.1) containing shRNA for PXR along with packaging plasmid (psPAX2) and envelope plasmid (pMD2.G) in the ratio of 4: 3: 1 respectively, in HEK293T cells using calcium-phosphate transfection method. 12-15 hour post-transfection virus was collected, filtered and stored at -80°C until used. This virus filtrate was then added to adherent LS180 cells in the presence of 8µg/ml of polybrene (Sigma). A pool of infected cells was selected in the presence of Puromycin (1µg/ml). Endogenous PXR mRNA levels were confirmed by quantitative RT-PCR using Taqman probes from Applied Biosystems (Foster city CA). This cell line was propagated in the presence of puromycin and called PXRsi. Similarly, the negative control cell line, called GFPsi, was created by using shRNA for GFP. Because of the lack of GFP in LS180 cells, shRNA for GFP should not affect the expression levels of any specific target gene in LS180 cells and would be a control for off-target silencing.

Drug treatment of LS180 or PXRsi or GFPsi cells for mRNA analysis: Cells (0.5 million) were seeded in 6-well plate and allowed to attach at 37°C in a humidified atmosphere of 5% CO_2. Except for nelfinavir, stock solutions of the PIs or rifampin (500X prepared in dimethyl sulfoxide, DMSO) were diluted in medium prior to use. Because of limited solubility, nelfinavir stock solution (500X prepared in DMSO) was mixed with β-cylcodextrin (0.05%) prior to diluting in the medium. Therefore, β-cylcodextrin (0.05%) was added to the medium containing the other drugs as well. Twenty-four h after cell seeding, media containing various drugs were added to respective treatment wells at a final DMSO concentration of 0.2%. DMSO at this concentration had no effect on expression levels of various enzymes and was not cytotoxic. The media was not renewed for the duration of the experiment. Cells were harvested at different time points (24, 48, 72 or 96 h), as specified, following treatment, to determine the time point for maximal induction. To determine the level of induction of the target genes by the various PIs, cells were treated with the drug (10 μM) for 96 h followed by RNA extraction. Detailed concentration-response studies were performed for ritonavir, nelfinavir or rifampin to obtain the EC_{50} and Emax of induction of CYP3A and MDR1 transcripts. Cells were treated with various concentrations of the drug (0-75 μM) for 96 h followed by harvesting the cells in RNA extraction lysis buffer (provided in RNeasy mini kit). RNA was extracted from the harvested cells using RNeasy mini kit according to manufacturer's protocol and stored at -80ºC for mRNA analysis by real-time assay. The concentration of purified RNA was determined by a spectrophotometer (SmartSpec Plus

Spectrophotometer, BioRad), as was the purity, using the 260/280 absorbance ratio (ratio of 1.8 to 2.0).

Quantification of mRNA induction: 2 µg (in 50 µl reaction) of the total RNA was reverse transcribed into cDNA using Applied Biosystem's Taqman reverse transcription reagents according to the manufacturer's instructions. The resulting cDNA was used for real time PCR (qPCR) analysis for all the target genes.

qPCR assays for various CYP (1A1, 1A2, 2B6, 3A4, 3A5, 2C8, 2C9, 2C19, 2D6) enzymes and transporters (MDR1, MRP2, BCRP, OATP1A2) were carried out using gene-specific primers and FAM-labeled fluorescent MGB probes in an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). β-glucuronidase was used as the endogenous control. The real-time reaction contained 10 µl 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 40 ng of RNA equivalent cDNA, and primers (200 nM) and probes (100 nM) in a final volume of 20 µL. The PCR reaction was as follows: 95°C hot start for 10 minutes, followed by 40 cycles at 95°C for 15 sec and then 60°C for 60 seconds. Each sample was analyzed in triplicate. The mRNA levels of each test gene were normalized to *β-glucuronidase*, according to the following formula: C_T (*test gene*) - C_T (*β-gal*) = ΔC_T. Thereafter, the relative mRNA induction levels of each gene were calculated using the $\Delta\Delta C_T$ method: ΔC_T (test gene) - ΔC_T (test gene in the DMSO control) = $\Delta\Delta C_T$ (test gene). The fold-changes of mRNA levels were expressed as the relative expression $2^{-\Delta\Delta C_T}$.

CYP 3A activity assay: CYP3A activity was measured by estimating 1'-OH midazolam (MDZ) formation in drug treated intact cells. Cells (0.5 million) were seeded in a 6-well plate in DMEM containing 10% FBS. After 24 h, cells were treated with DMSO (vehicle) or various drugs as described above. Following 96 h drug treatment, cells were washed twice with phosphate buffered saline (PBS) and then incubated in DMEM (without FBS) at 37 ºC for 4 h (time optimized in a time course study). Following washing, the cells were incubated with media containing 8 µM MDZ. The media was sampled at 1 h and stored at 4 ºC pending analysis for 1'-OH-MDZ. In any given experiment, each reaction was performed in duplicate. A calibration curve ranging from 2.5 to 250 ng/ml of 1'-OH MDZ was prepared in blank cell media. The samples were subjected to liquid-liquid extraction and 1'-OH MDZ amount was measured by LC-MS as described previously (Kirby et al., 2006b) with the following minor modifications. To a 0.5 ml aliquot of cell lysate, a stable labeled internal standard (d5-1′-OH midazolam, m/z 346.9, 25 ng) was added. Samples were acidified with 100 µl of ammonium hydroxide and vortexed. Then, five ml of n-tert butyl ether were used for extraction.

PXR, CAR1 or CAR3 activation assays: PXR activation assay- LS180 cells were seeded into 24 well plates at a density of 2 x 10^5 cells / well. Following 24 hrs of plating, overnight transfection was performed employing Lipofectamine and Plus reagent (Invitrogen), as per the manufacturer's protocol. Briefly, the transfection mixes contained 100 ng human PXR expression vector (pSG5-humanPXR), 400 ng luciferase reporter gene construct (XREM-CYP3A4 -tk-luc), 400 ng pCH110 (an expression vector containing β-galactosidase cDNA under T7 promoter). Following transfection, plasmid-

containing media was replaced with drug- or DMSO-containing media (replaced after 24 hours) and incubated for 48 hours. The final concentration of DMSO (0.2% (v/v)) was kept constant in each sample. In every experiment, rifampin (10 µM) was included as positive control. At the end of 48 hrs, the cell layers were washed twice with ice-cold phosphate buffer saline (pH 7.4), scraped and collected in 200 µl reporter lysis buffer provided with the ß-galactosidase assay system (Promega, Madison, WI). 10 µl of the cell lysate was used for determining the luciferase activity employing a Luciferase Assay System (Promega, Madison, WI). An aliquot of cell lysate (50 µl) was used to determine the β-galactosidase activity according to the manufacturer's recommendation. Luciferase activity was normalized to the β-galactosidase activity and expressed as fold-activation with respect to the solvent (0.2 % DMSO) treated controls. The potency (EC_{50}) and maximal activation of PXR (Emax) by ritonavir, nelfinavir or rifampin was determined by treating the transfected cells with various concentrations (0.1 µM – 50 µM) of the drug. PXR activation by the remaining PIs was studied at only 10 µM.

For CAR1 and CAR3 activation assay, the procedure was similar to the PXR activation assay with the following modifications. For CAR1or CAR3 activation, 100ng of human CAR1 (pCMV2-CAR1) or human CAR3 (pCMV2-CAR3) expression plasmids, respectively were added to the transfection mix containing 200 ng of luciferase reporter plasmid (CYP2B6-PBREM/XREM) and 200 ng of pCH110 (β-galactosidase expression plasmid). To test for RXR-dependent activation, 100 ng of human RXR expression plasmid (pcDNA3.1-RXR) were added to the transfection mix. Negative controls for each experiment involved using the empty plasmid in the transfection mix. After overnight transfection with plasmids (expressing CAR1 or CAR3 along with RXR,

2B6 response elements and β-galactosidase) using Lipofectamine 2000 (Invitrogen technologies, CA), the cells were treated with the drug containing medium for 24 h followed by determining luciferase activity and β-galactosidase activity as explained above. The data for each set of transfection was normalized to the respective DMSO treated controls.

Cell cytotoxicity (MTT) assay: Cell viability was quantified by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxicity of drugs in LS180 cells was tested in a collagen coated 96-well plate. Cells (5×10^4/ well) were seeded overnight followed by addition of rifampin (0-100 μM) or various PIs (0-50 μM) for 96h as described above (in drug treatment section). MTT assay was performed at the end of 96 h as per manufacturer's instructions.

Cell viability was also tested in LS180 cells following transfection and drug treatment. Collagen-coated 24-well plates (2×10^5 cells/ well) were used for toxicity determination. Following transfection (as described in PXR activation assay), cells were treated with various drugs (as described above) for 24 or 48 hr. At the end of the treatment, MTT assay was performed as per manufacturer's instructions. Value was expressed as a percentage of the control.

Statistical and data analysis: Results from real time assays, reporter gene assays and CYP3A activity assay are expressed relative to that observed in the vehicle control. Data are presented as mean ± standard deviation of 3-5 different experiments (triplicate determinations in each experiment unless otherwise indicated). Where appropriate,

statistical comparisons were made on log transformed data (Fig. 1, 2, 4, 5, 6) using ANOVA followed by the Bonferroni correction. The criterion of significance was set at $p< 0.05$ (or as indicated). For concentration-dependent mRNA induction experiments (Fig. 3), E_{max} and EC_{50} were determined using WinNonLin (Pharsight Corporation, Mountain View, CA).

To gain insight into whether rifampin, ritonavir or nelfinavir share a common mechanism for induction of CYP3A4 or MDR1 transcripts, we examined if the induction of each of these transcripts was correlated between the three drugs. In addition, to explore if each drug induced CYP3A4 and MDR1 transcripts by a common mechanism, we determined the correlation in induction of the two transcripts by each of the three drugs. To further confirm that rifampin and PIs induce MDR1 and CYP3A4 via PXR activation, we determined the correlation between PXR activation and CYP3A4 or MDR1 induction across all the drugs. These correlations were estimated using Microsoft Office Excel 2007 (Microsoft, Redmond, WA).

Results

Time course of induction of CYP3A4 and MDR1 transcripts by ritonavir, nelfinavir or rifampin: At 10 μM, the three drugs induced CYP3A4 mRNA to different extent (3-16 fold). This induction reached a plateau between 24-72 h, and then increased (10-30 fold) at 96 h (statistically significant for rifampin but not for ritonavir or nelfinavir) (Fig. 1). In contrast, MDR1 mRNA was maximally induced (4.5-14 fold) by all the three drugs at 24 h and this induction remained stable until 96 h. Since the 96 h time point showed the highest level of induction of CYP3A4 mRNA, all further induction studies

were carried out over this period. We did not investigate longer time points due to the concern of viability of the cells beyond 96 h.

Induction of CYP3A4 and MDR1 transcripts by the PIs or rifampin: Treatment of LS180 cells for 96 h with 10 μM of various PIs (RTV, NFV, APV, IDV, SQV, LPV, TPV, ATV) or RIF, significantly (n=3-5) induced CYP3A4 (2-28 fold) in the order, RIF≈TPV≈APV>RTV≈LPV>NFV≈SQV ≈IDV≈ATV and MDR1 (4-20 fold) in the order RIF≈APV>RTV≈LPV≈TPV>NFV≈ATV ≈SQV (Fig 2A). Indinavir did not significantly induce MDR1 transcripts. When these data were expressed with respect to induction by 10 μM rifampin, tipranavir and amprenavir were as potent as rifampin ($p > 0.05$) in inducing CYP3A4 transcripts followed by RTV≈LPV>NFV>SQV> IDV>ATV. For induction of MDR1 transcripts, ritonavir and amprenavir were as potent as rifampin followed by TPV≈LPV>NFV≈ATV >SQV (Fig 2B). At 10 μM, the transcripts of none of the other CYPs and transporters tested were significantly induced by the PIs. This observation was despite the fact their transcript expression was robust and easily detected (threshold cycle, C_t ranging from 29-35 as estimated in cDNA synthesized from 40 ng of mRNA in qRT-PCR) and followed the order 2B6>2D6>2C9>1A1=1A2 >3A5>3A4=2C19 or MDR1>MRP2> BCRP. However, the expression of CYP2C8 and OATP1A2 transcripts was low or undetectable. At the concentrations used, the cytotoxicity (determined by the MTT assay) of the drugs was minimal with estimated cell survival of 85-100%.

Concentration-dependent induction of CYP3A4 and MDR1 by rifampin, ritonavir or nelfinavir in LS180 cells: In order to allow future *in vitro* to *in vivo* prediction of CYP- and transporter-based inductive drug interactions by ritonavir and nelfinavir, we conducted a concentration-dependent study to estimate the Emax and EC_{50} of induction of CYP3A4 and MDRI transcripts by these PIs and rifampin. Transcript levels of CYP3A4 and MDR1 were determined in cells treated with varying concentrations of ritonavir (0-25 μM), nelfinavir (0-15 μM) or rifampin (0-50 μM). The concentration range for the three drugs were based on obtaining greater than 85% cell survival (estimated by MTT assay) in the presence of the drugs. All the three drugs induced CYP3A4 and MDR1 transcripts in a concentration dependent manner (Fig 3). Emax and EC_{50} of CYP3A4 transcript induction by nelfinavir could not be estimated due to cytotoxicity observed at the higher concentrations (> 15 μM) of the drug. The EC_{50} of induction of CYP3A4 (~ 7 μM) or MDR1 (~ 1.5 μM) transcripts by ritonavir and rifampin were remarkably similar with MDR1 transcripts being more inducible at a lower concentration of the drugs than CYP3A transcripts. The maximal induction of transcripts, as determined by Emax (Fig. 3), differed between the drugs and decreased in the order: CYP3A4- RIF>RTV and MDR1- RIF>RTV>NFV. Maximal induction of CYP3A4 transcripts was higher than MDR1 transcripts by rifampin (49-fold and 24-fold, respectively) at 50 μM or by nelfinavir (15-fold and 6-fold, respectively) at 15 μM concentration. However, maximal induction of CYP3A4 and MDR1 transcripts by ritonavir (25 μM) was comparable (~ 13 fold).

CYP3A activity in LS180 cells in the presence of various PIs or rifampin: To test whether the above induction in CYP3A4 transcripts translated into activity, we optimized a CYP3A activity assay in LS180 cells using midazolam as a substrate. Because the inducers could act as CYP3A inhibitors, after induction, and prior to measuring CYP3A activity, we incubated the cells with drug free media for 4 h to promote drug efflux (a time determined to be optimal in a time course experiment ranging from 0-12 h). Rifampin induced CYP3A activity by two-fold (when compared to DMSO control) at 10 μM but not at other concentrations tested (1 and 5 μM) (Fig 4). At the concentration tested (1, 5 and 10 μM), ritonavir decreased CYP3A activity by 7-fold while nelfinavir decreased CYP3A activity by approximately 4-fold. At 10 μM, saquinavir decreased CYP3A activity by 3-fold while lopinavir, tipranavir and atazanavir decreased this activity by only 1.5-fold. At 10 μM, amprenavir and indinavir had no effect on CYP3A activity.

Role of PXR in the induction of CYP3A4 and MDR1 transcripts in LS180 cells: In order to gain insight into the mechanisms involved in induction of CYP3A4 and MDR1 transcripts by the PIs, we measured the activation of PXR as well as the induction of CYP3A4 and MDR1 transcripts in PXR competent and PXR-knockdown LS180 cells by the PIs or rifampin.

PXR activation by PIs or rifampin in a reporter gene assay in LS180 cells - At 10 μM, the order of PXR activation was as follows: RIF (20-fold) > RTV (11-fold) ≈ APV (12-fold) > LPV (5-fold) ≈ TPV (5-fold) ≈ SQV (4-fold) ≈ATV (4-fold) ≈ IDV (2.4-fold) (Fig 5). Statistically significant activation was observed with all the PIs except 10 μM

nelfinavir. This was because nelfinavir was significantly more toxic to the transfected cells (~10% cell survival) than the other PIs where the cytotoxicity was minimal (>90% cell survival observed). When nelfinavir was studied in a concentration-response study, significant PXR activation (2.7–11.7 fold) was observed at lower concentrations (0.1-5 μM). This demonstrates that nelfinavir was more toxic in this assay than the induction assay possibly because of treatment of the cells with lipofectamine combined with 48 h of drug exposure. Concentration-response studies for PXR activation were performed by treating transfected cells with various concentrations of ritonavir (0.1-50 μM), nelfinavir (0.1-5 μM) or rifampin (0.1-50 μM). All the three drugs activated PXR in a concentration dependent manner (data not shown). The EC_{50} of PXR activation (~ 0.4 μM) by ritonavir and nelfinavir were remarkably similar but lower than rifampin (~ 0.9 μM). The EC_{50} of PXR activation by rifampin was comparable to that reported previously (0.72 μM, (Lemaire et al., 2006)). The maximal PXR activation, as determined by Emax, was similar between ritonavir and nelfinavir (~ 13-fold) and slightly higher for rifampin (~ 16-fold).

Induction of CYP3A4 and MDR1 transcripts by various protease inhibitors and rifampin in PXR-knockdown LS180 cells- To confirm the predominant involvement of PXR in PI-mediated induction of CYP3A4 and MDR1 transcripts in LS180 cells, we established a PXR-knockdown LS180 cell line (PXRsi-LS180) using RNA interference. When compared with the negative vector transfected control cell line, GFPsi-LS180, we consistently achieved greater than 80% knockdown of PXR transcripts in this PXRsi-LS180 cell line. The basal levels of CYP3A4 and MDR1 decreased (1.5 to 3-fold; P>0.05) in PXRsi-LS180 cells compared with the control cells. When these cells were

treated with various PIs or rifampin (10 μM) for 96 h, induction of CYP3A4 and MDR1 transcripts by rifampin or the more potent PI inducers (RTV, NFV, APV, LPV, TPV; induction levels greater than 5- fold) was reduced by 60-80% in PXRsi-LS180 cells compared with the control cells (Figure 6). As expected, weak inducers (IDV, ATV and SQV) did not affect the induction of CYP3A4 and MDR1 transcripts in PXRsi-LS180 cells when compared with control cells (data not shown).

Role of CAR in the induction of CYP3A4 and MDR1 transcripts by the PIs in LS180 cells: Although PXR appeared to be the major player in the induction of CYP3A4 and MDR1 transcripts in LS180 cells, we also investigated the role of CAR1 in the induction of these transcripts because it has been previously shown to be involved in the transcriptional regulation of CYP3A4 (Goodwin et al., 2002) and intestinal MDR1 (Burk et al., 2005).

Expression of CAR1 transcript in LS180 cells - Prior to creating CAR1-knockdown cell line, we determined the expression of CAR1 in LS180 cells. To our surprise, we discovered that CAR1 was not expressed in LS180 cells. CITCO is a known activator of CAR and induces the prototypical CAR target gene, CYP2B6 (Maglich et al., 2003). To confirm the absence of CAR1 in LS180 cells, we treated LS180 cells with CITCO (1 μM and 5 μM) for 96 h. Consistent with our observation that CAR1 is not expressed in LS180 cells, CITCO did not induce CYP2B6 transcripts. These data indicate that the PIs or rifampin induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR and not CAR1 activation.

CAR1 and CAR3 activation by PIs or rifampin – Even though CAR1 is absent in LS180 cells, it is present in human hepatocytes where the PIs and rifampin are potent inducers of CYP2B6 (Dixit et al., 2007). Therefore, we determined if the PIs could activate CAR1. Transfection of LS180 cells with CAR1 resulted in high basal activity even in the absence of ligands (e.g. CITCO 5 μM), thus making it difficult to determine whether PIs are ligands of CAR1. A similar phenomenon has been reported by other groups in immortalized hepatic cell line such as HepG2 (Honkakoski et al., 1998). For this reason, we used a naturally occurring splice variant of CAR1, namely CAR3. This variant has low basal activity and has been successfully used to identify CAR ligands (Auerbach et al., 2003). None of the PIs activated CAR3 and CITCO activated CAR3 only 2.3 to 4-fold. CITCO's poor activation of CAR3 led us to test if co-expression of RXR was required for CAR3 activation. Indeed it was. When RXR (pcDNA3.1-RXR) was co-expressed with CAR3 (CMV2-CAR3), we observed a 35- to 48-fold activation by CITCO (5μM) of the CAR reporter (data not shown). This magnitude of CAR3 activation is consistent with CITCO-mediated RXR-dependent CAR3 activation observed in COS-1 cells (Auerbach et al., 2005). However, even in this refined activation assay, PIs (10 μM) did not activate CAR3 (data not shown).

Correlation in induction of transcripts: The correlation between the three drugs (RTV, NFV, RIF) in induction of CYP3A4 transcripts was excellent (r=0.8-0.86) as was that of the MDR1 transcripts (r=0.91-0.92) (Table 1A). In addition, the induction of CYP3A4 transcripts was highly correlated (r=0.88) with that of MDR1 transcripts across all eight PIs and rifampin. Similarly, across all the drugs (nelfinavir was not included due to

cytotoxicity), the correlation between PXR activation and induction of CYP3A4 (r=0.93) or MDR1 transcripts (r=0.93) was excellent and similar (Table 1B).

Discussion

LS180 cells are an intestinal colon carcinoma cell line that expresses some characteristics of the small intestine (Tom et al., 1976). We chose to use these cells as they have been previously used to study induction of CYP3A and P-gp and because they constitutively express CYP3A, P-gp and PXR (Pfrunder et al., 2003).

Although LS180 cells expressed transcripts of many of the CYPs at levels comparable to that of CYP3A4, only CYP3A4 transcripts were inducible by the various PIs and rifampin (Fig 2). This is surprising as we have previously shown that RTV, NFV and RIF are inducers of CYP2B6, 2C9, and 2C19 transcripts in human hepatocytes (Dixit et al., 2007). This observation suggests that LS180 cells lack the necessary transcriptional factors (or other cellular machinery) for induction of these CYP transcripts. As discussed below, one such factor is CAR1, which is particularly important in the induction of CYP2B6 (Wang et al., 2004). Interestingly, tissue specific induction of CYP3A expression and activity has been observed *in vivo*. Mouly et al., observed induction of *in vivo* hepatic CYP3A4 activity by efavirenz as measured by the erythromycin breath test (ERMBT) without a change in intestinal CYP3A4 or P-glycoprotein expression (Mouly et al., 2002). Similarly, chronic administration of NFV to rats induced hepatic but not intestinal CYP3a expression (Huang et al., 2001).

RTV, APV, LPV, TPV were more potent inducers of CYP3A4 and MDR1 transcripts (>50% of induction produced by RIF) than NFV, which was a modest inducer,

or SQV, IDV and ATV, which were weak inducers (Fig 2). Amongst the transporters studied, the PIs and rifampin induced MDR1 transcripts (Fig. 2) but not BCRP, MRP2 or OATP1A2.

We compared the concentration-dependent induction of CYP3A4 and MDR1 transcripts by ritonavir and nelfinavir with that produced by rifampin. These two PIs were chosen as they differ in their frequency and magnitude of clinical drug interactions. Ritonavir produces more frequent and profound drug interactions when compared with nelfinavir. In addition, *in vivo*, nelfinavir appears to be a less potent inducer of CYP enzymes than ritonavir. The relative induction of CYP3A4 and MDR1 transcripts by the three drugs was concentration-dependent. The EC_{50} for induction of MDR1 and CYP3A4 transcripts by the inducers were remarkably consistent while the Emax differed (Fig 3). These data support the notion that these three drugs share a common mechanism, most likely PXR, in the induction of CYP3A4 and MDR1 transcripts (see below for further discussion). The EC_{50} value for CYP3A4 mRNA induction by rifampin obtained in our study is higher (~8 μM) than similar studies in either human hepatocytes (0.37 μM, (Faucette et al., 2004)) or the hepatic cell lines, (~1.9 μM in Fa2N-4 cells, (Ripp et al., 2006); ~0.25 μM in HepaRG cells (Kanebratt and Andersson, 2007)). The reasons for this discrepancy are not clear. It may be due to differences in the expression of transcription factors such as CAR1 (HepaRG and human hepatocytes express CAR1; Aninant et al., 2006). Also, differences in the cytotoxicity profile of some drugs (e.g. ritonavir) between the cell lines have been observed suggesting differences in drug permeability between the cells (for example due to differences in expression of transporters). This could result in differences in EC_{50} between the cell lines. The same

may be true for rifampin resulting in different EC_{50} values between cell types. Since we did not replenish the medium with drug during the 96 h treatment, there might have been some drug depletion during the period resulting in a shift in the concentration-response curve.

In contrast to the magnitude of induction of CYP3A4 transcripts by the three drugs, the magnitude of induction of CYP3A activity by rifampin was modest (~ 2-fold) while this activity was reduced in the presence of the majority of the PIs. For rifampin, the reason for the discrepancy between induction of CYP3A4 transcripts and CYP3A activity is not clear. The LS180 cells do not translate the increased CYP3A transcripts into activity either because they lack the necessary machinery to do so or the protein may be unstable in these cells. In addition, our assay for quantification of CYP3A activity did not distinguish between CYP3A4 and CYP3A5. Although CYP3A5 transcripts are expressed in LS180 cells at similar levels as CYP3A4 transcripts, CYP3A5 transcripts are less inducible by rifampin and negligibly induced by the PIs (data not shown). This could have dampened the overall induction in CYP3A activity, but is unlikely to completely explain the modest induction of this activity. Also, despite washing the cells for 4 h, residual amounts of rifampin in the cells could also have inhibited CYP3A activity. In contrast, the reduction in CYP3A activity by the majority of the PIs was probably due to inactivation of the protein as has been previously shown *in vitro* (Granfors et al., 2006) and *in vivo* (Fellay et al., 2005; Yeh et al., 2006). In addition, we cannot rule out the possibility that despite washing the cells for 4 h, residual intracellular PIs may have inhibited CYP3A activity since these drugs are potent inhibitors of CYP3A activity (Ernest et al., 2005). Consistent with the previous data (Ernest et al., 2005),

ritonavir was the most potent inhibitor of CYP3A activity while indinavir did not inhibit CYP3A activity. Despite our efforts to test if P-gp activity is induced by the PIs or rifampin, we were unable to measure P-gp activity in these cells possibly because of low levels of P-gp protein expression at the cell surface. Although Perloff et al. (Perloff et al., 2003) observed induction of P-gp activity (measured by RH123 accumulation) in the LS180 cells by ritonavir, they did so in cells selected (using vinblastine) for elevated expression of P-gp.

Rifampin has been shown to induce CYP3A and P-gp activity by activating PXR (Lehmann et al., 1998; Geick et al., 2001). Dussault et al. have reported that ritonavir is an excellent activator/ligand of PXR (comparable to rifampin) but not of CAR or VDR (Dussault et al., 2001). Interestingly, none of the other PIs tested in their study, saquinavir or indinavir, activated PXR. In contrast, we observed statistically significant PXR activation with all the PIs. We suspect that the discrepancy in the two studies could be due to Dussault et al. (Dussault et al., 2001) not accounting for the poor solubility of the PIs. We utilized β-cyclodextrin to improve the solubility of the PIs (data not shown). The significant correlation between PXR activation and induction of MDR1 or CYP3A4 transcripts (Table 1B) supports the notion that these drugs induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR activation.

The above correlation is not a proof. To directly test that PXR was involved in the induction of CYP3A4 and MDR1 transcripts in LS180 cells, we knocked-down PXR expression in these cells. In PXR knocked-down cells, the induction of CYP3A4 and MDR1 transcripts by the PIs and rifampin was significantly reduced and this reduction was proportionate to the reduction in PXR transcripts (Fig 6). These data unequivocally

show that PIs and rifampin induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR activation. Since CAR (constitutive androstane receptor) is also important in inducing CYP3A4 and MDR1 (Goodwin et al., 2002; Burk et al., 2005; Cerveny et al., 2007), we investigated if CAR1 is expressed in LS180 cells. Surprisingly, we found that CAR1 transcripts were not expressed in LS180 cells. These data further support our conclusion that induction of CYP3A4 and MDR1 transcripts by the PIs and rifampin in LS180 cells is PXR- and not CAR1-mediated.

While CAR1 is not expressed in LS180 cells, it is expressed in the human liver where it may play a role in the *in vivo* induction of CYP3A4 and MDR1 transcripts by the PIs. It is difficult to study CAR1 activation in hepatic-derived immortalized cells, as CAR1 spontaneously translocates to nucleus in the absence of ligand (Honkakoski et al., 1998) resulting in high constitutive CAR activity. The most frequently used cell system to study CAR1 activation are the primary human hepatocytes. Their limited availability, variability and lack of suitability for high-throughput assays prompted us to test whether LS180 cells could potentially be used for CAR1 activation. Unfortunately, results from our experiments suggest that similar to HepG2 cells, CAR1 translocates to the nucleus in the absence of ligand in LS180 cells, hence LS180 cells cannot be used for CAR1 activation studies. To overcome this problem, measurement of activation of CAR3, a splice variant of CAR1, has been proposed. CAR3 has low basal activity (Auerbach et al., 2005) and does not spontaneously translocate to the nucleus. Therefore, we determined the ability of the PIs to activate CAR3 in LS180 cells. None of the PIs tested were able to activate CAR3 indicating negligible involvement of CAR in PI-mediated induction of CYP3A4 and MDR1. These data further support our conclusion that PIs and

rifampin induce CYP3A4 and MDR1 transcripts by PXR- and not CAR-mediated activation.

Since the PIs inactivate CYP3A enzymes, both *in vitro* and *in vivo*, their ability to induce CYP3A4 transcripts will not translate into induction of CYP3A activity. Therefore such induction will not correlate with *in vivo* CYP3A activity after chronic administration of these drugs. However, does the ability of PIs to induce MDR1 transcripts in LS180 cells correlate with their ability to induce P-gp activity *in vivo*? In humans, single dose administration of ritonavir or lopinavir/ritonavir increased the plasma AUC (2.2-4 fold) and C_{max} of the P-gp substrate, fexofenadine. However, a moderate induction of P-gp activity was suspected after 12 days of lopinavir/ritonavir 400/100 mg twice daily, although a net inhibitory effect on P-gp activity was still maintained (van Heeswijk et al., 2006). Additionally, when digoxin (0.4 mg), a P-gp substrate, was administered orally before and after ritonavir treatment (200 mg twice daily for 14 days), digoxin AUC_{0-72} was increased by 22% and oral digoxin clearance was reduced by approximately 30% (Penzak et al., 2004). In another study, 300mg of ritonavir twice daily for 11 days resulted in increased $AUC_{0-\infty}$ (86%) and volume of distribution (77%) of digoxin (0.5mg) administered IV on day 3 of ritonavir administration. Furthermore, the non-renal (48%) and renal clearance (35%) of digoxin was reduced (Ding et al., 2004). Collectively, these data suggest that on chronic administration of the drug, ritonavir is a net inhibitor, not inducer, of intestinal P-gp.

In conclusion, this is the first comprehensive study of PI-mediated induction of CYP3A4 and MDR1 transcripts in LS180 cells. We show for the first time that nelfinavir and several other PIs such as amprenavir, tipranavir, lopinavir, atazanavir and saquinavir

induce CYP3A4 and MDR1 transcripts by PXR (but not CAR) activation. In addition, our results indicate that LS180 cells do not express CAR1 and therefore can serve as an excellent, inexpensive cell line to screen for PXR-mediated induction of CYP3A4 and MDR1 gene products.

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References:

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouët S, Morel F, Guguen-Guillouzo C, Guillouzo A (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos.* **34**:75-83.

Auerbach SS, Ramsden R, Stoner MA, Verlinde C, Hassett C and Omiecinski CJ (2003) Alternatively spliced isoforms of the human constitutive androstane receptor. *Nucleic Acids Res* **31:**3194-3207.

Auerbach SS, Stoner MA, Su S and Omiecinski CJ (2005) Retinoid X receptor-alpha-dependent transactivation by a naturally occurring structural variant of human constitutive androstane receptor (NR1I3). *Mol Pharmacol* **68:**1239-1253.

Back DJ (2006) Drug-drug interactions that matter. *Top HIV Med* **14:**88-92.

Barry M, Mulcahy F, Merry C, Gibbons S and Back D (1999) Pharmacokinetics and potential interactions amongst antiretroviral agents used to treat patients with HIV infection. *Clin Pharmacokinet* **36:**289-304.

Burk O, Arnold KA, Geick A, Tegude H and Eichelbaum M (2005) A role for constitutive androstane receptor in the regulation of human intestinal MDR1 expression. *Biol Chem* **386:**503-513.

Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, Ulrichova J, Anzenbacher P and Pavek P (2007) Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. *Drug Metab Dispos* **35:**1032-1041.

Ding R, Tayrouz Y, Riedel KD, Burhenne J, Weiss J, Mikus G and Haefeli WE (2004) Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. *Clin Pharmacol Ther* **76:**73-84.

Dixit V, Hariparsad N, Li F, Desai P, Thummel K and Unadkat J (2007) Cytochrome P450 Enzymes And Transporters Induced By Anti-HIV Protease Inhibitors In Human Hepatocytes: Implications For Predicting Clinical Drug Interactions. *Drug Metab Dispos* **35:**1853-1859.

Dussault I, Lin M, Hollister K, Wang EH, Synold TW and Forman BM (2001) Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. *J Biol Chem* **276:**33309-33312.

Ernest CS, 2nd, Hall SD and Jones DR (2005) Mechanism-based inactivation of CYP3A by HIV protease inhibitors. *J Pharmacol Exp Ther* **312:**583-591.

Faucette SR, Wang H, Hamilton GA, Jolley SL, Gilbert D, Lindley C, Yan B, Negishi M and LeCluyse EL (2004) Regulation of CYP2B6 in primary human hepatocytes by prototypical inducers. *Drug Metab Dispos* **32:**348-358.

Fellay J, Marzolini C, Decosterd L, Golay KP, Baumann P, Buclin T, Telenti A and Eap CB (2005) Variations of CYP3A activity induced by antiretroviral treatment in HIV-1 infected patients. *Eur J Clin Pharmacol* **60:**865-873.

Geick A, Eichelbaum M and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* **276:**14581-14587.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* **62:**359-365.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56:**1329-1339.

Granfors MT, Wang JS, Kajosaari LI, Laitila J, Neuvonen PJ and Backman JT (2006) Differential inhibition of cytochrome P450 3A4, 3A5 and 3A7 by five human immunodeficiency virus (HIV) protease inhibitors in vitro. *Basic Clin Pharmacol Toxicol* **98:**79-85.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18:**5652-5658.

Huang L, Wring SA, Woolley JL, Brouwer KR, Serabjit-Singh C and Polli JW (2001) Induction of P-glycoprotein and cytochrome P450 3A by HIV protease inhibitors. *Drug Metab Dispos* **29:**754-760.

Kanebratt K and Andersson T (2007) HepaRG Cells as an in Vitro Model for Evaluation of Cytochrome P450 Induction in Humans. *Drug Metab Dispos* **36**:137-145.

Kirby B, Collier A, Thummel K, Kharasch E and Unadkat J (2006a) Effect of multiple dose administration of nelfinavir or rifampin on in-vivo CYP1A2, 2C9, 2D6, 3A, and P-gp activity In HIV-negative volunteers. *Drug Metabolism Reviews* **38:** 110.

Kirby B, Kharasch ED, Thummel KT, Narang VS, Hoffer CJ and Unadkat JD (2006b) Simultaneous measurement of in vivo P-glycoprotein and cytochrome P450 3A activities. *J Clin Pharmacol* **46:**1313-1319.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate

CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102:**1016-1023.

Lemaire G, Mnif W, Pascussi JM, Pillon A, Rabenoelina F, Fenet H, Gomez E, Casellas C, Nicolas JC, Cavailles V, Duchesne MJ and Balaguer P (2006) Identification of new human pregnane X receptor ligands among pesticides using a stable reporter cell system. *Toxicol Sci* **91:**501-509.

Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM and Moore JT (2003) Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278:**17277-17283.

Malaty LI and Kuper JJ (1999) Drug interactions of HIV protease inhibitors. *Drug Saf* **20:**147-169.

Mouly S, Lown KS, Kornhauser D, Joseph JL, Fiske WD, Benedek IH and Watkins PB (2002) Hepatic but not intestinal CYP3A4 displays dose-dependent induction by efavirenz in humans. *Clin Pharmacol Ther* **72:**1-9.

Penzak SR, Shen JM, Alfaro RM, Remaley AT, Natarajan V and Falloon J (2004) Ritonavir decreases the nonrenal clearance of digoxin in healthy volunteers with known MDR1 genotypes. *Ther Drug Monit* **26:**322-330.

Perloff MD, Stormer E, von Moltke LL and Greenblatt DJ (2003) Rapid assessment of P-glycoprotein inhibition and induction in vitro. *Pharm Res* **20:**1177-1183.

Perloff MD, von Moltke LL, Fahey JM, Daily JP and Greenblatt DJ (2000) Induction of P-glycoprotein expression by HIV protease inhibitors in cell culture. *Aids* **14:**1287-1289.

Perloff MD, von Moltke LL and Greenblatt DJ (2004) Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats. *Xenobiotica* **34:**133-150.

Pfrunder A, Gutmann H, Beglinger C and Drewe J (2003) Gene expression of CYP3A4, ABC-transporters (MDR1 and MRP1-MRP5) and hPXR in three different human colon carcinoma cell lines. *J Pharm Pharmacol* **55:**59-66.

Profit L, Eagling VA and Back DJ (1999) Modulation of P-glycoprotein function in human lymphocytes and Caco-2 cell monolayers by HIV-1 protease inhibitors. *Aids* **13:**1623-1627.

Ripp SL, Mills JB, Fahmi OA, Trevena KA, Liras JL, Maurer TS and de Morais SM (2006) Use of immortalized human hepatocytes to predict the magnitude of clinical drug-drug interactions caused by CYP3A4 induction. *Drug Metab Dispos* **34:**1742-1748.

Storch CH, Theile D, Lindenmaier H, Haefeli WE and Weiss J (2007) Comparison of the inhibitory activity of anti-HIV drugs on P-glycoprotein. *Biochem Pharmacol* **73:**1573-1581.

Tom BH, Rutzky LP, Jakstys MM, Oyasu R, Kaye CI and Kahan BD (1976) Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro* **12:**180-191.

Unadkat JD and Wang Y (2000) Antivirals., in: *Metabolic Drug Interactions* (Levy RH, Thummel KE, Trager WF, Hansten PD and Eichelbaum M eds), pp 421-434., William Wilkins.

van Heeswijk RP, Bourbeau M, Campbell P, Seguin I, Chauhan BM, Foster BC and Cameron DW (2006) Time-dependent interaction between lopinavir/ritonavir and fexofenadine. *J Clin Pharmacol* **46:**758-767.

Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M and LeCluyse E (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. *J Biol Chem* **279:**29295-29301.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* **278:**14146-14152.

Yeh RF, Gaver VE, Patterson KB, Rezk NL, Baxter-Meheux F, Blake MJ, Eron JJ, Jr., Klein CE, Rublein JC and Kashuba AD (2006) Lopinavir/ritonavir induces the hepatic activity of cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP1A2 but inhibits the hepatic and intestinal activity of CYP3A as measured by a phenotyping drug cocktail in healthy volunteers. *J Acquir Immune Defic Syndr* **42:**52-60.

Footnotes:

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Figure Legends

Fig 1: Induction of CYP3A4 and MDR1 transcripts by 10 μM rifampin (RIF), ritonavir (RTV) or nelfinavir (NFV) was significant at 24, 48, 72 and 96 h. For RIF, induction of CYP3A4 transcripts was constant over 24-72 h and then increased at 96 h ($p<0.05$) while that of RTV and NFV showed a similar pattern but induction levels at 96 h were not significantly different from that at 24-72 h. MDR1 transcripts remained unchanged from 24 to 96 h. Data are presented as mean ± S.D. (n=4).

Fig. 2: When expressed relative to DMSO (A) or 10 μM rifampin (B), protease inhibitors (PIs; 10 μM) significantly induced CYP3A4 (open bar) transcripts with rifampin (RIF), tipranavir (TPV), amprenavir (APV), ritonavir (RTV) and lopinavir (LPV) producing >10-fold induction; NFV and SQV producing >5-fold induction; IDV and ATV producing <5-fold induction. The induction of MDR1 (solid bar) transcripts was significant ($p<0.05$) for rifampin and the PIs (except IDV) with RIF, APV, RTV, LPV, TPV producing > 10-fold induction; NFV and ATV producing >5-fold induction; and SQV producing <5-fold induction. Data are presented as mean±S.D. (n=3-5). LS180 cells were treated with various PIs (10 μM) for 96 h and mRNA levels were measured as described in materials and methods.

Fig. 3: Induction of CYP3A4 and MDR1 transcripts by rifampin (RIF), ritonavir (RTV) or nelfinavir (NFV) is concentration-dependent. LS180 cells were treated with RIF (square, 0-50 μM), RTV (triangle, 0-25 μM) or NFV (inverted triangle, 0-15 μM) for 96 h and mRNA levels were measured as described in materials and methods. Data (mean± S.D., n=3-5) were expressed relative to DMSO. EC_{50} and Emax were estimated by WinNonLin (Pharsight, CA). The EC_{50} was comparable between the compounds for MDR1(~1.5 μM) and CYP3A4 (~7 μM). The Emax of induction differed in the following order - CYP3A4, RIF> RTV; MDR1, RIF> RTV> NFV. The Emax and EC_{50} of CYP3A4 transcript induction by nelfinavir could not be estimated because of cytotoxicity at concentrations greater than 15 μM. n.d. - Could not be determined.

Fig. 4: Protease inhibitors (PIs) affect CYP3A activity as measured by 1'-OH midazolam formation (pg/µg protein). LS180 cells were treated with PIs or rifampin at various concentrations for 96 h, cells were washed for 4 h and MDZ activity was measured as described in materials and methods. Rifampin treatment at 10 µM resulted in 2-fold increase in CYP3A activity whereas PIs treatment resulted in decreased activity in the following order ritonavir (1, 5, 10 µM) > nelfinavir (1, 5, 10 µM) > saquinavir (10 µM) > lopinavir (10 µM) = tipranavir (10 µM) = atazanavir (10 µM). Indinavir (10 µM) and amprenavir (10 µM) treatment did not affect CYP3A activity. Data are presented as mean±S.D. (n=4).

Fig. 5: Protease inhibitors (PIs) or rifampin activate PXR. LS180 cells were transfected with PXR expression plasmid and CYP3A4-PXRE/XREM followed by treatment with the test drugs for 48 h. Luciferase activity was measured following treatment and data were normalized to β-galactosidase activity. PIs (10 µM) activated PXR in the following order - ritonavir ≈ amprenavir > lopinavir ≈ tipranavir ≈ saquinavir ≈ atazanavir ≈ indinavir. Data are expressed relative to DMSO control treatment and presented as mean±S.D. (n=3).

Fig. 6: Induction of CYP3A4 and MDR1 transcripts is reduced in PXR knocked-down (PXRsi) cells when compared with negative vector transfected (GFPsi) cells. PXRsi (PXR levels ~20% of control cells) cells were treated for 96 h with moderate to strong inducers (fold induction > 5) namely rifampin (RIF), ritonavir (RTV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV) or tipranavir (TPV). For all drugs, the magnitude of induction of CYP3A4 (open bar) and MDR1 (solid bar) transcripts in PXRsi cells was 20-40% of that in control cells. Data are presented as mean±S.D. (n=4).

Table 1: Correlation (r) between (A) induction of CYP3A4 and MDR1 transcripts by ritonavir (RTV), nelfinavir (NFV) or rifampin (RIF) and (B) PXR activation and induction of CYP3A4 or MDR1 by various PIs and rifampin.

A

mRNA		RIF	RTV
		3A4	3A4
RTV	3A4	0.8	
	MDR1	0.91	
NFV	3A4	0.86	0.83
	MDR1	0.91	0.92

B

mRNA/activation	MDR1	PXR
CYP3A4	0.88	0.93
MDR1		0.93

Figure 1

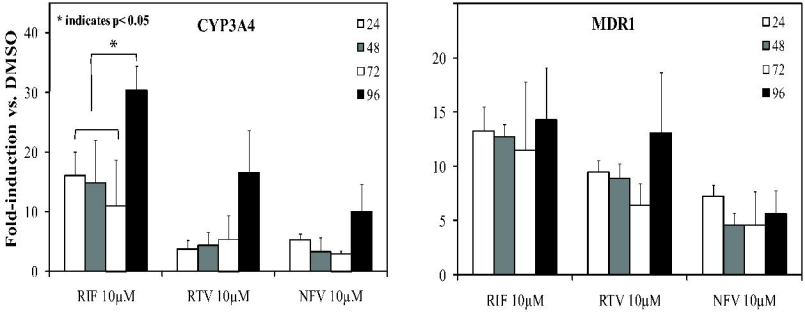

* indicates p< 0.05
CYP3A4
MDR1
24
48
72
96
Fold-induction vs. DMSO
RIF 10μM
RTV 10μM
NFV 10μM

Figure 2

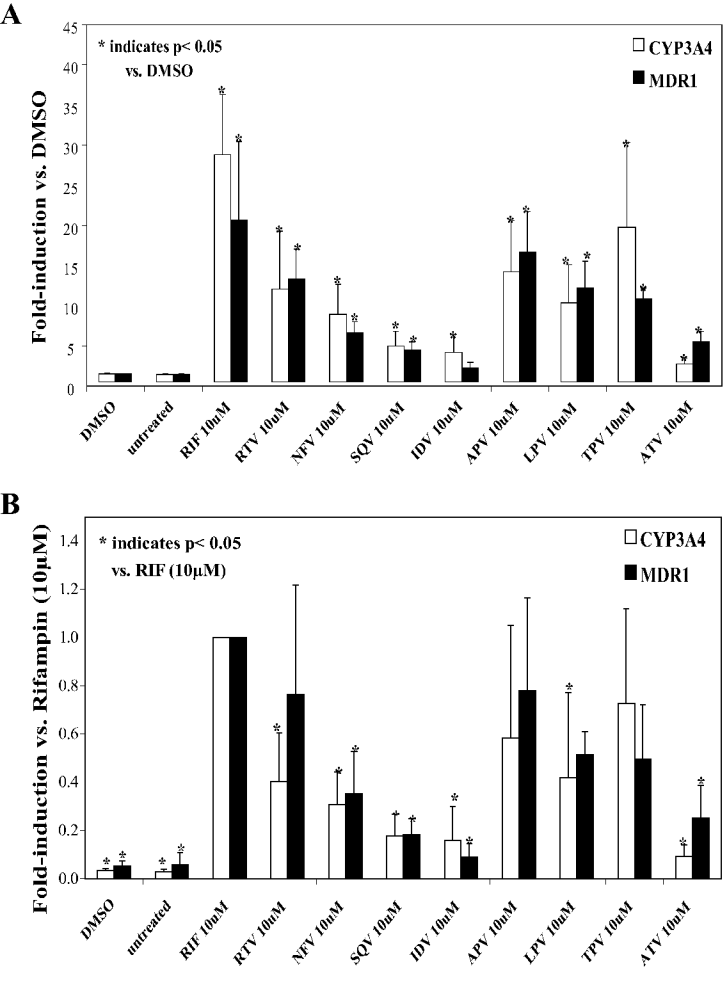
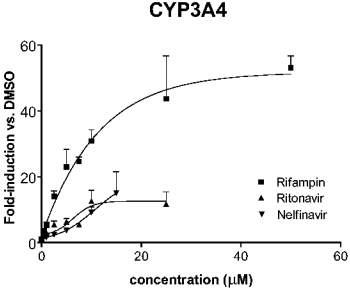
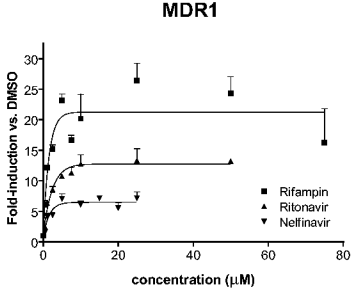

A
* indicates p< 0.05
vs. DMSO
CYP3A4
MDR1
Fold-induction vs. DMSO
45
40
35
30
25
20
15
10
5
0
DMSO
untreated
RIF 10uM
RTV 10uM
NFV 10uM
SQV 10uM
IDV 10uM
APV 10uM
LPV 10uM
TPV 10uM
ATV 10uM
B
* indicates p< 0.05
vs. RIF (10µM)
CYP3A4
MDR1
Fold-induction vs. Rifampin (10µM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
untreated
RIF 10uM
RTV 10uM
NFV 10uM
SQV 10uM
IDV 10uM
APV 10uM
LPV 10uM
TPV 10uM
ATV 10uM

Figure 3





	Emax (fold induction vs. control)	EC50 (μM)	Emax (fold induction vs. control)	EC50 (μM)
Ritonavir	13.3±5.4	7.3±6.7	13.4±1.1	1.7±0.1
Nelfinavir	n.d.	n.d.	6.4±0.7	1.2±0.6
Rifampin	48.9±7.5	7.3±0.7	24.3±4.2	1.3±0.4

Figure 4

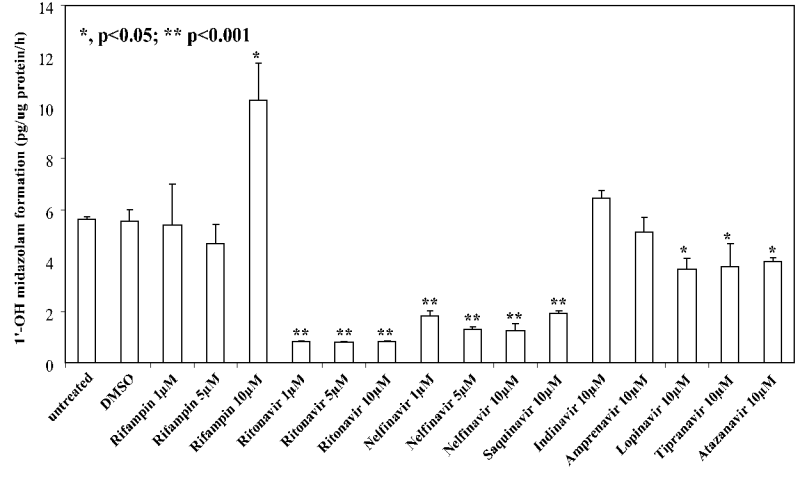

*, p<0.05; ** p<0.001
1'-OH midazolam formation (pg/ug protein/h)
0
2
4
6
8
10
12
14
untreated
DMSO
Rifampin 1μM
Rifampin 5μM
Rifampin 10μM
Ritonavir 1μM
Ritonavir 5μM
Ritonavir 10μM
Nelfinavir 1μM
Nelfinavir 5μM
Nelfinavir 10μM
Saquinavir 10μM
Indinavir 10μM
Amprenavir 10μM
Lopinavir 10μM
Tipranavir 10μM
Atazanavir 10μM

Figure 5
PXR ACTIVATION

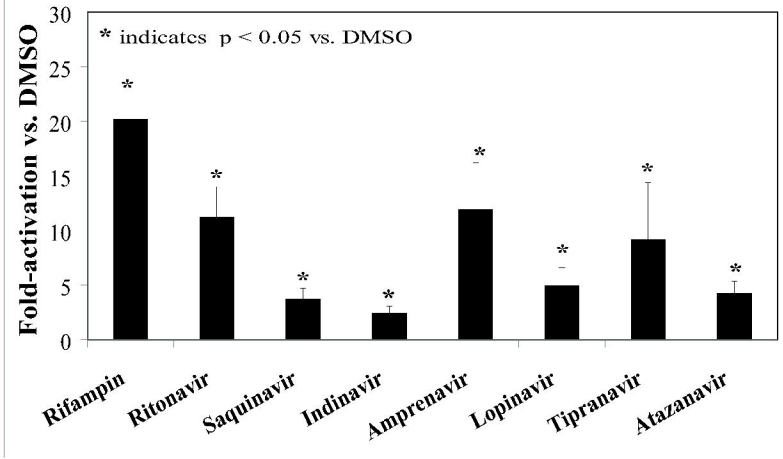
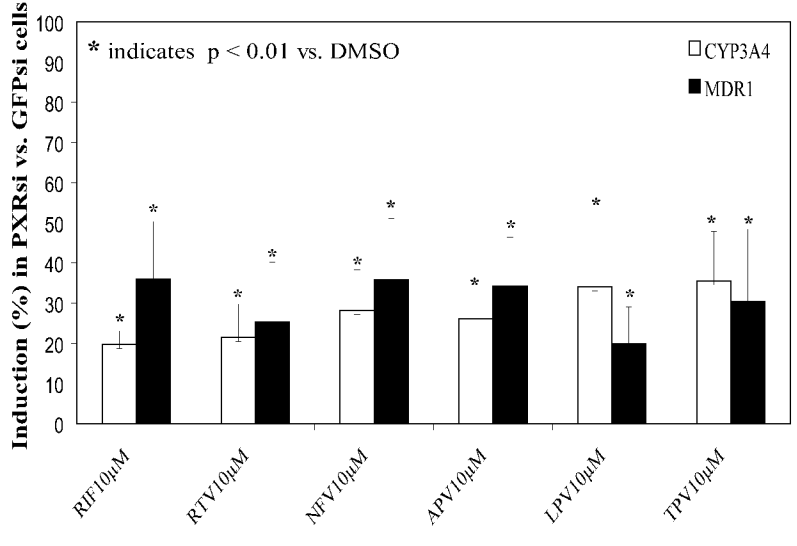


Figure 6


Induction (%) in PXRsi vs. GFPsi cells
* indicates p < 0.01 vs. DMSO
CYP3A4
MDR1
100
90
80
70
60
50
40
30
20
10
0
RIF10μM
RTV10μM
NFV10μM
APV10μM
LPV10μM
TPV10μM