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Cytochrome P450 Enzymes And Transporters Induced By Anti-HIV Protease Inhibitors In Human Hepatocytes: Implications For Predicting Clinical Drug Interactions

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Running Title: Induction of CYPs by ritonavir, nelfinavir or rifampin.

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Abbreviations:
PI, anti-HIV Protease inhibitors; CYP, cytochrome P450; P-gp, P-glycoprotein; AUC, area under the plasma concentration-time curve; PXR, pregnane X receptor; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; OATP, organic anion transporter; BCRP, breast cancer resistance protein; PCR, polymerase chain reaction; Rit, ritonavir; Rif, rifampin; Nel, nelfinavir

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Abstract

Although many of the clinically significant drug interactions of the anti-HIV protease inhibitors (PIs) can be explained by their propensity to inactivate CYP3A enzymes, paradoxically these drugs cause (or lack) interactions with CYP3A substrates that cannot be explained by this mechanism (e.g. alprazolam), To better understand these paradoxical interactions (or lack thereof), we determined the CYPs and transporters induced by varying concentrations (0-25 μM) of two PIs, ritonavir and nelfinavir, and rifampin (positive control), in primary human hepatocytes. At 10 μM, ritonavir and nelfinavir suppressed CYP3A4 activity, but induced its transcripts and protein expression (19- & 12-, and 12- & 6-fold respectively; >2-fold change over control were interpreted as induction). At 10 μM, rifampin induced CYP3A4 transcripts, CYP3A protein and activity by 23-, 12- and 13-fold respectively. Rifampin's induction of CYP3A activity was significantly correlated with its induction of CYP3A4 transcripts ($r=0.96$, $p<0.05$) and CYP3A protein ($r=0.89$, $p<0.05$). All three drugs (10 μM) induced CYP2B6 activity by 2-4 fold, 2C8 and 2C9 activity by 2-4 fold and the transcripts of CYP2B6, 2C8 and 2C9 by >3-, 5- and 3-fold respectively. CYP2C19 and 1A2 activity and transcripts were modestly induced (2-fold), whereas, as expected, CYP2D6 was not induced by any of the drugs. Of the transporters studied, protease inhibitors moderately induced MDR1 (ABCB1) and MRP2 (ABCC1) transcripts, but had no or minimal effect on the transcripts of BCRP (ABCG2), OATP1B1 (SLCO1B1) or OATP1B3 (SLCO1B3). Based on these data, we conclude that many of the paradoxical drug interactions (or lack thereof) with the PIs are metabolic- rather than transporter-based and are due to induction of CYP2B6 and 2C enzymes.

Anti-HIV protease inhibitors (PIs) are frontline drugs in the treatment of HIV infection, and are routinely administered in combination with additional anti-HIV drugs such as other protease inhibitors, nucleosides, e.g. azidothymidine and non-nucleoside reverse transcriptase inhibitors, e.g. delavirdine (Barry et al., 1999). In addition, HIV-infected patients are also prescribed medications for other concomitant conditions, supportive care, opportunistic infections, and immunomodulation (Yeh et al., 2006). Due to this polytherapy, the potential for clinically significant drug interactions in this patient population is high. This potential is compounded by the fact that PIs are high affinity substrates and potent inhibitors of CYP3A enzymes (Ernest et al., 2005) and the multi-drug resistance (MDR) transporters, encoded by MDR1, P-glycoprotein (P-gp), (Bachmeier et al., 2005) and MRP2 (Huisman et al., 2002; Bachmeier et al., 2005) . As many of the PIs, including ritonavir, are inactivators of CYP3A4/5 (Yeh et al., 2006), interactions of the PIs with drugs that are cleared predominately by CYP3A enzymes are profound and clinically significant (Culm-Merdek et al., 2006). In fact, ritonavir is now almost exclusively used, in combination with other PIs, for its ability to inactivate CYP3A enzymes and therefore to “pharmacologically boost” the bioavailability of other PIs (e.g. lopinavir, nelfinavir, amprenavir (Zeldin and Petruschke, 2004)).

Although many of the clinically significant drug interactions of the PIs can be explained by their propensity to inactivate CYP3A enzymes, paradoxically these drugs cause (or lack) some interactions that cannot be explained by this mechanism. For example, acute administration of ritonavir profoundly decreases the clearance of the CYP3A substrate, alprazolam (Greenblatt et al., 1999), but chronic administration has no effect on alprazolam clearance (product labeling). Others have shown that chronic

administration of ritonavir increases the oral clearance of methadone (Gerber et al., 2001), phenytoin (Lim et al., 2004), and ethinyl estradiol (Ouellet et al., 1998). Likewise, nelfinavir, also a PI, increases the oral clearance of ethinyl estradiol and zidovudine (Viracept® package insert). These data suggest that the PIs are inducers as well as inhibitors of CYP enzymes. Consequently, package inserts of the PIs suggest no change in the dose of some drugs co-administered with PIs while recommending an increase in the dose of others (Malaty and Kuper, 1999). Currently, no logical basis or unifying mechanisms exists to predict drug interactions with the PIs. To more effectively predict such interactions due to concurrent inhibition and induction, it is important to determine both enzymes and transporters that are induced by these drugs. Therefore, the primary objective of this study was to determine the CYPs and transporters induced by the two prototypic PIs, ritonavir and nelfianvir, using primary human hepatocytes. Also, because this study is part of a larger study to determine if *in vivo* inductive drug interactions (Kirby et al., 2006) can be predicted from *in vitro* studies using human hepatocytes (this study) and human intestinal cell lines (Gupta et al., 2006), we included in our study rifampin as a prototypic inducer and an internal biological control.

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Materials and Methods

Ritonavir and nelfinavir were obtained from NIH AIDS Research and Reference Reagent Program. Rifampin was purchased from Bedford laboratories, Bedford OH. Bupropion, dextromethorphan, tolbutamide, testosterone, phenacetin, omeprazole, acetaminophen, nicotinamine adenine diphosphate (NADPH) were purchased from Sigma-Aldrich (St.Louis, MO). Hydroxybupropion, dextrorphan, and 4-hydroxytolbutamide, and desethylamodiaquine were purchased from GenTest (Woburn, MA). Hydroxyomeprazole was a gift from AstraZeneca (Mölndal, Sweden). 6-β hydroxytestosterone was purchased from Steraloids Inc (Newport, RI). HPLC grade solvents (methanol, acetonitrile and water) were purchased from Fisher Scientific. Universal PCR master mix was purchased from Applied Biosystems, Foster city CA. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburg, PA), and were of highest purity available.

Hepatocyte cultures and drug treatment*:* Primary human hepatocytes, isolated from lobes of liver from 4 donors (Table 1), were provided by the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), funded by National Institutes of Health (NIH) contract N01-DK-9-2310.

Hepatocytes were plated in collagen-coated T25 cm^2 flasks (4×10^6 cells/flask) for the determination of CYP activity and immunoreactive protein concentrations. In parallel, cells were plated in collagen-coated 6-well plates (1×10^6 cells/well) for mRNA analysis of CYP enzymes. Collagen-coated 24-well plates (1.25×10^5) were used for toxicity determination by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] assay. Hepatocytes were maintained in Williams' E medium (Caisson Laboratories, Inc. North Logan, UT) supplemented with 100nM dexamethasone and insulin-transferrin-selenium (ITS-G; Invitrogen Corporation).

Stock drug solutions (1000X) were prepared in dimethyl sulfoxide (DMSO) and diluted prior to use. Forty-eight hours after plating, hepatocytes were treated at 37^{o}C for 72 hrs with vehicle (DMSO, 0.2% and cyclodextran, 0.05 %), or vehicle containing nelfinavir (1-25 μM), ritonavir (1-25 μM), or rifampin (0.1-25 μM). During this period, drug-containing medium was replaced every 24 hrs. Cell viability was assessed daily during the course of drug exposure with the MTT assay (Carmichael and Ozols, 1997). No significant difference was observed in the viability of cells treated with the vehicle and cells treated with rifampin, ritonavir or nelfinavir. Previous studies from our laboratories (Nallani et al., 2001) and those of others (Kostrubsky et al., 1999) have shown that DMSO at concentrations ranging from those in our experiments to concentrations twice as high do not alter the expression of CYP enzymes. At the end of the treatment period, using a cell scraper, cells were harvested in phosphate-buffered saline (PBS) and fractionated to isolate microsomes.

CYP activity assays: Microsomal activities of CYP1A2, 3A4/5, 2B6, 2C8, 2C9, 2C19, 2D6 were determined using two validated cocktail assays developed in our laboratory (Dixit et al., 2007). We conducted these assays in microsomes, rather than in human hepatocytes, to minimize the potential of these drugs to inhibit the enzymes competitively or non-competitively.

Briefly, one assay contained phenacetin (1A2), bupropion (2B6), amodiaquine (2C8), and omeprazole (2C19) and the other assay contained testosterone (3A),

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tolbutamide (2C9), dextromethorphan (2D6) as probe substrates. Substrates were incubated with 0.25 mg/ml of microsomal protein, 0.1 M phosphate buffer, 1mM NADPH, 5mM $MgCl_2$ in a final volume of 100 µl. The incubation reaction was quenched with 100 µl of acetonitrile containing the internal standard, deuterated midazolam (6 ng/100 µl). The sample was then vacuum dried (Jouan RC1010), reconstituted in 25% acetonitrile (60 µl) and 20 µl was injected onto the LC-MS to quantify the concentration of metabolites formed by individual CYP enzymes. Calibrators, containing the CYP substrate metabolites in 25% acetonitrile/0.1 M phosphate buffer and the internal standard were assayed along with the samples. Quality control samples were processed as described above and consisted of the above microsomal mix without NADPH but did contain metabolites of each CYP substrate.

Quantification of mRNA induction: Total RNA from the hepatocytes was isolated using the Qiagen RNeasy mini kit (Qiagen Sciences, MD) according to the manufacturer's instructions. 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). 0.5 µg of the total RNA was reverse transcribed into cDNA using Applied Biosystems Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The resulting cDNA was used for real time PCR (qPCR) analysis.

qPCR assays for CYP enzymes, MDR1, BCRP, MRP2, 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). *hGus* was used as the endogenous control. The real-time reaction contained 10 µl 2X TaqMan

Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 10 ng of RNA equivalent cDNA, and primers (200 nM) and probes (100 nM) in a final volume of 20 µL. The reactions were conducted 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. Quantification of OATP1B1, OATP1B3 was conducted using gene specific primers designed by Briz et.al, 2003 (Briz et al., 2003) and synthesized by IDT Inc, Coralville. Detection of the amplification products was carried out using SYBR green I. Non specific products of PCR, as determined by melting point curves, were not detected. Each sample was analyzed in duplicate. The mRNA levels of each test gene were normalized to *hGus*, according to the following formula: C_T (*test gene*) - C_T (*hGus*) = ΔC_T. Thereafter, the relative mRNA 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 $2^{-\Delta\Delta C_T}$.

Quantification of CYP protein by Western blotting: 10 µg of microsomal protein was resolved employing a 10% SDS-PAGE and transferred to PVDF membrane. The membrane was then blocked with Odyssey blocking reagent (LI-COR biosciences, Lincoln NE) for 1 hr, incubated with primary antibodies (1:1000 fold dilution) for CYP3A, 2B6, (Gentest, Woburn, MA) and β-actin (Sigma,) overnight. The membrane was then incubated for 1 hr with IRDye secondary antibodies; diluted 1:20000 (Licor biosciences, Lincoln, NB) raised against mouse (CYP3A, β-actin) and rabbit (CYP2B6) antigens. The blots were washed with TBS containing 0.05% tween for 30 minutes to remove excess antibody, followed by a final wash in PBS and then imaged on Odyssey Infrared Imaging Systems (Licor biosciences, Lincoln, NB). Integrated intensity of the

bands, as calculated by the Odyssey version 2.1, was used for quantification. Only CYP3A and CYP2B6 enzymes were quantified by western blotting due to limited quantity of microsomes and the low level of induction of mRNA and activities of the remaining CYPs.

Statistical and data analysis: CYP activity, immunoreactive protein content and mRNA levels in the treated groups were expressed relative to that observed in the vehicle control (negative control). Although the magnitude of induction of CYPs and transporters was large, due to the significant inter-individual variability observed, experiments in a large number of human hepatocyte batches would need to be conducted to reach statistical significance. Given the limited availability of hepatocytes, we defined a greater than 2-fold change in mRNA, protein or activity, of CYPs and transporters, relative to the negative control, to be induction. We also expressed the data relative to the induction produced by the positive control (rifampin).

To gain insight into whether rifampin, ritonavir and nelfinavir share a common mechanism for induction of the highly inducible CYP3A, 2B6 or 2C8 transcripts, we examined if the induction of each of these transcripts was correlated across the three drugs. In addition, to explore if each drug induced the three enzymes by a common mechanism, we determined the correlation in induction of the three transcripts by each drug. Correlation analysis of data across all concentrations of study drugs was conducted using GraphPad Prism version 4.0. Correlations with r values <0.5 were designated as weak or poor, r values of 0.51-0.7 were designated modest, and r values of 0.71-1.0 were designated as strong or excellent.

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Results

The MTT assay showed that PIs and rifampin were not significantly toxic to the hepatocytes; cell viability ranged from 70-100%.

Induction of CYP3A mRNA, protein and activity: The magnitude of induction of CYP3A4 transcripts and CYP3A protein by the PIs (and rifampin) was large and concentration-dependent with potency of induction decreasing in the order rifampin ≈ ritonavir > nelfinavir (Fig 1A). While rifampin induced the CYP3A activity in a concentration-dependent manner, the PIs reduced this activity, most likely due to inactivation of the enzymes. At 10 μM, rifampin induced CYP3A4 transcripts, CYP3A protein and activity by 23-, 12- and 13-fold respectively. At the same concentration, ritonavir and nelfinavir induced CYP3A4 transcripts and CYP3A protein by 19- and 12-, and 13- and 7-fold respectively. In addition, ritonavir inhibited CYP3A activity by 55% while nelfinavir did not appear to affect this activity. However, at higher concentrations, ritonavir further reduced CYP3A activity. In contrast, nelfinavir appeared to be a weaker inactivator of CYP3A activity, as this activity could be measured at all concentrations studied but, unlike protein expression, was not induced with increasing concentration of the drug. CYP3A5 transcripts were modestly induced by the three drugs in a concentration-dependent manner. Rifampin's ability to induce CYP3A activity was strongly and significantly correlated with its ability to induce CYP3A4 transcripts ($r=0.96$, $p<0.05$), and CYP3A protein ($r=0.89$, $p<0.05$). However, the magnitude of induction of CYP3A4 transcripts was always larger than the magnitude of induction of either protein or activity (Fig 2A). Ritonavir and nelfinavir inactivated CYP3A activity

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and hence showed negative or no correlation between induction of activity and transcripts.

Induction of CYP2B6 mRNA, protein and activity: After CYP3A, the induction of CYP2B6 enzyme activity was the next highest. All the three drugs induced transcripts, protein and activity of this enzyme in a concentration-dependent manner (Fig 1B). At 10 μM, CYP2B6 transcripts, protein and activity were induced 3-fold, 5-fold and 3-fold by ritonavir, 2-fold, 3-fold and 2-fold by nelfinavir and 4-fold, 6-fold and 3-fold by rifampin respectively. Regression analysis of the data showed modest to strong correlation between CYP2B6 transcripts and activity for rifampin ($r=0.52$, $p<0.05$), ritonavir ($r=0.52$, $p<0.05$) or nelfinavir ($r=0.81$, $p<0.05$). Activity and protein of 2B6 were strongly correlated for rifampin ($r=0.81$), but modestly correlated for ritonavir ($r=0.52$, $p<0.05$) or nelfinavir ($r=0.65$, $p<0.05$)

Induction of CYP2C8, 2C9, and 2C19 mRNA and activity: Except for the effect of ritonavir on CYP2C activity, induction of CYP2C transcripts and activity by the three drugs was concentration-dependent. Of the three CYP2C enzymes examined, 2C8 transcripts (Fig 2C) were induced to a greater extent than those of 2C9 or 2C19 (Fig 1D, 1E). At 10 μM, ritonavir, nelfinavir or rifampin caused a 7-, 5-, and 7- fold induction of 2C8 transcripts respectively. At the same concentration, ritonavir and nelfinavir induced 2C8 activity by 2-fold while rifampin induced such activity by 4-fold. At ritonavir concentration of greater than 1 μM, CYP2C8 activity appeared to be inhibited (Fig 1C). The transcripts and activity of CYP2C8 were strongly correlated for rifampin ($r=0.81$, $p<0.05$), modestly correlated for ritonavir ($r=0.61$, $p<0.05$), and weakly correlated for nelfinavir ($r=0.35$, $p>0.05$).

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CYP1A2 and CYP2D6 mRNA and activity: At 10 μM**,** CYP1A2 transcripts were induced up to 6-fold by rifampin and 4-fold by ritonavir and nelfinavir. However, CYP1A2 activity was only modestly induced (1.5- to 3-fold) by rifampin, ritonavir or nelfinavir (Fig 1F). CYP2D6 was not induced by either the PIs or rifampin (Fig 1G).

Induction of transporter mRNA by protease inhibitors and rifampin: Induction of MDR1 and MRP2 transcripts by PIs and rifampin was quite variable (Fig 2). In general, the magnitude of induction of MDR1 was greater than that of MRP2 and occurred at lower concentrations of the drugs. The magnitude of induction followed the order ritonavir > rifampin > nelfinavir. BCRP, another ABC efflux transporter, was not inducible by any of the drugs. Amongst the hepatic uptake transporters studied, OATP1A2, 1B1 and 1B3, only transcripts of OATP1B1 and 1B3 were expressed in hepatocytes and the induction of these transcripts was modest (Fig 2). The magnitude of induction of OATP1B3 was greater than that of OATP1B1.

Correlation Analysis: The induction of CYP3A4 transcripts was strongly correlated between the three drugs ($r=0.88$-0.95, $p<0.05$) but modestly to strongly correlated for CYP2B6 ($r=0.55$-0.59, $p<0.05$) and CYP2C8 ($r=0.6$-0.8, $p<0.05$) (Table 2B). In addition, the induction of CYP2B6 transcripts by ritonavir was weakly correlated with that of 2C8 ($r=0.4$ $p>0.05$) and strongly correlated with that of 3A4 ($r=0.76$, $p<0.05$). The induction of CYP2C8 transcripts by nelfinavir was strongly correlated with that of 2B6 ($r=0.82$, $p<0.05$) and with that of 3A4 ($r=0.85$, $p<0.05$). In addition, the induction of CYP2B6 transcripts by nelfinavir was weakly correlated with that of 3A4 ($r=0.4$, $p>0.05$). Similarly, the induction by rifampin of CYP2C8 transcripts was strongly

correlated with that of 2B6 ($r=0.77$, $p<0.05$) and 3A4 ($r=0.88$, $p<0.05$), while that of 2B6 was weakly correlated with that of 3A4 ($r=0.5$, $p<0.05$).

Discussion

In vivo inhibitory drug interactions are now routinely and quantitatively predicted using human liver and recombinant enzyme microsomes. Based on the success of this *in vitro* model, FDA has established guidelines for using this model to predict such interactions (http://www.fda.gov/cder/guidance/6695dft.pdf). However, a validated model and methodology to quantitatively predict *in vivo* inductive drug interactions has not been established. Our long term goal is to validate such a model using human hepatocytes. We used primary human hepatocytes to determine the CYPs and transporters induced by the PIs as this *in vitro* model is routinely used to investigate induction of CYPs and transporters (Meneses-Lorente et al., 2007). In addition, to determine if induction studies in the human hepatocytes could predict the magnitude of *in vivo* induction of CYPs and transporters, we included in all our experiments, rifampin, a prototypical inducer, as our biological control. We chose to study the induction of ritonavir and nelfinavir as these two protease inhibitors differ in their frequency and magnitude of clinical drug interactions (Unadkat and Wang, 2000). 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.

We found that PIs (and rifampin) were potent inducers of CYP3A4 transcripts and protein but less potent inducers of CYP3A5 transcripts. The magnitude of induction of the CYP3A4 transcripts was always larger than of CYP3A protein or activity. The discrepancy between transcripts and protein or activity may be due to a time lag between the synthesis of transcripts and protein or due to the fact that our assay for quantification

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of CYP3A proteins and their activities did not distinguish between CYP3A4 and 3A5 due to lack of selective substrates for each enzyme. The discrepancy between CYP3A protein and activity was the largest for the PIs, because these drugs induced expression of the proteins but either reduced their activities or produced no change. Ritonavir was more potent than nelfinavir at reducing this activity. These data are consistent with the observation that ritonavir and nelfinavir are both inactivators of CYP3A enzymes *in vitro* and *in vivo* (Malaty and Kuper, 1999). Interestingly, ritonavir (200-500 mg bid) induces its own clearance (Hsu et al., 1997) and that of others such as ethinyl estradiol (Ouellet et al., 1998). At clinical doses (1200 mg bid), nelfinavir does not induce *in vivo* CYP3A activity (Hsyu et al., 2001). Yet, it induces its own clearance and that of other drugs such as phenytoin and amprenavir (Unadkat and Wang, 2000; Pfister et al., 2002). Our data in human hepatocytes and those cited above suggest that this induction of *in vivo* clearance by ritonavir and nelfinavir is NOT due to induction of CYP3A enzymes, but due to induction of other enzymes or transporters.

In investigating other enzymes induced by the PIs, we found that CYP2B6 and CYP2C8 enzymes were the second most induced enzymes by the PIs (and rifampin). The induction potency of CYP2B6 decreased in the order rifampin > ritonavir ≈ nelfinavir. In contrast to CYP3A enzymes, the magnitude of induction of CYP2B6 transcripts, protein and activity correlated for all three drugs. Indeed clinical studies have shown that ritonavir enhances the clearance of drugs such as methadone, a drug which is cleared in part by CYP2B6 (Beauverie et al., 1998). The role of CYP2B6 in the clearance of drugs which are metabolized by both CYP3A4 and 2B6, such as meperidine (Ramirez et al., 2004), will increase when CYP3A is inactivated by the protease

inhibitors. If, upon chronic administration of the PIs, the induction of CYP2B6 is significant, it could nullify the inactivation of CYP3A enzymes by the PIs and therefore result in no change in the clearance of the drug.

Since the CYP2C family is important in metabolizing a wide variety of drugs, we also investigated the induction of CYP2C8, 9 and 19 by the PIs (and rifampin). Except for the effect of ritonavir on CYP2C8 activity, the induction of transcripts and activity of all three isoforms was concentration-dependent and decreased in the order CYP2C8>2C9≈2C19. For all three isoforms, induction of transcripts was greater than that of activity. Even though the activity of each of the CYP2C isoforms is modestly induced *in vivo* by the PIs, if a drug is metabolized by more than one of these isoforms or the drug has a narrow therapeutic window, the degree of induction observed here could lead to clinically significant inductive drug interactions. This may explain the observation that lopinavir/ritonavir (400/100 mg bid) and nelfinavir (750 mg tid) have been observed to induce the clearance of warfarin (Knoell et al., 1998; Newshan and Tsang, 1999) and phenytoin (Honda et al., 1999; Lim et al., 2004).

The least inducible enzyme was CYP1A2 while CYP2D6 was not inducible. Consistent with this observation, lopinavir-ritonavir (400/100 mg bid) modestly induced CYP1A2 (43%) activity *in vivo* (Yeh et al., 2006). CYP1A isoforms are primarily regulated by the AhR receptor (Li et al., 1998). However, rifampin and ritonavir have not been shown to be ligands of AhR. These data are consistent with the very modest induction of CYP1A2 observed in our human hepatocytes most likely due to the indirect cross-talk between AhR and PXR (Maglich et al., 2002). Based on these data, we predict that the PIs will not produce clinically significant interactions with drugs that are

predominantly cleared by CYP1A2. We included CYP2D6 in this study as a negative control since CYP2D6 is not induced by xenobiotics, including rifampin. Consistent with these data, we did not observe induction of transcripts or activity of this enzyme by the PIs or rifampin.

The recent FDA draft guidelines (http://www.fda.gov/cder/guidance/6695dft.pdf) suggest that "a drug that produces a change that is equal to or greater than 40% of the positive control" (e.g. rifampin) "can be considered as an enzyme inducer and *in vitro* and *in vivo* evaluation is warranted". Based on these guidelines, both ritonavir and nelfinavir would be classified as inducers of CYP2B6 and 2C enzymes (Fig 2H).

PXR has been shown to be important in xenobiotic (including rifampin) induction of several CYPs, such as CYP3A4, 2B6 and 2C8, and transporters such as MDR1, OATP1B1(Xu et al., 2005). Both ritonavir and rifampin are known ligands of PXR (Dussault et al., 2001). If the PIs and rifampin also induce enzymes and transporters via a similar mechanism, the concentration dependent induction (but not necessarily the magnitude) of a given gene product should be correlated across the three drugs. Therefore, we investigated the correlation between the three drugs in their propensity to induce the transcripts of the highly inducible genes, CYP2B6, 3A4 or 2C8.

The induction of transcripts was strongly correlated between the three drugs for CYP3A4, and modestly correlated for CYP2B6 and CYP2C8 (Table 2B). These data corroborate previous evidence that these drugs induce CYP3A4 by a common mechanism, most likely transcriptional activation of PXR. These data also suggest that divergent mechanisms (e.g other transcriptional factors) are likely involved in the

induction of CYP2B6 and 2C8 by the three compounds. Indeed, evidence in the literature suggests that CAR is another nuclear receptor important in the induction of CYP2B6 and 2C8 (Ferguson et al., 2005). To gain further insight into this possible mechanism, we also determined if the induction of the three transcripts by a single drug was correlated. For ritonavir, there was strong correlation between CYP2B6 and 3A4 transcripts. However, for nelfinavir and rifampin, although induction of CYP2C8 and 2B6 transcripts was strongly correlated, induction of CYP2B6 and 3A4 transcripts was poorly correlated. These data are consistent with the notion that induction of CYP2B6 and 3A4 transcripts by the PIs and rifampin appears to occur via different mechanism(s), perhaps one predominantly via CAR while the other predominately via PXR. The induction of CYP2C8 transcripts showed good to excellent correlation with 2B6 transcripts for rifampin and nelfinavir suggesting that these genes share common induction mechanism(s). However, activation studies of PXR and CAR need to be conducted to definitively test these hypotheses.

Besides CYP enzymes, drug transporters can significantly affect drug disposition such as hepatic uptake and biliary clearance. Protease inhibitors are transported by efflux transporters such as P-gp (Bachmeier et al., 2005) and MRP2 (Huisman et al., 2002) and possibly by uptake transporters such as OATPs (Su et al., 2004). At 10 μM, the PIs (and rifampin) induced OATP1B1 and OATP1B3 modestly. At this concentration, MDR1 transcripts were induced to a greater extent by ritonavir and rifampin, but nelfinavir was a weaker inducer. Correlation analysis showed a poor correlation between induction of MDR1 gene transcripts by rifampin and ritonavir ($r=0.19$, $p>0.05$) and by rifampin and nelfinavir ($r=0.23$, $p>0.05$). This may be due to the significant variability in the data, or

perhaps due to different mechanisms of induction of these drugs. We did not measure the activities of P-gp or MRP2 as our experiments did not use the sandwich-cultured hepatocytes necessary for targeting P-gp and MRP2 transporters to the canalicular membrane (Kipp and Arias, 2000; Hoffmaster et al., 2004).

In conclusion, our data show that despite induction of CYP3A transcripts and protein, CYP3A activity is suppressed by the PIs or unchanged, most likely due to inactivation of these enzymes. However, the PIs do induce several other CYPs with potency comparable to that of rifampin. Of these, the most inducible are CYP2B6 and 2C8. While the hepatic basal expression of these CYPs is highly variable and on average lower than that of other major CYP enzymes, they are highly inducible (Gerbal-Chaloin et al., 2001; Wang and Negishi, 2003). After induction, they are likely to play a significant role in metabolism of substrates that are cleared by these enzymes. Based on these data we predict that many of the paradoxical interactions observed of the PIs with CYP3A substrates (i.e. induction rather than inhibition) are due to induction of these two enzymes. While the PIs are more modest inducers of CYP2C9 and 2C19, collectively the induction of these enzymes could also play a role in some of these paradoxical interactions. Our data also suggest that inductive drug interactions with ritonavir and nelfinavir are likely to be metabolic-based rather than transporter-based. Studies are presently ongoing on our laboratory to determine the *in vivo* induction of CYPs and transporters by these PIs and rifampin (Kirby et al., 2006). Preliminary data obtained from these studies show excellent agreement between the data obtained in human hepatocytes and *in vivo*.

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Footnotes

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Figure Legends

Fig 1: Except for CYP3A and 2C8 activity, induction of CYP2B6, 2C9, 2C19 activity, protein and/or transcripts by ritonavir (Rit), nelfinavir (Nel) or rifampin (Rif) was concentration-dependent. All the three drugs were potent inducers of 3A4 transcripts and protein. The potency of induction decreased in the order Rif > Rit > Nel. While Rif induced CYP3A activity, Rit and Nel inhibited it (A). Induction of 2B6 activity and protein by Rit and Rif was similar but greater than that by Nel (B). Rit and Nel were less potent in inducing 2C8 activity (C). All the 3 drugs modestly induced 2C9, 2C19 and 1A2 activity (D, E and F) but did not affect 2D6 activity or transcripts (G). When expressed relative to the corresponding concentration of rifampin, except for CYP3A and 2C8 activity, Rit and Nel were almost as potent as Rif in inducing CYP transcripts, protein and activity (H). In all instances, except for panel H, only changes greater than 2-fold over negative control were considered as induction. N=4, mean±SEM.

Fig 2: MDR1 transcripts were more inducible by the PIs and Rif than MRP2 transcripts. OATP1B3 and 1B1 transcripts were only modestly induced by the PIs. OATP1A2 transcripts were not detected. BCRP transcripts were not induced by either PIs or rifampin. Only changes greater than 2-fold over negative control were considered induction. N=4 except for BCRP where N=3, mean±SEM.

Table 1: Demographic characteristics of the liver donors.

Patient	Age	Sex	Disease Status	Medication	Source	Cause of Death
HL1247	3	M	Maple syrup urine disease	NA	Liver transplant	NA
HL1233	32	M	Diabetes, steatosis, bipolar disorder	Insulin, nor epinephrine	Organ donor	Gun shot
HL1294	14	M	Depression	NA	Organ donor	Anoxia
HL1298	78	M	NA	NA	Liver resection	NA

NA – not available

Table 2: (A) Correlation (r) between induction of CYP3A4, CYP2B6, CYP2C8 transcripts by ritonavir (Rit), nelfinavir (Nel) or rifampin (Rif). (B) Correlation (r) between drugs in their ability to induce CYP3A, 2B6 and 2C8 transcripts.

A

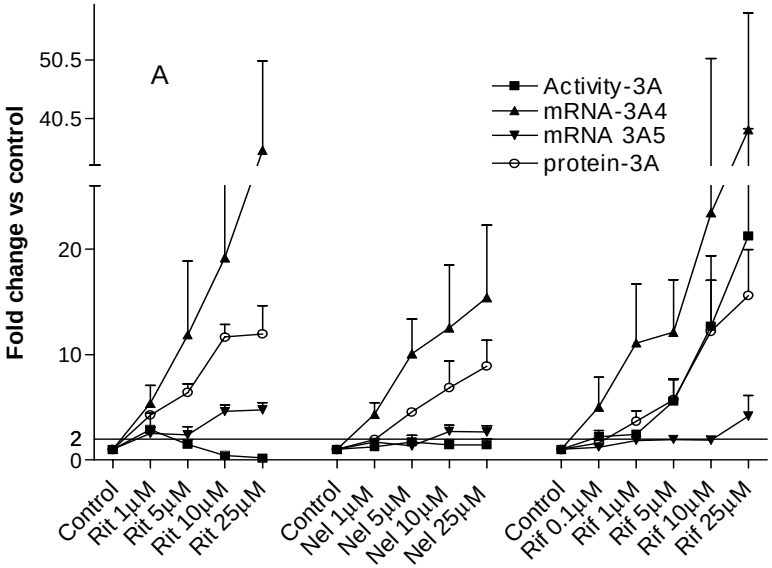
mRNA		Rif		Rit		Nel	
		2B6	2C8	2B6	2C8	2B6	2C8
Rif	2B6		0.77*				
	3A4	0.50*	0.88*				
Rit	2B6				0.4		
	3A4			0.76*	0.85*		
Nel	2B6						0.76*
	3A4					0.40	0.8*

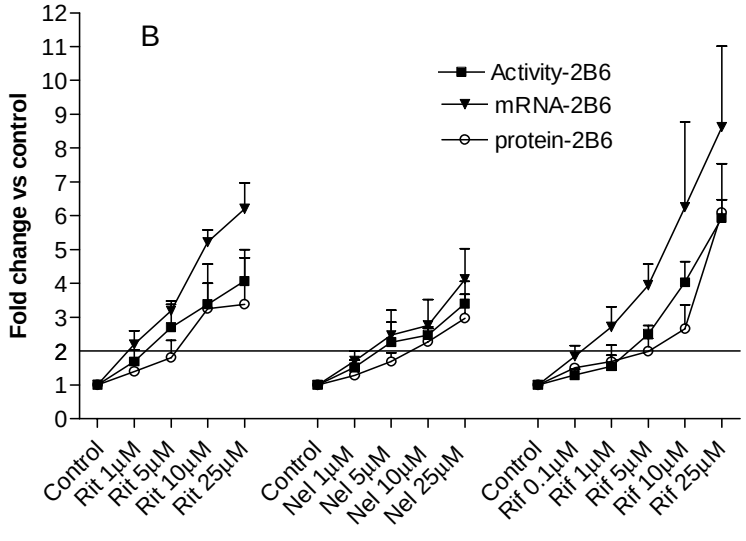
* $p<0.05$

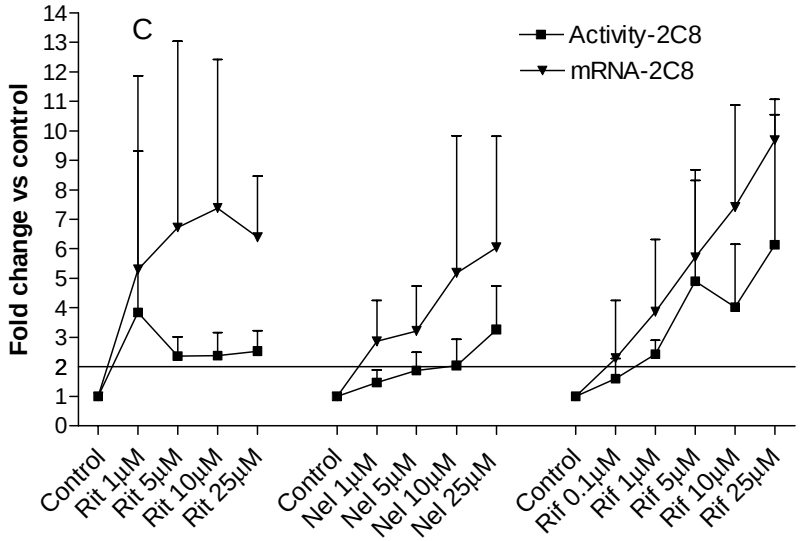
B

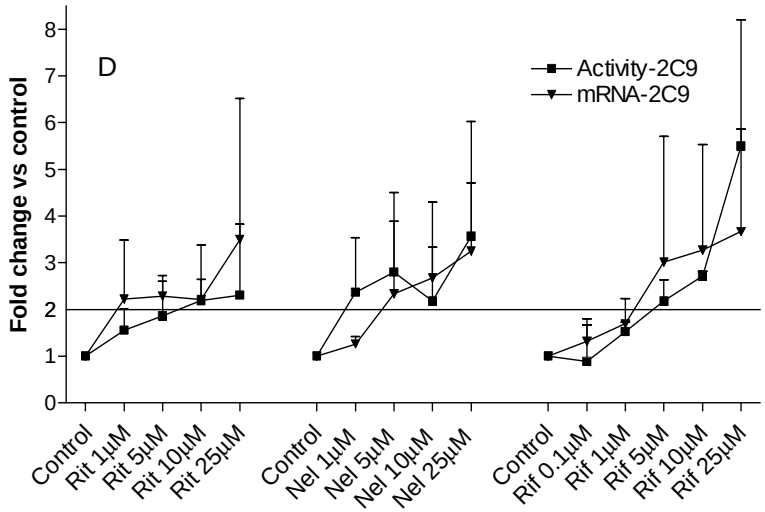
mRNA		3A4		2B6		2C8	
		Rif	Rit	Rif	Rit	Rif	Rit
3A4	Rif		0.91*				
	Nel	0.95*	0.88*				
2B6	Rif				0.55*		
	Nel			0.56*	0.59*		
2C8	Rif						0.80*
	Nel					0.78*	0.59*

Figure 1









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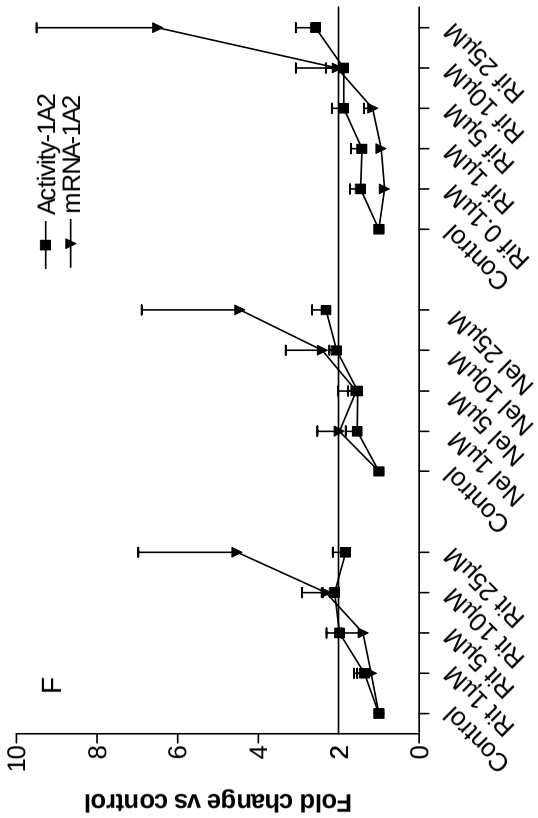

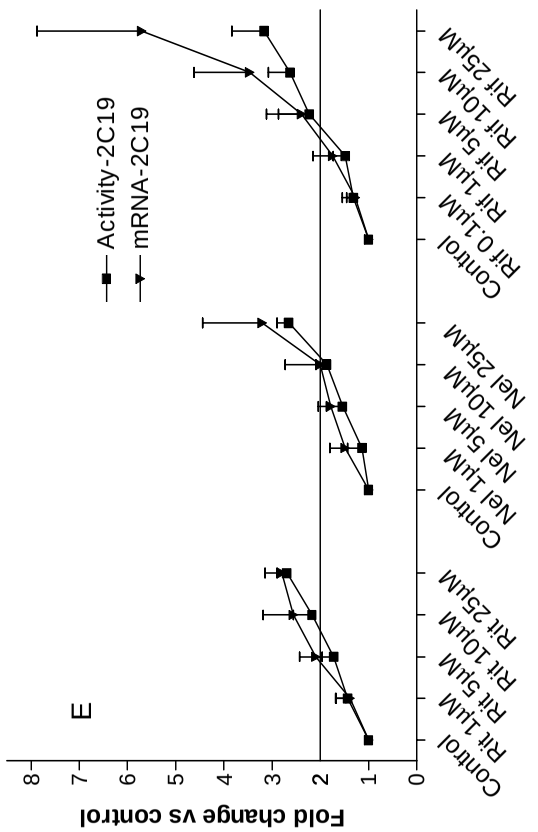

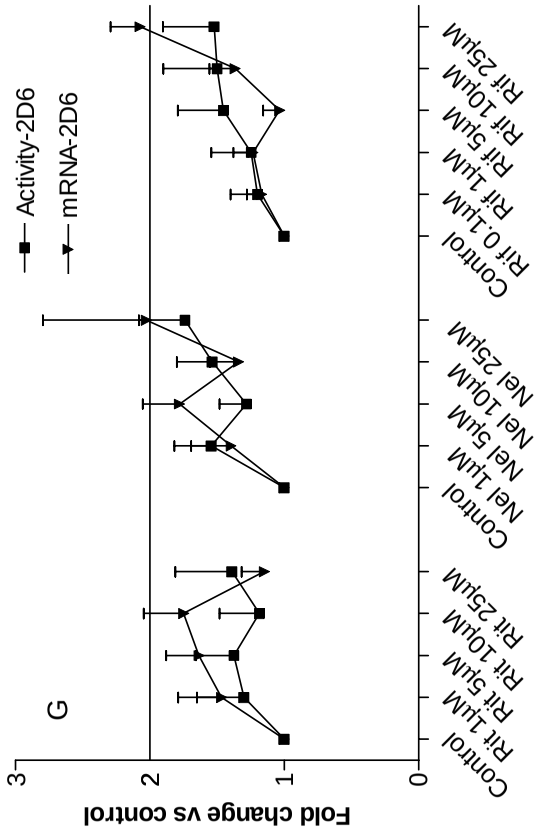

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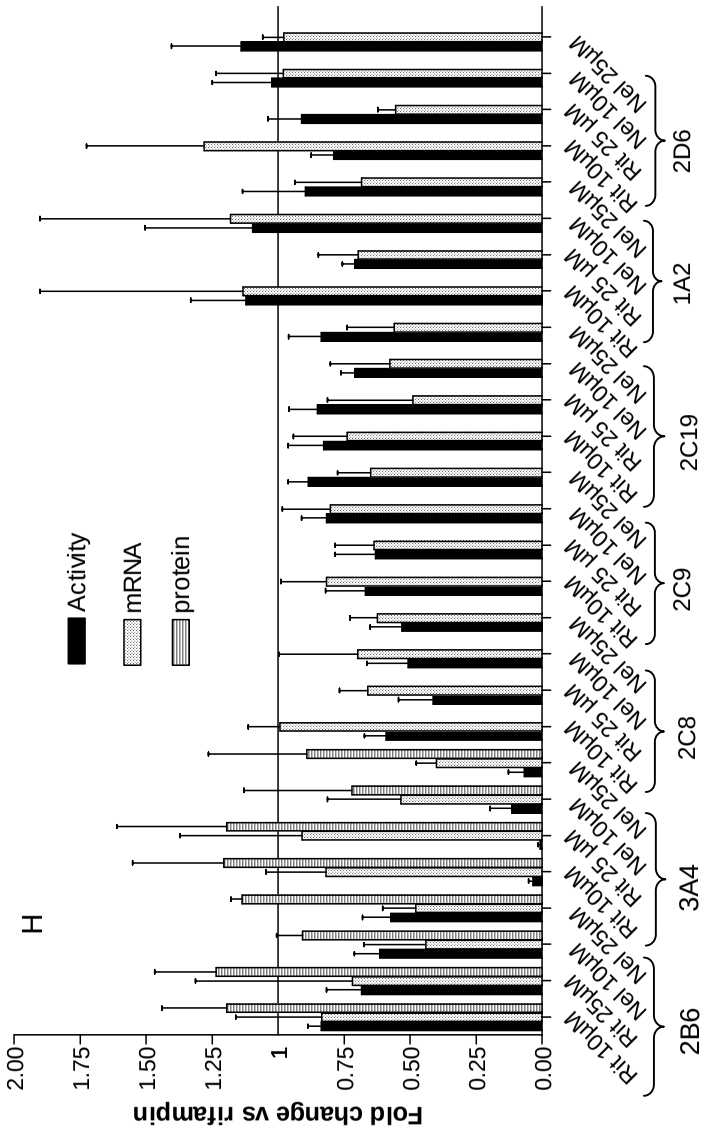

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Figure 2