Cytochrome P450 Enzymes and Transporters Induced by Anti-Human Immunodeficiency Virus Protease Inhibitors in Human Hepatocytes: Implications for Predicting Clinical Drug Interactions

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ABSTRACT:

Although many of the clinically significant drug interactions of the anti-human immunodeficiency virus (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 cytochromes P450 and transporters induced by various 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- and 12- and 12- and 6-fold, respectively; a >2-fold change over control was interpreted as induction). At 10 μM, rifampin induced CYP3A4 transcripts, CYP3A protein, and activity by 23-, 12-, and 13-fold, respectively. The induction by rifampin 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- to 4-fold, CYP2C8 and 2C9 activity by 2- to 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 multidrug resistance 1 (ABCB1) and multidrug resistance-associated protein (ABCC1) transcripts but had no or minimal effect on the transcripts of breast cancer resistance protein (ABCG2), organic anion-transporting peptide (OATP) 1B1 (SLCO1B1), or OATP1B3 (SLCO1B3). On the basis of these data, we concluded that many of the paradoxical drug interactions (or lack thereof) with the PIs are metabolism- rather than transporter-based and are due to induction of CYP2B6 and 2C enzymes.
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 P450 enzymes. Consequently, package inserts of the PIs suggest no change in the dose of some drugs coadministered with PIs, whereas increases in doses of others are recommended (Malaty and Kuper, 1999). Currently, no logical basis or unifying mechanisms exist to predict drug interactions with the PIs. To more effectively predict such interactions because of 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 P450s and transporters induced by the two prototypic PIs, ritonavir and nelfinavir, using primary human hepatocytes. Also, because this study is part of a larger study to determine whether 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.

Materials and Methods

Ritonavir and nelfinavir were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Ritonavir was purchased from Bedford Laboratories (Bedford OH). Bupropion, dextromethorphan, tolbutamide, testosterone, phenacetin, omeprazole, acetaminophen, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxybu- propion, dextropropor, 4-hydroxytolbutamide, and desethylamodiaquine were purchased from BD GenTest (Woburn, MA). Hydroxyomeprazole was a gift from AstraZeneca (Mölndal, Sweden). 6β-Hydroxytestosterone was purchased from Steraloids (Newport, RI). High-performance liquid chromatography-grade solvents (methanol, acetonic, and water) were purchased from Fisher Scientific Co. (Pittsburgh, PA). Universal PCR master mix was purchased from Applied Biosystems (Foster City CA). All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific, PA) and were of highest purity available.

Hepatocyte Cultures and Drug Treatment. Primary human hepatocytes, isolated from lobes of liver from four donors (Table 1), were provided by the Liver Tissue Procurement and Distribution System (Pittsburgh, PA).

Hepatocytes were plated in collagen-coated T25 cm² flasks (4 × 10⁶ cells/flask) for the determination of P450 activity and immunoreactive protein concentrations. In parallel, cells were plated in collagen-coated six-well plates (1 × 10⁶ cells/well) for mRNA analysis of P450 enzymes. Collagen-coated 24-well plates (1.25 × 10⁶ cells/well) were used for toxicity determination by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hepatocytes were maintained in Williams E medium (Caisson Laboratories, Inc., North Logan, UT) supplemented with 100 μM dexamethasone and insulin-transferrin-selenium (Invitrogen, Carlsbad, CA).

Stock drug solutions (1000×) were prepared in dimethyl sulfoxide (DMSO) and diluted before use. Forty-eight hours after plating, hepatocytes were treated at 37°C for 72 h with vehicle (0.2% DMSO and 0.05% cyclodextran) 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 h. Cell viability was assessed daily during the course of drug exposure with the MTT assay (Carmichael and Oozes, 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 (Nal- lani 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 does not alter the expression of P450 enzymes. At the end of the treatment period cells were harvested in phosphate-buffered saline using a cell scraper and fractionated to isolate microsomes.

P450 Activity Assays. Microsomal activities of CYP1A2, 3A4/5, 2B6, 2C8, 2C9, 2C19, and 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 noncompetitively.

Briefly, one assay contained phenacetin (1A2), bupropion (2B6), amodia- quine (2C8), and omeprazole (2C19) and the other assay contained testosterone (3A), tolbutamide (2C9), and dextromethorphan (2D6) as probe substrates. Substrates were incubated with 0.25 mg/ml microsomal protein, 0.1 M phosphate buffer, 1 mM NADPH, and 5 mM MgCl₂ in a final volume of 100 μl. The incubation reaction was quenched with 100 μl of acetoniclrite containing the internal standard, deuterated midazolam (6 ng/100 μl). The sample was then vacuum dried (Jouan RC101D) and reconstituted in 25% acetonitrile (60 μl), and 20 μl was injected onto the liquid chromatograph-mass spectrometer to quantify the concentration of metabolites formed by individual P450 enzymes. Calibrators, containing the P450 substrate metabolites in 25% aceto- nitrite-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 P450 substrate.

Quantification of mRNA Induction. Total RNA from the hepatocytes was isolated using the IQIAGEN RNeasy mini kit (IQIAGEN Sciences, German- town, MD) according to the manufacturer’s instructions. The concentration of purified RNA was determined by a spectrophotometer (SmartSpec Plus Spec- trophotometer; BioRad, Hercules, CA), as was the purity, using the 260/280 nm absorbance ratio (ratio of 1.8 to 2.0). The total RNA 0.5 μg was reverse- transcribed into cDNA using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer’s instructions. The resulting cDNA was used for real-time quantitative PCR (qPCR) analysis.

qPCR Assays for P450 enzymes, MDR1, BCRP, MRP2, and OATP1A2 were carried out using gene-specific primers and 6-carboxyfluorescin-labeled fluorescent minor groove border probes in an ABI 7000 Sequence Detection System (Applied Biosystems). hGus was used as the endogenous control. The real-time reaction contained 10 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 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 min, followed by 40 cycles at 95°C for 15 s and then 60°C for 60 s. Quantification of OATP1B1 and OATP1B3 was conducted using gene-specific primers designed by Briz et al., (2003) and synthesized by Integrated DNA Technologies, Inc. (Corvalde, IA). Detection of the amplification products was carried out using SYBR green I. Nonspecific 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: Cₖ (test gene) − Cₖ (hGus) = ΔCₖ. Thereafter, the relative mRNA levels of each gene were calculated using the ΔΔCₖ method: ΔCₖ (test gene) − ΔCₖ (internal control) = ΔΔCₖ (test gene). The fold-changes of mRNA levels were expressed as the relative expression 2^−ΔΔCₖ.

### TABLE 1

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<th>Patient</th>
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<td>Insulin, norepinephrine</td>
<td>Liver transplant</td>
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<tr>
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<td>32 M</td>
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<tr>
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<td></td>
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</tbody>
</table>

NA, not available.

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Quantification of P450 Protein by Western Blotting. Micromolar protein (10 μg) was resolved using 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with Odyssey blocking reagent (Li-Cor Biosciences, Lincoln, NE) for 1 h, incubated with primary antibodies (1:10,000 dilution) for CYP3A, CYP2B6, (BD GenTest), and β-actin (Sigma-Aldrich) overnight. The membrane was then incubated for 1 h with IRDye secondary antibodies, diluted 1:20,000 (Li-COR Biosciences), and raised against mouse (CYP3A and β-actin) and rabbit (CYP2B6) antigens. The blots were washed with Tris-buffered saline containing 0.05% Tween for 30 min to remove excess antibody, followed by a final wash in phosphate-buffered saline and then imaged on Odyssey Infrared Imaging Systems (Li-COR Biosciences). Integrated intensity of the bands, as calculated by Odyssey version 2.1, was used for quantification. Only CYP3A and CYP2B6 enzymes were quantified by Western blotting because of the limited quantity of microsomes and the low level of induction of mRNA and activities of the remaining P450s.

Statistical and Data Analysis. P450 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 P450s and transporters was larger, because of the significant interindividual 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 P450s 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 ritonavir, ritonavir, and nelfinavir share a common mechanism for induction of the highly inducible CYP3A, 2B6, or 2C8 transcripts, we examined whether the induction of each of these transcripts was correlated across the three drugs. In addition, to explore whether 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 performed using GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA).

Correlations with r values >0.5 were designated as weak or poor, correlations with r values of 0.51 to 0.7 were designated as modest, and correlations with R values of 0.71 to 1.0 were designated as strong or excellent.

Results

The MTT assay showed that PIs and rifampin were not significantly toxic to the hepatocytes. Cell viability ranged from 70 to 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 ritonavir > nelfinavir (Fig. 1A). Whereas ritonavir induced CYP3A activity in a concentration-dependent manner, the PIs reduced this activity, most likely due to inactivation of the enzymes. At 10 μM, ritonavir 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%, whereas 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 concentrations of the drug. CYP3A5 transcripts were modestly induced by the three drugs in a concentration-dependent manner. The ability of ritonavir 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. 1A). Ritonavir and nelfinavir inactivated CYP3A activity 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 enzymatic activity was the next highest. All 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-, 5-, and 3-fold by ritonavir, 2-, 3-, and 2-fold by nelfinavir, and 4-, 6-, and 3-fold by rifampin, respectively. Regression analysis of the data showed modest to strong correlation between CYP2B6 transcripts and activity for ritonavir (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 CYP2B6 were strongly correlated for rifampin (r = 0.81), but modestly correlated for nelfinavir (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. 1C) were induced to a greater extent than those of 2C9 or 2C19 (Fig. 1, D and E). At 10 μM, ritonavir, nelfinavir, or rifampin caused a 7-, 5-, or 7-fold induction of 2C8 transcripts, respectively. At the same concentration, ritonavir and nelfinavir induced 2C8 activity by 2-fold, whereas rifampin induced such activity by 4-fold. At ritonavir concentrations of >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).

CYP1A2 and CYP2D6 mRNA and Activity. At 10 μM, CYP1A2 and CYP2D6 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 ATP-binding cassette efflux transporter, was not inducible by any of the drugs. Among 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 among 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, Table 2A). 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, Table 2A). In addition, the induction of CYP2B6 transcripts by nelfinavir was weakly correlated with that of 3A4 (r = 0.4, p > 0.05, Table 2A). 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), whereas that of 2B6 was weakly correlated with that of 3A4 (r = 0.5, p < 0.05).
FIG. 1. Except for CYP3A and 2C8 activity, induction of CYP2B6, 2C9, and 2C19 activity, protein and/or transcripts by ritonavir (Rit), nelfinavir (Nel), or rifampin (Rif) was concentration-dependent. All three drugs were potent inducers of 3A4 transcripts and protein. The potency of induction decreased in the order Rif > Rit > Nel. Whereas 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 three 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 P450 transcripts, protein and activity (H). In all instances, except for H, only changes greater than 2-fold over negative control were considered as induction. Mean ± S.E.M.; n = 4.
FIG. 2. MDR1 transcripts were more inducible by the PIs and rifampin (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. Mean ± S.E.M.; n = 4 except for BCRP for which n = 3. Rit, ritonavir; Nel, nelfinavir.

Discussion

In vivo inhibitory drug interactions are now routinely and quantitatively predicted using human liver and recombinant enzyme microsomes. On the basis of the success of this in vitro model, the Food and Drug Administration 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 inhibitory drug interactions have not been established. Our long-term goal is to validate such a model using human hepatocytes. We used primary human hepatocytes to determine the P450s and transporters induced by the PIs as this in vitro model is routinely used to investigate induction of P450s and transporters (Meneses-Lorenzo et al., 2007). In addition, to determine whether induction studies in human hepatocytes could predict the magnitude of in vivo induction of P450s and transporters, we included rifampin, a prototypical inducer, in all our experiments as our biological control. We chose to study the induction of ritonavir and nelfinavir as these two protease inhibitors differ in their frequency and variety of drugs, we also investigated the induction of CYP2C8, 9, and 19 by the PIs (and rifampin). The induction potency of 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 that is cleared in part by CYP2B6 (Beauverie et al., 1998). The role of CYP2B6 in the clearance of drugs that are metabolized by both CYP3A4 and 2B6, such as meperidine (Ramírez 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.

Because 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 if 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 b.i.d.) and nelfinavir (750 mg t.i.d.) have been observed to induce the clearance of warfarin (Knoll et al., 1998; Newshan and Tsang, 1999) and phenytoin (Honda et al., 1999; Lim et al., 2004).

TABLE 2

<table>
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<tr>
<th>mRNA</th>
<th>Rif</th>
<th>Rit</th>
<th>Nel</th>
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Rif, rifampin; Rit, ritonavir; Nel, nelfinavir. *P < 0.05.
The least inducible enzyme was CYP1A2, and CYP2D6 was not inducible. Consistent with this observation, lopinavir-ritonavir (400/100 mg b.i.d.) modestly induced CYP1A2 (43%) activity in vivo (Yeh et al., 2006). CYP1A isoforms are primarily regulated by the AhR (Li et al., 1998). However, rifampin and ritonavir have not been shown to be ligands of AhRs. 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 AhRs and PXR (Maglich et al., 2002). On the basis of 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 because 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 Food and Drug Administration draft guidelines (http://www.fda.gov/cder/guidance/6695dft.pdf) suggested 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.” On the basis of these guidelines, both ritonavir and nelfinavir would be classified as inducers of CYP2B6 and 2C enzymes (Fig. 1H).

The PXR has been shown to be important in xenobiotic (including rifampin) induction of several P450s, such as CYP3A4, 2B6, and 2C8 and transporters such as MDR1 and 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 among 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 among 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 the PXR. These data also suggest that divergent mechanisms (e.g., other transcriptional factors) are probably involved in the induction of CYP2B6 and 2C8 by the three compounds. Indeed, evidence in the literature suggests that the 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 whether the induction of the three transcripts by a single drug was correlated. For ritonavir, there was a strong correlation between CYP2B6 and 3A4 transcripts (Table 2A). 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 the CAR with the other predominately via the 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 P450 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 MRPs (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 poor correlations 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). These results 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 because of inactivation of these enzymes. However, the PIs do induce several other P450s with potency comparable to that of rifampin. Of these, the most inducible are CYP2B6 and 2C8. Whereas the hepatic basal expression of these P450s is highly variable and, on average, lower than that of other major P450 enzymes, they are highly inducible (Gerbal-Chaloin et al., 2001; Wang and Negishi, 2003). After induction, they likely 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 for the PIs with CYP3A substrates (i.e., induction rather than inhibition) are due to induction of these two enzymes. Although 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 metabolism-based rather than transporter-based. Studies are presently ongoing on our laboratory to determine the in vivo induction of P450s 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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References
Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N.

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