Complex Drug Interactions of HIV Protease Inhibitors 2: In Vivo Induction and In Vitro to In Vivo Correlation of Induction of Cytochrome P450 1A2, 2B6 and 2C9 by Ritonavir or Nelfinavir


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Running Title: Ritonavir, Nelfinavir or Rifampin CYP Induction.

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DDI, drug-drug interaction, RTV, ritonavir; RIF, rifampin; NFV, nelfinavir; PI, anti-HIV Protease inhibitors; CYP, cytochrome P450; AUC, area under the plasma concentration-time curve; HLMs, human liver microsomes.
ABSTRACT

Drug-drug interactions (DDIs) with the HIV protease inhibitors (PIs) are complex, paradoxical (e.g. ritonavir/alprazolam), and involve multiple mechanisms. As part of a larger study to better understand these DDIs and to devise a framework for in vitro to in vivo prediction of these DDIs, we determined the inductive effect of ~ 2 weeks of administration of two prototypic PIs, nelfinavir (NFV), ritonavir (RTV), and rifampin (RIF, induction positive control), on cytochrome P450 enzymes, CYP1A2, 2B6, 2C9 and 2D6 and the inductive or inductive plus inhibitory effect of NFV, RTV or RIF on CYP3A and P-glycoprotein in healthy human volunteers. Statistically significant induction of CYP1A2 (2.1, 2.9 and 2.2-fold), 2B6 (1.8, 2.4 and 4.0-fold) and 2C9 (1.3, 1.8 and 2.6-fold) was observed after NFV, RTV or RIF treatment respectively (as expected, CYP2D6 was not induced). Moreover, we accurately predicted the in vivo induction of these enzymes by quantifying their induction by the PIs in human hepatocytes and by using RIF as an in vitro to in vivo scalar. Based on the modest in vivo induction of CYP1A2, 2B6 or 2C9, the in vivo paradoxical DDIs with the PIs are likely explained by mechanisms other than induction of these enzymes such as induction of other metabolic enzymes, transporters, or both.
INTRODUCTION

Clinical use of the HIV protease inhibitors (PIs) is complicated by their unpredictable but profound drug-drug interactions (DDIs). Many of the protease inhibitors, most notably ritonavir (RTV), are known to potently inhibit and/or inactivate CYP3A enzymes (Ernest et al., 2005). For this reason, RTV is almost exclusively used in combination with other PIs to pharmacologically boost their bioavailability by inactivating CYP3A (Cooper et al., 2003). Based on in vitro metabolism and the in vivo effect of RTV boosting, CYP3A is believed to be the major clearance mechanism of many of the PIs including RTV (Kumar et al., 1996; Koudriakova et al., 1998; Unadkat and Wang, 2000). But, on chronic administration, RTV and other PIs are capable of inducing their own clearance in spite of potent CYP3A inactivation (Hsu et al., 1998). Similarly, amprenavir, another potent CYP3A inactivator (Ernest et al., 2005), has little effect (18% increase) on the clearance of saquinavir, a CYP3A substrate (Unadkat and Wang, 2000). Likewise, after multiple dose administration of RTV, the oral clearance of alprazolam (a CYP3A probe drug) remains unchanged (Norvir product labeling), whereas on acute administration RTV decreases the oral clearance of alprazolam as expected (Greenblatt et al., 2000). While these observations could be explained by net induction of in vivo CYP3A activity, we have shown this is not the case. In fact, CYP3A activity is reduced by ~90% after multiple dose RTV treatment which is accurately predicted from in vitro data using sandwich cultured human hepatocytes (Kirby et al., 2011). Therefore, these data suggest induction of other clearance mechanisms, likely other CYPs and/or drug efflux pumps such as P-glycoprotein (P-gp) or multidrug resistance protein 2 (MRP2) (Su et al., 2004; Ye et al., 2010). In agreement with this hypothesis, there are sporadic in vivo reports that RTV appears to induce CYP1A2 (Norvir product labeling), 2B6 and 2C9 (Fichtenbaum and Gerber, 2002; Hughes et al., 2007).
In an attempt to provide a mechanistic framework to accurately predict the multifaceted and seemingly unpredictable DDIs elicited by the PIs, we conducted in vivo studies in healthy volunteers with two prototypic PIs (RTV and nelfinavir, NFV) and the well known inducer, rifampin (RIF). Our studies were designed to assess the inductive effect of multiple dose treatment (~14 days) with RTV (400 mg bid), NFV (1250 mg bid) or RIF (600 mg qd) on the major drug metabolizing CYP enzymes (CYP1A2, 2B6, 2C9, 2D6, 3A4) and P-gp by administration of probe drug cocktails. We then asked if these DDIs can be predicted from in vitro experiments using human liver microsomes and hepatocytes using the broad spectrum inducer RIF as an in vitro to in vivo scalar for induction of multiple enzymes. We have previously reported and predicted the CYP3A-mediated DDIs from in vitro data (Kirby et al., 2011). In this manuscript we demonstrate and predict the in vivo induction of CYP1A2, 2B6, 2C9 and 2D6 by RTV, NFV or RIF. CYP2D6 was included in our study as a negative control as it is not inducible by xenobiotics (Benedetti, 2000). The effect of the PIs on P-gp activity will be the subject of another manuscript as that study also revealed an interesting and clinically significant drug-drug interaction between the CYP2B6 probe drug bupropion and digoxin.
METHODS

Study Design

The study design, subject selection criteria, and subject safety monitoring have been described in detail in our previous manuscript focusing on the CYP3A mediated DDIs with RTV, NFV and RIF (Kirby et al., 2011). The design of the study with respect to the CYP1A2, 2B6, 2C9 and 2D6 mediated DDIs with RTV, NFV or RIF is described here (Figure 1). Briefly, two studies were conducted in healthy volunteers. In study 1, the drugs used to measure in vivo CYP1A2 (caffeine, 200 mg PO), 2C9 (tolbutamide, 500 mg PO) and 2D6 (dextromethorphan, 30 mg PO) activity were administered as part of a CYP phenotyping cocktail (Wang et al., 2001). Study 1 was conducted in two arms (RTV and RIF treatment or NFV and RIF treatment). In study 2, bupropion (150 mg PO) was administered to measure in vivo CYP2B6 activity and all subjects were treated with RTV, NFV and RIF. The phenotyping drugs were administered prior to and after ~14 day treatment with oral RTV (dose escalation to 400 mg bid), NFV (1250 mg bid) or RIF (600 mg qd). The order of treatment was randomized in all studies. Our studies utilized 400 mg bid RTV since they were initiated prior to the exclusive use of low dose RTV (100 mg) as a “booster”, and many of the unpredictable DDIs described above were observed with the higher doses of RTV (>200 mg bid). Subjects fasted after midnight prior to each study session and until two hours after administration of the phenotyping drugs. The phenotyping drugs were given approximately 12 hours after the last dose of RTV, NFV or RIF to minimize reversible inhibition and thereby more accurately estimate the fold-induction of CYP enzymes. Blood and urine samples were collected prior to and up to 48 hours after phenotyping drug administration. Plasma and urine samples were stored at -20°C until analysis.
Study Drugs, Chemicals and Reagents

All study drugs were supplied via the University of Washington Investigational Drug Services. See Table 1 for provider information for study drugs and drug and metabolite reference standards. Optima grade water, methanol and methyl t–butyl ether (MTBE) were purchased from Fisher (St. Louis, MO). β-glucuronidase was purchased from MP biomedicals (Solon, OH). All other chemicals used were reagent grade or higher.

Drug and Metabolite Analysis

Caffeine, bupropion, 4-hydroxy bupropion (4-OH BUP), tolbutamide, 4-hydroxy tolbutamide (4-OH TOLB), carboxy tolbutamide, dextromethorphan (DEX), dextrorphan (DOR), 3-methoxy morphinan (3MM) and 5-hydroxy morphinan plasma concentrations were determined by a UPLC/MS/MS method. Briefly, 50 μL of an internal standard mixture containing D4-midazolam, D4-hydroxy midazolam, 13C-caffeine and chlorpropamide was added to plasma samples (1 mL) followed by 100 μL of concentrated ammonium hydroxide and 4 mL of MTBE. Samples were mixed for 30 min then centrifuged for 10 min at 2000g and the organic layer was removed. Two hundred μL of concentrated HCL was added to the remaining sample which was again extracted with 4 ml of MTBE. The organic layer was removed and combined with the previous extract and evaporated to dryness under vacuum. The residue was reconstituted in 100 μL of 50:50 0.1% acetic acid in water:0.1% acetic acid in methanol. Fifteen μL of this solution was analyzed by a previously described UPLC/MS/MS method(Kirby et al.,2011). See Table 1 for ion collection parameters. A calibration curve and quality control samples were prepared in fetal bovine serum owing to difficulty in obtaining caffeine free plasma from the local blood bank. Urine samples were diluted 1/10 or 1/50 then pretreated with 1000 units of β-
glucuronidase in a pH 5.0 100 mM acetic acid solution overnight (~16 hours) prior to being extracted using the method described above. A set of calibrators and controls were similarly subjected to the β-glucuronidase treatment for quantification of the urine samples. Precision and accuracy of the controls was less than 20% CV and 20% error respectively.

Stereospecific analysis of the urinary (S,S)-4-OH BUP/S-BUP ratio was conducted as described previously (Coles and Kharasch, 2007; Kharasch et al., 2008) with minor modifications including optimized LC/MS/MS parameters and dilution of urine samples rather than solid phase extraction. With these modifications, comparable sensitivity, linearity, reproducibility and enantiomeric separation were achieved as previously published. Urinary caffeine, paraxanthine, 1-methyl uric acid, 1,7-dimethyl uric acid, 1 methyl xanthine and 5-acetylamino-6-amino-3-methyluracil (AAMU, a combination of AAMU and 5-acetylamino-6-formylamino-3-methyluracil, AFMU) concentrations were measured using a previously validated HPLC/UV method (Nyeki et al., 2001).

Pharmacokinetic Analysis

Noncompartmental analysis of the plasma concentration-time profiles of caffeine, bupropion, tolbutamide, dextromethorphan and dextrophan was performed using WinNonlin Professional v 5.0 (Pharsight Corp, Mountain View, CA). Parameters estimated included area under the plasma concentration-time curve (AUC$_{0-t}$), where t is the last sample time, AUC$_{0-\infty}$, terminal plasma half life ($t_{1/2}$) and oral clearance (CL$_{PO}$, Dose / AUC$_{0-\infty}$). Renal clearance of BUP and 4-OH-BUP were calculated as the ratio of the amount of unconjugated drug excreted in the urine to the plasma AUC over the urine collection interval. The (S,S)-4-OH-BUP/S-BUP urinary ratio (UR) was calculated as the molar ratio of total (after deconjugation) (S,S)-4-OH-
BUP to S-BUP in the 24 hour urine. Formation clearances (Cl_{form}) of paraxanthine, 4-OH BUP and 4-OH TOLB, were estimated by the ratio of total amount of metabolite plus downstream metabolites (paraxanthine plus downstream metabolites, 4-OH BUP and 4-OH TOLB plus carboxy tolbutamide) excreted in the 24 hour urine and the AUC_{0-24} of the parent (caffeine, bupropion and tolbutamide) respectively. Dextrorphan/dextromethorphan AUC ratio (DOR/DEX) was calculated as the ratio of DOR and DEX plasma AUC_{0-24}. URs for DOR/DEX and 3MM/DEX were calculated by the ratio of total amount of DOR or 3MM respectively and the total amount of DEX excreted in the 24 hour urine. All subjects with a DOR/DEX UR less than 3.3 prior to treatment were deemed phenotypic CYP2D6 poor metabolizers and were excluded from further analysis (Schmid et al., 1985). All urinary metabolite data were corrected for equivalent mass of the parent compound.

**Statistical analyses**

As pharmacokinetic (PK) parameters are typically log-normally distributed, statistical analysis was conducted on log-transformed PK parameters. This was done by calculating the geometric mean ratio (GMR) by exponentiation of the average difference of log transformed PK parameters. If the 90% confidence interval (CI) of this GMR included unity, the treatment was considered to not have significantly altered the PK parameter.

Using historical data of caffeine, bupropion, and tolbutamide pharmacokinetics in healthy volunteers, we conducted an a priori power analysis using plasma AUC as the primary outcome measure. Assuming equal variance between control and treatment groups, our analysis indicated that n=7 would provide 80% power (α< 0.05) to discern a 100%, 40% and 59% change in plasma AUC of caffeine, bupropion and tolbutamide respectively.
In Vitro to In Vivo Prediction of CYP Induction

In vitro induction of CYP activity and mRNA expression was estimated from our previously published studies in human hepatocytes (Dixit et al., 2007). Briefly, human hepatocytes were treated with increasing concentrations (0-25 μM) of RTV, NFV or RIF for 72 hours (in protein-free media), the cells were harvested, microsomal CYP activity was evaluated using a validated in vitro phenotyping cocktail (Dixit et al., 2007). The maximum fold-induction ($E_{\text{max}}$) and concentration resulting in half maximal induction ($EC_{50}$) for RTV, NFV and RIF induction of CYP1A2, 2B6, 2C9 and 2D6 were estimated by fitting Eq. 1 (Fahmi et al., 2008) to the data where $I$ is the total inducer concentration.

$$FoldInduction = 1 + \frac{E_{\text{max}} \times I}{EC_{50} + I}$$

Eq. 1

In vivo induction of CYP enzymes was predicted using the in vitro derived $E_{\text{max}}$ and $EC_{50}$ values for each enzyme using Eq. 2 (Fahmi et al., 2008). Where $I_u$ is the unbound average inducer concentration and $d$ is the in vitro to in vivo scaling factor for induction.

$$f_{\text{Induction}}^{\text{Rep}} = 1 + \frac{d \times E_{\text{max}} \times I_u}{I_u + EC_{50}}$$

Eq. 2

The in vitro to in vivo induction scaling factor for each enzyme was estimated by determining the $d$ value that provided accurate prediction of the in vivo CYP activity GMR (CYP1A2; paraxanthine $Cl_{\text{form}}$, CYP2B6; 4-OH BUP $Cl_{\text{form}}$ and CYP2C9; 4-OH TOLB $Cl_{\text{form}}$) as
a result of RIF treatment. These scaling factors were used for the in vitro to in vivo prediction of these CYP enzymes by RTV or NFV using Eq. 2.
RESULTS

Subject demographics, treatment periods for RTV, NFV and RIF and cocktail administration are described in detail in our previous manuscript addressing the CYP3A mediated DDIs (Kirby et al., 2011). Briefly 16 healthy volunteers (33 ± 9 yr, 78 ± 14 kg, 5 males and 11 females) completed Study 1 and 9 (29 ± 9 yr, 79 ± 14 kg, 3 males and 6 females) completed Study 2.

CYP1A2 (Caffeine)

Two subjects in the RTV arm of study 1 were excluded from CYP1A2 analysis because of suspected ingestion of caffeine overnight (24 hour plasma caffeine concentrations greater than at 12 hours) during the control phase. All treatments resulted in modest induction of in vivo CYP1A2 (paraxanthine formation clearance \( Cl_{\text{form}} \) geometric mean ratios \( \text{GMR} \) greater than unity), with RTV being the most effective inducer (2.9-fold) and NFV and RIF inducing 2.1- and 2.2-fold respectively (Figure 2A and Table 2).

CYP2B6 (Bupropion)

All three treatments, nelfinavir, ritonavir and rifampin significantly induced the CYP2B6 marker 4-hydroxy bupropion (4-OH BUP) \( Cl_{\text{form}} \) by 1.8-, 2.4- and 4.0-fold respectively (Figure 2B, Table 2). The magnitude of CYP2B6 induction by NFV, RTV and RIF was highly variable, and in general those subjects that showed the greatest degree of CYP2B6 induction by RIF also had the greatest degree of induction by RTV or NFV. Since the racemic 4-OH-BUP \( Cl_{\text{form}} \) is a combination of the formation-rate limited (S,S)-4-OH BUP and the elimination rate limited (R,R)-4-OH BUP metabolites, we evaluated the stereospecific 0-24 hr urinary ratio of the
formation-rate limited metabolite (S,S)-4-OH-BUP ((S,S)-4-OH BUP/S-BUP UR; Table 2) to confirm the magnitude of CYP2B6 induction observed after RTV, NFV or RIF treatment as assessed by the racemic 4-OH BUP \( C_{\text{form}} \). Both of these markers showed comparable increases in CYP2B6 activity, with the (S,S)-4-OH-BUP/S-BUP UR being a slightly more sensitive measure for RTV and RIF treatments. Rifampin was the only treatment that significantly altered the bupropion clearance (66% increase) and AUC (40% decrease), which is likely due to RIF inducing the non-CYP2B6 clearance of bupropion.

When calculating the 4-OH BUP \( C_{\text{form}} \) we used only the 24 hour urine because we observed an unexpected mutual DDI between BUP and the P-glycoprotein probe, digoxin, during the 24-48 hr period after bupropion administration (see Figure 1 for study design). This interaction was observed when comparing BUP urinary excretion during the 24-48 hr period, (when digoxin was present) with 0-24 hr (when it was absent; Figure 3). When digoxin was present, BUP \( C_{\text{renal}} \) did not change, but the racemic 4-OH-BUP \( C_{\text{renal}} \), 4-OH-BUP \( C_{\text{form}} \), racemic 4-OH-BUP/BUP UR and RR-4-OH-BUP/R-BUP UR were significantly increased. Interestingly, there was no significant effect of digoxin on the SS-4-OH-BUP/S-BUP UR. The dramatic effect of BUP or OH-BUP on digoxin pharmacokinetics will be described in our future manuscript.

**CYP2C9 (Tolbutamide)**

All treatments resulted in statistically significant induction of in vivo CYP2C9 activity measured by tolbutamide AUC, plasma clearance or 4-hydroxytolbutamide (4-OH TOLB) \( C_{\text{form}} \) (Table 2 and Figure 2C). 4-OH TOLB \( C_{\text{form}} \) is a good measure of CYP2C9 activity as literature data indicate very little contribution of other enzymes (CYP2C19 and 2C8) to this
pathway (Komatsu et al., 2000). In vivo CYP2C9 activity was increased 2.6-, 1.8-, and 1.3-fold by RIF, RTV and NFV respectively. Induction of CYP2C9 was more variable with RIF (range 1.0- to 7.4-fold) compared to RTV (range 1.2- to 2.2-fold) or NFV (range 0.98- to 1.95-fold).

CYP2D6 (Dextromethorphan)

One subject with a dextrorphan/dextromethorphan urinary ratio (DOR/DEX UR) < 3.3 prior to treatment was deemed a CYP2D6 poor metabolizer and therefore removed from further analysis of CYP2D6 activity. Treatment with NFV did not significantly alter any measured pharmacokinetic parameters of DEX (Table 2 and Figure 2D-F, note the log Y-scale). Ritonavir significantly increased the DEX AUC (4.5-fold) and decreased the clearance of DEX (GMR 0.22). Two commonly used CYP2D6 markers, the plasma AUC ratio of dextrorphan/dextromethorphan (DOR/DEX) and the urinary ratio of DOR/DEX (DOR/DEX UR) were significantly decreased by RTV (GMR 0.11 and 0.13 respectively). Two subjects in the RTV arm had plasma dextrorphan (DOR) concentrations that were below the limit of quantification, therefore for the comparison of DOR/DEX AUC ratio the number of subjects were six. Rifampin significantly decreased the DEX AUC (GMR 0.27) and increased DEX clearance (3.7-fold). Rifampin treatment significantly affected the CYP2D6 markers (DOR/DEX AUC ratio and DOR/DEX UR) but in an opposing fashion (GMR 0.5 and 1.8 respectively). The DOR/DEX AUC ratio was decreased implying inhibition of CYP2D6 whereas the DOR/DEX UR was increased implying induction of CYP2D6. This discrepancy is likely the result of the effect of RIF on the non-CYP2D6 clearance of both DEX and DOR (CYP3A and glucuronidation).
The urinary ratio of 3-methoxy morphinan/dextromethorphan (3MM/DEX UR) has been proposed as a marker of in vivo CYP3A activity. This parameter was significantly affected only by treatment with RIF (GMR 2.48; Table 2, Fig. 2F) which is in contrast to our previously reported data where RTV, NFV and RIF all significantly altered CYP3A activity as measured by IV and oral midazolam clearance (Kirby et al., 2011).

**Correlation of Basal CYP Activity and Observed Fold-Induction**

Previous reports have shown the magnitude of in vivo induction of CYP3A is correlated with CYP3A activity prior to treatment (Gorski et al., 2003). Therefore, we examined if this was true for the in vivo activity of CYP1A2, 2B6 or 2C9 after RIF, NFV or RTV treatment (Figure 4). There was a modest to strong non-linear inverse correlation of activity prior to treatment and fold-induction of CYP1A2 ($R^2= 0.44, 0.70$ and 0.39) or CYP2B6 ($R^2=0.47, 0.45$ and 0.82) activity by RIF, NFV or RTV respectively. In contrast, no such correlation was observed for CYP2C9 activity ($R^2 <0.05$ for all treatments).

**In Vitro to In Vivo Prediction of CYP Induction.**

The estimated parameters ($E_{max}$ and $EC_{50}$) for RTV, NFV or RIF induction of CYP1A2, 2B6, 2C9 enzyme activity and mRNA expression in human hepatocytes (Table 3) were obtained by re-analysis of our previously published data (Dixit et al.,2007). The in vitro to in vivo induction scaling factors for RIF were found to be 20, 8 and 24 (mRNA expression) or 10, 18 and 4 (activity) for CYP1A2, 2B6 and 2C9 respectively (Figure 5C). These scaling factors scale the fold-induction of the CYP mRNA expression or activity measured in human hepatocytes.
with the fold-induction of the CYP activity observed in vivo assuming the average unbound concentration (\(C_{\text{ave,u}}\)) of the inducer is the driving force for induction. Using these scaling factors 4 of 6 induction interactions with RTV or NFV were accurately predicted (within the observed 90% CI) using mRNA expression (Figure 5A) and 5 of 6 using enzyme activity (Figure 5B).
DISCUSSION

All treatments resulted in 2- to 3-fold-induction of CYP1A2 activity (caffeine Cl_{PO} or paraxanthine Cl_{form}). There was excellent correlation between the fold-change in caffeine CL_{PO} and paraxanthine Cl_{form} (R^2 = 0.90, slope = 1.01). Thus caffeine CL_{PO} is an adequate marker of change in CYP1A2 activity in vivo. Induction of CYP1A2 by the PIs and RIF is somewhat surprising because RIF has not been shown (or is not known) to be an aryl hydrocarbon receptor (AHR) ligand and RTV and NFV are low affinity AHR ligands (EC_{50} > 20 \mu M) (Frotschl et al., 1998). Previous studies showed that RIF (500-600 mg qd) induces in vivo CYP1A2 ~20-30\% (Branch et al., 2000; Backman et al., 2006; Kanebratt et al., 2008), using a single point caffeine/paraxanthine plasma concentration ratio which is confounded by increased clearance of paraxanthine (mediated by multiple enzymes including CYP1A2) (Lelo et al., 1989). Our results indicate greater induction of CYP1A2 by RIF (120\%) using paraxanthine Cl_{form}. Therefore, we speculate that in vivo induction of CYP1A2 by the PIs and rifampin may be a result of nuclear receptor cross-talk (Pascussi et al., 2008) or insensitivity of the in vitro nuclear receptor activation experiments to mimic the in vivo response.

All treatments (NFV, RTV and RIF) significantly induced CYP2B6 activity measured by 4-OH BUP Cl_{form} (1.8-, 2.4- and 4-fold respectively). In human liver microsomes, CYP2B6 contributes ~93\% to the formation of 4-OH BUP, with minor contribution from CYP2C19 (Chen et al.). Thus CYP2C19 contribution to our CYP2B6 measure is expected to be minimal. A previous study with RTV (400 mg bid) increased 4-OH BUP Cl_{form} (2.1-fold) and decreased BUP AUC 33\% (Kharasch et al., 2008). RTV has been shown to cause a dose-dependent decrease in BUP AUC (66\% and 22\% for 600 mg bid and 100 mg bid respectively) (Park et
al., 2010). The reason we did not observe a statistically significant decrease in BUP AUC is unclear. CYP2B6 expression is known to vary as much as 200-fold (Wang and Tompkins, 2008) perhaps a result of genetic polymorphisms and/or variable expression of CYP2B6 and/or the nuclear receptors Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR), or environmental factors (Mo et al., 2009). Induction of CYP2B6 is mediated by PXR and CAR (Faucette et al., 2006). NFV, RTV and RIF are known PXR ligands (Dussault et al., 2001). RTV and NFV are not ligands of the CAR splice variant (CAR3) (Gupta et al., 2008) while RIF is believed not to be a CAR ligand (Faucette et al., 2006). This implies that CYP2B6 induction by RTV, NFV or RIF is PXR mediated. One subject in our study (with the second highest basal 4-OH BUP Cl\text{\textsubscript{form}}) showed no increase in CYP2B6 activity after NFV, RTV or RIF treatment, but showed the expected response for PXR mediated CYP3A induction after RIF treatment (Kirby et al., 2011). These data reiterate that induction of CYP2B6 is quite variable.

All treatments (NFV, RTV and RIF) modestly induced CYP2C9 activity with GMRs of 1.3, 1.8 and 2.6 respectively. Consistent with this observation, RTV decreases the anticoagulant effect of warfarin and acenocoumarol, (Hughes et al., 2007; Bonora et al., 2008). CYP2C9 expression is regulated, at least in part, by PXR (Sahi et al., 2009). A correlation analysis of fold-induction of CYP1A2, 2C9, 2B6 and 3A showed no significant correlations ($R^2 \leq 0.1$, data not shown) implying minimal coregulation of these enzymes or an insufficient dynamic range to discern a correlation.

NFV had no significant effect on DEX pharmacokinetics. Ritonavir on the other hand, significantly increased the DEX AUC (4.5-fold), decreased the clearance (0.22-fold), and significantly decreased the DOR/DEX AUC and UR GMRs (~0.10) implying potent inhibition
of CYP2D6. Based on the unbound plasma concentration of RTV at the time of DEX dosing (~0.03 μM) and the modest reversible inhibition \( K_i \) (~4μM)(von Moltke et al.,1998), reversible inhibition of CYP2D6 by RTV should be minimal. Therefore, we speculate that RTV is either a mechanism-based inactivator of CYP2D6, or a long-lasting metabolite of RTV is a more potent CYP2D6 inhibitor. RIF decreased the AUC and increased the clearance of DEX, likely by induction of CYP3A or conjugation of DEX because CYP2D6 is not inducible by xenobiotics(Benedetti,2000). However, the DOR/DEX AUC ratio decreased (GMR = 0.5) implying inhibition of 2D6, but the DOR/DEX UR increased (GMR = 1.77) implying induction of 2D6. These conflicting data suggest that these purported measures of CYP2D6 activity may not be reliable under induced conditions.

Our data show RTV and NFV are in vivo inducers of CYP enzymes other than CYP3A. Therefore, we asked the question, “can in vivo induction of CYP enzymes be predicted from in vitro hepatocyte experiments and can these data be used to explain some of the unpredictable DDIs with the PIs”. To answer this question we used our previously published human hepatocyte RIF induction data(Dixit et al.,2007) (mRNA expression and activity) and in vivo induction of CYP activity (this study) to estimate an in vitro to in vivo induction scaling factor for RIF, an approach similar to that previously used for CYP3A induction(Kirby et al.,2011). Using the induction scaling factors for mRNA or activity, 4 of 6 and 5 of 6 DDIs were well predicted (prediction fell within the 90% CI of the observed DDI) respectively. In contrast, when the induction scaling factor wasn’t used, the greatest predicted fold-induction was 1.4 (RIF - CYP2C9) which was below the observed 90% CI (2.02-3.22). For all of the other DDIs, the prediction without scaling was no induction (≤20%). The difference between scaling factors for
mRNA and activity may result from a number of factors including differing degrees of mRNA
translation into active protein in vitro and/or different shape of the in vitro fold-induction vs.
inducer concentration profile ($E_{\text{max}}$ and $EC_{50}$).

The induction scaling values being greater than 1 and variable across enzymes, implies
the in vitro sensitivity of CYP induction in human hepatocytes is substantially less than observed
in vivo and a “one size fits all” approach to induction scaling across CYP enzymes is
unacceptable. For example, the in vitro induction $E_{\text{max}}$ of CYP1A2 was less than 2-fold for all
three inducers whereas greater than 2-fold-induction of CYP1A2 activity was observed in vivo
for all three inducers. Many factors may contribute to these differences, including: 1) different
inducer exposure profiles in vitro and in vivo; 2) different expression profiles of the nuclear
receptors in vitro and in vivo; 3) an intrinsic insensitivity of the in vitro hepatocytes as a result of
being in an artificial environment lacking potentially important paracrine factors; and/or 4) a
lack of three dimensional architecture in the in vitro hepatocytes (our previous experiments were
conducted in plated rather than sandwich cultured hepatocytes) which may alter the uptake or
efflux transport and thereby affect hepatic accumulation of the inducer. Our analysis illustrates
the need for in vitro experimental design modifications to mitigate the insensitivity of in vitro
hepatocytes relative to in vivo, potentially alleviating the need for scaling factors and/or the
variability of scaling factors across enzymes.

In summary, we have shown that multiple dose treatment of RTV (400 mg bid) or NFV
(1250 mg bid) significantly, but modestly induce CYP1A2, 2B6 and 2C9 activity. Thus co-
administration of this higher dose RTV or NFV with narrow therapeutic index drugs
predominantly cleared by CYP1A2, 2B6 or 2C9 may require dose adjustment to maintain
efficacy. Since RTV is now almost exclusively administered at low doses (100-200 mg) we used our scaled in vitro to in vivo prediction method to predict the fold-induction of CYP1A2, 2B6 or 2C9 by low dose RTV (100-200 mg bid, with average plasma concentrations ranging from 1-2 \( \mu \text{M} \)). We predicted \( \leq 30\% \) induction of CYP1A2, 2B6 or 2C9 at this low RTV dose, a magnitude of induction unlikely to necessitate dose adjustment. But, these predictions account only for induction by RTV and not for the coadministered PIs. In addition, our data show that the magnitude of induction of CYP1A2, 2B6 and 2C9 can be predicted using in vitro human hepatocytes if an in vitro to in vivo induction scaling factor based on RIF is implemented.

Additional analyses of in vitro and in vivo data are needed to see if this approach can be applied to predict in vivo CYP induction by other inducers and if other scalars provide better prediction. However, our results don’t explain the more perplexing DDIs observed with the PIs, such as the lack of RTV-alprazolam interaction after chronic RTV administration or autoinduction of RTV clearance. The modest induction of CYP1A2 or 2C9 by RTV (or for that matter CYP2B6) is unlikely to contribute significantly to the clearance of alprazolam (contribution of CYP1A2, 2C9 or 2B6 is \(<1\%\)\(\text{(Gorski et al.,1999)}\) or the autoinduction of RTV. Thus, induction of other clearance mechanisms of alprazolam or other unknown mechanisms must contribute to this paradoxical DDI. Clearly additional studies are needed to determine how the PIs or alprazolam are cleared despite net inactivation of CYP3A and only modest induction of other minor contributing CYP enzymes.
ACKNOWLEDGEMENTS

We wish to thank Eric Helgeson, RN and Christine Hoffer, CCRC for clinical study coordination.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Collier, Desai, Dixit, Kharasch, Kirby, Thummel and Unadkat

Conducted experiments: Dixit, Kirby and Whittington

Contributed new reagents or analytical tools: Whittington

Performed data analysis: Dixit, Kirby and Whittington

Wrote or contributed to the writing of the manuscript: Collier, Desai, Dixit, Kharasch, Kirby, Thummel, Unadkat and Whittington
REFERENCES


FOOTNOTES

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**FIGURE LEGENDS**

Figure 1: Study design showing administration of phenotyping drugs and the dosing regimen of RTV, NFV or RIF treatment. Cocktail A: midazolam (2 mg PO), digoxin (0.5 mg PO). Cocktail B: midazolam (1 mg IV), caffeine (200 mg PO), tolbutamide (500 mg PO), dextromethorphan (30 mg PO). Bupropion: bupropion (150 mg PO). Treatment ~14 days: ritonavir (RTV, escalating dose to 400 mg bid), nelfinavir (NFV, 1250 mg bid), rifampin (RIF, 600 mg qd).

Figure 2: Induction of in vivo CYP activity by NFV, RTV or RIF. Panels A-F show the fold-change in in vivo CYP activity for each subject (circles) and the GMR with 90% CIs (horizontal bars). CYP1A2 activity (paraxanthine CL\textsubscript{form}) was significantly induced by NFV (2.1-fold), RTV (2.9-fold) or RIF (2.2-fold) (A). CYP2B6 activity (4-OH BUP CL\textsubscript{form}) was variably and significantly induced by NFV (1.8-fold), RTV (2.4-fold) or RIF (4.0-fold); subjects that showed the greatest induction with RIF also showed the greatest induction with RTV or NFV (B). CYP2C9 activity (4-OH TOLB CL\textsubscript{form}) was significantly induced by NFV (1.3-fold), RTV (1.8-fold) or RIF (2.6-fold) (C). CYP2D6 activity (DOR/DEX AUC ratio or DOR/DEX UR, (D and E respectively), as expected, was not significantly induced by NFV or RIF. RTV significantly decreased both CYP2D6 phenotype markers to ~ 10% of basal activity. The purported CYP3A phenotype marker (3MM/DEX UR) was induced by RIF (2.5-fold) but was unchanged by NFV or RTV (F).

Figure 3: The effect of an unexpected DDI between the P-glycoprotein probe drug, digoxin, on bupropion pharmacokinetics. Relevant pharmacokinetic parameters of BUP and 4-OH-BUP, in the presences (24-48hr) and absence of digoxin (0-24 hr), were compared as GMR and 90% CI.
Digoxin caused a significant increase (* 90% CI does not include unity) in the 4-OH-BUP Cl\text{renal} (1.6-fold), racemic 4-OH-BUP Cl\text{form} (2.4-fold), racemic 4-OH-BUP/BUP UR (2.0-fold) and RR-4-OH-BUP/R-BUP UR (2.0-fold), but not the SS-4-OH-BUP/S-BUP UR.

Figure 4: Correlation analysis of observed in vivo induction of CYP1A2 (A), 2B6 (B) and 2C9 (C) after treatment with RIF, NFV or RTV relative to control CYP activity (4-OH BUP, paraxanthine or 4-OH TOLB Cl\text{form}, respectively). In vivo CYP2B6 (A) and CYP1A2 (B) but not CYP2C9 (C) fold-induction by RTV, NFV or RIF treatment was inversely and nonlinearly correlated with basal activity. Those subjects with the highest basal CYP2B6 or 1A2 activity tended to show only modest induction of in vivo activity of these enzymes after treatment with the inducers. Note the log scale for control CYP2B6 activity whereas CYP1A2 and 2C9 are displayed on a linear scale. Regression analysis results for CYP1A2 (RIF: $y = 12.7X^{-0.44}$ $R^2 = 0.44$, NFV: $y = 24.1X^{-0.60}$ $R^2 = 0.70$, RTV: $y = 29.9X^{-0.55}$ $R^2 = 0.39$) and CYP2B6 (RIF: $y = 17.4X^{-0.37}$ $R^2 = 0.47$, NFV: $y = 7.1X^{-0.34}$ $R^2 = 0.45$, RTV: $y = 37.3X^{-0.68}$ $R^2 = 0.83$).

Figure 5: In vitro to in vivo prediction of CYP induction. The GMR and 90% CI of the observed in vivo induction of CYP1A2, 2B6 and 2C9 are shown in grey bars and error bars respectively. Prediction of the in vivo CYP induction without using RIF in vitro to in vivo scaling (open circles) generally resulted in minimal to no predicted in vivo induction. In contrast, incorporating the RIF in vitro to in vivo induction scaling factor (closed circles) generally resulted in predicted in vivo CYP induction within the 90% CI of that observed.
Table 1: Study drugs and reference standard provider and analytical method information

<table>
<thead>
<tr>
<th>Study Drugs</th>
<th>Supplier Information</th>
<th>Analytical Method</th>
<th>Detection Parameters: m/z transition, cone voltage, collision energy</th>
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<tr>
<td>Caffeine 100 mg tablets</td>
<td>GlaxoSmithKline (Philadelphia, PA)</td>
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<td>Bupropion 150 mg ER tablets</td>
<td>Watson Pharmaceuticals (Corona, CA)</td>
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<tr>
<td>Tolbutamide 500 mg tablets</td>
<td>Mylan (Pittsburgh, PA)</td>
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<td>Dextromethorphan 15 mg cough gels</td>
<td>Wyeth Consumer Healthcare (New York, NY)</td>
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<table>
<thead>
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<th>Reference Standards</th>
<th>Supplier Information</th>
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<th>Detection Parameters: m/z transition, cone voltage, collision energy</th>
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<td>Caffeine</td>
<td>Cerilliant (Round Rock, TX)</td>
<td>UPLC/MS/MS and HPLC/UV</td>
<td>195.1&lt;137.9, 15, 20</td>
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<td>1-methyl uric acid</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
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<td>1,7-dimethyl uric acid</td>
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<td>1-methyl xanthine</td>
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<td>5-acetylamino-6-amino-3-methyluracil (AAMU)</td>
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<td>D4-OH-midazolam</td>
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<td>13C-caffeine</td>
<td>Toronto Research Chemicals (North York, Ontario, Canada)</td>
<td>UPLC/MS/MS</td>
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<td>Chlorpropamide</td>
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<td>T-β OH-propyl theophylline</td>
<td>Sigma-Aldrich</td>
<td>HPLC/UV</td>
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Table 2: In vivo CYP1A2, 2B6, 2C9 or 2D6 activity measured by disposition of caffeine, bupropion, tolbutamide or dextromethorphan respectively before and after multiple dose administration of nelfinavir, ritonavir or rifampin

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nelfinavir</th>
<th>GMR (90%CI)</th>
<th>Ritonavir</th>
<th>GMR (90%CI)</th>
<th>Rifampin</th>
<th>GMR (90%CI)</th>
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<td><strong>CYP1A2</strong> (Caffeine)</td>
<td></td>
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<td>n= CON 14, NFV 7, RTV 6, RIF 14</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
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<tr>
<td>AUC 0-∞ (hr*ug/ml)</td>
<td>56.7 ± 46.6</td>
<td>30.8 ± 12.9</td>
<td>0.49 (0.29-0.84)</td>
<td>13.90 ± 6.8</td>
<td>0.35 (0.26-0.45)</td>
<td>22.6 ± 7.3</td>
<td>0.47 (0.38-0.58)</td>
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<tr>
<td>Clearance (ml/min)</td>
<td>85.4 ± 45.0</td>
<td>131 ± 69.0</td>
<td>2.03 (1.20-3.45)</td>
<td>285 ± 128</td>
<td>2.90 (2.22-3.80)</td>
<td>168 ± 73.0</td>
<td>2.14 (1.73-2.66)</td>
</tr>
<tr>
<td>ClFormation (ml/min)</td>
<td>64.3 ± 44.5</td>
<td>108 ± 71.2</td>
<td>2.13 (1.20-3.81)</td>
<td>211 ± 88.8</td>
<td>2.89 (2.02-4.12)</td>
<td>132 ± 76.4</td>
<td>2.23 (1.79-2.78)</td>
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<tr>
<td>SS-OH-BUP/S-BUP UR</td>
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<tr>
<td><strong>CYP2B6</strong> (Bupropion)</td>
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<tr>
<td>n= 9</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
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<td>Ave ± SD</td>
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</tr>
<tr>
<td>AUC 0-∞ (hr*ng/ml)</td>
<td>1786 ± 1543</td>
<td>1648 ± 1117</td>
<td>1.10 (0.75-1.61)</td>
<td>1201 ± 775</td>
<td>0.98 (0.57-1.71)</td>
<td>962 ± 761</td>
<td>0.60 (0.50-0.73)</td>
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<tr>
<td>Clearance (L/min)</td>
<td>5.66 ± 8.80</td>
<td>5.49 ± 11.2</td>
<td>0.91 (0.62-1.34)</td>
<td>2.82 ± 1.39</td>
<td>1.01 (0.58-1.77)</td>
<td>8.97 ± 15.7</td>
<td>1.66 (1.36-2.02)</td>
</tr>
<tr>
<td>ClFormation (ml/min)</td>
<td>192 ± 347</td>
<td>279 ± 590</td>
<td>1.80 (1.08-3.00)</td>
<td>174 ± 149</td>
<td>2.40 (1.13-5.09)</td>
<td>619 ± 1310</td>
<td>3.98 (2.33-6.80)</td>
</tr>
<tr>
<td>SS-OH-BUP/S-BUP UR</td>
<td>5.7 ± 5.3</td>
<td>9.0 ± 7.5</td>
<td>1.77 (1.18-1.84)</td>
<td>17.7 ± 16.1</td>
<td>3.30 (1.97-5.53)</td>
<td>34.4 ± 34.3</td>
<td>4.84 (2.88-8.12)</td>
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<tr>
<td><strong>CYP2C9</strong> (Tolbutamide)</td>
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<tr>
<td>n= CON 16, NFV 7, RTV 8, RIF 15</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
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<tr>
<td>AUC 0-∞ (hr*ug/ml)</td>
<td>596 ± 203</td>
<td>455 ± 229</td>
<td>0.68 (0.54-0.85)</td>
<td>295 ± 106</td>
<td>0.50 (0.43-0.58)</td>
<td>216 ± 101</td>
<td>0.34 (0.28-0.42)</td>
</tr>
<tr>
<td>Clearance (ml/min)</td>
<td>15.7 ± 6.0</td>
<td>21.6 ± 8.3</td>
<td>1.47 (1.18-1.84)</td>
<td>33.2 ± 16.7</td>
<td>2.00 (1.73-2.31)</td>
<td>48.3 ± 25.4</td>
<td>2.94 (2.39-3.62)</td>
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<tr>
<td>ClFormation (ml/min)</td>
<td>6.92 ± 3.59</td>
<td>9.80 ± 3.86</td>
<td>1.34 (1.13-1.58)</td>
<td>13.4 ± 9.61</td>
<td>1.80 (1.56-2.08)</td>
<td>19.5 ± 11.1</td>
<td>2.55 (2.02-3.22)</td>
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<td><strong>CYP2D6</strong> (Dextromethorphan)</td>
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<tr>
<td>n= CON 15, NFV 7, RTV 7, RIF 14</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
<td>Ave ± SD</td>
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<tr>
<td>AUC 0-∞ (hr*ng/ml)</td>
<td>39.4 ± 56.0</td>
<td>46.3 ± 58.4</td>
<td>0.87 (0.41-1.82)</td>
<td>65.1 ± 67.0</td>
<td>4.54 (2.35-8.77)</td>
<td>10.3 ± 19.0</td>
<td>0.27 (0.17-0.42)</td>
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<tr>
<td>Clearance (ml/min)</td>
<td>97.1 ± 124</td>
<td>102 ± 157</td>
<td>1.16 (0.55-2.42)</td>
<td>19.8 ± 26.3</td>
<td>0.22 (0.11-0.43)</td>
<td>285 ± 292</td>
<td>3.73 (2.38-5.86)</td>
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<td>DOR/DEX AUC Ratio</td>
<td>3.78 ± 3.99</td>
<td>1.17 ± 1.30</td>
<td>0.64 (0.35-1.20)</td>
<td>0.94 ± 1.44</td>
<td>0.11 (0.04-0.31)</td>
<td>1.97 ± 2.36</td>
<td>0.50 (0.28-0.88)</td>
</tr>
<tr>
<td>CYP3A? (Dextromethorphan) N=CON 15, NFV 7, RTV 8, RIF 15</td>
<td>DOR/DEX UR Ratio</td>
<td>GMR – Geometric Mean Ratio (Treatment/Control)</td>
<td>Bold values are statistically significant (90%CI does not include 1.00)</td>
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<td>3MM/DEX UR Ratio</td>
<td>0.72 ± 0.75</td>
<td>0.76 ± 1.00</td>
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<td>DOR/DEX UR Ratio</td>
<td>306 ± 313</td>
<td>375 ± 650</td>
<td>0.13 (0.05-0.32)</td>
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<td>37.1 ± 34.1</td>
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<td>493 ± 407</td>
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<td>1.00 (0.44-2.26)</td>
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<td>0.46 (0.20-1.09)</td>
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<td>1.49 ± 1.35</td>
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Table 3: Parameters describing the in vitro induction of CYP1A2, 2B6 and 2C9 mRNA expression and activity in plated human hepatocytes by rifampin, ritonavir or nelfinavir

<table>
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<tr>
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<th>Rifampin mRNA</th>
<th>Activity</th>
<th>Ritonavir mRNA</th>
<th>Activity</th>
<th>Nelfinavir mRNA</th>
<th>Activity</th>
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<tbody>
<tr>
<td>CYP1A2</td>
<td>0.160 ± 0.034*</td>
<td>1.7 (36)</td>
<td>0.145 ± 0.032*</td>
<td>1.1 (22)</td>
<td>0.134 ± 0.034*</td>
<td>1.6 (30)</td>
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<tr>
<td></td>
<td>5.4 (107)</td>
<td></td>
<td>1.3 (111)</td>
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<td>5.4 (90)</td>
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<td>CYP2B6</td>
<td>10.9 (43)</td>
<td>9.4 (31)</td>
<td>7.0 (16)</td>
<td>3.7 (29)</td>
<td>4.3 (45)</td>
<td>3.0 (33)</td>
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<tr>
<td></td>
<td>11.1 (94)</td>
<td></td>
<td>8.0 (41)</td>
<td>5.5 (87)</td>
<td>11.0 (102)</td>
<td>7.9 (84)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.165 ± 0.015*</td>
<td>3.0 (34)</td>
<td>2.9 (52)</td>
<td>1.4 (35)</td>
<td>2.0 (28)</td>
<td>2.8 (43)</td>
</tr>
<tr>
<td></td>
<td>2.7 (133)</td>
<td></td>
<td>7.5 (135)</td>
<td>1.9 (157)</td>
<td>1.0 (153)</td>
<td>6.4 (119)</td>
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* Estimates of EC₅₀ were above the highest tested concentration (25μM), therefore the slope of the line with intercept forced to 0 was used for extrapolation purposes. Reported value is mean slope ± SD

Estimated value (% CV of the estimate) in n=4 pooled hepatocyte lots
**Figure 1**

**Study 1**
Staggered

Study day 1

Arm 1

Cocktail A

Cocktail B

RTV or RIF Treatment

Washout

RTV or RIF Treatment

Arm 2

Cocktail A

Cocktail B

NFV or RIF Treatment

Washout

NFV or RIF Treatment

**Study 2**
Simultaneous

Study day 1

Arm 1

Cocktail A

Cocktail A

RTV, NFV or RIF Treatment

Washout

RTV, NFV or RIF Treatment

Arm 2

Cocktail A

Cocktail A

NFV or RIF Treatment

Washout

NFV or RIF Treatment
Figure 2:

A  Paraxanthine Cl\textsubscript{form}  

B  4-OH BUP Cl\textsubscript{form}  

C  4-OH TOLB Cl\textsubscript{form}  

D  DOR/DEX AUC Ratio  

E  DOR/DEX UR  

F  3MM/DEX UR
Figure 3:
Figure 4:

A

CYP1A2 Fold Change (Treatment/Control)

Control CYP1A2 Activity (Paraxanthine Cl\text{form}, ml/min)

B

CYP2B6 Fold Change (Treatment/Control)

Control CYP2B6 Activity (4-OH BUP Cl\text{form}, ml/min)

C

CYP2C9 Fold Change (Treatment/Control)

Control CYP2C9 Activity (4-OH TOLB Cl\text{form}, ml/min)
Figure 5:

A

**Prediction Using In Vitro mRNA Expression**

- **Fold Change in In Vivo CYP Activity**
- **Observed**
- **Predicted with scaling**
- **Predicted without scaling**

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<th>RIF</th>
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B

**Prediction Using In Vitro Activity**

- **Fold Change in In Vivo CYP Activity**
- **Observed**
- **Predicted with scaling**
- **Predicted without scaling**

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C

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