Title Page

MECHANISM-BASED INACTIVATION OF CYTOCHROME P450 3A (CYP3A) BY HIV PROTEASE INHIBITORS

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List of non-standard abbreviations: CYP, cytochrome P450; b5, cytochrome b5; AUC, area under the plasma concentration time curve; ki, reversible inhibition constant; kdeg, rate constant for enzyme degradation; k\text{inact}, the maximal rate constant for inactivation; K\text{I}, inactivator
concentrations that reduces half the maximal rate of inactivation; MIC, metabolic intermediate complex; P-gp, P-glycoprotein.

e) Absorption, Distribution, Metabolism & Excretion
Abstract

HIV protease inhibitors (PI's) are inhibitors of CYP3A enzymes but the mechanism is poorly defined. In this study, time- and concentration-dependent decreases in activity as defined by $k_{\text{inact}}$ (maximum rate of inactivation) and $K_i$ (inhibitor concentration that gives 50% maximal inactivation) of CYP3A by amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir were quantified using testosterone 6β-hydroxylation as a marker for CYP3A activity with recombinant CYP3A4(+b5), recombinant CYP3A5, and pooled human liver microsomes (HLM’s). All the PI’s, except indinavir, displayed inactivation with CYP3A4(+b5) and HLM’s. Ritonavir was the most potent ($K_i = 0.10$ and $0.17 \mu M$) and demonstrated high $k_{\text{inact}}$ values (0.32 and 0.40 min$^{-1}$) with both CYP3A4(+b5) and HLM’s. Ritonavir was not significantly depleted by high-affinity binding with CYP3A4(+b5) and confirmed that estimation of reversible inhibition was confounded with irreversible inhibition. For CYP3A5, nelfinavir exhibited the highest $k_{\text{inact}}$ (0.47 min$^{-1}$) but ritonavir was the most potent ($K_i = 0.12 \mu M$). Saquinavir and indinavir did not show time- and concentration-dependent decreases in activity with CYP3A5. Spectrophototometrically determined metabolic intermediate complex formation was observed for all of the PI’s with CYP3A4(+b5), except for lopinavir and saquinavir. The addition of nucleophilic and free aldehyde trapping agents, free iron and reactive oxygen species scavengers did not prevent inactivation of CYP3A4(+b5) by ritonavir, amprenavir or nelfinavir, but glutathione decreased the inactivation by saquinavir (17%) and catalase decreased the inactivation by lopinavir (39%). In conclusion, all the PI’s exhibited mechanism-based inactivation and predictions of the extent and time-course of drug interactions with PI’s could be underestimated if based solely on reversible inhibition.
Introduction

A major advance in the anti-retroviral therapy has been the introduction of the HIV protease inhibitors. These HIV protease inhibitors, when administered in high doses and in combination with other antivirals, dramatically reduce HIV levels in the blood (Hammer et al., 2002). Highly active antiretroviral therapy (HAART) generally involves combination therapy and consists typically of three or four drugs for the treatment of disease (Yeni et al., 2002). Despite the effectiveness of HAART, HIV-infected individuals experience a broad array of comorbidities as a result of an impaired immune response and develop metabolic disturbances related to therapy and HIV infection (Fichtenbaum and Gerber, 2002). Consequently, pharmacokinetic interactions are common when HIV protease inhibitors are administered in combination with other AIDS related therapeutic agents, such as hypolipidemics, macrolide antibiotics, calcium channel blockers, and hormones. For example, in healthy volunteers, simvastatin and atorvastatin exposure increased 3059% and 79%, respectively, after 15 days of combination ritonavir/saquinavir treatment (Fichtenbaum et al., 2002). In a group of HIV positive men, the exposure of sildenafil increased approximately 4-fold, 11-fold, and 3-fold after multiple doses of indinavir (Merry et al., 1999), ritonavir (Muirhead et al., 2000), and saquinavir (Muirhead et al., 2000), respectively. In addition, standard HAART employs intentional drug interactions whereby ritonavir is used to boost concentrations of lopinavir, indinavir, saquinavir, and amprenavir (Yeni et al., 2002). This pharmacoenhancement reflects the fact that the currently available HIV protease inhibitors are both substrates and inhibitors of CYP3A and, thus, compete for the same metabolic enzyme at both hepatic and intestinal sites (Eagling et al., 1997; Lillibridge et al., 1998; Kumar et al., 1999; Singh et al.,1996). Despite the fact that these HIV protease inhibitors demonstrate highly potent competitive inhibition, predictions of in vivo...
drug-drug interactions are sometimes underestimated using *in vitro* $K_i$ values, and the inhibitory effect can persist after discontinuation and protease inhibitor concentrations are below detectable levels (Washington et al., 2003).

These observations suggest that additional mechanisms of inhibition may occur *in vivo* other than competitive inhibition. Using spectrophotometry, Lin et al. demonstrated that an analogue of indinavir converted approximately 40 to 60% of the cytochrome P450 in human liver microsomes to a metabolic intermediate complex (Lin et al., 1995). Thus, HIV protease inhibitors have the potential to inactivate CYP3A enzymes by the formation of a metabolic intermediate complex (MIC). Compounds that inactivate CYP via mechanism-based inactivation are typically substrates for the target enzyme and are converted to an intermediate that inactivates the enzyme, thus, removing the enzyme from the pool of CYP (Silverman, 1995). Mechanism-based inactivation is distinguished from reversible inhibition in that decreased enzyme activity persists after the compound has been cleared from the body (Kanamitsu et al., 2000).

This report quantifies the capabilities of several HIV protease inhibitors to act as mechanism-based inactivators at clinically relevant steady-state concentrations through the formation of a metabolic intermediate complex.
Methods

Chemicals. Amprenavir, ritonavir, nelfinavir mesylate, saquinavir, lopinavir and indinavir were donated by GlaxoSmithKline (Research Triangle Park, NC), Abbott Laboratories (Chicago, Ill), Agouron Pharmaceuticals, Inc. (New York, NY), Hoffmann-La Roche Inc (Nutley, NJ), Abbott Laboratories (Chicago, Ill), and Merck & Co., Inc (Whitehouse Station, NJ), respectively. Testosterone, 6β-hydroxytestosterone, temazepam, catalase, glutathione, defereroxamine, N-acetylcysteine, superoxide dismutase, methoxylamine, and semicarbazide were purchased from Sigma-Aldrich (St. Louis, MO). NADPH was purchased from Boehringer Mannheim (Indianapolis, IN). All other reagents were of high-performance liquid chromatography (HPLC) grade.

cDNA-Expressed Human CYP3A4(+b3), CYP3A5, and Human Liver Microsomes (HLM). Insect cell microsomes containing baculovirus cDNA-expressed CYP3A4(+b3) and CYP3A5 were purchased from Gentest Corp. (Woburn, MA). Pooled adult human liver microsomes (HLM) were prepared from five human liver tissues obtained at surgery in accordance with protocols approved by the Institutional Review Board of IUPUI/Clarian at Indianapolis, Indianapolis, IN. The handling, preparation, and storage of microsomes have been previously described (Gorski et al., 1994). The total protein concentration of the pooled HLM’s was 20 mg/mL and cytochrome P450 concentration was 0.3 nmol/mg protein (Lowery et al., 1951 and Omura and Sato, 1964).

Enzyme Inactivation and High-Affinity Binding. Testosterone 6β-hydroxylase activity was determined to quantify time- and concentration-dependent loss of CYP3A activity of microsomes in the presence of protease inhibitors. HIV protease inhibitors in methanol were added to sample tubes and evaporated to dryness prior to addition of HLM (50 µg of total
protein), CYP3A4(+b5) (20 pmol), or CYP3A5 (50 pmol). Microsomal buffer (100 mM phosphate buffer containing 5 mM MgCl₂, pH 7.4) was added to bring the total volume to 45 µL. After equilibration at 37°C for 1 min, inactivation was initiated by the addition of 5 µL of NADPH (10mM). At the end of the pre-incubation period, 950 µL of a mixture that consisted of microsomal buffer, testosterone (final concentration 200 µM), and NADPH (final concentration 10 mM) was added to the sample tubes. After a 2-min incubation at 37°C, testosterone metabolism was terminated by the addition of 1 mL ice-cold acetonitrile. Temazepam (800 ng) was then added to each test tube as an internal standard and each tube was centrifuged at 2500 rpm for 5 min. The supernatant was removed, placed in a screw cap glass tube, combined with 6 mL of ethyl acetate, and mixed for 30 minutes. After mixing, each tube was centrifuged at 2500 rpm for 5 min and the organic phase was removed, placed in a glass culture tube, and evaporated to dryness. The residues were re-suspended in 150 µL of the HPLC mobile phase [20 mM phosphate buffer containing 0.1% (v/v) triethylamine adjusted to pH 6.3 with orthophosphoric acid/MeOH (40:60, v/v)]. The concentration of 6β-hydroxytestosterone for duplicate samples was determined by HPLC using a 5 µm C-18 column (Luna; Phenomenex, Torrace, CA) that was eluted at a flow rate of 1 mL/min and ultraviolet detection at a wavelength of 254 nm, as previously described (Zhao et al., 2002).

To test for depletion of unbound inhibitor by high-affinity binding to CYP3A4(+b5), testosterone (final concentration 200 µM) and ritonavir (0.05, 0.10, 0.20, 0.50 and 1 µM) in methanol were added to sample tubes and evaporated to dryness. CYP3A4(+b5) (10, 20, 50, 100, and 200 pmol) and microsomal buffer were added to bring the total volume to 900 µL. After equilibration at 37°C for 1 min, 100 µL of a 10 mM NADPH solution was added to initiate metabolism. After a 2-min incubation at 37°C, the testosterone metabolism was terminated by
the addition of 1mL ice-cold acetonitrile. The addition of internal standard and extraction of 6β-
hydroxytestosterone were performed as described above. The organic solvents were evaporated
to dryness and residues were re-suspended in 150 μl of the HPLC mobile phase [Phosphate
buffer (pH 6.3)/MeOH (40:60)] and 6β-hydroxytestosterone formation was determined for
duplicate samples, as described above.

MIC Formation. HLM, CYP3A4(+b5), or CYP3A5 was used to characterize MIC formation
associated with the metabolism of the protease inhibitors. MIC formation was observed with
dual-beam spectroscopy (Uvikon 933 double-beam UV/VIS spectrophotometer; Research
Instruments International, San, Diego, CA) by scanning from 380 to 500 nm at 0, 5, 10, 15, 20,
25, 30, 40, 50 and 60 min to monitor the formation of an absorbance maximum at ~455 nm.
Each cuvette contained 1 mg total protein of HLM’s, 200 pmol of CYP3A4(+b5), or 500 pmol of
CYP3A5 and 1mM NADPH. The test sample cuvette contained 10 μM protease inhibitor in
vehicle and the reference cuvette contained vehicle without protease inhibitor. Quantities of
protein were chosen to allow detection of MIC formation. All MIC formation experiments were
maintained at 37°C with a final volume of 1 mL for each cuvette. The extent of MIC formation
was quantified based on a previous reported extinction coefficient (455-490 nm) value of 65 M⁻¹
(Pershing and Franklin, 1982).

Effects of Nucleophilic and Free Aldehyde Trapping Agents, Free Iron and Reactive
Oxygen Species Scavengers. The effects of nucleophilic and free aldehyde trapping agents, free
iron and reactive oxygen species scavengers on the inactivation of CYP3A4(+b5) were
determined, as previously described (Koenigs et al., 1997). These effects were examined by co-
incubation of amprenavir (2 μM), ritonavir (0.50 μM), nelfinavir (30 μM), saquinavir (5 μM) or
lopinavir (30 μM) in the presence of NADPH, and nucleophilic and free aldehyde trapping
agents, free iron and reactive oxygen species scavengers with CYP3A4(+b5), in the pre-incubation step, as described above. The concentration of each protease inhibitor and pre-incubation time with CYP3A4(+b5) and NADPH was chosen to produce the maximum inactivation that followed pseudo-first order kinetics as obtained from the enzyme inactivation experiments. Activities in the presence of HIV protease inhibitors and nucleophilic and free aldehyde trapping agents, free iron and reactive oxygen species scavengers were expressed as percentage of control activity (HIV protease inhibitors alone).

**Analysis of Enzyme Inactivation Kinetics.** Kinetic parameters for enzyme inactivation were obtained by nonlinear regression without weighting using WinNonlin Professional Version 3.1 (Pharsight; Mountain View, CA). Appropriateness of fit was determined by visual inspection of the lines of best-fit and precision of the parameter estimates. Mechanism-based inactivation parameters were obtained from the pseudo-first order decline in percent remaining CYP3A activity after pre-incubation with protease inhibitors. Preliminary experiments determined inhibitor concentrations and pre-incubation times that followed pseudo-first order kinetics by plotting the natural logarithm of the percentage of the remaining CYP3A activity for each concentration of protease inhibitor against the pre-incubation time. Only data that were determined to follow pseudo-first order kinetics, as assessed by a linear decline in activity over time relative to initial enzyme activity, were used. For each inhibitor concentration, the percentage of remaining testosterone 6β-hydroxylase activity vs. pre-incubation time was fit to estimate the observed inactivation rate constant (k_{obs}), using the following equation:

\[
\% \text{ of remaining enzyme activity} = 100 \times e^{-\left(k_{\text{obs}} \times t\right)}
\]

(1)

Prior to addition of inhibitor, percent activity of enzyme is 100%, and k_{obs} is related to the inhibitor concentration (I) during the pre-incubation according to the following relationship:
where $k_{\text{inact}}$ is the maximal inactivation rate constant, $K_I$ is the inhibitor concentration at 50% of $k_{\text{inact}}$. Equations 1 and 2 assume that loss of enzyme is solely due to inactivation by the inhibitor and there is negligible change in I during the incubation period. The mechanism-based inactivation constants, $k_{\text{inact}}$ and $K_I$, were estimated by simultaneously fitting the time dependent loss of testosterone 6β-hydroxylase activity at all inhibitor concentrations for a given protease inhibitor substituting $k_{\text{obs}}$ in equation 1 for $(k_{\text{inact}} \times I)/(K_I + I)$ from equation 2. Preliminary experiments identified inhibitor concentrations that resulted in less than ~60% of control initial activity. This range of inhibitor concentrations allowed the inactivation rate constants to be accurately estimated despite possible substantial reversible inhibition by some HIV protease inhibitors (ritonavir and indinavir). There were at least three pre-incubation time points used at a given inhibitor concentration and the appropriate range of pre-incubation times were determined from preliminary experiments.

**Kinetics for High-Affinity Binding.** In order to address the potential for depletion of unbound inhibitor during pre-incubations due to high affinity binding, the effect of CYP concentration on the ritonavir concentration dependent inhibition of CYP3A was evaluated with CYP3A4(+b5) graphically and by linear regression. If unbound inhibitor was not depleted then the potency of ritonavir as a reversible inhibitor would be independent of protein concentration. The percent inhibition was determined as $[(1-v_i/v_o) \times 100]$, where $v_i$ is the velocity of 6β-hydroxytestosterone formation in the presence of inhibitor and $v_o$ is the velocity of 6β-hydroxytestosterone formation in the absence of inhibitor. Multiple linear regression analysis using S-PLUS 2000 (Insightful; Seattle, WA) was performed to assess the significance ($p<0.05$) of CYP3A4(+b5) concentration...
on the ritonavir concentration dependent inhibition of 6β-hydroxytestosterone formation, as previously described using log-transformed data (Gibbs et al., 1999). The bound fraction of inhibitor ($f_b$) forming an enzyme-inhibitor complex $[EI]$ was determined as follows:

$$[EI] = \frac{1 - v_i}{v_o} \times E_i$$

where $E_i$ is the total enzyme concentration and $I_i$ is the total inhibitor concentration. The apparent $k_i$ (equilibrium inhibition constant for reversible inhibition) of ritonavir was estimated by simultaneously fitting the inhibition data from 10 and 20 pmol CYP3A4(+b5) in the presence of ritonavir to the following non-competitive inhibition model (Segel, 1975):

$$\frac{I}{1 - \frac{v_i}{v_o}} = K_i \times \frac{v_o}{v_i} + [E]$$

where $I$ is the concentration of unbound inhibitor estimated from the difference of $I_i$ and $[EI]$, and $E$ is the unbound concentration of enzyme estimated from the difference of $E_i$ and $[EI]$. 

**Quantitative Predictions of Drug Interactions In Vivo based on Mechanism-based Inactivation.** Administration of a mechanism-based inactivator reduces the amount of enzyme available to metabolize a co-administered drug that relies on that enzyme for elimination. Thus, predictions of the extent of drug-drug interactions become important in assessing the combined administration of these two drugs. The theoretical background on the prediction of in vivo CYP3A inhibition using mechanism-based inactivation has been previously described (Mayhew et al., 2000; Wang et al., 2004).

Wang et al. have demonstrated that the extent of a drug-drug interaction can be described by the ratio of oral drug exposure in the inactivated state ($AUC_{po}^-$) over drug exposure in the absence of inhibitor ($AUC_{po}$). Assuming the unbound fraction of substrate is not altered in the
presence of inhibitor, the extent of interaction can be predicted as follows based on hepatic intrinsic clearance and intestinal wall availability:

$$\frac{AUC'_{po}}{AUC_{po}} = \frac{F'_G}{F_G} \times \frac{1}{1 + \frac{k_{inact} \times I_u}{I_k + I_u} + (1 - f_m) + \frac{I_k}{k_{deg} x (K_i + I_u)}}$$

(6)

where $K_I$ is the concentration at 50% of $k_{inact}$, $I_u$ is the unbound, steady-state inhibitor concentration, $f_m$ represents the fraction of the total hepatic elimination that is due to the CYP3A pathway in the absence of inhibitor, $F_G$ is the intestinal wall bioavailability in the absence of inhibitor, $F'_G$ is the intestinal wall bioavailability in the presence of inhibitor, and $k_{deg}$ is the endogenous degradation rate constant of the enzyme. After multiple doses of a HIV protease inhibitor, it was assumed that maximal inhibition of intestinal wall CYP3A occurred resulting in complete bioavailability ($F'_G = 1$), and $k_{deg}$ was assigned values of 0.00128 and 0.00026 min$^{-1}$ based on rat CYP3A and human CYP3A4 in CaCO-2 cells (Correia, 1991; Malhotra et al., 2001).
Results

Enzyme Inactivation. Co-incubation of a protease inhibitor (amprenavir, lopinavir, nelfinavir, or saquinavir) and testosterone for 2 min in the presence of NADPH with HLM, CYP3A4(+b5) or CYP3A5 did not demonstrate any appreciable inhibition of initial activity for the concentrations used to estimate the inactivation rate constants. However, significant loss of initial activity (~60%) due to inhibition of CYP3A activity with HLM, CYP3A4(+b5), and CYP3A5 was observed in the presence of ritonavir or indinavir at relatively low concentrations (Figure 1). In the co-incubation of ritonavir, the IC$_{50}$ (concentration that produces 50% inhibition) was approximately 0.05 µM for all tissues (Figure 1). In the co-incubation of indinavir (Figure 1), the IC$_{50}$, ~ 1 µM with HLM and CYP3A5, was relatively close to the reported K$_{i}$ values that range from 0.2 to 0.9 µM (Levy et al., 2000), and the IC$_{50}$ with CYP3A4(+b5) was slightly higher (~1.5 µM). Therefore, concentrations of ritonavir and indinavir, which upon a 20-fold dilution maintained at least 60% initial activity relative to control prior to pre-incubation, were used to estimate the inactivation rate constants.

The loss of CYP3A activity as a function of protease inhibitor concentration and time was determined with HLM, CYP3A4(+b5), and CYP3A5. The initial concentration of the HIV protease inhibitors ranged from 0.03 to 50 µM and pre-incubations times ranged from 0 to 10 minutes. The rates of inactivation showed a clear dependence on inactivator concentration and pre-incubation time. Figure 2 (A, B, and C) illustrate the decline in percent remaining of testosterone 6β-hydroxylase activity versus pre-incubation time with HLM, CYP3A4(+b5) and CYP3A5 in the presence of ritonavir. In addition, the expected hyperbolic relationship between $k_{obs}$ values and ritonavir concentration is presented in Figure 2 (D, E, and F) along with the line of best fit from the simultaneous fitting of all data to Equations 1 and 2. Relatively low
concentrations of ritonavir were required to produce the maximal rate of inactivation. Similar decreases in percent remaining testosterone 6β-hydroxylation activity versus pre-incubation time and hyperbolic relationships between k_{obs} values and protease inhibitor concentration were observed for amprenavir, lopinavir, and nelfinavir with HLM, CYP3A4(+b5), and CYP3A5. Saquinavir demonstrated time- and concentration-dependent loss of activity with HLM and CYP3A4(+b5) but not with CYP3A5. The corresponding estimates of k_{inact} and K_{I} are presented in Table 1. For indinavir, the rate of 6β-hydroxytestosterone formation by HLM, CYP3A4(+b5), and CYP3A5 without prior exposure to indinavir, i.e. no pre-incubation, at concentrations greater than 0.05, 1.25 and 0.05 μM, respectively, was reduced by at least 60% of initial activity (Figure 1). In addition, at indinavir concentrations that maintain at least 60% of initial activity, concentration and time-dependent loss of 6β-hydroxytestosterone formation with HLM, CYP3A4(+b5), and CYP3A5 was not observed. Thus, indinavir was a potent reversible inhibitor and due to the limitations of the experimental conditions, the estimation of k_{inact} and K_{I} for indinavir was precluded.

Among the HIV protease inhibitors examined in the current study, ritonavir was the most potent (lowest K_{I}) and most efficient inactivator (highest k_{inact}/K_{I}) with HLM, although, efficient inactivation was also exhibited by amprenavir, saquinavir, nelfinavir, and lopinavir (Table 1). A similar pattern of inactivation was observed with CYP3A4(+b5) (Table 1) and there was a high correlation between inactivation efficiency values obtained from HLM’s and CYP3A4(+b5) (r^2=0.94; Figure 3). On average the inactivation efficiency of HLM's was approximately 60% of that observed with CYP3A4(+b5). When compared to CYP3A4(+b5), there was less efficient inactivation of CYP3A5 by ritonavir, amprenavir and lopinavir but more efficient inactivation by...
nelfinavir (Table 1). No correlation between CYP3A5 and HLM or CYP3A4(+b5) was observed.

**MIC Formation.** Spectrophotometric peak absorbance difference at ~455 nm was examined to identify MIC formation of HIV protease inhibitors with HLM, CYP3A4(+b5), or CYP3A5 and NADPH. The observed MIC formation with CYP3A4(+b5) at 10 µM of protease inhibitor increased with time and was close to 95% of the total CYP3A in the sample for amprenavir followed by ritonavir (~62%), nelfinavir (~49%), and indinavir (~47%) after 60 minutes of incubation. As the concentration of HIV protease inhibitors were ~ 20-fold higher than the Kᵢ values obtained during the inactivation experiments, the percent of MIC formation relative to total CYP3A in the sample correlated closely with the kᵢnact values for amprenavir, ritonavir and nelfinavir. Thus, amprenavir demonstrated the highest kᵢnact value and the highest percent of MIC formation. MIC formation with CYP3A5 was only detectable with amprenavir, accounting for ~23% of the total CYP3A in the sample. The HIV protease inhibitors did not form detectable MIC with HLM, which may be due to low CYP3A content or limit of detection for MIC. The limit of quantification of MIC formation is 23pmols of enzyme using a 1 mL incubation volume with a 1 cm cuvette. This value corresponds to an absorbance difference of 0.0015 using an extinction coefficient of 65 M⁻¹. The time courses of MIC formation for amprenavir and indinavir with CYP3A4(+b5) are illustrated in Figure 4. In the absence of NADPH, no characteristic peak at ~455nm was observed. These results indicate that for several HIV protease inhibitors inactivation occurred through MIC formation. In addition, these results demonstrate that potent reversible inhibitors such as indinavir may form MIC at concentrations much higher than reported Kᵢ values even though concentration and time-dependent experiments could not detect mechanism-based inactivation.
Effect of Nucleophilic and Free Aldehyde Trapping Agents, Free Iron and Reactive Oxygen Species Scavengers. Agents were tested to determine if inactivation by HIV protease inhibitors was confined to the active site of the enzyme or through liberation of a reactive metabolite. In assessing the impact of nucleophilic and free aldehyde trapping agents, free iron and reactive oxygen species scavengers on the capacity to inhibit the ability of HIV protease inhibitors to inactivate CYP3A4(+b5), the concentrations of amprenavir, ritonavir, nelfinavir, saquinavir and lopinavir were chosen from the previous experiments so the maximum inactivation that followed pseudo-first order kinetics was attained. Nucleophilic and free aldehyde trapping agents, free iron and reactive oxygen species scavengers failed to protect inactivation (defined as less than a 10% change) of CYP3A4(+b5) by ritonavir, nelfinavir, and amprenavir. Saquinavir inactivation of CYP3A4(+b5) decreased by 17% with the addition of glutathione, and lopinavir inactivation of CYP3A4(+b5) decreased by 39% with the addition of catalase in the pre-incubation mixture.

High-Affinity Binding. Ritonavir was characterized by a low IC\textsubscript{50} value (~0.05 µM, vide supra), and therefore, the potential for high-affinity binding of ritonavir to CYP3A4(+b5), as this would bias estimates of K\textsubscript{i}, was investigated. The inhibition of 6β-hydroxytestosterone formation increased with increasing concentrations of ritonavir, and the inhibitory effect, as defined by (v\textsubscript{i}/v\textsubscript{o}), decreased with increasing concentrations of CYP3A4(+b5) (Figure 5A). In the absence of ritonavir, the formation of 6β-hydroxytestosterone was approximately linear from 10 to 50 pmol of CYP3A4(+b5) (Figure 5B). Whereas, above 50 pmol of enzyme, the formation of 6β-hydroxytestosterone was non-linear, which suggests a metabolic depletion of testosterone (data not shown).

Multiple linear regression of the ritonavir concentration dependent inhibition of 6β-hydroxytestosterone formation indicated that amounts of 50 to 200 pmol CYP3A4(+b5) were
significant determinants of inhibition ($p<0.05$). In contrast, enzyme amounts of 10 and 20 pmol CYP3A4(+b5) were not significant determinants of inhibition ($p<0.70$). Thus, inhibition of 6β-hydroxytestosterone formation by ritonavir with 10 to 20 pmol CYP3A4(+b5) was not appreciably influenced by CYP concentrations and the calculation of high-affinity binding of ritonavir could be assessed for 20 pmol CYP3A4(+b5) with minimal non-specific binding. The impact of the fraction of ritonavir bound (i.e. ~7% with 20 pmol CYP3A4(+b5)) did not significantly affect the $K_I$ value obtained for the mechanism-based inactivation experiments, and thus, did not violate the assumption that unbound inhibitor was in excess to total enzyme concentration.

By visual inspection, estimates of $IC_{50}$ for 10 to 20 pmol CYP3A4(+b5) were approximately 0.06 to 0.08 $\mu$M, respectively (Figure 5A). Using equation 5, the $K_I$ of ritonavir, calculated from data obtained for 10 and 20 pmol CYP3A4(+b5), was 0.053 $\mu$M assuming only non-competitive reversible inhibition (Levy et al., 2000). To confirm that the estimates of reversible inhibition were not influenced by mechanism-based inactivation, linear regression of 6β-hydroxytestosterone formation versus CYP3A4(+b5) from 10 to 50 pmol of CYP3A4(+b5) for each ritonavir concentration was performed (Figure 5B). As the slopes change and do not intersect the origin with increased ritonavir and CYP3A4(+b5) concentrations, these results indicate that substantial irreversible inhibition is occurring at ritonavir concentrations above 0.05 $\mu$M (Segel, 1975). Therefore, estimation of reversible inhibition by ritonavir with CYP3A4(+b5) was confounded with mechanism-based inactivation inhibition, and accurate estimates of reversible inhibition may not be possible.

Quantitative Predictions of Drug Interactions In Vivo based on Mechanism-based Inactivation. Quantitative predictions of drug interactions in vivo are based on the unbound
steady-state concentration of the inactivators and the contribution of CYP3A involved in the metabolism of the substrate. Several literature sources used for the predictions did not provide unbound steady-state concentration of ritonavir and these values were estimated from the mean concentration-time profiles. Based on inactivation parameters estimated with HLM and pharmacokinetic properties of each HIV protease inhibitor (de Maat et al., 2003), the predicted increase of $\frac{\text{AUC'}_{\text{po}}}{\text{AUC}_{\text{po}}}$ (Equation. 6) of several co-administered CYP3A substrates in the presence of saquinavir and ritonavir is presented in Table 2.
Discussion

Current HAART is aimed at suppression of viral replication through the use of multiple agents such as protease inhibitors. Yet, this aim is complicated by a variety of drug interactions with co-administration of protease inhibitors. These interactions provide a challenge to optimized therapy. Clinical studies have shown that HIV protease inhibitors inhibit the metabolism of CYP3A substrates, such as rifabutin, clarithromycin, ketoconazole, midazolam, simvastatin, and sildenafil (Hsu et al., 1998; Hsyu et al., 2001; Muirhead et al., 2000; Palkama et al., 1999). In previous attempts to predict the effectiveness of these HIV protease inhibitors as inhibitors of CYP3A in vivo, equilibrium inhibition constants (K_i) for reversible inhibition of CYP3A were determined (Levy et al., 2000). However, based on the ratio of unbound steady-state plasma concentrations (de Maat et al., 2003) to K_i, conventional reversible inhibition models typically fail to predict inhibition of the metabolism of co-administered CYP3A substrates. In addition, inhibition persists after discontinuation of HIV protease inhibitors when plasma concentrations are below detectable levels (Washington et al., 2003), and a structural analogue to the HIV protease inhibitor indinavir demonstrates that cytochrome P450 was inactivated by the formation of MIC (Lin et al., 1995). These factors suggest that in vivo, mechanism-based inactivation may be occurring in addition to reversible inhibition.

In the present study, time- and concentration-dependent decrease of microsomal CYP3A activity with protease inhibitors, except indinavir, provide evidence that inhibition can occur by mechanism-based inactivation (Silverman, 1995). In addition, when compared with other inactivators, such as diltiazem, verapamil and tamoxifen, the studied HIV protease inhibitors are efficient inactivators in vitro as reflected in the ratios of k_inac/K_I (Jones et al., 1999; Wang et al., 2004; Zhao et al., 2002). For example, the k_inac/K_I ratio for amprenavir is 40 and 11 times
greater than that for R-verapamil and S-verapamil, respectively. These results indicate that these HIV protease inhibitors are substantially more efficient inactivators than known mechanism-based inactivators that have been reported to cause significant drug-drug interactions.

In some cases, inhibition caused by a mechanism-based inactivator occurs through the formation of a metabolic intermediate complex (MIC). Ultimately, the inhibitor is catalytically oxidized by the enzyme into the actual inactivating species that coordinates tightly and/or chelates to the prosthetic heme of the enzyme resulting in a more stable (ferrous) state of iron that can be measured using spectrophotometric methods to detect an absorbance maximum of 445-455 nm (Silverman, 1995). It has been shown that primary amines produce metabolic intermediate complex (MIC) formation, although secondary and tertiary amines are appropriate precursors (Franklin, 1977; Bensoussan et al., 1995). In this study, four of the six HIV protease inhibitors, ritonavir, amprenavir, indinavir and nelfinavir, formed measurable MIC formation with CYP3A4(+b3). The structural moieties that form a MIC may be primary amines present in amprenavir or metabolites from amprenavir and ritonavir metabolism (Denissen et al., 1997; Singh et al., 1996). Metabolites of indinavir, lopinavir, saquinavir and limited data for circulating metabolites of nelfinavir (Chiba et al., 1997; Eagling et al., 2002; Kumar et al., 1999; Zhang et al., 2001) do not display primary, secondary or tertiary amines in any of the metabolized structures. However, indinavir and nelfinavir displayed MIC formation. Consequently, the structures for indinavir and nelfinavir that coordinate tightly with the heme cannot be identified. Thus, while data suggest that mechanism-based inactivation is occurring through MIC formation for ritonavir, amprenavir, indinavir and nelfinavir, the mechanism of inactivation for lopinavir and saquinavir cannot be confirmed through MIC formation.
Relatively few examples in literature of HIV protease inhibitors with good CYP3A substrates are available. However, quantitative prediction of drug interactions in vivo based on unbound steady-state plasma concentrations of saquinavir and ritonavir through the use of a mechanism-based inactivation model (Equation 6) demonstrated that several fold increases in AUC/\textsubscript{po}/AUC\textsubscript{po} were expected (Table 2). Accurate predictions of the extent of exposure increase in the presence of saquinavir and ritonavir were obtained as estimated values are relatively close to observed values for most substrates. In addition, based on the low, unbound plasma concentrations of nelfinavir (0.07 µM) relative to the reported range of K\textsubscript{i} values of 0.31 to 4.8, competitive inhibition predicts an increase of the AUC ratio by only 10-20% (de Maat et al., 2003; Levy et al. 2000). Whereas in vivo predictions based on mechanism-based inactivation with nelfinavir anticipate significant interactions such as that observed for simvastatin (5 to 7-fold) and terfenadine (9-fold) increases in AUC/\textsubscript{po}/AUC\textsubscript{po} (Hsyu et al., 2001; Lillibridge et al., 1998). Thus, these in vivo predictions and observed changes in AUC with HIV protease inhibitors support the use of the mechanism-based inactivation model over simple, competitive inhibition.

An important aspect of this predictive approach was to assume the inhibitor at the active site of the enzyme is equivalent to the unbound concentrations in plasma. But this assumption may be invalid as several HIV protease inhibitors have been shown to be substrates and inhibitors of P-gp (Alsenz et al., 1998; Kim et al., 1998; Srinivas et al., 1998; Washington et al., 1998). P-gp functions as an efflux pump that lowers intracellular drug concentrations up to 10-fold in human carcinoma cells (Schuetz et al., 1996). Therefore, the intracellular concentrations of HIV protease inhibitors may be lower than the unbound plasma concentrations. Secondly, the model assumes the inhibitor has no effect on the rate of CYP3A synthesis. However, nelfinavir,
Lopinavir and ritonavir have been reported to induce the rate of expression of CYP3A (de Maat et al., 2003). Since this induction was not included for the predictions, the higher enzyme concentration could decrease the inhibitory effect and the predicted AUC increase will be higher than actually observed. Additionally, unbound $K_I$ was assumed equivalent to $K_I$. As assessed in this study, high-affinity binding depleted a small fraction of ritonavir. As it was assumed the greatest impact for depletion would occur for ritonavir due to the low $K_I$ and depletion was negligible, these results would lead to indicate that it is unlikely depletion would occur for the other HIV protease inhibitors. Thus, the approximation that unbound $K_I$ is equivalent to $K_I$ appears appropriate. In addition, predictions were based on the assumption that the concentration of enzyme at steady-state in presence of inhibitor ($E^\prime_{ss}$) was achieved. If $E^\prime_{ss}$ was not achieved, this could explain additional differences noted between predicted and observed AUC increase. The dosing of ritonavir and saquinavir used for several of the predictions might not have been sufficiently long to produce this maximal level of enzyme inactivation. Thus, the predictions would tend to overestimate the extent of interaction. Finally, the predictions are affected by the enzyme degradation rate, $k_{deg}$, which was based on rat CYP3A or human CYP3A4 in CaCO-2 cells (Correia, 1991; Malhotra et al., 2001) as a value for the CYP3A4 degradation rate for humans is not available. Thus, these values are likely to be different from humans.

In conclusion, our study demonstrated that in vivo drug interactions with HIV protease inhibitors are most likely due to mechanism-based inhibition of CYP3A. Several of these HIV protease inhibitors demonstrated MIC formation indicating that a metabolic reactive intermediate complexes with the heme group at the active site of the enzyme. This inactivation of CYP3A is also consistent with the persistent effect observed after discontinuation of therapy. The
mechanism-based inactivation model predicted a more accurate increase in exposure of co-
administered CYP3A substrates than using conventional reversible inhibition. Understanding
the impact of inactivation of CYP3A by these HIV protease inhibitors could lead to better
predictions of potential drug-drug interactions.
References


Footnotes

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Legends for figures

Figure 1. Percentage of Control CYP3A activity in presence of ritonavir or indinavir with HLM (black bar), CYP3A4(+b₃) (light gray bar) and CYP3A5 (dark gray bar)

Initial testosterone 6β-hydroxylase activity without pre-incubation (time zero) in presence of inhibitors (ritonavir or indinavir) was determined and expressed as a percentage relative to control activity (absence of inhibitor). Each bar shown represents the mean from duplicate samples.

Figure 2. Time and concentration dependent inhibition of CYP3A activity by ritonavir with HLM (A), CYP3A4(+b₃) (B), and CYP3A5 (C) and hyperbolic plots of k_{obs} vs ritonavir concentration with HLM (D), CYP3A4(+b₃) (E), and CYP3A5 (F).

(A, B, C) Remaining testosterone 6β-hydroxylase activity in presence of ritonavir concentrations was determined and expressed as a percentage relative to control activity. The solid line represents the predicted % remaining activity versus time of pre-incubation for each ritonavir concentration from the simultaneous fit of all data. Each point represents the mean of duplicate samples. (D, E, F) The k_{obs} (●) value for the percent remaining testosterone 6β-hydroxylase activity, expressed as a percentage relative to control activity, for each ritonavir concentration was estimated utilizing Eq. 1, and plotted against inhibitor concentration. Each point represents the mean of duplicate samples. The solid line represents the predicted k_{obs} values using the estimates of the parameters from the simultaneous fits (Eq. 2).

Figure 3. Correlation of k_{inac}/K_{I} between HLM and CYP3A4(+b₃)

Ratios of k_{inac}/K_{I} obtained with HLM and CYP3A4(+b₃) were plotted for ritonavir (●), amprenavir (○), saquinavir (■), nelfinavir (□), and lopinavir (▲). Linear regression (–) for the correlation of ratios between HLM and CYP3A4(+b₃) was k_{inac}/K_{I} with HLM = 0.59 x k_{inac}/K_{I} with CYP3A4+(b₃).

Figure 4. Metabolic Intermediate Complex (MIC) Formation by (A) Amprenavir and (B) Indinavir with CYP3A4(+b₃)

The sample cuvette contained CYP3A4(+b₃), protease inhibitor in methanol, and NADPH, whereas the reference cuvette contained CYP3A4(+b₃), methanol, and NADPH, as described in Methods. MIC formation with CYP3A4(+b₅) was detected spectrophotometrically from the absorbance difference for the incubates with and without inhibitor at wavelengths between 400 and 500 nm. Absorbance was monitored 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes. The solid line represents the smoothed
fit of the absorbance from 5- to 60-min. For the purpose of clarity only a few selected lines are exhibited in this figure. The
peak absorbance difference was observed at ~455nm.

Figure 5. (A) v_i/v_o versus Ritonavir Concentration and (B) velocity of 6β-hydroxytestosterone
formation versus CYP3A4(+b5)

(A) The ratio of v_i/v_o after a 2-minute co-incubation of ritonavir and testosterone with CYP3A4(+b5) (pmol) were plotted against
ritonavir concentration. The 2-minute co-incubation was terminated by the addition of 1mL ice-cold acetonitrile as described
under Methods. Each point shown represents the mean from duplicate samples. (B) The velocity of 6β-hydroxytestosterone
formation after a 2-minute co-incubation of ritonavir and testosterone were plotted against CYP3A4(+b5) (10 - 50 pmol). The 2-
minute co-incubation was terminated by the addition of 1mL ice-cold acetonitrile as described under Methods. Each point shown
represents the mean from duplicate samples. Linear regression (-) for each ritonavir concentration (µM) between 6β-
hydroxytestosterone formation and CYP3A4(+b5) was performed.
### Table 1. Kinetic Parameters (% coefficient of variation) for Enzyme Inactivation

<table>
<thead>
<tr>
<th>Protease Inhibitor</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$K_I$ (µM)</th>
<th>$k_{\text{inact}}/K_I$ (min$^{-1}$ x nM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Liver Microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.40 (21)</td>
<td>0.17 (38)</td>
<td>2353</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>0.59 (75)</td>
<td>0.37 (24)</td>
<td>1595</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>0.11 (9.3)</td>
<td>1.00 (25)</td>
<td>110</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.26 (5.1)</td>
<td>0.65 (17)</td>
<td>400</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>0.22 (7.1)</td>
<td>1.00 (27)</td>
<td>220</td>
</tr>
<tr>
<td>Indinavir</td>
<td>--$^a$</td>
<td>--$^a$</td>
<td>--</td>
</tr>
<tr>
<td><strong>CYP3A4(+ b5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.32 (7.5)</td>
<td>0.10 (20)</td>
<td>3200</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>0.73 (9.1)</td>
<td>0.26 (18)</td>
<td>2808</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>0.10 (4.3)</td>
<td>0.41 (14)</td>
<td>244</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.31 (6.8)</td>
<td>0.17 (25)</td>
<td>1824</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>0.22 (8.5)</td>
<td>0.48 (30)</td>
<td>458</td>
</tr>
<tr>
<td>Indinavir</td>
<td>--$^a$</td>
<td>--$^a$</td>
<td>--</td>
</tr>
<tr>
<td><strong>CYP3A5</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ritonavir</td>
<td>0.08 (15)</td>
<td>0.12 (39)</td>
<td>667</td>
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<tr>
<td>Amprenavir</td>
<td>0.35 (5.7)</td>
<td>0.20 (27)</td>
<td>1750</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>0.05 (6.5)</td>
<td>1.00 (27)</td>
<td>50</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>--$^a$</td>
<td>--$^a$</td>
<td></td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>0.47 (4.5)</td>
<td>0.57 (20)</td>
<td>825</td>
</tr>
<tr>
<td>Indinavir</td>
<td>--$^a$</td>
<td>--$^a$</td>
<td>--</td>
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</table>

$^a$ Parameters not estimated; no inactivation detected.
### Table 2. Pharmacokinetic Parameters and Predicted Extent of Interaction of HIV Protease Inhibitors and CYP3A Substrates

<table>
<thead>
<tr>
<th>Protease Inhibitor</th>
<th>( I_u ) (µM)</th>
<th>CYP3A Substrate</th>
<th>( f_m )</th>
<th>( F_G )</th>
<th>( \frac{AUC'}{AUC_{po}} )&lt;br&gt;Predicted&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( \frac{AUC'}{AUC_{po}} )&lt;br&gt;Observed&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saquinavir SGC</td>
<td>0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sildenafil&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.7 – 8.9</td>
<td>3.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saquinavir SGC</td>
<td>0.004-0.038&lt;sup&gt;e&lt;/sup&gt;</td>
<td>IV Midazolam&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>--</td>
<td>1.8-6.5</td>
<td>1.0 – 5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saquinavir SGC</td>
<td>0.004-0.038&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Oral Midazolam&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4 - 14.3</td>
<td>3.5 – 9&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.21&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Sildenafil&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.7 - 11.8</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.08&lt;sup&gt;i&lt;/sup&gt;</td>
<td>IV Fentanyl&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.5 – 0.70&lt;sup&gt;e,h&lt;/sup&gt;</td>
<td>--</td>
<td>2.0 – 3.3</td>
<td>1.5 – 5.2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.02&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Triazolam&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.8&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;j&lt;/sup&gt;</td>
<td>10 - 17</td>
<td>20&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.02&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Zolpidem&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.6&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.67&lt;sup&gt;j&lt;/sup&gt;</td>
<td>3.5 – 3.6</td>
<td>1.3&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.02&lt;sup&gt;k&lt;/sup&gt;</td>
<td>Alprazolam&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.8&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.88&lt;sup&gt;k&lt;/sup&gt;</td>
<td>5.1 – 5.3</td>
<td>2.5&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.03&lt;sup&gt;l&lt;/sup&gt;</td>
<td>Trazodone&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;m&lt;/sup&gt;</td>
<td>0.75&lt;sup&gt;n&lt;/sup&gt;</td>
<td>2.0</td>
<td>2.4&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: \( I_u \)– unbound average steady-state concentration; SGC– soft gelatin capsule; \( f_m \)– fraction metabolized via CYP3A; \( F_G \)– bioavailability in absence of inhibitor.

<sup>a</sup> Predicted values were based on range of protease inhibitor concentrations, estimated inactivation parameters estimated with HLM, pharmacokinetic properties of each CYP3A substrate and \( k_{deg} \) values (Correia, 1991; Malhotra et al., 2001).

<sup>b</sup> Muirhead et al., 2000; <sup>c</sup> Warrington et al., 2000; <sup>d</sup> Thummel and Shen, 2001; <sup>e</sup> Palkama et al., 1999; <sup>f</sup> Olkkola et al., 1999; <sup>g</sup> Tateishi et al., 1995; <sup>h</sup> Guitton et al., 1997; <sup>i</sup> Greenblatt et al., 2000(a); <sup>j</sup> Rodrigues et al., 2001; <sup>k</sup> Greenblatt et al., 2000(b); <sup>l</sup> Greenblatt et al., 2003; <sup>m</sup> Jauch et al., 1976; <sup>n</sup> Nilsen and Dale, 1992.
Figure 1

![Graph showing percentages of initial activity relative to control activity for different concentrations of Ritonavir and Indinavir.](image-url)
Figure 2
Figure 3
Figure 4
Figure 5