**Introduction**

HIV protease inhibitors (PIs), such as ritonavir, saquinavir, and amprenavir, produce profound and clinically significant drug-drug interactions (DDIs) by time-dependent inactivation of CYP3A4 enzymes. Therefore, it is surprising that these PIs occasionally do not produce a clinically significant DDI with some CYP3A substrates when one is expected. For example, chronic administration of ritonavir significantly increases midazolam AUC but has no effect on alprazolam AUC and this has been shown not to be due to CYP3A4 induction. Since CYP3A4 has multiple binding sites, we hypothesized that these PIs inactivate CYP3A enzymes in a substrate-dependent manner. However, in the present study, we evaluated the in vitro CYP3A inactivation kinetics of ritonavir, saquinavir, and amprenavir with several model CYP3A probe substrates, namely alprazolam, testosterone, nifedipine, alfentanil, or midazolam. Inactivation of CYP3A enzymes by the different inhibitors or CYP3A4 was quantified by determining the maximum inactivation rate constant (k_{inact}) and the inactivation constant (K_{I}).

**Materials and Methods**

Chemicals and Reagents

Alprazolam and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO); amprenavir, saquinavir, and ritonavir were purchased from Toronto Research Chemicals (North York, Ontario, Canada); alfentanil was purchased from US Pharmacopeia (Rockville, MD); CYP3A4 was purchased from Cypex (Dundee, Scotland, UK). The sources of the other reagents used in this study have been described elsewhere. 3-5.

**Test System**

Pooled human liver microsomes (n = 16, mixed gender) were prepared from nontransplantable livers and characterized at Xenotech, LLC (Lexington, KY) as described previously. 7.

**Determination of CYP3A4 Inactivation Kinetics**

To determine K_{I} and k_{inact} for the inactivation of CYP3A4, incubations were performed with NADPH-primed pooled human liver microsomes at 0.3 mM or CYP3A4 (20 pmol/mL) for 1.5 min with ritonavir (0.05-1 µM), 3.0 min with saquinavir (0.3-3 µM), and 2.5-2.0 min for amprenavir (0.3-10 µM) at 37°C. After the preincubation step, duplicate samples were diluted 10-fold (to give a final protein concentration of 0.03 mg/mL or 2.0 µM CYP3A4) into ice-cold medium containing marker substrate at the approximate K_{m} and an NADPH regeneration system. The approximate K_{m} of each substrate was used (rather than multiple-fold K_{m}) so that the kinetics between substrates could be accurately compared. The diluted samples were incubated for 5 min reactions were stopped with an equal volume of acetonitrile containing internal standard. Samples were then centrifuged for 10 min at 9200 RCF to precipitate protein and residual CYP3A4 activity was measured by LC/MS/MS.

**Analytical Methods**

Relevant CYP3A4 activity was assessed by LC/MS/MS. An AP2000, 3000 or 4000 (AB Sciex) was used with Shimadzu HPLC pumps and autosampler systems to quantify alprazolam 4-hydroxylation, testosterone 6β-hydroxylation, nifedipine metabolism, alfentanil hydroxylation, midazolam 1-hydroxylation and midazolam 4-hydroxylation, respectively. MRM transitions were 325/239 (4-hydroxyalprazolam), 305/297 (6β-hydroxytestosterone), 345/284 (oxidized nifedipine), 146/92 (1'-hydroxymidazolam), and 342/297 (4-hydroxy-midazolam).

**Data Processing**

The inactivation kinetics were processed with GraFit 4.0.21 (Eithaus Software Ltd., Horley, Surrey, UK). To determine the rate of enzyme inactivation at each inhibitor concentration tested, the data were analyzed by a two-step method incorporating nonlinear regression. The first step calculated the apparent rate of the kinetic expression (Eo - En) described by Jones et al. 27.

The equation is analogous to the Michaelis-Menten equation, where k_{inact} represents the rate of enzyme inactivation at each inhibitor concentration. E_{o} is the initial (predilution) inhibitor concentration, K_{I} is the inhibitor concentration that produces half the maximum rate of enzyme inactivation (analogous to Km), and k_{inact} represents the maximum rate of enzyme inactivation. For this equation, based in part on a method described by Kitz and Wilson 7, the natural log of the ratio of residual activity (E_{o}) to the control activity (E_{o} - En) (where the residual activity is the rate after a defined preincubation period with the test article) is plotted against preincubation time for each concentration of inhibitor.

In the second step, K_{I} and k_{inact} were calculated by solving the nonlinear equation (eq. 4) described by Jones et al. 27.

**Results**

The kinetics of CYP3A4 inactivation by ritonavir, saquinavir, and amprenavir are summarized in Table 1. For ritonavir, the inactivation potencies (k_{inact}/K_{I}) in human liver microsomes (HLM) spanned over 3-fold across all substrates (as shown in Figure 1) with the highest potency of inactivation seen with alprazolam (0.25 mM-1 min-1), followed by alfentanil (0.35 mM-1 min-1), midazolam 1'-hydroxylation (0.54 mM-1 min-1), midazolam 4-hydroxylation (0.34 mM-1 min-1), testosterone (0.34 mM-1 min-1), and nifedipine (0.29 mM-1 min-1). Saquinavir K_{I} values were similar to human liver microsomes (1.6-3.4×10^-3 M) across all substrates (with the exception of midazolam which was omitted due to unusual sigmoidal inactivation kinetics). As shown in Figure 2, amprenavir K_{I} values in human liver microsomes and CYP3A4 were within 3-fold across all substrates (148-405×10^-3 M and 536-1154×10^-3 M) respectively.

When the inactivation by amprenavir was compared between human liver microsomes and CYP3A4 as seen in Figure 2, we observed a trend towards larger K_{I}/k_{inact} with CYP3A4. This trend (shown in Figure 2) will need to be confirmed with additional replicates with CYP3A4. Further analysis of amprenavir and saquinavir K_{I} values (as shown in Table 1) revealed that the human liver microsomal values were similar (0.1111-0.1560×10^-3 M and 0.0370-0.0631×10^-3 M) across the substrates used whereas the K_{m} value was considerably variable. These data will also need to be confirmed by additional replicates with HLM. For ritonavir, both K_{I} and k_{inact} were considerably variable across substrates.

**Conclusions**

- In HLM, the potency of CYP3A4 inactivation by ritonavir appears to be substrate-dependent, with the largest difference in k_{inact}/K_{I} between nifedipine and alfentanil (3.2 fold). For amprenavir, the largest difference in potency was between alfentanil and midazolam 4-hydroxylation (over 2 fold). However, in some cases, there was high variability in the inactivation parameters highlighting the intrinsic variability in the k_{inact}/K_{I} assay.

- CYP3A4 inactivation by ritonavir was highly test system dependent, with a broad increase (3.6-5 fold) in k_{inact}/K_{I} across all substrates with CYP3A4 when compared to human liver microsomes. This may be due to metabolic involvement of other CYP enzymes in human liver microsomes, or due to the high reductase content in CYP3A4 assays (and consequently higher rate of inactivation).

- Future experiments are needed to build upon our initial data and clarify whether substrate-dependent inactivation of CYP3A4 by protease inhibitors plays a role in their clinical drug interactions.

**References**