Substrate-Specific Inactivation of CYP3A by the HIV Protease Inhibitors Ritonavir, Saquinavir and Amprenavir

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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 CYP3A 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 [1, 2]. Since CYP3A4 has multiple binding sites, we hypothesized that the PIs inactivate CYP3A enzymes in a substrate-dependent manner. Therefore, in the present study, we evaluated the in vitro CYP3A inactivation kinetics of ritonavir, saquinavir or amprenavir with several model CYP3A probe substrates, namely alprazolam, testosterone, nifedipine, alfentanil, or midazolam. Inactivation of CYP3A enzymes in human liver microsomes or rCYP3A4 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); rCYP3A4 was purchased from Cypex (Dundee, Scotland, UK). The sources of the other reagents used in this study have been described elsewhere [3, 4].

Test System

Pooled human liver microsomes (n = 16, mixed gender) were prepared from nontransplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously [5, 6].

Determination of CYP3A4 Inactivation Kinetics

To determine K_I and k_{inact} for the inactivation of CYP3A4, inactivation incubations were performed with NADPH-fortified pooled human liver microsomes at 0.3 mg/mL or rCYP3A4 (20 pmol/mL) for 1-5 min with ritonavir (0.03-1 µM), 5-30 min for saquinavir (0.3-30 µM), and 2.5-20 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 pmol/mL rCYP3A4) into incubation medium containing marker substrate at the approximate K_m and an NADPH regenerating 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 and reactions were stopped with an equal volume of acetonitrile containing internal standard. Samples were then centrifuged for 10 min at 920 RCF to precipitate protein and residual CYP3A4 activity was measured by LC/MS/MS.

Analytical Methods

Residual CYP3A4 activity was assessed by LC/MS/MS. An API2000, 3000 or 4000 (AB Sciex) was used with Shimadzu HPLC pumps and autosampler systems to quantify alprazolam 4-hydroxylation, testosterone 6β-hydroxylation, nifedipine oxidation, alfentanil *N*-dealkylation, midazolam 1'-hydroxylation and midazolam 4-hydroxylation, respectively. MRM transitions were 325/239 (4-hydroxyalprazolam), 305/269 (6β-hydroxytestosterone), 345/284 (oxidized nifedipine), 148/92 (*N*-phenylpropionamide), 342/324 (1'-hydroxymidazolam), and 342/297 (4-hydroxymidazolam).

Data processing

The inactivation kinetics were processed with GraFit 4.0.21 (Erithacus 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 slope of enzyme inactivation (k_{obs}) for each inhibitor concentration based on the following formula:

$$\ln\left(\frac{E_t}{E_o}\right) = -k_{obs} \cdot t$$

For this equation, based in part on a method described by Kitz and Wilson [7], the natural log of the ratio of the residual activity (E_t) to the control activity (E_0) (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. [8]:

$$k_{obs} = \frac{(k_{inact} \cdot [I])}{(K_I + [I])}$$

This equation is analogous to the Michaelis-Menten equation, where k_{obs} represents the rate of enzyme inactivation at each inhibitor concentration, [I] is the initial (predilution) inhibitor concentration, K_I is the inhibitor concentration that produces half the maximum rate of enzyme inactivation (analogous to K_m), and k_{inact} represents the maximum rate of enzyme inactivation (analogous to V_{max}). This equation assumes there is negligible change in inhibitor concentration during the incubation period and that the loss of enzyme activity is due solely to enzyme inactivation. The potency of inactivation was determined by k_{inact}/K_I.

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 (925 mM^{-1} min^{-1}), followed by alfentanil (835 mM^{-1} min^{-1}), midazolam 1'-hydroxylation (554 mM^{-1} min^{-1}), midazolam 4-hydroxylation (524 mM^{-1} min^{-1}), testosterone (434 mM^{-1} min^{-1}), and nifedipine (292 mM^{-1} min^{-1}). Saquinavir k_{inact}/K_I values were similar in human liver microsomes (1.56-3.43 mM^{-1} min^{-1}) across all substrates (with the exception of midazolam which was omitted due to unusual sigmoidal inactivation kinetics). As shown in **Figure 2**, amprenavir k_{inact}/K_I values in human liver microsomes and rCYP3A4 were within 3-fold across all substrates (148-405 mM^{-1} min^{-1} and 536-1154 mM^{-1} min^{-1}) respectively.

When the inactivation by amprenavir was compared between human liver microsomes and rCYP3A4 as seen in **Figure 2**, we observed a trend towards larger k_{inact}/K_I with rCYP3A4. This trend (driven by larger k_{inact}) will need to be confirmed with additional replicates with rCYP3A4. Further analysis of amprenavir and saquinavir k_{inact} values (as shown in **Table 1**) revealed that the human liver microsome values were similar (0.1111- 0.1506 min^{-1} and 0.0357-0.0631 min^{-1} respectively) across the substrates used whereas the K_I value was considerably variable. These data will also need to be confirmed by additional replicates with HLM. For ritonavir, both k_{inact} and K_I were considerably variable across substrates.

Tables and Figures

Figure 1. The kinetics (k_{inact}/K_I) of CYP3A4 inactivation by ritonavir with several substrates of CYP3A4 in pooled human liver microsomes

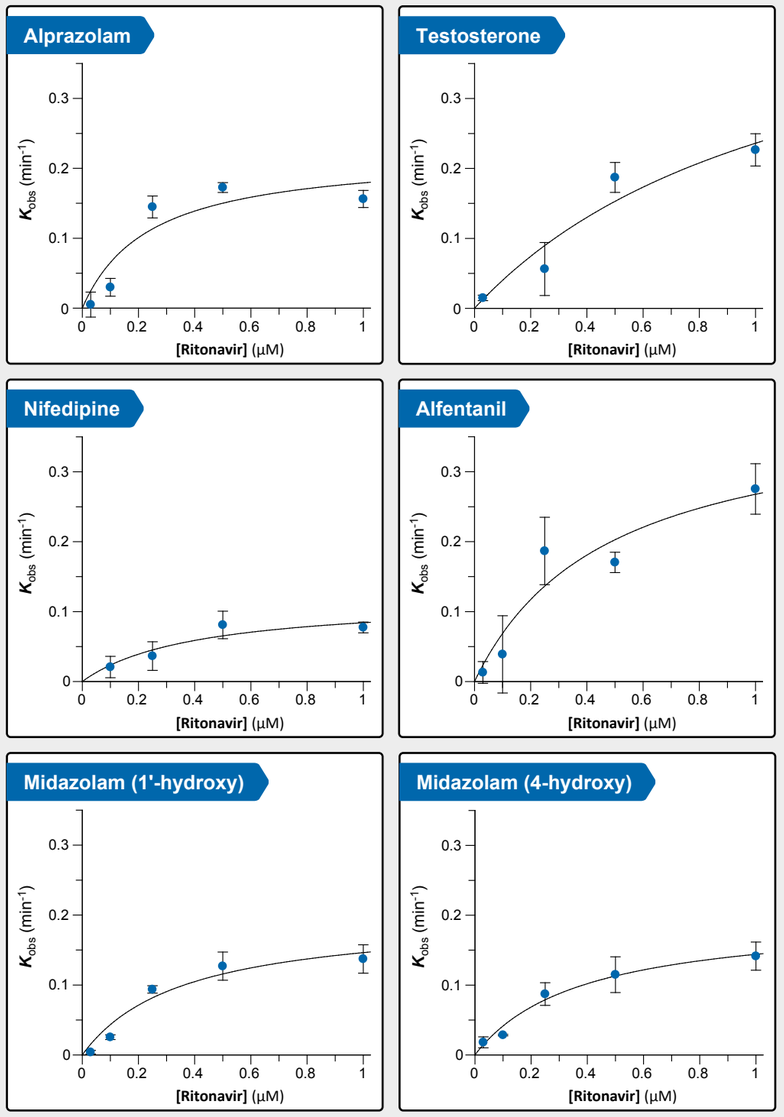

Figure 2. The kinetics (k_{inact}/K_I) of CYP3A4 inactivation by amprenavir with several substrates of CYP3A4 in pooled human liver microsomes or rCYP3A4

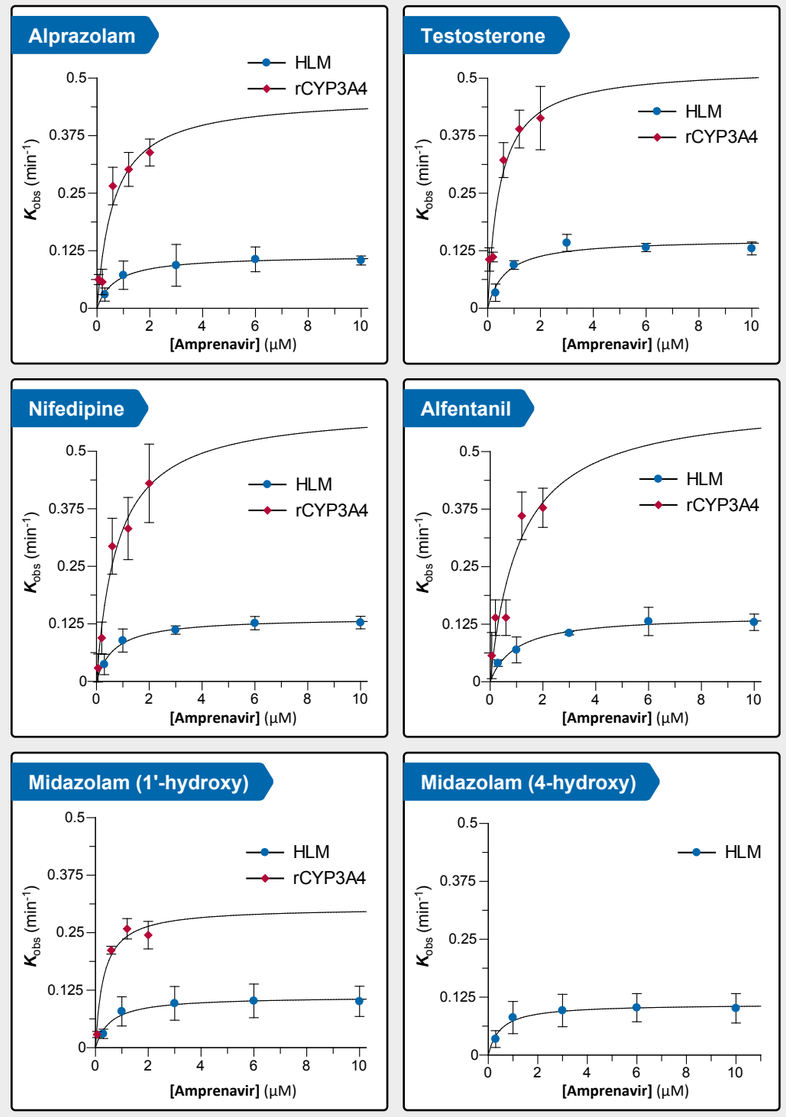

Table 1. Summary of the CYP3A4 inactivation kinetics by protease inhibitors

Substrate	[Substrate] (µM)	k_{inact} (min^{-1})	SEM	K_I (µM)	SEM	k_{inact}/K_I (mM^{-1} min^{-1})	Test system	(n)
Amprenavir								
Alprazolam	1000	0.115	± 0.004	0.701	± 0.116	164	HLM	n = 3
		0.460	± 0.098	0.630	± 0.357	731	rCYP3A4	n = 1
Testosterone	70	0.151	± 0.014	0.673	± 0.276	224	HLM	n = 4
		0.523	± 0.075	0.453	± 0.197	1154	rCYP3A4	n = 1
Nifedipine	10	0.139	± 0.005	0.680	± 0.105	204	HLM	n = 3
		0.595	± 0.092	0.803	± 0.299	741	rCYP3A4	n = 1
Alfentanil	40	0.145	± 0.007	0.974	± 0.182	148	HLM	n = 3
		0.612	± 0.252	1.143	± 0.984	536	rCYP3A4	n = 1
Midazolam (1'-hydroxy)	4	0.111	± 0.006	0.566	± 0.152	196	HLM	n = 5
		0.304	± 0.040	0.309	± 0.168	986	rCYP3A4	n = 1
Midazolam (4-hydroxy)	4	0.138	± 0.007	0.342	± 0.096	405	HLM	n = 4
Ritonavir								
Alprazolam	1000	0.222	± 0.069	0.240	± 0.209	925	HLM	n = 1
Testosterone	70	0.517	± 0.381	1.190	± 1.411	434	HLM	n = 1
Nifedipine	10	0.118	± 0.041	0.404	± 0.329	292	HLM	n = 1
Alfentanil	40	0.394	± 0.135	0.472	± 0.351	835	HLM	n = 1
Midazolam (1'-hydroxy)	4	0.199	± 0.043	0.358	± 0.189	554	HLM	n = 4
Midazolam (4-hydroxy)	4	0.199	± 0.026	0.380	± 0.117	524	HLM	n = 3
Saquinavir								
Alprazolam	1000	0.0631	± 0.0003	30.4	± 0.2	2.07	HLM	n = 1
Testosterone	70	0.0684	± 0.0055	19.9	± 3.2	3.43	HLM	n = 1
Nifedipine	10	0.0667	± 0.0263	42.7	± 26.3	1.56	HLM	n = 1
Alfentanil	40	0.0357	± 0.0122	22.1	± 14.6	1.62	HLM	n = 1
Midazolam (1'-hydroxy)	4	NA	± NA	NA	± NA	NA	HLM	NA
Midazolam (4-hydroxy)	4	NA	± NA	NA	± NA	NA	HLM	NA

SEM = standard error of the measurement

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 alprazolam (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 amprenavir was highly test system dependent, with a broad increase (3.6-5 fold) in k_{inact}/K_I across all substrates with rCYP3A4 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 rCYP 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

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