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Apparent mechanism-based inhibition of human CYP3A in-vitro by lopinavir

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Abstract

The influence of the viral protease inhibitor lopinavir on the activity of six human cytochrome P450 (CYP) enzymes was evaluated in a model system using human liver microsomes. Column chromatography methodology was developed to separate lopinavir from ritonavir starting from the commercially available lopinavir–ritonavir combination dosage form. Lopinavir produced negligible or weak inhibition of human CYP1A2, 2B6, 2C9, 2C19 and 2D6. However, lopinavir was an inhibitor of CYP3A. At 250 μM triazolam (the CYP3A index substrate), the mean ($\pm$ s.e., n = 4) IC50 versus triazolam α-hydroxylation (where IC50 is the concentration producing a 50% decrement in reaction velocity) was 7.3 ($\pm$ 0.5) μM. Pre-incubation of lopinavir with microsomes prior to addition of triazolam yielded a significantly lower IC50 of 4.1 ($\pm$ 0.5) μM. This is consistent with mechanism-based inhibition of human CYP3A by lopinavir. Although lopinavir is less potent than ritonavir as an inhibitor of CYP3A, lopinavir is nonetheless likely to contribute to net CYP3A inhibition in-vivo during treatment with the lopinavir–ritonavir combination.

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Introduction

The viral protease inhibitor lopinavir is currently available as part of a combination preparation with ritonavir (Kaletra, Abbott Laboratories, N. Chicago, IL) (Magnum & Graham 2001). The rationale for the combination is that lopinavir, biotransformed mainly by CYP3A isoforms, has low systemic availability when administered orally by itself, presumably due to extensive presystemic extraction by hepatic and/or enteric CYP3A (Kumar et al 1999a, b), as well as to possible efflux transport by P-glycoprotein (P-gp) or other transport proteins present in enteric mucosal cells (van Heeswijk et al 2001). Co-administration of lopinavir with relatively low doses of ritonavir greatly increases oral bioavailability and systemic plasma levels of lopinavir (Sham et al 1998), probably attributable to inhibition of CYP3A and P-gp by ritonavir (von Moltke et al 1998b, 2000; Perloff et al 2002). The lopinavir–ritonavir combination has served as a component of effective antiretroviral therapy for individuals with HIV infection, and reduces the medication-taking burden for these patients (Benson et al 2002; Walmsley et al 2002).

Viral protease inhibitors have complex effects on human CYPs and on transporter systems. Ritonavir, for example, inhibits CYP3A and P-gp with acute exposure (Kumar et al 1996; Eagling et al 1997; Iribarne et al 1998; von Moltke et al 1998b, 2000; Greenblatt et al 1999, 2000a, b; Muirhead et al 2000; Warrington et al 2000; Zalma et al 2000; Perloff et al 2002), but may induce expression and activity of both of these proteins with extended exposure (Greenblatt et al 2000a; Perloff et al 2000; Venkatakrishnan et al 2001). The contribution of lopinavir to net CYP3A or P-gp induction or inhibition attributable to the lopinavir–ritonavir combination is not fully established. This study assessed the effect of lopinavir alone on the activity of six human CYPs in-vitro, and evaluated alterations of CYP3A inhibition associated with pre-incubation of lopinavir with liver microsomes.

Materials and Methods

Isolation of lopinavir from the dosage form

Kaletra capsules (Abbott Laboratories, N. Chicago, IL) were opened, and the interior viscous liquid was harvested. This material was treated with water–chloroform, the organic phase dried over magnesium sulfate, separated and evaporated to dryness. The residue was then subjected to column chromatography on silica with a step gradient of methanol (0–3%) in a mixture of chloroform–acetic acid–ethanol (96:3:1). The fractions were collected, the organic solvent was evaporated and three co-evaporation steps with chloroform were performed to remove acetic acid. The chloroform solution was filtered, evaporated and dried under high vacuum.

The fractions containing lopinavir and ritonavir were verified as homogenous using laser desorption mass spectrometry with azathiothymine as the matrix (Figure 1). The structure of lopinavir was confirmed using high-field ^{1}H NMR (Figure 2). Homogeneity of at least 99% was estimated.

In-vitro studies of CYP isoforms

Liver samples from individual donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN) or the National Disease Research Interchange (Philadelphia, PA). All samples were of the CYP2D6 and CYP2C19 normal metabolizer phenotype based on previous in-vitro phenotyping studies.

Microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80 °C until use (von Moltke et al 1993, 1994). Lopinavir was prepared as described above. Other chemical reagents and drug entities were purchased from commercial sources or kindly provided by their pharmaceutical manufacturers.

Incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg^{2+}, 0.5 mM $NADP^+$ and an isocitrate/isocitric dehydrogenase regenerating system. Index substrates (Table 1), with and without inhibitor in methanol solution, were added to a series of incubation tubes. The solvent was evaporated to dryness at 40 °C under conditions of mild vacuum. Reactions were initiated by addition of microsomal protein ($0.25\ mg\,mL^{-1}$); the total reaction volume was 250 μL. After an appropriate duration of incubation at 37 °C, reactions were stopped by cooling on ice and addition of 100 μL of acetonitrile (for bupropion hydroxylation studies, reactions were stopped using 50 μL of 1 M HCl). Internal standard was added, the incubation mixture was centrifuged and the supernatant

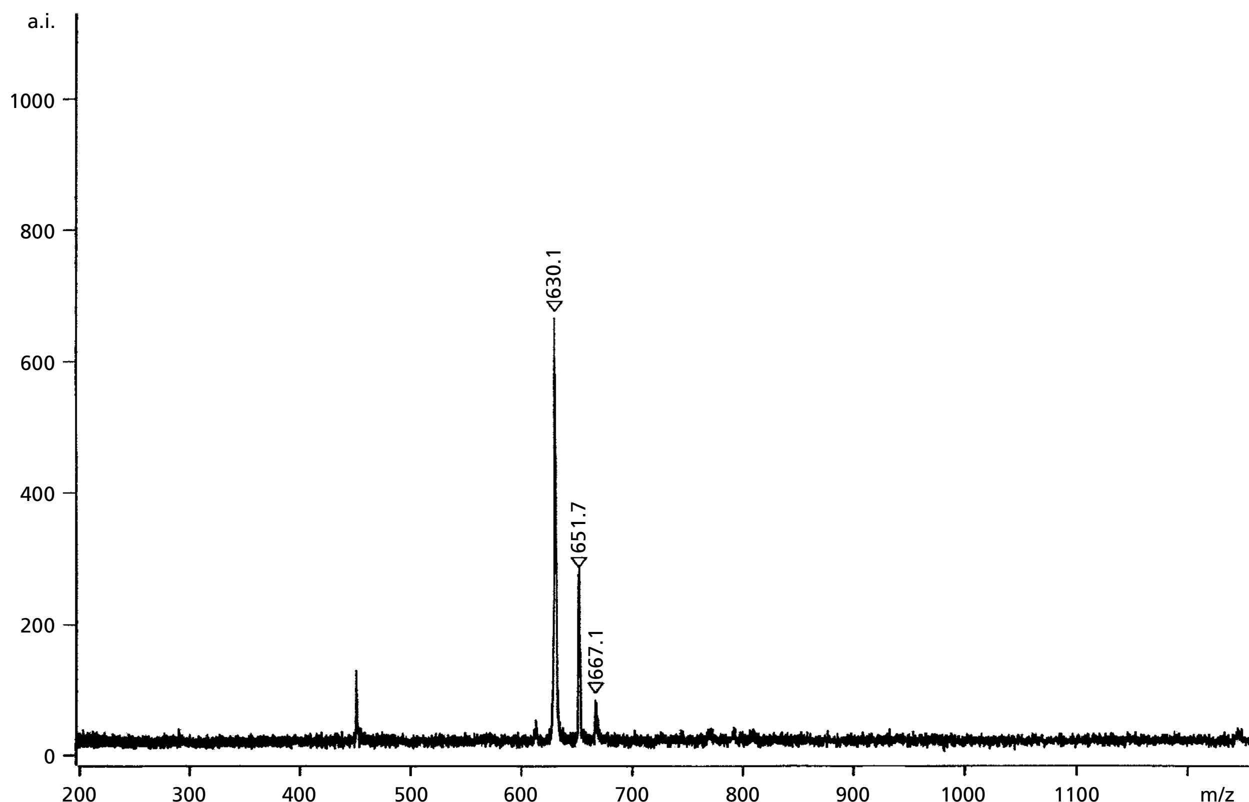

Figure 1 Matrix-assisted laser desorption ionization time-of-flight detection mass spectrum (linear, positive mode, azathiothymine matrix) of lopinavir solution obtained through column chromatography. Peaks of pseudomolecular ions ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) of the unfragmented molecule are labelled.

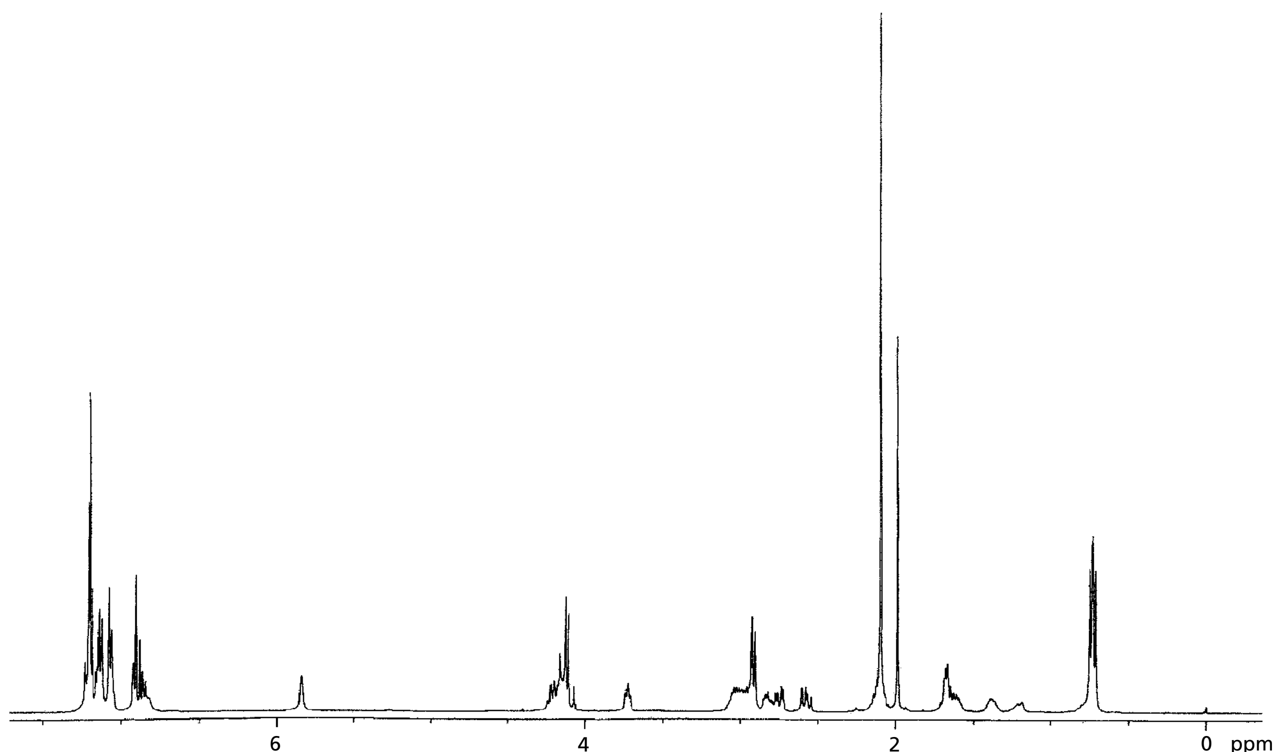

Figure 2 ^{1}H NMR spectrum (chloroform, 400 MHz) of lopinavir as purified from Kaletra by column chromatography.

Table 1 Effect of lopinavir and positive control inhibitors on human CYP activity in human liver microsomes.

CYP isoform	Substrate (concn)	Product	Positive control	% of control velocity	
				Lopinavir 10 μg mL^{-1}	Positive control
CYP1A2	Phenacetin (100 μM)	Paracetamol	α-Naphthoflavone (1 μM)	81 ($\pm$5)	17 ($\pm$1)
CYP2B6	Bupropion (50 μM)	OH-Bupropion	Anti-2B6 antibody (80 μg mL^{-1})	92 ($\pm$3)	3 ($\pm$1)
CYP2C9	Flurbiprofen (5 μM)	OH-Flurbiprofen	Sulfaphenazole (5 μM)	106 ($\pm$5)	11 ($\pm$1)
CYP2C19	*S*-mephenytoin (25 μM)	4′-OH-Mephenytoin	Omeprazole (10 μM)	78 ($\pm$2)	6 ($\pm$1)
CYP2D6	Dextromethorphan (25 μM)	Dextrorphan	Quinidine (5 μM)	85 ($\pm$3)	18 ($\pm$2)
CYP3A	Triazolam (250 μM)	α-OH-Triazolam	Ketoconazole (1 μM)	41 ($\pm$6)	5 ($\pm$1)

Data are means ($\pm$s.e.).

transferred to an autosampling vial for HPLC analysis. The mobile phase consisted of a combination of acetonitrile and 50 mM phosphate buffer as specific for each assay. The analytical column was stainless steel, 30 cm × 3.9 mm, containing reverse-phase C-18 microBondapak, or 15 cm × 3.9 mm, containing reverse-phase C-18 Novapak (Waters Associates, Milford, MA). Column effluent was monitored by ultraviolet absorbance at the appropriate wavelength, or by fluorescence detection.

The potential inhibitory effect of lopinavir on the activity of six human cytochromes was evaluated using index reactions (Venkatakrishnan et al 2001; von Moltke et al 2002) and methods as follows (Table 1): CYP1A2, phenacetin (100 μM) to paracetamol (acetominophen); CYP2B6, bupropion (50 μM) to hydroxybupropion; CYP2C9, flurbiprofen (5 μM) to hydroxyflurbiprofen; CYP2C19, *S*-mephenytoin (25 μM) to 4′-OH-mephenytoin; CYP2D6, dextromethorphan (25 μM) to dextrorphan; CYP3A, triazolam (250 μM) to α-OH-triazolam.

Lopinavir (10 μg mL^{-1}, 15.9 μM) was co-incubated with a fixed concentration of each index substrate. This concentration of lopinavir is consistent with plasma levels generally achieved during clinical use. Also studied in each system were positive control index inhibitors (Table 1). Reaction velocities with co-addition of inhibitor were expressed as a percentage ratio (R_v) of the control velocity with no inhibitor present.

For studies of triazolam hydroxylation (the CYP3A index reaction), R_v was also determined at multiple lopinavir concentrations. Both ketoconazole and troleandomycin

served as positive control inhibitors. These studies were performed both without and with 20 min of pre-incubation of lopinavir with microsomes prior to addition of substrate (von Moltke et al 2000, 2001).

Analysis of data

The relation of R_v to lopinavir concentration was analysed by nonlinear regression to determine the concentration producing a 50% decrement in reaction velocity (IC50) (von Moltke et al 1998b). Differences in lopinavir IC50 values between studies done without and with pre-incubation were evaluated using Student's paired *t*-test.

Results

All positive control index inhibitors produced extensive inhibition of their respective CYP isoforms, thereby providing internal validation of the individual index systems. Lopinavir was a weak inhibitor of all CYP index reactions except triazolam hydroxylation (Table 1). At $10\,\mu g\,mL^{-1}$ lopinavir, α-OH-triazolam formation was reduced to 46% of control. Evaluation of CYP3A activity at variable concentrations of lopinavir without pre-incubation indicated a mean ($\pm$ s.e., n = 4) IC50 value of 7.3 ($\pm$ 0.5) μM (Figure 3). When lopinavir was pre-incubated with microsomes prior to addition of substrate, the IC50 was significantly reduced ($P < 0.01$) to 4.1 ($\pm$ 0.5) μM (Figure 3). In a representative liver sample, the IC50 for troleandomycin inhibition of triazolam hydroxylation was reduced from 5.3 μM to 1.7 μM by pre-incubation (Figure 4). However, pre-incubation actually caused an increase in the IC50 for ketoconazole (Figure 4).

Discussion

In-vitro models have been of value in anticipating clinical pharmacokinetic drug interactions involving antiretroviral agents, when such interactions are attributable to CYP inhibition. In-vitro studies of ritonavir have demonstrated inhibition of CYP3A with K_i or IC50 values in the nanomolar range (Kumar et al 1996; Eagling et al 1997; Iribarne et al 1998; von Moltke et al 1998b, 2000; Merry et al 1999; Warrington et al 2000; Zalma et al 2000), and inhibition of CYP2D6 with K_i or IC50 in the low micromolar range (von Moltke et al 1998a). These values are consistent with clinical studies demonstrating that short-term exposure to low-dose ritonavir causes extensive impairment of clearance of CYP3A substrates including alprazolam, triazolam, trazodone and sildenafil (Greenblatt et al 1999, 2000a, b, 2003; Muirhead et al 2000), and of the CYP2D6 substrate desipramine (von Moltke et al 1998a). However, scaling procedures also have significant limitations, as may happen when acute inhibition occurs by a non-reversible mechanism, or when the inhibitor becomes an inducer with extended exposure (von Moltke et al 1998c; Greenblatt et al 1999; Venkatakrishnan et al 2001). Both of these limitations may apply to ritonavir.

We evaluated the effect of lopinavir alone, as opposed to the lopinavir–ritonavir combination, on the activity of six human CYP isoforms using a standard in-vitro model. Column chromatography was used to separate and isolate lopinavir from the lopinavir–ritonavir combined dosage

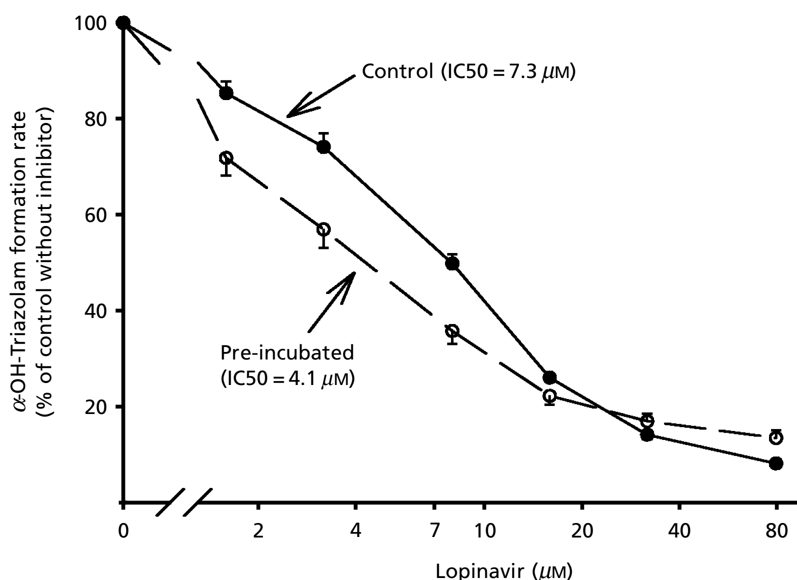

Figure 3 Effect of lopinavir on triazolam α-hydroxylation activity, an index of CYP3A activity, by human liver microsomes in-vitro. Reaction velocities with co-addition of lopinavir were expressed as a percent of the control velocity without inhibitor. Each point is the mean ($\pm$ s.e.) of four microsomal preparations. Incubations were conducted both without pre-incubation (control) and with pre-incubation of lopinavir with microsomes and cofactors. The pre-incubation procedure caused a significant reduction in the mean IC50 value for lopinavir compared with the control value without pre-incubation (7.3 $\pm$ 0.5 μM vs 4.1 $\pm$ 0.5 μM, $P < 0.05$).

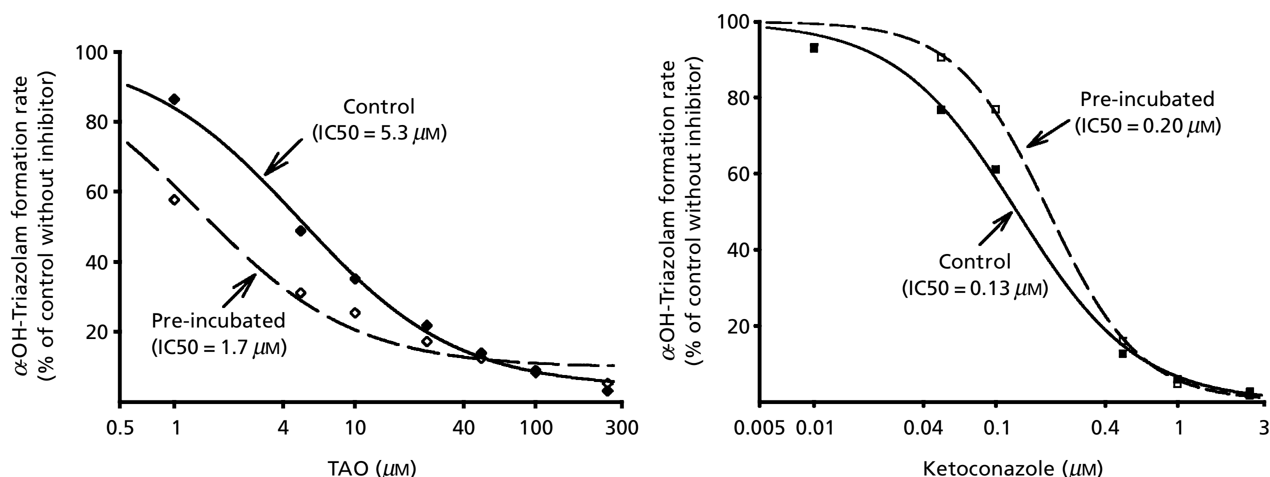

Figure 4 Effect of troleandomycin (TAO, left) and ketoconazole (right) on triazolam α-hydroxylation activity in a representative human liver microsomal preparation. Studies were conducted as described in Figure 3. Pre-incubation caused a reduction in the IC50 value for TAO compared with control, whereas pre-incubation actually increased the IC50 for ketoconazole.

preparation. Lopinavir alone had weak or negligible inhibitory activity versus human CYP1A2, 2B6, 2C9, 2C19 and 2D6. This is consistent with a previous report (Kumar et al 1999a). In this study lopinavir was a moderately potent inhibitor of CYP3A, with a mean IC50 value of 7.3 μM versus triazolam α-hydroxylation. However, pre-incubation of lopinavir with microsomes and cofactors prior to addition of substrate yielded a significantly lower mean IC50 value of 4.1 μM. This is consistent with mechanism-based inhibition, as demonstrated to occur with troleandomycin but not with the reversible inhibitor ketoconazole (Venkatakrishnan et al 2000; von Moltke et al 2000). The increased inhibitory potency of lopinavir as a consequence of pre-incubation is similar to that observed in some studies with ritonavir (Koudriakova et al 1998; von Moltke et al 2000). In any case, the inhibitory potency of lopinavir vs CYP3A is approximately an order of magnitude lower than that of ritonavir, suggesting that clinical CYP3A inhibition associated with acute exposure to the lopinavir–ritonavir combination will be largely attributable to ritonavir.

Quantitative prediction of drug interactions based on in-vitro inhibition will be complicated by the apparent mechanism-based inhibitory process for both lopinavir and ritonavir. Furthermore, lopinavir is both an inhibitor and an inducer of P-glycoprotein function (Vishnuvardhan et al 2002), as is the case with ritonavir (Perloff et al 2000, 2001, 2002). Nonetheless, the findings suggest that lopinavir may contribute to net CYP3A inhibition during treatment with the lopinavir–ritonavir combination.

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