Contributions of CYP3A4, P-glycoprotein, and Serum Protein Binding to the Intestinal First-Pass Extraction of Saquinavir

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ABSTRACT

Using CYP3A4-expressing Caco-2 cell monolayers, we assessed the roles of CYP3A4-mediated metabolism, P-glycoprotein (P-gp)-mediated efflux, and serum protein binding in determining the extent of the intestinal first-pass extraction (Ei) of saquinavir. Saquinavir (5–40 μM) was added to the apical compartment of culture inserts. After 3 h, apical and basolateral media and cell scrapings were analyzed for saquinavir and a major CYP3A4-mediated metabolite (M7). The intracellular concentration of saquinavir was estimated from the degree of inhibition of CYP3A4 catalytic activity (midazolam 1-hydroxylation). Compared with vehicle, the P-gp inhibitor LY335979 (zosuquidar trihydrochloride) (0.5 μM, apical) increased saquinavir cell content and M7 formation rate, but decreased the Ei by ~50% due to a >90% increase in the amount of saquinavir recovered in the basolateral compartment. Compared with LY335779, physiological concentrations of basolateral serum proteins [human serum albumin and α1-acid glycoprotein (AAG)] increased saquinavir permeability by a similar degree but decreased the Ei by ~50% due to a marked reduction in M7 formation. Increasing AAG concentration (1.0–2.5 g/l) had no additional effect on permeability or Ei. An estimate of the range of the Ei of saquinavir (7–60%) was less than has been predicted based on in vitro data (>99%) but was consistent with a clinical study involving grapefruit juice. The incidental finding of greater M7 formation after basolateral compared with apical dosing could not be explained by differences in saquinavir cell content. We conclude that variable intestinal first-pass extraction of saquinavir in human immunodeficiency virus-infected patients could reflect variation in P-gp-mediated efflux and/or CYP3A4-catalyzed metabolism, but not in blood AAG levels.

Saquinavir, the first of the HIV protease inhibitors to reach the market (Perry and Noble, 1998), remains one of the most widely prescribed agents in the treatment of AIDS. Attainment of effective and sustained blood levels of saquinavir can be challenging, however, due to an extremely low and variable oral bioavailability. For example, the oral bioavailability of the hard gelatin capsule formulation (Invirase) averaged 4% (range 1–9%) in healthy volunteers (Williams et al., 1992) and has been estimated to be approximately 10% in HIV-infected patients at steady state (Holladay et al., 2001). Although the soft gelatin formulation (Fortovase) provides roughly a 3-fold higher extent of systemic exposure (as measured by the AUC) compared with Invirase, a wide interindividual variation in oral pharmacokinetics remains (Kilby et al., 2000).

One contributing factor to the low and variable oral bioavailability of saquinavir is extensive and variable first-pass metabolism. Incubations with human liver or intestinal microsomes demonstrated that saquinavir is efficiently metabolized to multiple products, essentially entirely by the prominent enzyme cytochrome P450 3A4 (Fitzsimmons and Collins, 1997; Eagling et al., 2002). Inhibition of CYP3A4-mediated first-pass metabolism is the presumed primary mechanism underlying the significant increase in saquinavir systemic exposure when saquinavir is administered in combination with the known potent CYP3A4 inhibitor ritonavir (Hsu et al., 1998; Buss et al., 2001). The relative contributions by hepatic and intestinal CYP3A4 to the first-pass metabolism of saquinavir are not known. Human intestinal microsomes exhibited a high and variable intrinsic clearance (V_{max}/K_{m}) for saquinavir metabolism (0.6–8.8 ml/min/mg), suggesting that intestinal CYP3A4 could play a major role in the metabolism of saquinavir.

ABBREVIATIONS: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; AUC, area under the plasma versus concentration time curve; P-gp, P-glycoprotein; AAG, α1-acid glycoprotein; 1α,25-(OH)2-D3, 1α,25-dihydroxyvitamin D3; HSA, human serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; NEAA, nonessential amino acid; FBS, fetal bovine serum; HPLC, high-pressure liquid chromatography; DMSO, dimethyl sulfoxide; LY335979, zosuquidar trihydrochloride.
(Fitzsimmons and Collins, 1997; Eagling et al., 2002). Indeed, based on estimates of the total amount of immunoreactive CYP3A protein in the entire small intestine (70 nmol) (Paine et al., 1997), the turnover of saquinavir by CYP3A4, and the average duration of saquinavir absorption (T_{\text{max}}), Thummel et al. (1997) predicted that the high extraction ratio of saquinavir observed in vivo (0.96) (Williams et al., 1992) could be entirely attributed to first-pass metabolism in the intestine (i.e., the contribution by the liver need not be considered).

Besides undergoing extensive first-pass metabolism, saquinavir, like several other HIV protease inhibitors, is actively secreted by P-gp (Kim et al., 1998a,b), an ATP-dependent efflux pump located, among other cell types, in the luminal (apical) membrane of epithelial cells lining the small intestine (enterocytes) (Thiebaut et al., 1998). As such, P-gp could limit the extent of saquinavir absorption. A role for P-gp in limiting saquinavir oral bioavailability has been suggested from studies involving mice with homologous deletion ("knockout") of one or both genes that encode for P-gp (mdr1a and mdr1b) (Washington et al., 2000; Huisman et al., 2001). Compared with mdr1a(+)/- mice, mdr1a(-/-) mice demonstrated a 5-fold increase in the AUC of saquinavir after oral administration (Washington et al., 2000). However, double knockout mice [(mdr1a(-/-) mdr1b(-/-)] demonstrated only a 1.5-fold increase compared with their wild-type counterparts, suggesting a more limited role for this transporter. In addition, neither study could distinguish the intestinal from the hepatic contribution.

Extensive plasma protein binding is a third factor that could contribute to the broad and variable first-pass extraction of saquinavir. Saquinavir is highly bound to plasma proteins (>97%), primarily to AAG (Kageyama et al., 1994). AAG is an acute phase reactant, and levels can more than double in severe disease states, including AIDS (Mouly et al., 2000; Holladay et al., 2001). HIV-infected patients demonstrated an approximately 2-fold higher AUC and C_{\text{max}} of saquinavir compared with healthy volunteers (Fortovase product information; Roche Diagnostics, Indianapolis, IN). In addition, severely ill AIDS patients demonstrated a significantly higher (-3-fold) dose-adjusted AUC of saquinavir compared with less ill patients (Kodjo et al., 1997). Increased serum AAG may in part account for these observations, because mice genetically engineered to express high levels of human AAG demonstrated marked reductions in the systemic clearance of saquinavir (Holladay et al., 2001). In theory, elevated AAG could also increase the rate and extent of the passive absorption of saquinavir across the enterocytes due to a reduced unbound concentration of drug after it enters the portal circulation.

The human intestinal cell line Caco-2 forms polarized monolayers that structurally resemble enterocytes (Meunier et al., 1995). Although these cells constitutively express several drug-metabolizing enzymes and transporters (including P-gp) characteristic of enterocytes, they are lacking in CYP3A4. However, previous work from our laboratory demonstrated that exposure of Caco-2 cells to the hormone 1α,25-(OH)_{2}-D_{3} significantly increased CYP3A4 immunoreactive protein and catalytic activity (Schmiedlin-Ren et al., 1997). As such, these modified Caco-2 cells have been used to study the interplay between transport and metabolism during the intestinal first-pass extraction of indinavir (Hochman et al., 2000) and the immunosuppressant sirolimus (Paine et al., 2002). Accordingly, we reasoned that these modified Caco-2 cells would provide an appropriate model to assess the roles of CYP3A4, P-gp, and AAG in determining the extent of the intestinal first-pass extraction of saquinavir. Results confirm roles for P-gp and CYP3A4 but not AAG.

Materials and Methods

Materials and Chemicals

Biocao control culture inserts (4.2 cm², 1-µm pore size) and murine laminin were purchased from BD Biosciences Labware (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM) (containing 25 mM d-glucose and 4 mM l-glutamine) and nonessential amino acids (NEAA) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). Vitamin E, zinc sulfate, sodium selenite, gentamicin, all-trans-retinoic acid, HSA, AAG, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). 1α,25-(OH)_{2}-D_{3} was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Midazolam was a gift from Roche Applied Science (Nutley, NJ). Saquinavir base was kindly provided by Dr. Guy Aymard (La Salpêtrière Hospital, Paris, France). Indinavir was a gift from Merck Research Laboratories (Rahway, NJ). The selective P-gp inhibitor LY335979 (Dantzig et al., 1999) was a gift from Eli Lilly & Co. (Indianapolis, IN). cDNA-expressed CYP3A4 was purchased from BD Gentest (Woburn, MA). All other chemicals and materials were of tissue culture, HPLC, or reagent grade where appropriate.

Caco-2 Cell Experiments

Cell Culture Conditions. Cell culture inserts were coated with murine laminin (5 µg/cm²) as described previously (Fisher et al., 1999), onto which the Caco-2 cell clone P27.7 (Schmiedlin-Ren et al., 1997) (passage 22–33) was seeded at a density of ~5 × 10⁵ cells/cm². The cell cultures were maintained in complete growth medium (consisting of DMEM, 20% heat-inactivated FBS, 0.1 mM NEAA, 50 µg/ml gentamicin, and 45 mM vitamin E) until reaching confluence, as assessed by transepithelial electrical resistance values ≥250 Ω·cm² (mammal permeability was not measured). Thereafter, the cell monolayers were treated for 2 weeks with differentiation medium (consisting of DMEM, 5% heat-inactivated FBS, 0.1 mM NEAA, 50 µg/ml gentamicin, 45 nM vitamin E, 0.1 µM sodium selenite, and 3 µM zinc sulfate) supplemented with 1α,25-(OH)_{2}-D_{3}, as described previously (Schmiedlin-Ren et al., 1997). To enhance CYP3A4 expression and catalytic activity, differentiation medium was additionally supplemented with 0.2 µM all-trans-retinoic acid, and the concentration of 1α,25-(OH)_{2}-D_{3} was increased to 0.5 µM (Schmiedlin-Ren et al., 2001). The cell cultures were maintained in a humidified incubator at 37°C with a 5% carbon dioxide atmosphere.

Saquinavir Disposition. Saquinavir was dissolved as 100-fold concentrated solutions in DMSO. Warm incubation medium (differentiation medium devoid of 1α,25-(OH)_{2}-D_{3} and FBS) was spiked with drug just before its addition (1.5 ml) to the apical or basolateral compartment of duplicate or triplicate culture inserts. For A→B translocation, spiked incubation medium was added to the apical compartment, followed by 1.5 ml of plain incubation medium to the basolateral compartment. For B→A translocation, plain incubation medium was added to the apical compartment, followed by spiked incubation medium to the basolateral compartment. The final concentration of saquinavir was 0, 5, 10, 20, or 40 µM. (Solubility limitations in aqueous medium precluded testing concentrations greater than 40 µM). Culture inserts were incubated at 37°C for up to 4 h, after which the apical and basolateral media were collected and placed on dry ice. The remaining cell monolayer was quickly washed with cold DMEM, scraped into 0.4-ml cold incubation medium, and placed on dry ice. All collections were stored at ~80°C pending analysis for saquinavir and a major metabolite (M7) by
HPLC (described under “HPLC Analysis for Saquinavir and Metabolites”).

Effect of P-gp Inhibition on Saquinavir Disposition. Based on results from the aforementioned experiment (maintenance of sink conditions and linearity of M7 formation), an incubation time of 3 h was chosen for all ensuing experiments. LY335979 was dissolved as a 1000-fold concentrated solution in DMSO. For A→B translocation, incubation medium containing saquinavir (0–40 μM) and LY335979 (0.5 μM) was added to the apical compartment, followed by incubation medium containing appropriate vehicle to the basolateral compartment. For B→A translocation, incubation medium containing LY335979 and vehicle was added to the apical compartment, followed by incubation medium containing saquinavir to the basolateral compartment.

Effects of Basolateral Serum Proteins on Saquinavir Disposition. To evaluate the influence of basolateral serum proteins on saquinavir translocation, incubation medium containing saquinavir (0 to 40 μM) was added to the apical compartment, followed by incubation medium containing one of the following to the basolateral compartment: HSA (40 g/l), AAG (1 g/l), AAG (2.5 g/l), or a combination of HSA (40 g/l) and AAG (1 g/l).

Inhibition of Midazolam 1’-Hydroxylation by Saquinavir. Midazolam and ketoconazole were dissolved as 1000-fold concentrated solutions in DMSO and ethanol, respectively. Incubation medium containing saquinavir (40 μM), with or without midazolam (4 μM), was added to the apical or basolateral compartment. As a positive control for inhibition of midazolam 1’-hydroxylation and M7 formation, saquinavir and midazolam were each coincubated with ketoconazole (2 μM). 1’-Hydroxymidazolam was measured by gas chromatography/mass spectrometry as described previously (Schmiedlin-Ren et al., 1997).

Determination of the Apparent K, for Saquinavir-CYP3A4

The inhibitory potency (K) of saquinavir toward CYP3A4 catalytic activity (midazolam 1’-hydroxylation) was determined using cDNA-expressed CYP3A4 and in 4 × 4 matrix of substrate (midazolam) and inhibitor (saquinavir) concentrations. Midazolam was dissolved as 1000-fold concentrated solutions in methanol. Saquinavir was dissolved as 100-fold concentrated solutions in DMSO. Incubation mixtures consisting of 5 pmol/ml cDNA-expressed CYP3A4, substrate, inhibitor, and potassium phosphate buffer (0.1 M, pH 7.4) were prewarmed for 5 min in a shaking water bath at 37°C. Reactions were initiated with NADPH (final concentration, 1 mM) to yield a final volume of 0.5 ml. After 4 min, 0.5 ml of cold sodium carbonate (0.1 M, pH 12) was added to stop the reaction. Final midazolam concentrations were 1, 2, 4, and 8 μM; final saquinavir concentrations were 0, 0.2, 1, and 5 μM. 1’-Hydroxymidazolam was measured by gas chromatography/mass spectrometry as described previously (Painge et al., 1997). A Dixon plot was constructed to obtain an initial estimate of K. The mode of enzyme inhibition was assessed from a Dixon and a Cornish-Bowden plot (Cornish-Bowden, 1974). Final ketoconazole (2 μM) was added to the apical compartment, followed by incubation medium containing saquinavir to the basolateral compartment.

HPLC Analysis for Saquinavir and Metabolites

The monohydroxylated metabolite M7 has been reported to be the major CYP3A4-mediated metabolite of saquinavir, with a second monohydroxylated metabolite, M2, being the second most abundant (Fitzsimmons and Collins, 1997). Although multiple other metabolites have been identified (Fitzsimmons and Collins, 1997; Eagling et al., 2002), all are produced by CYP3A4, and their rates of formation, at least in microsomes, remain essentially constant relative to M7 (Fitzsimmons and Collins, 1997). For this reason, combined with the observation that M2 coeluted with LY335979, M7 was the only metabolite quantified.

Saquinavir and M7 were quantified by HPLC using liquid-liquid extraction. One hundred microliters of acetonitrile was added to 500 μl of apical or basolateral medium or the washed cell scrappings, to which 50 μl of 20 μM indinavir in acetonitrile (as internal standard) and 50 μl of 1 M sodium hydroxide were added. The mixtures were vortex mixed for 10 s, after which 5 ml of methyl-tert-butyl-ether was added. The mixtures were shaken for 25 min, and the precipitates were removed by centrifugation at 2500g for 10 min at 4°C. The organic layers were transferred to 10 ml borosilicate glass tubes and evaporated to dryness at 43°C with a SC110 Savant SpeedVac (Holbrook, NY) for 30 to 45 min. The residues were dissolved in 200 μl of 50% cold ACN in water, vortex mixed for 10 s, and 100 μl was injected into an Agilent 1100 Series HPLC system (Palo Alto, CA) coupled to a Keystone Prism column (2.0 × 150 mm; 5-μm particle size) maintained at 25°C. Saquinavir and its metabolites were eluted with water (A) and 0.1% (v/v) formic acid in acetonitrile (B) as follows: the initial eluant profile was held at 95% A for 5 min at 0.2 ml/min; B was increased linearly to 65% over 30 min and then B and the flow rate increased linearly to 85% and 0.25 ml/min, respectively, over 10 min; B and the flow rate were decreased linearly to 5% and 0.2 ml/min, respectively, over 5 min; and the column was equilibrated with 95% A for 15 min. The absorbance of the eluate was monitored at 240 nm. Saquinavir concentrations were calculated based on a standard curve containing known concentrations of saquinavir. M7 was calculated using the standard curve for saquinavir, because the UV absorption spectra for M7 was identical with that of saquinavir (Fitzsimmons and Collins, 1997). This method exhibited a linear range from 0.05 to 40 μM. The lower limit of quantification was 0.02 μM. Within and between-run coefficients of variation were ±7% over the concentration range studied.

Calculations

Apparent Permeability Coefficient of Saquinavir. The apparent permeability coefficient (Papp) of saquinavir in Caco-2 cell monolayers was calculated according to the following equation (Arтурsson, 1990):

\[
P_{\text{app}} = \frac{dQ}{dt} \frac{1}{A C_0}
\]

where dQ/dt is the flux (centimeters per second) of saquinavir from the apical compartment to the receiving compartment under sink conditions (i.e., before more than 10% of the initial concentration was recovered in the receiving compartment). A is the surface area of the culture insert (4.2 cm²), and C₀ is the initial concentration of saquinavir added to the dosing compartment.

Extraction Fraction. The intestinal first-pass extraction fraction (E₁) (the fraction of the initial dose of saquinavir metabolized relative to the total amount of drug (which includes drug-derived products) that traversed the cell monolayer) was calculated according to the following equation:

\[
E_1 = \frac{\sum \text{metabolites}_{A+B+C}}{\sum \text{metabolites}_{A+B+C} + \text{Parent}_{\text{rec}}}
\]

where A, B, and C represent apical, basolateral, and cellular amounts of metabolites, respectively, and rec represents the amount of parent recovered in the receiving compartment (Fisher et al., 1999).

Statistical Analysis

Data are expressed as means ± S.D. of at least three distinct cell experiments. Comparisons between treatment and between apical, basolateral, and cellular content of saquinavir were tested by multiple analysis of variance, including each tested concentration, followed by Scheffé’s post hoc test adjusted for multiple between-treatment comparisons when an overall significant difference ensued (Statview 5.01; SAS Institute Inc., Cary, NC). The effect of LY335979
on the apparent permeability ratio (B→A/A→B) of saquinavir was assessed at each tested concentration using the nonparametric Mann-Whitney U test for pairwise comparisons, because the obtained values were not normally distributed. A p value of <0.05 was considered significant.

Results

Apparent Permeability of Saquinavir. The rates of saquinavir translocation (A→B or B→A) were constant over time at each tested concentration (not shown). The P_{app} of saquinavir in the B→A direction exceeded that in the A→B direction at each tested concentration (Fig. 1A). Mean permeability ratios (B→A/A→B) ranged from 73 (at 5 μM) to 24 (at 40 μM) (Table 1). The selective P-gp inhibitor LY335979 significantly increased the P_{app} in the A→B direction and decreased the P_{app} in the B→A direction (Fig. 1A). Although mean permeability ratios significantly decreased, P_{app} in the B→A direction remained approximately 3-fold higher than that in the A→B direction at all concentrations (Table 1).

Saquinavir Content in Cell Scrapings. Whether saquinavir was applied to the apical or basolateral compart-

<table>
<thead>
<tr>
<th>Saquinavir</th>
<th>Control</th>
<th>(+)-LY335979a</th>
<th>p Valueb</th>
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</thead>
<tbody>
<tr>
<td>μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>73.4 ± 17.5</td>
<td>3.8 ± 1.2</td>
<td>0.008</td>
</tr>
<tr>
<td>10</td>
<td>75.4 ± 13.6</td>
<td>3.7 ± 1.1</td>
<td>0.004</td>
</tr>
<tr>
<td>20</td>
<td>44.1 ± 10.2</td>
<td>3.3 ± 1.1</td>
<td>0.004</td>
</tr>
<tr>
<td>40</td>
<td>23.9 ± 3.7</td>
<td>2.7 ± 0.8</td>
<td>0.006</td>
</tr>
</tbody>
</table>

a LY335979 (0.5 μM), a selective P-gp inhibitor, was applied to the apical compartment only.

b Mann-Whitney U test for each pairwise comparison.

ment, the percentage of the initial saquinavir dose detected in the cell scrapings remained relatively constant up to 20 μM saquinavir (~2.5 and 2% after apical and basolateral dosing, respectively); at 40 μM saquinavir, these percentages increased by at least 2-fold (Fig. 1B). Inhibition of P-gp by LY335979 (placed in the apical compartment) significantly increased the amount of saquinavir detected in the cell scrapings after either apical or basolateral administration (Fig. 1B), and the percentage of the dose detected in the cell scrapings was dose-independent. Although not significant, the amount of saquinavir detected in the cell scrapings was generally lower after basolateral compared with apical administration, and this difference remained with P-gp inhibition.

Metabolism of Saquinavir to M7. With either apical or basolateral dosing, in the absence of LY335979, M7 was not detected at the lowest applied concentration of saquinavir (5 μM) but was readily detected at the higher concentrations (Fig. 1C). The total amount of M7 formed was consistently greater after basolateral than after apical administration of saquinavir. This difference was most pronounced with saquinavir concentrations of 10 μM (54 ± 18 versus 20 ± 16 pmol; p = 0.05) and 20 μM (83 ± 22 versus 31 ± 9 pmol; p = 0.04), but was minimal at 40 μM (96 ± 30 versus 70 ± 11 pmol; p = 0.18). In the presence of LY335979, M7 was readily detected at 5 μM saquinavir. At each saquinavir concentration, the total amount of M7 formed was significantly greater than that in the absence of P-gp inhibition (Fig. 1C). With P-gp inhibition, there was also a trend for greater M7 formation after basolateral versus apical administration of saquinavir, but these differences were not significant. In the absence or presence of LY335979, total M7 formation, as a percentage of the initial saquinavir dose, decreased with increasing saquinavir concentration.

At all saquinavir concentrations greater than 5 μM, in the absence of LY335979, M7 was detected only in the apical compartment; M7 was not detected in the cell scrapings or in the basolateral medium (Fig. 2). However, M7 was readily detected in the basolateral compartment and in the cell scrapings when P-gp was inhibited. This occurred after both apical and basolateral administration of saquinavir. Even after P-gp inhibition, almost two-thirds of the total amount of M7 formed was recovered in the apical compartment.

With apical administration, the E_{50} of saquinavir decreased with increasing saquinavir concentration (Table 2, control). With basolateral administration, the E_{50} decreased only slightly with increasing saquinavir concentration (0.6 ± 0.2, 0.5 ± 0.2, and 0.4 ± 0.1 at 10, 20, and 40 μM saquinavir, respectively) and was less than 10% of the corresponding
value with apical administration. After apical administration, P-gp inhibition decreased the $E_{i}$ by $\sim 50\%$ at saquinavir concentrations $\geq 10 \mu M$ (Table 2; LY335979). In contrast, after basolateral administration, P-gp inhibition increased the $E_{i}$ relative to control at concentrations $\geq 10 \mu M$ ($7.6 \pm 1.7, 3.7 \pm 1.6, 2.1 \pm 0.5$, and $1.3 \pm 0.5$ at 5, 10, 20, and 40 $\mu M$, respectively).

**Interaction between Saquinavir and Midazolam.** To determine whether the amount of saquinavir measured in the cell scrapings reflected the intracellular content of saquinavir, the inhibitory effect of the highest concentration of saquinavir ($40 \mu M$) was examined on the 1'-hydroxylation of midazolam. In the absence of saquinavir, the extent of midazolam 1'-hydroxylation was comparable with that previously published using the same cell system (Fisher et al., 1999) (Table 3). As has also been published previously, the total amount of 1'-hydroxymidazolam formed was higher after apical compared with basolateral administration (Fisher et al., 1999), which was opposite to the trend observed for M7 formation from saquinavir (Table 3; Fig. 1C). In the presence of saquinavir, 1'-hydroxymidazolam formation decreased by $\sim 60$–70% after either apical or basolateral administration of saquinavir. In the presence of the control CYP3A inhibitor ketoconazole, 1'-hydroxymidazolam formation decreased by $\geq 90\%$ (Table 3). Midazolam had little effect on M7 formation (Table 3).

$K_{i}$ of Saquinavir toward CYP3A4. The apparent $K_{i}$ for the inhibitor-enzyme pair saquinavir-CYP3A4 was evaluated using cDNA-expressed CYP3A4 and varying concentrations of substrate (midazolam) and inhibitor (saquinavir). Dixon (Fig. 3) and Cornish-Bowden plots indicated that saquinavir was a mixed type inhibitor of midazolam 1'-hydroxylation. That is, the $K_{m}$ increased, whereas $V_{max}$ decreased with an increase in inhibitor concentration. Nonlinear regression of the data yielded a final $K_{i}$ value of 0.3 $\mu M$. Final $V_{max}$, $K_{m}$, and $\alpha$ values were 19 pmol/min/pmol, 4.4 $\mu M$, and 3.8, respectively.

**Effects of Serum Proteins on Saquinavir Disposition.** To render the modified Caco-2 cell system more physiologically relevant, basolateral medium was supplemented with HSA or AAG at normal physiological concentrations (40 g/l and 1 g/l, respectively). Compared with control, in which the basolateral medium was not supplemented with serum proteins, HSA increased the $P_{app}$ of saquinavir ($A \rightarrow B$) at all concentrations tested (Fig. 4A). AAG (1 g/l) also increased the permeability at all saquinavir concentrations, but its effect was consistently less than that of HSA. Increasing the concentration of AAG to 2.5 g/l (as has been reported for severely ill patients; Mouly et al., 2000) had minimal and no consistent effect on $A \rightarrow B$ permeability compared with AAG at 1 g/l. The combination of HSA and AAG (1 g/l) did not increase permeability over that with HSA alone.

Compared with control, HSA did not consistently alter the cell scraping content of saquinavir over the range of concentrations examined (Fig. 4B). In contrast, AAG (1 g/l) decreased cell content at each saquinavir concentration, and the effect was more pronounced when the AAG concentration was increased to 2.5 g/l. The combined effect of HSA and AAG (1 g/l) was similar to AAG alone. The effects of the serum proteins were independent of dose up to 20 $\mu M$ saquinavir.

In the presence or absence of basolateral serum proteins, M7 was not detected at 5 $\mu M$ saquinavir (Fig. 4C). At the higher saquinavir concentrations, M7 was readily detected, and the presence of HSA increased the total amount of M7 formed, although this was not significant. AAG (both high and low concentrations) also tended to increase M7 formation, except at 40 $\mu M$ saquinavir. The combination of HSA and AAG (1 g/l) slightly increased M7 formation at 10 $\mu M$ saquinavir (relative to HSA alone), but not at the two higher concentrations.

HSA, AAG (both concentrations), and the combination of HSA and AAG all decreased the $E_{i}$ of saquinavir to comparable extents relative to control at each saquinavir concentration (Table 2). When P-gp was inhibited in the presence of HSA, the $E_{i}$ of saquinavir markedly decreased, both relative to control (by at least 90%) and relative to HSA alone (by at least 60%).

**Discussion**

P-gp contributed prominently to the vectorial translocation of saquinavir in modified Caco-2 cells. At the highest applied concentration of saquinavir ($40 \mu M$), saturation of efflux was not apparent (Fig. 1A), and chemical inhibition of P-gp activity markedly reduced the apparent permeability ratio ($B \rightarrow A$; Table 1). These permeability ratios were generally higher than those reported by others (Alsenz et al., 1998; Kim et al., 1998b), which may reflect up-regulation of P-gp as a result of 1a,25-(OH)$_2$D$_3$ treatment (Schmiedlin-Ren et al., 1997; Paine et al., 2002). However, P-gp content in homogenate prepared from 1a,25-(OH)$_2$D$_3$-treated monolayers was comparable with that in human small intestinal mucosal homogenates (Paine et al., 2002). As such, the permeability ratios obtained in the current study may be representative of the in vivo situation. Non-P-gp-mediated vectorial translocation of saquinavir was also evident. At a concentration of LY335979 approximately 8 times higher than the reported $K_{i}$ for P-gp (Dantzig et al., 1999), the permeability ratio of saquinavir remained $\sim 3$ at all concentrations examined (Table 1). This may be explained by additional efflux transporters (e.g., MRP2) (Huismann et al., 2002) or by unidentified basolateral uptake transporters.
The current results confirm a role for CYP3A4 in determining the Eᵢ of saquinavir. The CYP3A4-mediated metabolite M7 was readily detected at initial saquinavir concentrations ≥10 μM. In the absence of P-gp inhibition or basolateral serum proteins, the Eᵢ of saquinavir after apical application ranged from 6 to 22% (Table 2). Interplay between P-gp and CYP3A4 was also apparent in that inhibition of P-gp led to a considerable increase in the rate of M7 formation at all concentrations of saquinavir tested (Fig. 1C). This likely resulted from increased cellular content of saquinavir due to reduced efflux (Fig. 1B), assuming CYP3A4 was not saturated in the absence of P-gp inhibition. Lack of enzyme saturation was supported by the interaction study with midazolam. CYP3A4-mediated midazolam 1’-hydroxylation was inhibited by just over 50% with 40 μM apically applied saquinavir (Table 3). This indicated that, at the highest dose examined, the concentration of saquinavir at the enzyme was near its IC₅₀ (0.2–1.0 μM; data not shown) and Kᵢ (0.3 μM) (Fig. 3), which are in the range of the published Kₘ (0.3–1 μM) (Fitzsimmons and Collins, 1997; Eagling et

TABLE 2
Effect of P-gp inhibition and basolateral serum proteins on the Eᵢ of apically applied saquinavir in modified Caco-2 cells
Each value represents the average ± S.D. of three to five experiments.

<table>
<thead>
<tr>
<th>Saquinavir</th>
<th>Control</th>
<th>LY335979*</th>
<th>HSA</th>
<th>AAG</th>
<th>AAG</th>
<th>HSA + AAG</th>
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<td>5</td>
<td>N.D.*</td>
<td>20.5 ± 4.0</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21.8 ± 16.3</td>
<td>10.4 ± 5.0</td>
<td>4.6 ± 1.4</td>
<td>7.7 ± 2.3</td>
<td>5.2 ± 1.8</td>
<td>7.9 ± 2.4</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td>10.9 ± 6.1</td>
<td>6.0 ± 3.9</td>
<td>2.8 ± 0.9</td>
<td>2.9 ± 0.9</td>
<td>2.2 ± 0.8</td>
<td>2.9 ± 2.4</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>40</td>
<td>5.5 ± 0.9</td>
<td>2.8 ± 1.3</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>0.5 ± 0.0</td>
</tr>
</tbody>
</table>

N.D., not determined.

*LY335979 (0.5 μM), a selective P-gp inhibitor, was applied to the apical compartment only.

TABLE 3
Interaction between saquinavir (40 μM) and midazolam (4 μM) in modified Caco-2 cells
Each value represents the average of duplicate culture inserts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Amount of Metabolite Formed/3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1’-OH MDZ M7</td>
</tr>
<tr>
<td>Saquinavir (apical dose)</td>
<td>70</td>
</tr>
<tr>
<td>+ Apical ketoconazole (2 μM)</td>
<td>BLD</td>
</tr>
<tr>
<td>Saquinavir (basolateral dose)</td>
<td>96</td>
</tr>
<tr>
<td>+ Basolateral ketoconazole (2 μM)</td>
<td>BLD</td>
</tr>
<tr>
<td>Midazolam (apical dose)</td>
<td>310 (100)</td>
</tr>
<tr>
<td>+ Vehicle (1% DMSO)</td>
<td>130 (45)</td>
</tr>
<tr>
<td>+ Basolateral saquinavir</td>
<td>100 (33)</td>
</tr>
<tr>
<td>+ Apical ketoconazole (2 μM)</td>
<td>20 (6)</td>
</tr>
<tr>
<td>Midazolam (basolateral dose)</td>
<td>230 (100)</td>
</tr>
<tr>
<td>+ Vehicle (1% DMSO)</td>
<td>110 (47)</td>
</tr>
<tr>
<td>+ Basolateral saquinavir</td>
<td>80 (34)</td>
</tr>
<tr>
<td>+ Basolateral ketoconazole (2 μM)</td>
<td>17 (8)</td>
</tr>
</tbody>
</table>

BLD, below limit of detection.

Fig. 3. Dixon plot showing the inhibitory effect of saquinavir toward midazolam 1’-hydroxylation activity in cDNA-expressed CYP3A4. MDZ, midazolam. Symbols denote the means of duplicate incubations.

Fig. 4. Effect of basolateral serum proteins on the P_app of saquinavir (A), cellular content of saquinavir (B), and total amount of M7 formed (C) in modified Caco-2 cells. Saquinavir (5 to 40 μM) was applied to the apical compartment alone or with the various serum proteins (applied to the basolateral compartment) and incubated for 3 h. Bars and error bars denote means and standard deviations, respectively, of at least three distinct experiments. At 5 μM saquinavir, M7 was below the limit of detection. *, significantly different from control (p < 0.05). †, significantly different from HSA (A and B) or from HSA + AAG (A) (p < 0.05).
al., 2002). Despite an increased extent of metabolism with P-gp inhibition (Fig. 1C), the corresponding Eᵢ values were reduced by roughly 50% (Table 2). This was likely the consequence of saturation of CYP3A4, as suggested by the reduction in the percentage of the dose converted to M7 with increasing saquinavir concentration (Fig. 1C).

In some individuals, CYP3A5, a polymorphic enzyme closely related to CYP3A4, is also expressed in enterocytes (Paine et al., 1997; Lin et al., 2002). The potential role of CYP3A5 could not be evaluated in the current studies because the concentration of CYP3A5 is very low relative to CYP3A4 in the modified Caco-2 cells (Schmiedlin-Ren et al., 1997).

The current findings also support a role for serum protein binding in determining the Eᵢ of saquinavir. When the basolateral compartment was supplemented with HSA, AAG, or both, the A→B permeability of saquinavir increased to values comparable with those achieved with P-gp inhibition (1 to 4 × 10⁻⁶ cm/s) (Figs. 1A and 4A). The Eᵢ values, however, were roughly one-half the values observed with P-gp inhibition, because the rates of M7 formation in the presence of serum proteins were roughly one-third those observed with P-gp inhibition (Figs. 1C and 4C). The lower rates of M7 formation in the presence of serum proteins relative to P-gp inhibition likely reflected lower intracellular saquinavir concentrations. Combining HSA and P-gp inhibition greatly reduced the Eᵢ (Table 2), which seemed to reflect a greater increase in permeability compared with metabolism (≥20- and 3-fold, respectively, relative to control) (data not shown).

Because saquinavir exists in plasma bound predominately to AAG (Kageyama et al., 1994), the finding that AAG reduced the cellular content of saquinavir and increased A→B permeability (Fig. 4B) was anticipated. This presumably reflected increased passive diffusion with an uncharged rate of active efflux. The effects of AAG were similar at 1 and 2.5 g/l, suggesting that higher AAG levels likely do not account for the increased saquinavir AUC reported in severely ill patients (Kodjo et al., 1997). The apparent increase in permeability caused by HSA was not associated with a reduced cellular content of saquinavir. In retrospect, this may have been an artifact of the system if HSA had displaced saquinavir adsorbed to the basolateral apparatus, as has been described for other lipophilic compounds (Krishna et al., 2001).

In the presence of serum proteins, the Eᵢ values of saquinavir were very low, ranging from 1.4 to 7.9% at 40 and 10 μM, respectively. M7 has been reported to represent 6% (Eagling et al., 2002) and ~20% (Fitzsimmons and Collins, 1997) of total saquinavir metabolism in human liver and intestinal microsomes, respectively. The “true” Eᵢ would thus have ranged from ~20 to 60% (assuming the 6% estimate) or from 7 to 30% (assuming the 20% estimate). These values are considerably less than the extraction ratio predicted for the human intestine in vivo (~99%) by Thummel et al. (1997).

One potential explanation for this discrepancy is that the modified Caco-2 cells have been shown to contain only 16 to 30% of the CYP3A4 present in human intestinal mucosal homogenates (Paine et al., 2002). Low CYP3A4 content in the cells would account for why the Eᵢ of midazolam calculated from modified Caco-2 cell data were about one-third of the average Eᵢ determined in vivo during liver transplantation surgery (15 versus 43%, respectively) (Fisher et al., 1999). However, these findings with midazolam may not apply to saquinavir, because saquinavir is more slowly absorbed than midazolam (average T₁/₂ of 4 h versus 0.5–1 h) (Thummel et al., 1997). The Caco-2 cell content of CYP3A4 may be more representative of that in distal small intestine, because CYP3A4 expression in this location is less (22–66%) than that in proximal small intestine (Paine et al., 1997).

An additional consideration is that the concentrations of saquinavir achieved in the small intestine could greatly exceed the highest concentration that could be tested (40 μM) without exceeding solubility limitations. Assuming a volume of dilution of about 1 liter when the highest oral dose of saquinavir (800 mg) leaves the stomach, the maximum concentration would approximate 1 mM (mol. wt. = 767 for the saquinavir mesylate formulation). Although the actual concentration of saquinavir cannot be estimated at the enterocytes, it seems likely that the true conditions would further favor saturation of CYP3A4. If this is the case in vivo, our estimates of the Eᵢ in the Caco-2 cells might overestimate the Eᵢ in vivo.

In spite of these reasons why the experimental Eᵢ values might over- or underestimate the Eᵢ in vivo, our estimate of 7 to 60% agrees with the observation of only a 2-fold increase in the oral bioavailability of saquinavir when taken with grapefruit juice (Kupferschmidt et al., 1998), which essentially abolishes intestinal CYP3A4 activity while having little effect on hepatic activity (Lown et al., 1997). These clinical observations are thus consistent with our cell-based data and suggest that intestinal and hepatic CYP3A4 may, on average, contribute equally to the first-pass extraction of saquinavir.

Unexpectedly, the rate of M7 formation was consistently greater after basolateral compared with apical administration of saquinavir (Fig. 1C), despite that the cellular content of saquinavir was consistently higher after apical administration (Fig. 1B). Higher cellular content with apical dosing has also been reported for sirolimus in this same cell system, in which the apical-basolateral difference was postulated to result from saturation of P-gp by sirolimus after apical but not basolateral administration (Paine et al., 2002). In addition, the extent of sirolimus metabolism was greater with the apical route, as would be expected based on cellular content. Increased metabolism after apical versus basolateral dosing has also been reported for cyclosporine (Gan et al., 1996), indinavir (Hochman et al., 2000), and midazolam (Fisher et al., 1999). To our knowledge, greater metabolism after basolateral dosing has not been described previously.

The mechanism whereby M7 formation was higher after basolateral dosing cannot be determined from the current results. There was a trend for saquinavir to inhibit midazolam metabolism to a greater extent after basolateral compared with apical dosing (Table 3). This may indicate that the concentration of substrate at the enzyme was higher after basolateral administration, the opposite of what would be assumed based on cell content alone. We speculate this could occur if increased transcellular flux of saquinavir decreases concentration gradients of parent in the microenvironment of the enzyme (i.e., a “stirring” effect). It may also be important that M7 was efficiently secreted across the apical membrane, largely by P-gp (Fig. 2). Because M7 is subject to secondary and tertiary metabolism by CYP3A4, (Fitzsimmons and Collins, 1997; Eagling et al., 2002), the increased amount of M7 formed with basolateral dosing could be explained if secondary metabolism of M7 was greater after apical dosing (per-
haps due to partial inhibition of apically located efflux transporters by saquinavir).

In summary, the current results support roles for P-gp and CYP3A4, but not physiologic variation in AAG concentration, as determinants of the intestinal first-pass extraction of saquinavir. The contribution by CYP3A4 was less than has been previously suggested for the intestine in vivo but was consistent with the magnitude of the interaction between saquinavir and grapefruit juice reported in a clinical study. It seems that inhibition of both CYP3A4 and P-gp would be required to optimally enhance the oral bioavailability of saquinavir.

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References


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