Differential Roles of P-Glycoprotein, Multidrug Resistance-Associated Protein 2, and CYP3A on Saquinavir Oral Absorption in Sprague-Dawley Rats

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ABSTRACT:

The objective of this investigation was to differentiate the roles of P-glycoprotein (Pgp), multidrug resistance-associated protein 2 (Mrp2), and CYP3A on saquinavir (SQV) oral absorption. With use of single-pass jejunal perfusion (in situ) and portal vein-cannulated rats (in vivo), SQV absorption was studied under chemical inhibition of Pgp [N-4-[(2,1,3,4-tetrahydro-6,7-dimethoxy-2isoquinoliny1)-ethenyl]phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), Mrp2 [3-[[3-(2-chloro-2-quinolinyl)-(E)-ethenyl]phenyl]-(3-dimethylamino-3-oxopropyl)thio)methyl)-thio) propanoic acid (MK571), and/or CYP3A (midazolam). Plasma concentrations of SQV and related metabolites were analyzed by liquid chromatography-tandem mass spectrometry. When given alone, SQV absorption was extremely low both in situ (F = 0.07%) and in vivo (Cmax = 0.068 μg/ml; area under the curve (AUC) = 6.8 μg · min/ml). Co-administration of GF120918 boosted SQV absorption by more than 20-fold with decreased variation in AUCs (percent coefficient of variation = 30% versus 100%). In contrast, co-administration of MK571 or midazolam increased SQV absorption only 2- to 3-fold without improving the variation in AUCs. SQV oral absorption was not further improved when it was given with GF120918 and midazolam or with GF120918 and MK571. The current results provide, for the first time, direct and explicit evidence that the low oral absorption of SQV is controlled by a secretory transporter, Pgp, and not by limited passive diffusion owing to its poor physicochemical properties. Pgp-mediated transport is also responsible for the highly variable oral bioavailability of SQV. In contrast, intestinal Mrp2 and intestinal CYP3A appear to play minor roles in SQV oral bioavailability. Given the differential and complex roles of Pgp and CYP3A in SQV oral absorption, the optimization of AIDS boosting regimens requires careful consideration to avoid therapy-limiting drug-drug transporter and enzyme interactions.

Saquinavir [SQV, (N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[(N-2-quinolylicarbonyl)-L-asparaginyl-amino]butyl]-[4aS,8aS]-isoquinoline-3(S)-carboxamide, Ro 31-8959; HIV, human immunodeficiency virus; CV%, percent coefficient of variation; MDR/Mdr, multidrug resistance; Mrp/Mrp, multidrug resistance-associated protein in rodents (e.g., Mrp1–Mrp9); CYP3A, cytochrome P-450 3A (CYP3A4 for humans and CYP3A1/2 for rodents); Pgp, P-glycoprotein (ABCB1 or MDR1 for humans and abcb1 or Mdr1 for rodents); MR2 (ABCC2)/Mrp2 (abcc2), multidrug resistance-associated protein 2; RTV, ritonavir; GF120918, N-4-[(2,1,3,4-tetrahydro-6,7-dimethoxy-2 isoquinoliny1)-(E)-ethenyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; MK571, 3-[[3-(2-chloro-2-quinolinyl)-(E)-ethenyl]phenyl]-(3-dimethylamino-3-oxopropyl)thio)methyl)-thio) propanoic acid; MDZ, midazolam; MES, 4-morpholineethanesulfonic acid; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

SQV is a known substrate of several intestinal transporters and CYP3A. In MDCKII cell lines overexpressing ABCB1 and ABCC2, our group and others found that MDR1 and MRP2 were responsible for SQV secretory transport, respectively (Huisman et al., 2002; Williams et al., 2002), whereas MRP1 appeared to be involved in SQV absorptive transport (Williams et al., 2002). A recent study by our group showed that SQV is also a substrate of organic anion-transporting polypeptide OATP-A (SLC21A3) (Su et al., 2004). In human liver and small intestinal microsomes, SQV was metabolized mainly to monohydroxylated compounds (Fitzsimmons and Collins, 1997; Eagling et al., 2002). The metabolism of SQV occurs primarily by CYP3A4. In the presence of a specific CYP3A4 inhibitor, ketoconazole, SQV metabolism in small intestinal microsomes was completely inhibited (Fitzsimmons and Collins, 1997; Eagling et al., 2002).

Pgp is known to be a major secretory transporter in the intestine, brain, and many other organs. The impact of Pgp on oral bioavailability of SQV has not been clearly defined. This study provides new evidence that Pgp is a major limiting step for SQV absorption. Pgp-mediated transport is responsible for the highly variable oral bioavailability of SQV. The results of this study also indicate that Pgp, Mrp2, and CYP3A each play minor roles in SQV oral bioavailability.
ability, intestinal excretion, and tissue distribution has been shown in mdr1a knockout mice (Schinkel et al., 1997a; Sparreboom et al., 1997). In humans, Pgp inhibition caused a significant decrease in digoxin intestinal excretion (Drescher et al., 2003), whereas rifampin-up-regulated intestinal Pgp expression appeared to be responsible for a decrease in digoxin oral bioavailability (Greiner et al., 1999).

MRP2, expressed primarily in the canalicular membrane of hepatocytes and the apical membrane of enterocytes (Suzuki and Sugiyama, 2002), is believed to be a major secretory transporter in the biliary excretion of glutathione, glucuronide, and sulfate conjugates and many nonconjugated organic anions (Keppeler et al., 1997; Williams et al., 2002). MRP2 deficiency, as seen in TR + rats or Eisai hyperbilirubinemic rats, results not only in decreased biliary secretion of organic anions and conjugates but also in significantly reduced bile flow (Paulusma et al., 1996; Suzuki and Sugiyama, 2002). MRP2/ MRP2 appears to play a minor role in intestinal absorption and secretion, especially when Pgp is involved even though they have similar intestinal localization. Furthermore, the oral absorption of gatifloxacin, a MRP2 and Pgp substrate, was not significantly enhanced by the inhibition of MRP2; however, biliary excretion was significantly reduced, suggesting differential roles for Pgp and MRP2 in the intestine (Buss et al., 2001; Williams et al., 2002).

Although it is known that Pgp, MRP2, and CYP3A4 contribute to the oral bioavailability and disposition of many drugs, their differential roles in SQV oral absorption and disposition are not yet fully understood. In AIDS therapy, SQV is often given with a boosting agent, such as ritonavir (RTV), to improve SQV oral bioavailability and to reduce its individual variation (Buss et al., 2001; Plosker and Scott, 2003). Addition of 1% Tween 80 and 10% dimethyl sulfoxide was necessary for dissolving MK571 and preventing MK571 from precipitation when mixed with SQV and GF120918. All dosing regimens were studied: SQV alone, SQV + GF120918 (SQV + GF), SQV + MK571 (SQV + MK), SQV + MDZ, SQV + GF + MK, or SQV + GF + MDZ. Each group consisted of three or more rats. The concentrations of the inhibitors in the perfusate were 20 μM (GF) and 100 μM (MK and MDZ). Selection of a 100 μM concentration for SQV, the solubility in the buffer system at pH 6.5, in the perfusate was to mimic the possible drug concentration at the absorption site during oral absorption in vivo. It was reported that GF120918 at 10 μM produced complete inhibition of Pgp function in MDR1-MDCKII cells (Polli et al., 2001). However, whether the boosting effect resulting from Pgp inhibition is due to shortened elimination t1/2 (Buss et al., 2001; Plosker and Scott, 2003). The mechanism of boosting therapy is widely thought to be the result of the inhibition of CYP3A (Kempf et al., 1997; Plosker and Scott, 2003). However, the shortened elimination t1/2 of SQV in boosting regimens cannot be fully explained by this hypothesis. Because RTV is a substrate and inhibitor of CYP3A4, Pgp, and MRP2 (Gutmann et al., 1999; Huis- man et al., 2001, 2002; Williams et al., 2002), the RTV boosting effect is likely to be a combined result of drug-drug interactions mediated by Pgp, MRP2, and CYP3A4. Recent evidence suggests that Pgp-mediated secretory transport may also play an important role in SQV oral bioavailability (Huisman et al., 2001; Sinko et al., 2004). It is unclear whether the boosting effect resulting from Pgp inhibition is due to enhanced SQV oral absorption or diminished clearance or disposition. Furthermore, SQV is excreted mainly into the bile in humans (Roche Laboratories, 2001). Because SQV is a MRP2 substrate, MRP2 inhibition may also affect its pharmacokinetic behavior.

The present study was designed specifically to differentiate the roles of Pgp, MRP2, and CYP3A4 on SQV oral absorption. In situ single-pass jejunal perfusion and portal vein–cannulated rats were used as study systems to exclude or minimize the confounding influence of first-pass hepatic metabolism.

Materials and Methods

Materials. Saquinavir (N-(tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[1-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-4aS,8aS)-quinoline-3(S)-carboxamide, Ro 31-8959) was a gift from Roche Laboratories (Nutley, NJ). GF120918 and MK571 were gifts from GlaxoSmithKline, Inc. (Research Triangle Park, NC) and Merck and Co., Inc. (West Point, PA), respectively. The chemical structures of GF120918 and MK571 (sodium salt) can be found in the literature (Planting et al., 2005), and product information is provided by Cayman Chemical (Ann Arbor, MI; www.caymanchem.com), respectively. Midazolam (MDZ) (Versed, 5 mg/ml; Roche Laboratories) was purchased through the Rutgers University Health Center Pharmacy.
MDZ, SQV + GF + MK, or SQV + GF + MDZ. The dosing volume was 2 ml. Blood samples (0.5 ml) were collected before (predose) and after dosing (10, 20, 40, 60, 90, 150, 240, and 360 min). After each sample was taken, the catheter was flushed with 0.5 ml of saline containing 50 U of heparin to compensate for blood loss and to prevent the catheter from clogging. At the end of the study, animals were euthanized using an i.v. dose of pentobarbital at ~100 mg/kg. Plasma was harvested after centrifugation at ~1000 g for 10 min. All samples were stored at ~80°C until analysis.

The selection of an SQV dose of 20 mg/kg was based on the quantitation limit for the plasma samples on LC-MS/MS and its clinical relevance (SQV clinical dose: 600–1200 mg/dose or 8.6–17 mg/kg for a 70-kg man) (Plosker and Scott, 2003). On the basis of the mass ratios between SQV (100 μM) and the inhibitors (20 μM GF120918, 100 μM MK571, and 100 μM MDZ), doses of 3 mg/kg for GF120918, 14 mg/kg for MK571, and 10 mg/kg for MDZ were selected for SQV at 20 mg/kg.

Sample Analysis. Plasma samples were purified by solid-phase extraction using a literature method with modification (Frappier et al., 1998). Briefly, 0.25 ml of plasma samples were mixed with 20 μl of internal standard (2 μg/ml quinidine) and 0.5 ml of 0.18 M ammonium acetate (pH 6.8) before extraction. After conditioning the C18 solid-phase extraction columns (SPEC·Plus·3 mł·C18) with 1 ml of methanol and 1 ml of deionized and distilled water, plasma samples were loaded onto the columns, washed with 1 ml of water, and then eluted twice with 0.25 ml of methanol. The eluate (10 μl) was injected directly onto a LC-MS/MS system.

The LC-MS/MS system consisted of a ThermoQuest Surveyor MS pump (ThermoQuest Co., San Jose, CA), a Surveyor autosampler, and a Finnigan LCQ DECA mass spectrometer (ThermoQuest Co.). SQV and quinidine (internal standard) were eluted from a C18 column (Eclipse XDB-C18, Zorbax 2.1 × 50 mm, 3.5 μm, Agilent Technologies, Palo Alto, CA) using an isocratic mobile phase of 35% 2 mM ammonium acetate and 65% acetonitrile at a flow rate of 0.2 ml/min. The retention times of the analytes were 1.5 min (internal standard) and 2.2 min (SQV). By electron spray ionization and under positive ion mode, SQV was detected at mlz 671.4 → mlz 570.4, its major metabolites (M2, M3, and M7, where M2 and M3 are stereo-isomers) (Fitzsimmons and Collins, 1997) were detected at mlz 687.4 → mlz 568.4 (M7) and at mlz 671.4 → mlz 586.4 (M2/M3 isomers eluted as a single peak), and quinidine was detected at mlz 325.2 → mlz 307.1 and mlz 264.2. The quantitation limit of the assay was 1 ng/ml. The recovery of SQV in plasma samples after extraction was ~80%.

Data Analysis. Plasma concentrations of SQV were determined from calibration standard curves. Because of the unavailability of purified metabolites, the plasma concentrations of M2/M3 and M7 were estimated using SQV calibration curves and denoted as SQV equivalent concentrations (e.g., nanogram-equivalents per milliliter). Mesenteric blood concentration levels were relatively constant under the constant perfusion rate; hence, the average plasma concentrations of SQV and its metabolites in the mesenteric blood samples were used as a standard parameter for the extent of SQV intestinal absorption. Because M2, M3, and M7 account for ~90% of SQV metabolites formed in the intestinal tissues (Fitzsimmons and Collins, 1997), the sum of unchanged SQV and these metabolites in the blood was considered as the total amount absorbed. On the basis of the cumulative total drug amount (SQV + M237) in the blood (SQV blood/plasma concentration ratio ~1), the fraction absorbed (F\textsubscript{a}) was determined as a fraction of perfused drug amount. The fraction of dose that survived from intestinal metabolism (F\textsubscript{gut}) was estimated from the ratio of cumulative drug amounts between the parent and total SQV-related compounds.

Pharmacokinetic parameters for oral studies, such as maximum plasma concentration (C\textsubscript{max}), the time to maximum concentration (T\textsubscript{max}) area under the plasma concentration curve to the last measurable concentration (AUC\textsubscript{last}) or to infinity (AUC\textsubscript{oo}), elimination half-life (t\textsubscript{1/2}), apparent volume of distribution (V\textsubscript{d}/F), and apparent clearance (CL/F) were analyzed using a noncompartmental analysis (WinNonlin Professional v4.1; Pharsight, Mountain View, CA). The fraction of dose that survived from intestinal metabolism (F\textsubscript{gut}) was estimated from the ratio of cumulative drug amounts between the parent and total SQV-related compounds. F\textsubscript{gut} = AUC\textsubscript{SQV}/[AUC\textsubscript{SQV} + AUC\textsubscript{M237}], where M237 indicates M2/M3 and M7. The statistical differences between the parameters of dose groups were tested with one-way or two-way analysis of variance (GraphPad Prism v4; GraphPad Software, Inc., San Diego, CA), and statistical significance was defined as p < 0.05. All data are presented to three significant figures. The geometric means of C\textsubscript{max} and AUCs and their 90% confidence intervals were determined from log-transformed values and used for comparisons between groups.

Results

SQV Absorption and Metabolism in Rat Jejunum. After a single-pass jejunal perfusion, SQV and its metabolites, M2/M3 and M7, were detected in the mesenteric plasma (Fig. 1). The plasma concentrations were generally consistent within the 30-min drainage in each animal, demonstrating that the average plasma concentrations reflect the extent of SQV intestinal absorption. Minimal unexpected drug loss occurred during the experiments as the recovery of total radioactivity from the outlet perfusate and intestinal tissue was approximately 100% of perfused radioactivity.

When perfused alone, SQV absorption in the jejunal region was
extremely low (average plasma concentration <25 ng-Eq/ml and $F_a <0.1\%$) (Table 1). Moderate amounts of its hydroxylated metabolites, M2/M3 and M7, were also detected with $F_{\text{gut}}$ of 78\%. When perfused with GF120918, SQV absorption increased by ~24-fold, whereas MK only increased SQV $F_a$ by ~2-fold. The rank order of $F_a$ was SQV + GF > SQV + GF + MDZ > SQV + GF + MK > SQV + MDZ > SQV + MK > SQV. All GF groups (SQV + GF, SQV + GF + MK, and SQV + GF + MDZ) showed statistically higher plasma concentrations and $F_a$ than those in the control group ($p < 0.05$) (Table 1). Based on the rank order, a synergic effect between GF120918 and MK571 or MDZ was not observed. As expected, MDZ minimized SQV intestinal metabolism ($F_{\text{gut}}$ of ~96\%).

**Oral Pharmacokinetics of SQV.** After oral administration to rats, SQV and its metabolites were detected in the portal circulation (Fig. 2). SQV plasma concentrations were low and highly variable with $C_{\text{max}}$ of 0.0681 μg/ml (CV% = 110\%) and AUC$_{\text{Cmax}}$ of 6.83 μg·min/ml (CV% = 102\%) (Table 2). A large apparent volume of distribution (V$_{\text{d}/F}$) of SQV (539 liters/kg) and a moderate apparent clearance (CL/F) (5.29 liters/min/kg) were observed. The elimination $t_{\text{1/2}}$ of SQV was 2 h. The $C_{\text{max}}$ and AUC of M237 were also low and highly variable with $F_{\text{gut}}$ of 78\% (Table 3). The $T_{\text{max}}$ values for the metabolites were similar to those for the parent compound.

Consistent with the in situ data, $C_{\text{max}}$ and AUCs of SQV in GF-treated groups were significantly higher than those for the non-GF treated groups (Table 2; Fig. 3). GF alone enhanced the $C_{\text{max}}$ and AUC of SQV by more than 20-fold, whereas MK571 and MDZ showed little effect (~3-fold increase in AUC) (Table 2). The rank order of SQV AUC was SQV + GF120918 > SQV + GF + MK > SQV + GF + MDZ > SQV + MDZ > SQV + MK > SQV. All GF-treated groups showed significantly higher $C_{\text{max}}$ and AUCs than those from the control group ($p < 0.05$). In addition, the variations of $C_{\text{max}}$ and AUCs in all GF120918-treated groups were much less than those in the non-GF120918-treated groups (CV%: 30–50\% versus 80–110\%). However, the CV% of SQV $C_{\text{max}}$ and AUCs were not improved by the dual inhibition regimens (26–31\% for GF alone versus 26–50\% for the dual inhibition groups). Synergism between GF120918 and MK571 or between GF120918 and MDZ was not observed. These results once again demonstrate that Pgp controls SQV oral absorption and Pgp-mediated transport is responsible for its highly variable oral bioavailability.

In the presence of the inhibitors, M237 AUCs were approximately proportional to the AUCs of the parent with exceptions of SQV + MK and SQV + MDZ. Because MDZ is a CYP3A inhibitor, lowered metabolite AUC with coadministration of SQV and MDZ was expected. For the SQV + MK group, decreased metabolite AUC may be caused by the limited data points in the terminal phase.

**In Situ and in Vivo Correlation.** To examine whether in vivo AUC measured from portal circulation can be used as a surrogate for the extent of oral absorption, a linear correlation test was performed (Fig. 4). The observed $F_a$ values in situ and the AUC values measured from portal vein-cannulated rats were highly correlated ($r^2 = 0.876$), suggesting a strong role of the intestine in the overall bioavailability of SQV.

**Discussion**

SQV, a potent first-in-class HIV protease inhibitor, has low and variable oral bioavailability (Roche Laboratories, 2001). To understand the roles of secretory transporters and metabolizing enzymes in SQV boosting regimens, we investigated SQV intestinal and oral absorption in situ using a single-pass intestinal perfusion technique and in vivo in portal vein-cannulated rats under specific chemical inhibition of Pgp, Mrp2, and CYP3A. The individual roles of Pgp, Mrp2, and CYP3A were elucidated by specific chemical inhibition using GF120918 (a potent Pgp and breast cancer resistance protein inhibitor) (Hyafil et al., 1993), MK571 (a specific inhibitor of Mrps) (König et al., 1999), and MDZ (an inhibitor and substrate of CYP3A) (Cummins et al., 2003). Because SQV is an inhibitor but not a substrate of breast cancer resistance protein (Gupta et al., 2004) and Mrp2 is the primary Mrp involved in SQV transport (Huisman et al., 2002), the modulating effects of GF120918 and MK571 were considered to be approximations of Pgp and Mrp2 inhibition.

The in situ single-pass rat jejunal perfusion and in vivo portal vein-cannulated rats were selected as the study systems to exclude or minimize the confounding influence of first-pass hepatic metabolism and allow for the direct study of SQV oral absorption. The in situ single-pass intestinal perfusion system preserves the functions of intestinal transporters and enzymes better than in vitro systems, yet it isolates intestinal absorptive functions from possible systemic and hepatic interferences. The use of the perfusion system serves two purposes: to confirm putative modulation by Pgp, Mrp2, and CYP3A on SQV oral absorption and to validate whether the portal vein-
cannulated rats can be used as an in vivo system for oral absorption mechanism studies. The in vivo pharmacokinetic data were used mainly to draw conclusions related to SQV oral absorption rather than its elimination pharmacokinetics because inhibiting CYP3A would minimize the role of the liver altering SQV oral bioavailability (F) and the disposition parameters (such as CL/F and Vd/F). Furthermore, the elimination t1/2 measured in portal circulation may or may not be the same as that measured in systemic circulation, depending on the completeness of oral absorption. Our results show that SQV plasma concentrations in the mesenteric (in situ) and portal (in vivo) circulation resulting from the modulation of Pgp, Mrp2, and CYP3A were highly correlated, providing direct evidence that drug concentrations in the portal circulation reflect the extent of SQV oral absorption. By minimizing the role of the liver, we were able to show that the role of the intestine in determining the oral bioavailability and first-pass clearance of SQV is significant.

As expected, in situ and in vivo SQV intestinal/oral absorption was low and highly variable, similar to literature observations in which rats were given 20 mg/kg and bioavailability was low (4%) and low and highly variable, similar to literature observations in which rats were given 20 mg/kg and bioavailability was low (4%) and highly correlated, providing direct evidence that drug concentrations in the mesenteric (in situ) and portal (in vivo) circulation resulting from the modulation of Pgp, Mrp2, and CYP3A were highly correlated, providing direct evidence that drug concentrations in the portal circulation reflect the extent of SQV oral absorption. By minimizing the role of the liver, we were able to show that the role of the intestine in determining the oral bioavailability and first-pass clearance of SQV is significant.

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variable (Shibata et al., 2002). Moderate amounts of metabolites (1 – F₀₃₀ = 20%) seen in the mesenteric and portal circulation indicate that SQV intestinal metabolism is a not significant factor in its low oral bioavailability. However, decreased metabolism formation by MDZ reconfirmed that CYP3A is responsible for the intestinal first-pass loss of the drug.

The significant effect of GF120918 on SQV F₀₃₀, Cₘₐₓ, and AUCs revealed that SQV oral absorption is controlled by the secretory efflux transporter Pgp and not by limited membrane permeability because of poor passive diffusion resulting from the equally poor physicochemical properties of SQV. The highly variable Cₘₐₓ and AUCs in non-GF-treated groups compared with the reduced variability of the GF-treated groups supports the concept that Pgp-mediated transport is a key factor for causing individual variation in SQV oral bioavailability. In contrast, Mrp2 appears to be an insignificant player in SQV oral absorption. When compared with other groups, Mrp2 inhibition (SQV + MK and SQV + GF + MK) appears to have caused a longer t½. Knowing that SQV is excreted mainly in the bile in rats (Pau-lusma et al., 1996) and Mrp2 is a major transporter to facilitate the biliary excretion of many drugs (Keppler et al., 1997; Williams et al., 1996) and Mrp2 is a major transporter to facilitate the biliary excretion of many drugs (Keppler et al., 1997; Williams et al., 1996), thus making Mrp2 a relevant model for transporter function, thus broadening the current scientific perspective on optimizing clinical anti-HIV boosting strategies.

In conclusion, our study results provide, for the first time, direct and explicit evidence that Pgp controls SQV oral absorption and that Pgp-mediated transport is a key factor for causing the low, yet highly variable, oral bioavailability of the drug. It was also found that intestinal Mrp2 and intestinal CYP3A play minor roles in SQV oral bioavailability. The differential roles of Pgp, Mrp2, and CYP3A in SQV oral absorption strongly suggests that boosting occurs by means of transient alterations not only in metabolizing enzyme function but also in transporter function, thus broadening the current scientific perspective on optimizing clinical anti-HIV boosting strategies.

References


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