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Differential Roles of Pgp, Mrp2 and CYP3A on Saquinavir Oral Absorption in Sprague-Dawley Rats

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Running Title:

The Roles of P-gp, Mrp2 and CYP3A in Saquinavir Boosting

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List of abbreviations:

SQV, saquinavir; Pgp, P-glycoprotein (ABCB1 or MDR1 for human, and abcb1 or Mdr1 for rodents); MRP2/Mrp2, multidrug resistance-associated protein 2 (ABCC2 or MRP2 for humans, and abcc2 or Mrp2 for rodents); Mrps, multidrug resistance-associated proteins in rodents (e.g., Mrp1 to Mrp9); CYP3A, cytochome P-450 3A (CYP3A4 for humans and CYP3A1/2 for rodents); GF, GF120918; MK, MK571; MDZ, midazolam; C_{SQV}, $C_{M237,}$ $C_{total,}$ the average plasma concentrations of SQV, M237 and total SQV related species in mesenteric circulation; Fa, fraction of absorbed; Cmax, maximum plasma concentration; Tmax, the time at Cmax; AUC, area-under plasma concentration-curve (to last measurable concentration time: AUC_{0-t}, or to infinity: $AUC_{0-\infty}$); Vd/F, apparent volume of distribution; CL/F, apparent total clearance; $t_{1/2}$, elimination half-life; F_{gut}, fraction escaping intestinal metabolism; M237, SQV metabolites #2, #3 and #7; RTV, ritonavir.

Abstract

The objective of this investigation was to differentiate the roles of Pgp, Mrp2 and CYP3A on saquinavir (SQV) oral absorption. Using single-pass jejunal perfusion (in situ) and portal vein-cannulated rats (in vivo), SQV absorption was studied under chemical inhibition of Pgp (GF120918), Mrp2 (MK571) or/and CYP3A (midazolam). Plasma concentrations of SQV and related metabolites were analyzed by LC-MS/MS. When dosed alone, SQV absorption was extremely low both in situ (F_a = 0.07%) and in vivo (Cmax = 0.068 μg/ml, AUC = 6.8 μg•min/ml). Co-administration of GF120918 boosted SQV absorption for more than 20 fold with decreased variation in AUCs (CV% = 30% vs. 100%). In contrast, co-administration of MK571 or midazolam increased the SQV absorption only 2-3 fold without improving the variation of AUCs. SQV oral absorption was not further improved when dosed with GF120918 and midazolam or GF120918 and MK571. The current results provide, for the first time, direct and explicit evidence that SQV's low oral absorption is controlled by a secretory transporter, Pgp, and not by limited passive diffusion due 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 in order to avoid therapy limiting drug-drug transporter and enzyme interactions.

Introduction

Saquinavir (SQV), saquinavir mesylate, N-tertbutyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide methanesulfonate, is a first-in-class and potent HIV protease inhibitor (PI) for the treatment of HIV infection (Roche Laboratories, 2001). Following oral administration, SQV pharmacokinetics in humans are characterized by low bioavailability with high individual variation (Figgitt and Plosker, 2000). In healthy volunteers, the oral bioavailability of SQV when dosed alone was about 4% with a CV% > 100% (Roche Laboratories, 2001). In humans, SQV undergoes extensive first-pass metabolism by CYP3A4 and its major route of excretion is through the bile (Roche Laboratories, 2001).

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 (Williams et al., 2002; Huisman et al., 2002), while 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 OATP-A (SLC21A3) (Su et al., 2004). In human liver and small-intestinal microsomes, SQV was metabolized mainly to mono-hydroxylated 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, intestinal excretion and tissue distribution has been shown in mdr1a knockout mice (Sparreboom et al., 1997; Schinkel et al., 1997a). In

humans, Pgp inhibition caused a significant decrease in digoxin intestinal excretion (Drescher et al., 2003), while rifampin-upregulated intestinal Pgp expression appeared to be responsible for a decrease in digoxin oral bioavailability (Greiner et al., 1999).

MRP2, primarily expressed 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 non-conjugated organic anions (Keppler et al., 1997; Williams, et al., 2002). Mrp2 deficiency, as seen in TR- rats or EHBR rats, results not only in decreased biliary secretion of organic anions and conjugates, but also in significantly reduced bile flow (Suzuki and Sugiyama, 2002; Paulusma et al., 1996). 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. Further, the oral absorption of grepafloxacin, a Mrp2 and Pgp substrate, was not significantly enhanced by the inhibition of Mrp2, however; biliary excretion was significantly reduced suggesting that differential roles for Pgp and Mrp2 in the intestine and liver (Naruhashi 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 dosed with a boosting agent, such as ritonavir (RTV), in order to improve SQV oral bioavailability and to reduce its individual variation (Buss et al., 2001; Plosker and Scott, 2003). Such boosted SQV regimens result in increased Cmax and AUC, reduced inter-subject variability and decreased elimination $t_{1/2}$ (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 $t_{1/2}$ of SQV in boosting regimens can not be fully explained by this hypothesis. Since RTV is a substrate and inhibitor of CYP3A4, Pgp and MRP2 (Williams, et al., 2002; Huisman, et al., 2001; Huisman, et al., 2002; Gutmann, et al., 1999), 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 (Sinko et al., 2004; Huisman, et al., 2001). 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 mainly excreted into the bile in humans (Roche Laboratories, 2001). Since SQV is a MRP2 substrate, MRP2 inhibition may also affect its pharmacokinetic behavior.

The present study was specifically designed to differentiate the roles of Pgp, Mrp2 and CYP3A on SQV oral absorption. In situ single-pass jejunal perfusion and portal-vein cannulated rats were used as study systems in order 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)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4aS,8aS)-quinoline-3(S)-carboxamide; Ro 31-8959) were given as gifts by Roche Laboratories (Nutley, NJ). GF120918 (*N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2 isoquinolinyl)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide, GF) and MK571 (3-(((3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)phenyl) ((3-(dimethylamino-3-oxopropyl)thio)methyl)-thio) propanoic acid, MK) were gifts from GlaxoSmithKline, Inc., (Research Triangle Park, NC) and Merck & Co., Inc. (West Point, PA),

respectively. The chemical structures of GF120918 and MK571 (sodium salt) can be found in the literature (Planting AS, et al, 2005) and Product Information provided by Cayman Chemical (www.caymanchem.com), respectively. Midazolam (MDZ, Versed®, 5 mg/ml, Roche Laboratories, Nutley, NJ) was purchased through the Rutgers University Health Center Pharmacy. Other reagents and solvents used are the highest grade commercially available. C18 solid phase extraction cartridges (SPEC•Plus•3ml•C18) were purchased from Ansys Diagnostics, Inc. (Lake Forest, CA).

Animals. Male Sprague-Dawley rats with or without portal-vein cannula, weighing 200-400g and 2-3 months of age, were purchased from Hilltop Lab Animals (Scottsdale, PA). The rats were used in accordance with the protocols (01-014 and 03-033) approved by the Institutional Review Board- Use and Care of Animal Committee, and housed in AAALAC accredited facilities at Rutgers University. All animals were allowed to acclimate for a minimum of 2-3 days before the studies were initiated. Food and water were provided *ad libitum*. Before experiments, all animals were fasted overnight with free access to water.

In situ perfusion studies. The single pass perfusion was performed following a method published in the literature (Johnson BM, et al., 2003) with modification. Briefly, rats were anesthetized by an intramuscular injection of the ketamine (80 mg/kg) and xylazine (10 mg/kg) cocktail. Through a midline incision, the small intestine was exposed and approximately 10 cm jejunum segment was externalized. The segment was cannulated with Teflon tubing at both proximal and distal ends of the segment. The inlet jejunal cannula was connected to an infusion pump (Harvard Apparatus, Holliston, MA). The perfusate containing 100 μM saquinavir with or without the inhibitor(s) in MES Ringer's buffer (pH 6.5) and with a trace amount of ^{14}C-saquinavir was perfused through the intestinal segment at a flow rate of 0.2 ml/min. The system

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was equilibrated for approximately 20 minutes prior to blood collection. The collective mesenteric vein for the segment was cannulated with polyethylene tubing for blood drainage. Before the mesenteric vein cannulation, a tail vein was cannulated using a 24G IV catheter, and the animal was heparinized (~90U/kg). Freshly collected blood from naive rats and normal saline (8:2, v/v) was infused via a tail vein catheter at 0.3 ml/min. The blood drained from the mesenteric vein catheter were collected at 5 min interval for approximately 30 minutes. During the experiment, a heating mat and a lamp were used to maintain animal's body temperature. The exposed jejunal segment was covered with a saline-soaked gauze and plastic film. Blood samples were centrifuged at ~ 1000 *g* for 10 min to harvest plasma. All plasma samples were stored at -80°C until analysis.

Six dosing regimen were studied: SQV alone, SQV + GF, SQV+MK, SQV+MDZ, SQV + GF + MK or SQV + GF + MDZ. Each group consisted 3 or more rats. The concentrations of the inhibitors in the perfusate were 20 μM (GF) and 100 μM (MK and MDZ). Selection of SQV concentration at 100 μM, 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); Ki values of MK571 in various MRP2 overexpressed cell lines were < 15 μM (Leier et al., 2000; Shen et al., 1996); MDZ at 50 μM inhibited more than 80% metabolite formation of SQV in human intestinal microsomes (Fitzsimmons and Collins, 1997). To assure maximal inhibition, GF120918 at 20 μM, MK571 at 100 μM and midazolam at 100 μM were used in the perfusion studies.

Oral Dosing and Sampling. The SQV dosing solution (6 mg/ml) was prepared using a co-solvent mixture of ethanol-propylene glycol- distilled and deionized water (2:3:5,v/v/v). The

GF120918 dosing solution (1 mg/ml) used the same solvent system. The MK571 dosing solution (3 mg/ml) was prepared with 1% Tween-80 in a mixture of DMSO, ethanol, propylene glycol and saline (10:18:27:45, v/v/v/v). All solutions were prepared freshly before dosing.

The co-solvent of ethanol-propylene glycol-water has been commonly used as a dosing vehicle for SQV and GF1201918 in humans and animals (Kempf, et al., 1997; Sinko, et al., 2004). Addition of 1% Tween 80 and 10% DMSO was necessary for dissolving MK571 and preventing MK571 from precipitating when mixed with SQV and GF120918. All cosolvents in the dosing vehicles represent the minimal amounts necessary to achieve solubilization, and the percentage of each component was within the recommended range for rats (Swindle and Adams, 1988).

Portal-vein cannulated rats were dosed with SQV at 20 mg/kg via oral gavage. GF120918 (3 mg/kg), MK571 (14 mg/kg) or MDZ (10 mg/kg) were co-administered with SQV. The animals were divided into 6 groups (n = 3-7 rats/group) and dosed with SQV alone (control), SQV + GF, SQV+MK, SQV+MDZ, SQV+GF+MK or SQV+GF+MDZ. The dosing volume was ~2 ml. Blood samples (0.5 ml) were collected before (pre-dose) 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 saline containing 50 U heparin to compensate for blood loss and to prevent the catheter from clogging. At the end of the study, animals were euthanized using an intravenous dosing of pentobarbital at ~100 mg/kg. Plasma was harvested after centrifugation at approximately 1000 *g* for 10 min. All samples were stored at -80ºC until analysis.

The selection of 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). Based on the mass ratios between

SQV (100 μM) and the inhibitors (GF120918, 20 μM; MK571, 100 μM; and MDZ, 100 μM), a dosage 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 plasma samples were mixed with 20 μl internal standard (quinidine, 2 μg/ml) and 0.5 ml 0.18 M ammonium acetate (pH 6.8) before extraction. After conditioning the C18 SPE columns (SPEC•Plus•3ml•C18, Ansys Diagnostics, Inc., Lake Forest, CA) with 1 ml methanol and 1 ml deionized and distilled water, plasma samples were loaded onto the columns, washed with 1 ml water, and then eluted with 0.25 ml methanol twice. The eluate (10 μl) was injected directly onto a liquid chromatographic tandem mass spectrometry (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, San Jose, CA). SQV and quinidine (internal standard, IS) eluted from a C18 column (Eclipse XDB-C18, Zorbax 2.1 x 50 mm, 3.5 μm, Agilent Technologies, Inc., 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 (IS) and 2.2 min (SQV). By electron spray ionization and under positive ion mode, SQV was detected at m/z 671.4→ m/z 570.4, its major metabolites (M2, M3, and M7, where M2 and M3 are stereo-isomers. Fitzsimmons and Collins, 1997) were at m/z 687.4 → m/z 568.4 (M7) and at m/z 687.4 → m/z 586.4 (M2/M3, isomers eluted as a single peak), and quinidine was at m/z 325.2→ m/z 307.1 and m/z 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. Due to unavailability of purified metabolites, the plasma concentrations of M2/3 and M7 were estimated using SQV calibration curves and denoted as SQV- equivalent concentrations (e.g., ng-eq/ml). 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 parameter for the extent of SQV intestinal absorption. Since M2, M3 and M7 account for approximately 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. Based on cumulative total drug amount (SQV+M237) in the blood (SQV blood/plasma concentration ratio ~1), fraction absorbed (Fa) was determined as a fraction of perfused drug amount. The fraction of dose that survived from intestinal metabolism (F_{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 (Cmax), the time to maximum concentration (Tmax), area-under-plasma concentration curve to the last measurable concentration (AUC_{0-t}) or to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$), apparent volume of distribution (Vd/F) and apparent clearance (CL/F) were analyzed using a non-compartmental analysis (WinNonlin Professional v4.1, Pharsight Co., Mountain View, CA). The fraction of dose that survived from intestinal metabolism (F_{gut}) was estimated from the AUC ratios between the parent and total SQV related compounds: $F_{gut} = AUC_{SQV}/(AUC_{SQV}+AUC_{M237})$, where M237 is the abbreviation of M2/M3 and M7. The statistical differences between the parameters of dose groups were tested with one-way or two-way ANOVA (GraphPad Prism v4, GraphPad Software, Inc., San Diego, CA) and statistical

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significance was defined as $p < 0.05$. All data are presented to 3 significant figures. The geometric means of Cmax 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. Following a single pass jejunal perfusion, SQV and its metabolites, M2/M3 and M7, were detected in the mesenteric plasma (Figure 1). The plasma concentrations were generally consistent within the 30-minute 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 since 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 Fa < 0.1%, Table 1). Moderate amounts of its hydroxylated metabolites, M2/M3 and M7, were also detected with F_{gut} equal to 78%.

When perfused with GF, SQV absorption increased for ~ 24 fold while MK only increased SQV Fa ~ 2 fold. The rank order of Fa 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 Fa than those in the control group ($p < 0.05$, Table 1). Based on the rank order, a synergistic effect between GF and MK or MDZ was not observed. As expected, MDZ minimized SQV intestinal metabolism (F_{gut} ~96%).

Oral pharmacokinetics of SQV. Following oral administration to rats, SQV and its metabolites were detected in the portal circulation (Figure 2). SQV plasma concentrations were

low and highly variable with Cmax of 0.0681 µg/ml (CV = 110%) and $AUC_{0-\infty}$ of 6.83 µg•min/ml (CV = 102%) (Table 2). A large apparent volume of distribution (Vd/F) of SQV (539 L/kg) and a moderate apparent clearance (CL/F) (5.29 L/min/kg) were observed. The elimination $t_{1/2}$ of SQV was approximately 2 hrs. The Cmax and AUC of M237 were also low and highly variable with F_{gut} being 78% (Table 3). The Tmax of the metabolites were similar to those for the parent compound.

Consistent with the in situ data, Cmax and AUCs of SQV in GF treated groups were significantly higher than the non-GF treated groups (Table 2 and Figure 3). GF alone enhanced the Cmax and AUC of SQV for more than 20 fold while MK and MDZ showed little effect (< 3 fold increase in AUC) (Table 2). The rank order of SQV AUC was SQV+GF > SQV+GF+MK > SQV+GF+MDZ > SQV+MDZ > SQV+MK > SQV. All GF treated groups showed significantly higher Cmax and AUCs than those from the control group ($p < 0.05$). In addition, the variation of Cmax and AUCs in all GF treated groups were much less than the non-GF treated groups (CV: 30-50% vs. 80-110%). However, the CV% of SQV Cmax and AUCs were not improved by the dual inhibition regimens (26-31% for GF alone vs. 26-50% for the dual inhibition groups). Synergism between GF and MK or between GF 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. Since MDZ is a CYP inhibitor, lowered metabolite AUC with co-administration 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 conducted (Figure 4). The observed Fa 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). In order 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 GF, (a potent Pgp and BCRP inhibitor, Hyafil et al., 1993), MK (a specific inhibitor of Mrps, Konig et al., 1999) and MDZ (an inhibitor and substrate of CYP3A, Cummins et al., 2003). Since SQV is an inhibitor but not a substrate of BCRP (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 in order 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 PK data were mainly used to draw conclusions related to SQV oral absorption rather than its elimination pharmacokinetics since inhibiting CYP3A would

minimize the role of the liver altering SQV oral bioavailability (F) and the disposition parameters (such as CL/F, Vd/F). Furthermore, the elimination $t_{1/2}$ measured in portal circulation may or may not be the same as 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 where rats were dosed at 20 mg/kg and bioavailability was low (4%) and variable (Shibata et al., 2002). Moderate amounts of metabolites (1- F_{gut} = ~20%) seen in the mesenteric and portal circulation indicate that SQV intestinal metabolism is a not significant factor in its the low oral bioavailability. However, decreased metabolite formation by MDZ reconfirmed that CYP3A is responsible for the intestinal first-pass loss of the drug.

The significant effect of GF on SQV Fa, Cmax and AUCs revealed that SQV oral absorption is controlled by the secretory efflux transporter Pgp and not by limited membrane permeability due to poor passive diffusion resulting from the equally poor physico-chemical properties of SQV. The highly variable Cmax and AUCs in non-GF treated groups as compared to 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 t1/2. Knowing that SQV is mainly excreted in the bile in rats (Paulusma et al., 1996) and Mrp2 is a major transporter to facilitate the biliary excretion of many drugs (Keppler et al., 1997; Williams, et al., 2002), the prolonged $t_{1/2}$ could be related to decreased biliary excretion under Mrp2 inhibition. However, because of the limitation of portal-vein cannulated rats for characterizing disposition parameters, further investigation of the role of Mrp2 in SQV elimination with intravenous administration and in bile-duct cannulated rats may lead to more definitive conclusions.

Dual inhibition of Pgp and Mrp2 or Pgp and CYP3A did not further improve the oral absorption of SQV nor further increase the exposure levels of the drug when compared to Pgp inhibition alone. This may be explained by the dominating effect of Pgp on SQV intestinal absorption and the limitation of the test systems for minimizing the functions of the transporters and enzymes in the liver. This also demonstrates that intestinal Mrp2 and intestinal CYP3A play a minor role in SQV oral bioavailability.

Despite anatomical differences between humans and rats, the oral bioavailability of SQV in the two species is similar, both low (4%) and variable (Roche Laboratories, 2001; Shibata et al., 2002). Perhaps this is due to the structural and functional similarity between rodent Mdr1, Mrp2 and CYP3A and their human counterparts (i.e., MDR1, MRP2 and CYP3A4) (Schinkel, 1997b; Bogaards et al, 2000; Konig et al., 1999), thus making the rat a relevant model for investigating SQV oral bioavailability in humans. Therefore, the roles of Pgp, Mrp2 and CYP3A in SQV oral absorption and intestinal metabolism revealed in the present study may be applicable to their roles in SQV boosting therapy in humans.

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 its low and highly variable oral bioavailability. It was also found that intestinal Mrp2 and intestinal CYP3A play a minor role 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.

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Footnote

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Legends of Figures

Figure 1. LC-MS/MS chromatograms of saquinavir (1a) and its metabolites M2/M3 and M7 (1b) in mesenteric plasma following a single-pass infusion in rats with 100 µM saquinavir (SQV) for 20 minutes. SQV was detected at m/z 671.4→ m/z 570.4 (~2.2 min), M2 and M3 were detected as one peak at m/z 687.4 → m/z 586.4 (~ 0.9 min), and M7 was detected at m/z 687.4 → m/z 568.4 (~ 1.3 min).

Figure 2. Plasma concentrations of saquinavir (SQV) and its metabolites M2, M3, and M7 (M237) in portal-vein cannulated rats following oral administration of SQV at 20 mg/kg. Data presented are mean and standard errors, n = 7.

Figure 3. Cmax and AUC of saquinavir in potal-vein cannulated rats following oral administration of SQV at 20 mg/kg with or without Pgp, Mrp2 and CYP3A inhibitors. Data presented are mean and standard error, n = 3-7. Asterisk (*) denotes statistical difference from the control (SQV alone) ($p < 0.05$).

Figure 4. Linear correlation between total AUC (saquinavir and metabolites) in portal cannulated rats and measured Fa from in situ perfusion. The data presented are mean values from 3-7 rats/group. AUC = 89.9 (± 17) *Fa + 8.70 (± 13.8), r^2 = 0.876, Sy = 23.0, F = 28.3, df = 4, p = 0.0060. Dotted lines represent 95% confidence intervals.

Table 1. Absorbed saquinavir in the mesenteric circulation of male Sprague-Dawley rats following single pass jejunal perfusion at 100 μM (Mean ± SD, n = 3-4 rats/dose treatment).

	SQV	SQV +GF	SQV +MK	SQV +MDZ	SQV +GF+MK	SQV +GF+MDZ
C_{SQV} ng/ml	16.8 ± 17.6	763 [a] ± 516	37.6 ± 43.3	90.6 ±117.3	209 [a] ±55	233 [a] ± 81
C_{M237} ng-eq/ml	7.86 ± 5.58	141 [a] ± 56	8.56 ± 8.30	8.22 ± 13.9	68.8 [a] ± 19.1	83.3 [a] ± 42.2
C_{Total} ng-eq/ml	24.7 ± 20.0	901 [a] ± 570	46.0 ± 49.5	98.8 ± 131	278 [a] ± 37	316 [a] ± 44
Fa (%)	0.0702 ± 0.0465	1.69 [a] ± 1.17	0.145 ± 0.199	0.237 ± 0.243	0.630 [a] ± 0.132	0.819 [a] ± 0.151
F_{gut} (%)	77.6 ± 3.7	81.6 ± 5.4	78.4 ± 19.5	96.4 ± 4.5	72.7 ± 11.8	74.9 ± 15.0
n	3	4	4	3	3	3

[a] Statistically significantly higher than the control (SQV alone), $p < 0.05$.

Table 2. Pharmacokinetic parameters of unchanged saquinavir in male Sprague-Dawley rats with a portal-vein catheter following single oral administration of saquinavir at 20 mg/kg.

		SQV	SQV+GF	SQV+MK	SQV+MDZ	SQV+GF+MK	SQV+GF+MDZ
Cmax (μg/ml)	Mean ± SD	0.102 ± 0.113	1.85 ± 0.49 [a]	0.188 ± 0.171	0.472 ± 0.528	1.29 ± 0.54 [a]	1.64 ± 0.58 [a]
	Geo. mean [b]	0.0681	1.80	0.176	0.308	1.21	1.55
	90% CI [c]	0.0327 - 0.142	1.42 - 2.27	0.068 - 0.453	0.132 - 0.717	0.800 - 1.83	1.05- 2.29
Tmax (min)	Mean ± SD	15.0 ± 12.2	15.0 ± 5.8	46.0 ± 75.0	15.0 ± 5.8	30.0 ± 17.3	33.3 ± 23.1
AUC_{0-t} (μg•min/ml)	Mean ± SD	6.84 ± 7.21	143 ± 45 [a]	15.2 ± 11.8	26.2 ± 30.6	89.7 ± 30.4 [a]	72.9 ± 19.7 [a]
	Geo. mean [b]	4.86	138	13.7	16.8	86.3	71.0
	90% CI [c]	2.31-10.2	105 - 180	5.22 - 35.8	7.20 - 39.2	62.3 - 119	53.9 - 93.6
$AUC_{0-\infty}$ (μg•min/ml)	Mean ± SD	11.9 ± 12.1	157 ± 44 [a]	21.0 ± 16.0	27.8 ± 31.3	117 ± 64	74.4 ± 19.5 [a]
	Geo. mean [b]	6.83	152	17.8	18.7	107	72.5
	90% CI [c]	3.16 - 14.8	118 - 195	6.85 - 46.0	8.52 - 41.1	66.6 - 173	55.6 - 94.6
Vd/F (L/kg)	Mean ± SD	539 ± 536	21.8 [a] ± 13.6	696 ± 843	340 ± 362	77.9 ± 80.2	33.0 ± 10.0
CL/F (L/min/kg)	Mean ± SD	5.29 ± 6.21	0.137 ± 0.045 [a]	3.10 ± 4.17	1.38 ± 0.87	0.201 ± 0.085 [a]	0.283 ± 0.083 [a]
$t_{1/2}$ (min)	Mean ± SD	118 ± 99	98.5 ± 30.9 [d]	176 ± 37	141 ± 78	253 ± 180	80.6 ± 1.6 [d]
n		7	4	5	4	3	3

[a] Statistically significant from the control group (SQV), $p < 0.05$.
[b] Geometric mean
[c] 90% confidence interval of geometric mean
[d] Statistically significant from the SQV+MK group, $p < 0.05$.

Table 3. Pharmacokinetic parameters of the metabolites (M2/M3 and M7) [a] of saquinavir in male Sprague-Dawley rats with a portal-vein catheter following oral administration of saquinavir at 20 mg/kg (n = 3-7 rats/group).

		SQV	SQV+GF	SQV+MK	SQV +MDZ	SQV+GF+MK	SQV+GF+MDZ
Cmax (μg-eq/ml)	Mean ± SD	0.0405 ± 0.0676	0.431 ± 0.219[b]	0.046 ± 0.046	0.0435 ± 0.0484	0.351 ± 0.217	0.298 ± 0.273
	Geo. Mean	0.0171	0.390	0.0272	0.0240	0.313	0.191
	90% CI	0.0066-0.0444	0.254-0.599	0.0107-0.0693	0.0078-0.0756	0.182-0.537	0.0556-0.659
Tmax (min)	Mean ± SD	26.0 ± 19.5	20.0 ± 14.1	14.0 ± 5.5	15.0 ± 5.8	23.3 ± 15.3	13.3 ± 5.8
AUC_{0-t} (μg-eq•min/ml)	Mean ± SD	2.93 ± 4.89	43.9 ± 31.3	1.92 ± 2.22	2.13 ± 2.72	33.0 ± 29.6	15.6 ± 12.7
	Geo. Mean	1.11	33.1	0.850	0.828	25.5	11.7
	90% CI	0.378 - 3.28	14.9-74.0	0.236-3.06	0.167-4.12	11.4-57.3	4.56-30.2
F_{gut}[c] (%)	Mean ± SD	77.9 ± 21.2	78.9 ± 9.7	88.3 ± 9.5	93.7 ± 3.6	76.3 ± 10.2	83.2 ± 10.4
n		5	4	5	4	3	3

[a] The parameters were determined based on the concentrations of metabolites equivalent to SQV.

[b] Statistically significant from the control group (SQV), $p < 0.05$.

[c] Estimated from AUC_{0-t} of the parent and metabolites (M2/3 and M7, abbreviated as M237): $F_{gut} = AUC_{SQV}/(AUC_{SQV}+AUC_{M237})$.

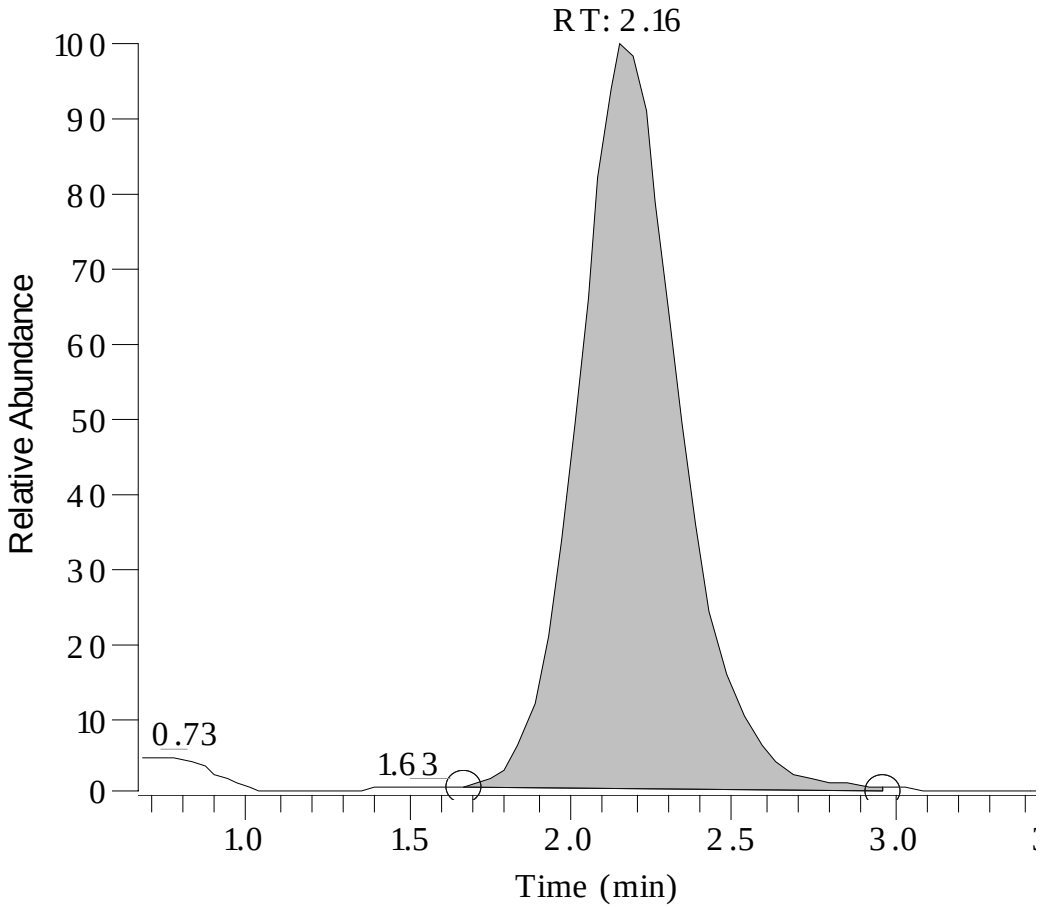

Figure 1a

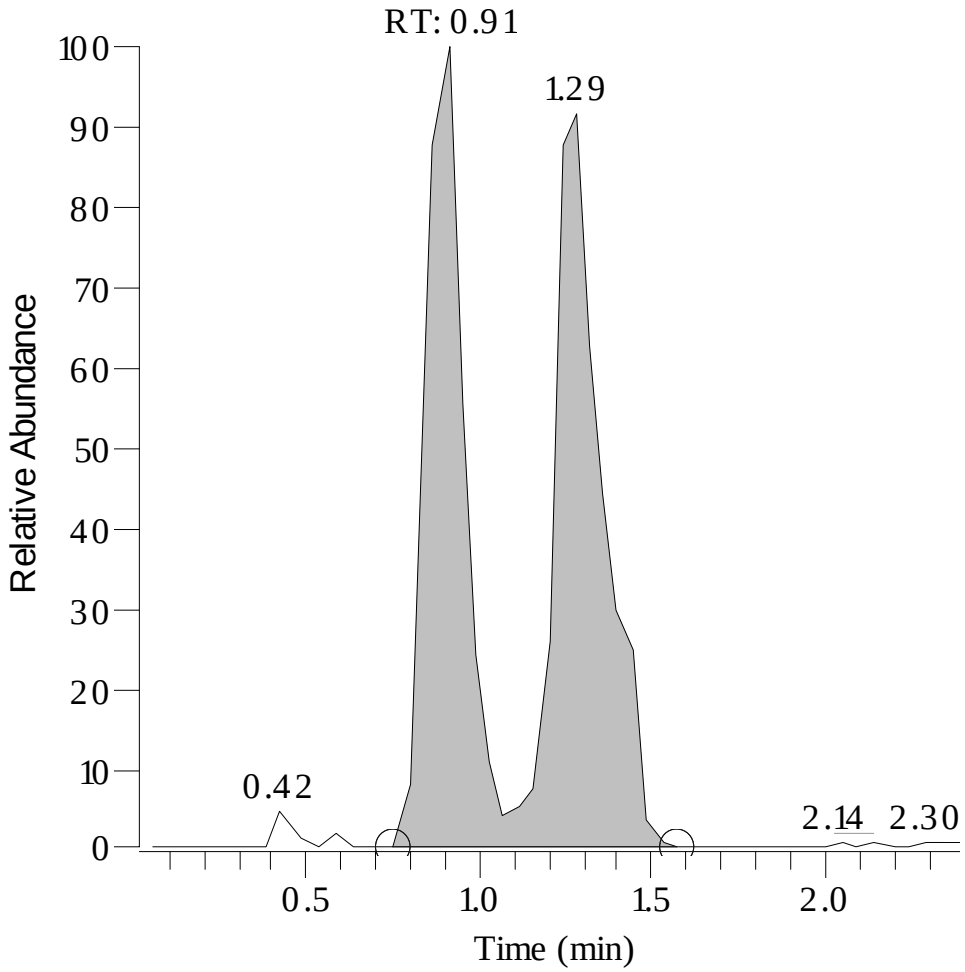

Figure 1b

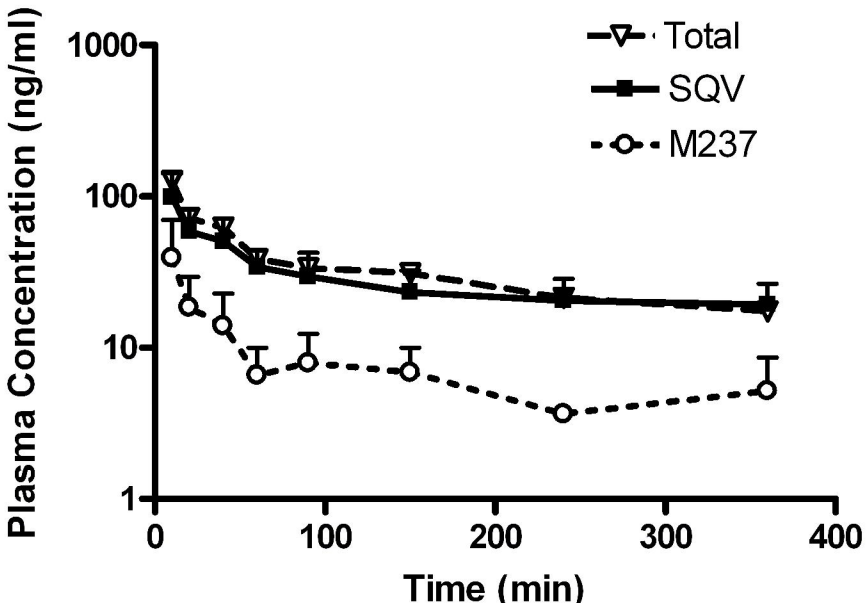
Plasma Concentration (ng/ml)
1000
100
10
1
0
100
200
300
400
Time (min)
Total
SQV
M237

Figure 2

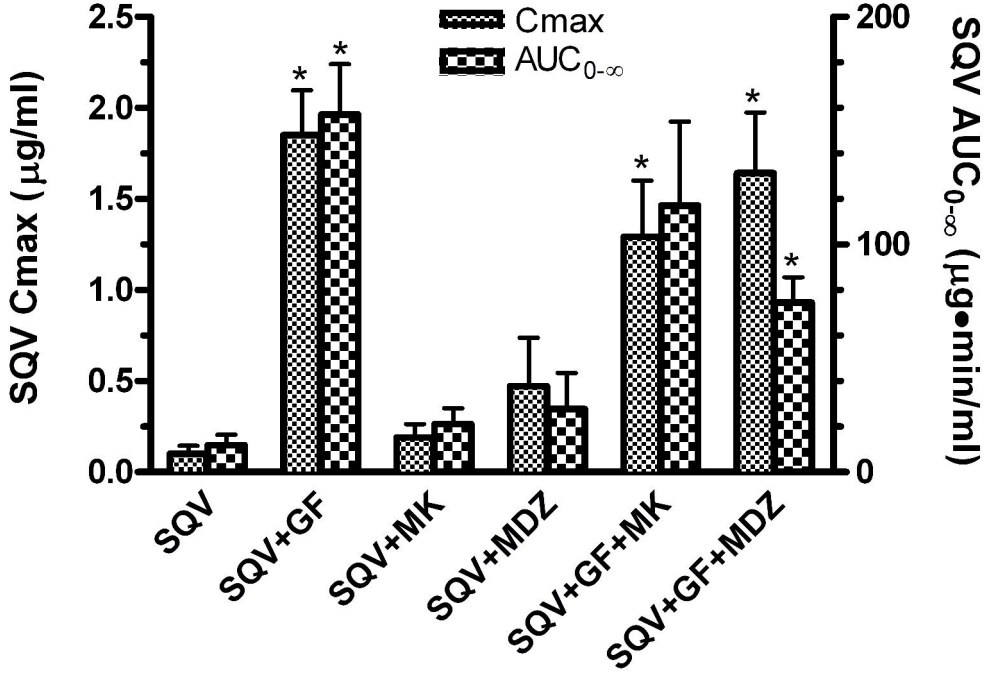
Cmax
AUC0-∞
SQV Cmax (μg/ml)
SQV AUC0-∞ (μg•min/ml)
2.5
2.0
1.5
1.0
0.5
0.0
200
100
0
*
SQV
SQV+GF
SQV+MK
SQV+MDZ
SQV+GF+MK
SQV+GF+MDZ

Figure 3

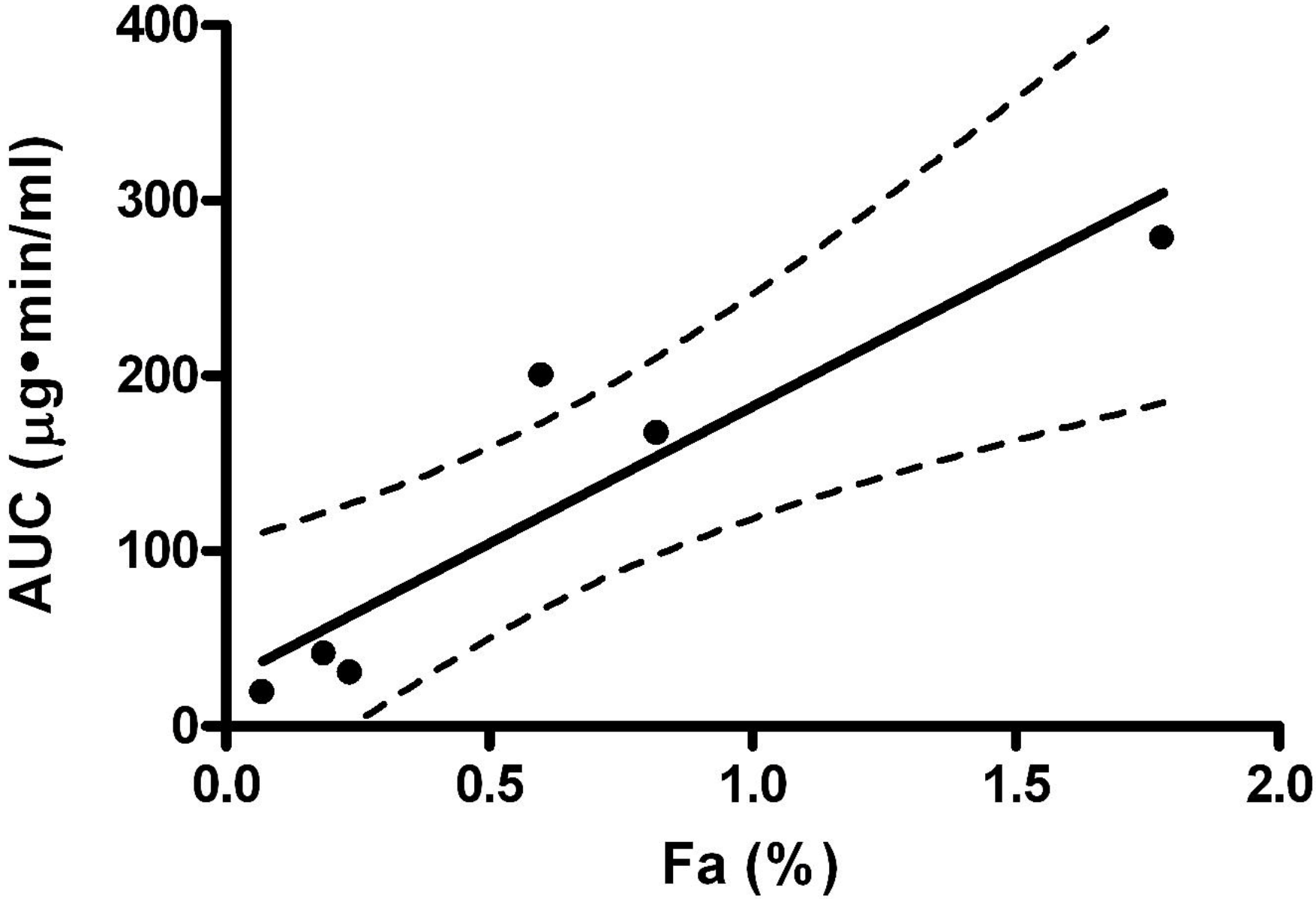

Figure 4