In Vivo Modulation of Intestinal CYP3A Metabolism by P-Glycoprotein: Studies Using the Rat Single-Pass Intestinal Perfusion Model

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ABSTRACT

P-Glycoprotein (P-gp) has been hypothesized to modulate intestinal drug metabolism by increasing the exposure of drug to intracellular CYP3A through repeated cycles of drug absorption and efflux. The rat single-pass intestinal perfusion model was used to study this interplay in vivo. N-Methyl piperazine-Phe-homoPhe-vinylsulfone phenyl (K77), a peptidomimetic cysteine protease inhibitor (CYP3A/P-gp substrate), and midazolam (CYP3A substrate) were each perfused through a segment of rat ileum alone and with the P-gp inhibitor N-4-{2-[1,2,3,4-tetrahydro-6,7-dimethoxy-2-isquinolinyl]-ethyl}-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine (GG918). Samples were obtained continuously from the outlet perfusate and the mesenteric vein at 5-min intervals for 40 to 60 min. The parent drug and two main metabolites of K77 (N-desmethyl and N-oxide) and midazolam (1-OH and 4-OH) were quantitated by liquid chromatography/mass spectrometry. K77 appearance in the mesenteric blood ($P_{\text{blood}} = 5 \pm 3 \times 10^{-6} \text{ cm/s}$) was increased 3-fold with GG918, whereas midazolam permeability ($P_{\text{blood}} = 1.1 \pm 0.3 \times 10^{-4} \text{ cm/s}$) was unchanged by GG918. K77 metabolites were preferentially excreted into the lumen, 4-OH midazolam was found equally in lumen and blood, and 1-OH was mainly excreted into blood. The extent of metabolism was estimated by calculating the fraction metabolized = $1 - P_{\text{blood}}/P_{\text{lumen}}$ and the extraction ratio (ER) determined from the direct measurement of known metabolites as ER = sum metabolites$_{\text{ur}}$/ (sum metabolites$_{\text{all}}$ + drug in blood). When P-gp was inhibited, the fraction metabolized for K77 was decreased (95 to 85%) and the ER tended toward a decrease, whereas no differences in either parameter were observed for midazolam (not a P-gp substrate). These data support a role for P-gp in modulating the extent of intestinal metabolism in vivo by controlling drug access to the enzyme.

Intestinal drug efflux by P-glycoprotein (P-gp), the multidrug resistance transporter, is widely recognized as a major determinant for low or variable oral absorption and bioavailability (Benet et al., 1996; Wacher et al., 2001). P-gp is a member of the ATP-binding cassette transporter superfamily (ABCB1) and is located on the apical membrane of intestinal enterocytes where it can actively efflux drugs from the cell back into the intestinal lumen (Ambudkar et al., 1999). Cytochrome P450 3A4 (CYP3A4) is the major oxidative drug-metabolizing enzyme found in the intestine and is localized to the endoplasmic reticulum of the enterocytes (de Waziers et al., 1990; Kolars et al., 1994). There is remarkable overlap between both the substrates and inhibitors of CYP3A4 and P-gp (Wacher et al., 1995; Kim et al., 1999). Recently, it has been discovered that CYP3A4 and P-gp are coregulated through the nuclear receptor steroid and xenobiotic receptor/pregnane X receptor (Synold et al., 2001).

The extensive overlap in the substrate specificities, tissue localization, and coinducibility of P-gp and CYP3A4 has led to the hypothesis that these two proteins work together to protect the body from absorption of harmful xenobiotics, including drugs (Wacher et al., 1998). We have hypothesized that, due to the spatial localization of P-gp and CYP3A4 in the intestine, P-gp controls the access of the drug to the metabolizing enzyme and results in increased metabolism from prolonged exposure to the enzyme through repeated cycles of absorption and efflux (Benet et al., 1996; Wacher et al., 2001).

Several investigators have attempted to examine the in

ABBREVIATIONS: P-gp, P-glycoprotein; K02, K11002, morpholine-urea-Phe-homoPhe-vinylsulfone phenyl; K77, K11777, N-methyl piperazine-Phe-homoPhe-vinylsulfone phenyl; GG918, GF120918, N-4-{2-[1,2,3,4-tetrahydro-6,7-dimethoxy-2-isquinolinyl]-ethyl}-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine; IS, internal standard; HPLC, high-performance liquid chromatography; ER, extraction ratio.
terplay between P-gp and CYP3A in intestinal drug metabolism using in vitro intestinal models. Indinavir metabolism was examined across vitamin D₃-induced Caco-2 cells and it was found that more M6 (main metabolite) was formed per indinavir molecule transported when P-gp was active compared with when it was inhibited with cyclosporine (Hochman et al., 2000). Studies of indinavir metabolism in a rat jejunal perfusion found decreased M6 formation in the presence of ketoconazole (Li et al., 2002). Cyclosporine and ketoconazole are inhibitors of both CYP3A and P-gp; therefore, the decreased metabolism, although supportive, could not definitively be shown to be due to inhibition of drug transport. Johnson et al. (2001) have examined the dynamics between CYP3A and P-gp by measuring verapamil metabolism across excised rat intestine at increasing drug concentrations. When P-gp was active (at low verapamil concentrations) the cellular residence time of the drug was increased, resulting in increased metabolism compared with the results at higher verapamil concentrations (when CYP3A and P-gp were saturated). Although these studies lend support to the proposed hypothesis, they do not address the influence of P-gp on CYP3A metabolism under nonsaturating conditions.

In vivo rat studies have also been performed in an attempt to study the role of intestinal P-gp in drug absorption and metabolism. The bioavailability of K02, a novel cysteine protease inhibitor (CYP3A/P-gp substrate), was increased from 3 to 30% in rats dosed with oral ketoconazole, with no corresponding change in the i.v. clearance (Zhang et al., 1998). A similar study performed with digoxin in rats showed that oral ketoconazole increased digoxin bioavailability from 68 to 84% but also altered the i.v. elimination of digoxin (Salphati and Benet, 1998). Lin et al. (1999) found that induction of CYP3A and P-gp in the rat using dexamethasone resulted in greater intestinal metabolism of indinavir than predicted based only on induction of CYP3A, suggesting that coinduction of P-gp was responsible for increasing the apparent metabolism beyond that expected. However, in the previously mentioned studies, only substrates, inhibitors, and inducers that affected both CYP3A and P-gp were used; therefore, true differentiation of the relative importance of CYP3A and P-gp on intestinal drug metabolism remains elusive.

Using relatively selective substrates and inhibitors of CYP3A and P-gp, we have recently demonstrated in CYP3A4-transfected Caco-2 cells that inhibition of P-gp (and not CYP3A) can decrease the extent of metabolism of a dual CYP3A and P-gp substrate even under nonsaturating conditions (Cummins et al., 2002). The goal of the current study was to determine whether the same phenomenon could be observed in an in vivo system, the rat single-pass intestinal perfusion with mesenteric cannulation. This system allows the in vivo determination of intestinal metabolism without concern for confounding effects from hepatic first-pass metabolism. Two substrates were tested: K77, a novel cysteine protease inhibitor and dual CYP3A and P-gp substrate (Cummins et al., 2002), and midazolam, an anesthetic agent that is a substrate of CYP3A only. Compounds were perfused alone and with the P-gp inhibitor GG918 to elucidate the role of P-gp in influencing intestinal metabolism.

Materials and Methods

Materials. K77 (K11777; N-methyl piperazine-Phe-homoPhe-vinylsulfone phenyl) and K02 (K11002; morpholine-urea-Phe-homoPhe-vinylsulfone phenyl) were kindly provided by Axys Pharmaceuticals (South San Francisco, CA). Midazolam and 1-OH midazolam were obtained from F. Hoffmann-La Roche (Nutley, NJ). Flurazepam, phenol red, and the ketamine/xylazine solution were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade and obtained from Fisher Scientific Co. (Santa Clara, CA).

Animals. Male Sprague-Dawley rats (300–350 g) were obtained from B&K Universal (Fremont, CA) and maintained on a 12-h light/dark cycle at the UCSF Animal Care Facility and acclimatized for at least 5 days before the study. Animals used in the intestinal perfusion experiments were fasted overnight. Rats were anesthetized with a ketamine (80 mg/ml) and xylazine (12 mg/ml) solution at a dose of 0.1 ml/kg by intraperitoneal injection. Animals were placed on a 37°C heating pad during surgery and throughout the in situ intestinal perfusion. Donor blood was obtained from two to three animals per experiment (total of 35–40 ml) by cardiac puncture. Blood was diluted 5:1 with saline before infusion. All animal experiments were approved by the Committee on Animal Research of the University of California San Francisco.

Surgical Procedures. For animals undergoing in situ intestinal perfusion with mesenteric cannulation, three procedures were performed: jugular vein cannulation for infusion of donor blood, isolation of an ileal segment for drug perfusion, and cannulation of the mesenteric vein for continuous collection of blood (Singhal et al., 1998). Immediately after implanting the right jugular vein cannula (10 cm Silastic tubing 0.012-inches i.d. × 0.025-inches o.d.; VWR Scientific, Brisbane, CA), a 5-cm-long midline incision was made and the ileum was located by using the ileocecal junction as a point of reference. A 7- to 11-cm-long segment of ileum was chosen based on the pattern of the mesenteric venules that would provide an optimal site for mesenteric cannulation. Incisions were made at both ends of the intestinal segment using an electrocautery (Harvard Apparatus, Holliston, MA) and then gently flushed using prewarmed saline. Intestinal cannulas (TEFZEL tubing, 0.62-inches i.d. × 0.125-inches o.d., 2 cm long, glued at the top to PEEK tubing; Western Analytical Products, Murrieta, CA) were inserted at each end and ligated using 3-0 silk suture. The mesenteric venules draining to the mesenteric vein to be cannulated that did not originate from the isolated intestinal segment were tied off using 4-0 silk suture. The tissue covering the top of the mesenteric vein to be cannulated was gently removed with tweezers. Two pieces of 4-0 silk suture were carefully placed underneath the vein at the desired cannulation site. A heparin solution (0.5 ml of 72 U/ml) was injected into the jugular vein 10 min before the mesenteric cannulation. Blank perfusion buffer or buffer containing 5 μM GG918 (for inhibition studies) was infused into the intestinal segment during this time at a rate of 0.2 ml/min from a syringe pump (22” pump; Harvard Apparatus) to test the free flow of solution across the segment. Stainless steel tubing was placed between the infusion syringe and the inlet cannula and at the exit of the outlet cannula to facilitate sample collection. Mesenteric cannulation was performed using the Angiocath 24G catheter (BD Biosciences, Franklin Lakes, NJ) fixed in place by tightly tying the silk sutures previously placed under the vein around the cannula. The cannula was cut just below the hub and attached to a 75-cm-long piece of polyethylene-50 (BD Biosciences) tubing that allowed blood to flow into vials placed approximately 25 cm below the plane of the animal. Donor blood was infused through the right jugular vein using a peristaltic pump (66” pump; Harvard Apparatus) and the rate was adjusted based on the outflow from the mesenteric blood (~0.4 ml/min). The experimental setup is illustrated in Fig. 1. At the end of the study, blank buffer was flushed through the segment and the length was measured from the end of the inlet cannula to the

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beginning of the exit cannula. Animals were sacrificed by performing a bilateral thoracotomy.

Single-Pass Intestinal Perfusion. The perfusion buffer consisted of 8.0 g/l NaH₂PO₄·H₂O and 11.3 g/l Na₂SO₄ with the pH adjusted to 7.4 using NaOH. All perfusion solutions contained 50 μM phenol red to act as a nonabsorbable marker for measuring water flux. The test compounds (K77 and midazolam) were infused individually at 50, 100, or 50 μM in the presence of 5 μM of the P-gp inhibitor GG918. All test solutions contained 0.5% dimethyl sulfoxide.

The intestinal perfusions were initiated by instilling drug solution from a 50-ml gastight syringe (Hamilton Co., Reno, NV) at 2000 g/min. The perfusion buffer and adding 300 μl of precipitating solution containing the internal standard (200 ng/ml flurazepam (IS) in 35:21:44 MeOH/acetonitrile/water). Samples were vortex mixed, centrifuged at 10,000 g, and 100 μl of the supernatant was injected. To determine midazolam metabolite levels in the perfusate, samples were processed as described above but no initial dilution of the sample was performed (60 μl of IS solution was added to 200 μl of sample). To quantitate midazolam metabolites in plasma, samples (150 μl) were precipitated with an equal volume of precipitating solution, vortex mixed, and centrifuged for 10 min at 10,000 g. The supernatant was filtered through a nylon filter (Alltech Associates) by centrifugation for 2 min at 10,000 g and transferred into HPLC vials from which 100 μl was injected into the LC/MS. To measure midazolam in plasma, samples had to be diluted 10-fold with water before precipitation due to the high permeability of the parent drug. All midazolam samples were placed in brown glass vials to prevent degradation of the light-sensitive internal standard during analysis.

Quantitation of Phenol Red for Water Flux Measurement. To correct for changes in the water flux across the intestine, phenol red was added to all perfusion solutions at a concentration of 50 μM. The quantitation of phenol red was performed colorimetrically with a dual wavelength endpoint reading (450-630 nm) using a 96-well plate reader (BTO2000 MicroKinetics reader; Fisher Scientific Co., Pittsburgh, PA). The unknown concentrations were determined from an external phenol red calibration curve prepared in perfusion buffer (linear for concentrations between 10 and 80 μM, r² = 0.995).

LC/MS Analysis of K77, Midazolam, and Their Metabolites. All samples were analyzed by HPLC/electrospray-MS in combination with an on-line column switching extraction step using an HP1100 LC connected to a 5989B mass spectrometer through a 59987A electrospray interface (all from Agilent Technologies Inc., Wilmington, DE). The solvents for the on-line column extraction step were delivered by a binary HPLC pump (PerkinElmer Instruments, Norwalk, CT) controlled by the external contacts of the HP1100 HPLC system. Analysis of K77 and its metabolites was performed as described previously (Jacobson et al., 2012) with modifications.

Briefly, the sample was loaded onto the precolumn (Hypersil ODS, 2 × 10 mm, 10 μm; Keystone Scientific, Bellafonte, PA) with 2 mM ammonium acetate at 6 ml/min for 1 min and then backflushed onto the analytical column (Capcell Pak CN, 4.6 × 35 mm, 5 μm; Phenomenex, Torrance, CA). The mobile phase consisted of 2 mM ammonium acetate and methanol. The column temperature was maintained at 40°C, the flow rate was 0.3 ml/min, and the total run time was 11.5 min. The following gradient was run: 0 min 60% methanol, 0.1 min 70% methanol, 4 min 80% methanol, and 5 min 90% methanol. Using selective ion monitoring, signals for [M + H]⁺ ions of K77 (m/z = 575, retention time 5.7 min), N-oxide K77 (m/z = 591, retention time 4.2 min), N-desmethyl K77 (m/z = 561, retention time 9.3 min), and the internal standard K92 (m/z = 562, retention time 4.6 min) were obtained. Because metabolite standards were not available for N-oxide K77 and N-desmethyl K77, an MS response factor was calculated for each using the ratio of peak areas for metabolite and parent peaks obtained by UV detection (λ = 230 nm) and comparing these to the ratio of peak areas of metabolite and parent obtained for the same sample when measured by MS. The MS response factor was obtained by dividing the UV metabolite to parent ratio by the MS metabolite-to-parent ratio. After adjusting the metabolite peak areas obtained by MS by their respective response factors, quantitation was performed using the K77 MS calibration curve.
Midazolam was analyzed using the same system, precolumn, and mobile phase but the analytical column was a Zorbax Eclipse XDB-C<sub>8</sub> (4.6 x 50 mm, 3.5 μm; Agilent Technologies Inc.). The total run time was 8 min. The following gradient was run: 0 min 60% methanol, 1 min 80% methanol, and 4 min 90% methanol. Using selective ion monitoring, signals for [M + H]<sup>+</sup> ions of midazolam (m/z = 326, retention time 5.4 min), 1-OH midazolam (m/z = 342, retention time 5.0 min), 4-OH midazolam (m/z = 342, retention time 4.8 min), and the internal standard flurazepam (m/z = 388, retention time 6.0 min) were obtained. Midazolam and 1-OH midazolam were quantitated in samples using external calibration curves for each. The MS peak area for 4-OH midazolam was adjusted by its response factor (calculated as described above for K77 metabolites) before quantitation using the midazolam MS calibration curve.

Data Analysis. The concentrations obtained from the perfusate were corrected for changes in the water flux at each time interval using eq. 1:

\[
\text{Conc}_{\text{corrected}} = \text{Conc}_{\text{measured}} \times \frac{[\text{phenol red}]_{\text{in}}}{[\text{phenol red}]_{\text{in}}} \tag{1}
\]

The permeabilities of K77 and midazolam across rat intestine were calculated based on the disappearance of the drug from the lumen (P<sub>lumen</sub>) as well as the appearance of drug in the blood (P<sub>blood</sub>) using the following equations (Singhal et al., 1998):

\[
P_{\text{lumen}} = \frac{Q}{2\pi rl} \ln \frac{C_i}{C_o} \tag{2}
\]

\[
P_{\text{blood}} = \frac{\Delta M_i}{2\pi rl < C > \Delta t} \tag{3}
\]

where r is the radius of the intestinal lumen (0.18 cm), l is the length of the segment (cm), Q is the flow rate of drug through the intestine (0.2 ml/min), C<sub>i</sub> is the concentration of drug at the start of perfusion (in the syringe), and C<sub>o</sub> is the steady-state concentration of drug exiting the lumen. The appearance of drug in the blood was measured as the steady-state amount of drug appearing in the blood with time (ΔM<sub>i</sub>/Δt) divided by the surface area of the intestine (2πrl) and the logarithmic mean concentration of drug in the lumen (<C>). The fraction of drug metabolized (Singhal et al., 1998) was estimated based on the difference between the appearance of unchanged drug in the blood and the disappearance of drug from lumen (assuming that the loss of drug from the lumen was solely due to absorption and intestinal metabolism):

\[
\text{Fraction metabolized} = 1 - \frac{P_{\text{blood}}}{P_{\text{lumen}}} \tag{4}
\]

The extent of metabolism was also determined from the quantitation of known CYP3A metabolites formed during the perfusion. An ER was calculated as the total amount of metabolites formed over the course of the experiment divided by the amount of unchanged drug appearing in the mesenteric vein plus the total amount of metabolites formed (assuming that the metabolites were formed from parent drug when inside the cell). The extraction ratio was normalized to the shortest segment used in these studies (7 cm) because more metabolism can occur over a longer intestinal segment (l):

\[
\text{ER} = \frac{\Sigma \text{metabolites perfusate + blood}}{\Sigma \text{parent drug perfusate + blood}} \times \frac{7}{l} \tag{5}
\]

Analysis of variance followed by multiple comparison testing using the Student-Newman-Keuls test was used to determine significance of data. The prior level of significance was set at p < 0.05. As the amount of drug absorbed (and metabolized) is dependent on the surface area of the intestinal segment, the drug and metabolite amounts were normalized to the calculated surface area from individual experiments when shown in the figures and table.

Results

Test of Drug Adsorption to Apparatus and Stability in Buffer and Intestinal Perfusate. No loss of K77 or midazolam was found when either drug was perfused individually through the intestinal perfusion apparatus, indicating that there was no significant adsorption to the tubing. The compounds were found to be stable in the perfusion buffer as well as intestinal perfusate at 37°C for at least 2 h (data not shown).

Stability of Midazolam in Plasma and Stability of K77 in Blood. To determine the best conditions for the collection of mesenteric blood samples, drug stabilities were tested in plasma and blood for midazolam and K77, respectively, and compared with their stabilities in water. No degradation of midazolam was observed over a 24-h time period tested in plasma (data not shown). K77 was found to be unstable in fresh blood with a half-life of 14 h; however, it was found that there was no loss of K77 when the blood was immediately frozen, stored at -80°C and then later thawed for analysis (data not shown). Although midazolam was also stable in blood (data not shown), the extraction of midazolam from plasma was simpler and therefore plasma was assayed for the studies with midazolam.

Absorption and Metabolism of K77 in the Rat Single-Pass Intestinal Perfusion Model. For intestinal perfusions performed with the dual CYP3A and P-gp substrate K77, samples were obtained from the outlet of the intestine as well as the mesenteric vein at 5-min intervals up to 60 min. The profiles of K77 disappearance from the intestinal lumen perfused alone at 50 μM or in the presence of the P-gp inhibitor GG918 are shown in Fig. 2A. The permeability of K77 based on the luminal disappearance of the drug was estimated using eq. 2 from the steady-state data (samples obtained between 45 and 60 min). The apparent permeability for K77 from perfusate data were relatively high (1.1 ± 0.7 × 10<sup>-4</sup> cm/s) and unchanged in the presence of GG918 (1.0 ± 0.2 x 10<sup>-4</sup> cm/s). In contrast, the appearance of K77 in the mesenteric blood (after a 50 μM dose) was increased 3-fold in the presence of GG918 (Fig. 2B). These data suggest that P-gp was effectively limiting the absorption of K77 across the intestine and was at least partially inhibited by 5 μM GG918. The permeability of K77 was calculated based on its appearance in mesenteric blood using eq. 3. P<sub>blood</sub> was 5 ± 3 × 10<sup>-6</sup> cm/s for 50 μM K77 and increased to 1.5 ± 0.7 × 10<sup>-5</sup> cm/s in the presence of GG918. These permeabilities were 20-fold lower than the estimated permeabilities from the luminal disappearance data indicating K77 was extensively metabolized in the intestine. In support of the importance of P-gp limiting drug absorption across the intestine, preliminary studies showed that 50 μM was the lowest concentration at which K77 could be detected in the mesenteric blood (lower limit of quantitation = 5 ng/ml). Furthermore, when a 2-fold higher concentration (100 μM) was tested, there was a non-linear increase (30% increase) in the appearance of unchanged K77 in mesenteric blood, indicating that P-gp was effectively pumping the drug back into the intestinal lumen and limiting drug absorption. These data are summarized for all groups in Table 1.
K77 metabolism was monitored with time and found to reach steady state in the intestinal perfusate after 40 min. N-desmethyl K77 and N-oxide K77 were found in the intestinal perfusate; however, only the N-desmethyl metabolite (at a low concentration) was detected in the blood, indicating preferential excretion of the metabolites toward the intestinal lumen (Fig. 3). At 50 μM, N-desmethyl K77 was observed in the mesenteric blood of only one in three animals, whereas it was found in the blood of all animals at the 100 μM dose. Figure 3 illustrates the appearance of K77 metabolites in perfusate and in blood at 50 μM alone and in the presence of GG918. Higher metabolite levels were observed in both perfusate and blood for K77 dosed with GG918. This general increase was consistent with higher drug levels crossing the membrane after inhibition of P-gp (Fig. 2B). The significant increase in the blood levels of N-desmethyl K77 when incubated with GG918 may indicate a change in the distribution pattern of the metabolite upon inhibition of P-gp because the metabolites are also thought to be transported by P-gp. The 100 μM concentration of K77 was tested to ensure that both the efflux transporters and drug-metabolizing enzymes were not saturated under the control condition (50 μM). Metabolite levels were increased 1.8-fold at the 100 μM dose compared with a 50 μM dose indicating that CYP3A was not saturated at the lower concentration tested (Table 1).

**Absorption and Metabolism of Midazolam in the Rat Single-Pass Intestinal Perfusion Model.** The intestinal perfusion of midazolam, a CYP3A substrate not transported by P-gp, was found to reach steady-state more rapidly than K77; therefore, blood and perfusate samples were obtained at 5-min intervals up to 40 min. Data obtained at steady state (between 25 and 40 min) were used in the calculation of midazolam permeabilities. The luminal permeability of midazolam was unchanged with GG918 (P_{\text{lumen}(control)} = 1.4 \pm 0.4 \times 10^{-4} \text{ cm/s} and P_{\text{lumen}(GG918)} = 1.5 \pm 0.2 \times 10^{-4} \text{ cm/s}) as was the blood permeability (P_{\text{blood}(control)} = 1.1 \pm 0.3 \times 10^{-4} \text{ cm/s} and P_{\text{blood}(GG918)} = 1.1 \pm 0.1 \times 10^{-4} \text{ cm/s}). Midazolam is not a P-gp substrate; therefore, GG918 was not anticipated to have an effect on its permeability across the ileum. The permeability values obtained for midazolam relative to K77 were plotted in Fig. 4 for comparison. The contrast in the relative permeabilities between the two drugs was only evident from the blood permeability measurement, P_{\text{blood}}, in which less unchanged drug was absorbed for K77.

Both known CYP3A-mediated midazolam metabolites, 1-OH and 4-OH midazolam, were found after incubation of 50 or 100 μM midazolam in rat ileum. The excretion patterns of both metabolites were monitored with time in perfusate and blood and are shown in Fig. 5. Unlike the preferential secretion of metabolites into the lumen as seen for K77, 4-OH midazolam showed no preference for directional secretion and 1-OH midazolam was mainly found in the blood (using plasma measurements) rather than the perfusate (Fig. 5). There was little difference in metabolite formation and excretion in the presence of GG918, with the exception of 1-OH midazolam in plasma, whereas although there was considerable variability, higher levels of metabolite were observed with GG918. The major metabolite observed after incubation of midazolam in the ileum was 1-OH midazolam. This contrasts with earlier findings demonstrating 4-OH midazolam as the major metabolite formed from rat liver microsomes (Ghosal et al., 1996). This result may reflect the limited presence of other P450 isoforms in the intestinal segment or the differential distribution of CYP3A isoforms in each of the organs (liver 3A1, 3A2; intestine 3A1 only) (Debri et al., 1995).

**Role of P-gp in Influencing CYP3A Drug Metabolism: Calculating the Extent of Metabolism.** The extent of metabolism for K77 and midazolam after rat single-pass intestinal perfusion was estimated using two measures: the fraction metabolized and the extraction ratio. The fraction metabolized is the commonly used method to estimate the extent of metabolism that is based on the assumption that all drug not accounted for through absorption was lost by metabolism (eq. 4). The second measure, the extraction ratio, is a more direct measure of metabolism because it includes in the calculation the amounts of known CYP3A metabolites detected after perfusion of the test drug (eq. 5). The extents
The fraction metabolized was unchanged for K77 at the two concentrations tested (95%) but was decreased to 85% \( (p < 0.05) \) in the presence of the P-gp inhibitor GG918 (Table 1).
These data support the proposed interplay between CYP3A and P-gp and show that the transporter can modulate the extent of metabolism. A similar trend was observed when we calculated the extraction ratio, a more direct measure of metabolism, where the ER decreased when P-gp was inhibited (from 49 to 37%; Table 1), but this difference did not reach statistical significance. In contrast, the extent of metabolism for midazolam (a CYP3A substrate not transported by P-gp) was unchanged in the presence of GG918 compared with control (27 versus 19%; Table 1). Very little of the known CYP3A-derived metabolites (1-OH and 4-OH midazolam) were found after midazolam perfusion compared with the amount of parent drug absorbed across the intestine. The concentration of midazolam tested did not seem to be saturating because more metabolites were observed at the higher concentration tested (total metabolites at 50 μM = 75 pmol/cm² versus at 100 μM = 187 pmol/cm²). The reasons for the low midazolam metabolism are unknown but could be related to the segment of the intestine chosen, because the ileum is known to have the lowest level of CYP3A compared with the jejunum in rats (Li et al., 2002) and humans (Paine et al., 1997). Nonetheless, the extraction ratio (calculated from the small amounts of metabolites that were observed) was unchanged for midazolam when P-gp was inhibited (control, 1.1 ± 0.3% versus GG918, 1.4 ± 0.9%; Table 1), indicating that GG918 was most likely not affecting other cellular processes related to metabolism at the concentration tested (5 μM).

The single-pass rat intestinal perfusion model provided an isolated in vivo system in which to study the role of the efflux transporter P-gp in controlling the extent of intestinal drug metabolism by CYP3A. These studies extend our previous findings obtained from an in vitro model of the intestine (CYP3A4-transfected Caco-2 cells) where we discovered that P-gp could increase the presystemic metabolism of dual CYP3A4/P-gp substrates by decreasing drug absorption and prolonging the exposure of the drug to the metabolizing enzymes (Cummins et al., 2002). Here, the absorption and metabolism of a dual substrate of P-gp and CYP3A4 (K77) and a substrate of CYP3A but not of P-gp (midazolam) were examined across a segment of rat ileum and transporter function was modulated using the P-gp inhibitor GG918.

From the appearance of drug in the mesenteric vein, we obtained permeability values (Pblood) for K77 and midazolam that corresponded to the relatively low and high permeabilities of these compounds seen in vitro. The permeability across CYP3A4-Caco-2 monolayers in the absorptive direction was 6.8 × 10⁻⁷ cm/s for K77 (10 μM) and 1.8 × 10⁻⁷ cm/s for midazolam (3 μM) (Cummins et al., 2002). In vivo, the Pblood of K77 and midazolam at 50 μM were 5 × 10⁻⁶ and 1.1 × 10⁻⁴ cm/s, respectively. Although the absolute permeability numbers differed between the in vitro and in vivo systems, the relative permeability ratios (Pmidazolam/PK77) of the two drugs were almost identical (26 in vitro and 22 in vivo). The transport activity of P-gp was inhibited in the perfusion study at a 5 μM dose of GG918. This was determined from the increased absorptive permeability of K77 with GG918 and the unchanged permeability of midazolam under similar conditions. A comparison of the formation of metabolites of K77 when dosed alone or in the presence of GG918 allowed for the assessment of P-gp’s role in modulating the extent of intestinal metabolism.

K77 metabolites were found primarily in the lumen of the intestine with small amounts of N-desmethyl K77 found in the mesenteric blood. This excretion pattern for metabolites was in agreement with data obtained from the CYP3A4-transfected Caco-2 cells, where a 6-fold preference for the metabolites to exit the apical side (corresponding to the lumen) was observed (Cummins et al., 2002). The formation of K77 metabolites increased after P-gp inhibition, consistent with the in vitro studies and rationalized by the higher levels of drug crossing the intestine after P-gp countertransport.

**Discussion**

Fig. 4. Permeabilities of K77 and midazolam across rat ileum alone (gray columns) or in the presence of the P-gp inhibitor GG918 (white columns) calculated using eq. 2 based on the disappearance of drug from the intestine (A) or using eq. 3 based on the appearance of unchanged drug in the mesenteric blood (B). Data shown are mean ± S.E.M. (n = 3). *, statistically different from control for that drug, p < 0.05.

Fig. 5. Formation of midazolam metabolites at 40 min after perfusion of midazolam (50 μM) alone (gray columns) or in the presence of 5 μM GG918 (white columns). 1-OH midazolam was preferentially excreted to the blood, whereas 4-OH midazolam was found at similar levels in the blood and lumen. Data shown are mean ± S.E.M. (n = 3).
was inhibited. The increased appearance of N-desmethyl K77 into the blood in the presence of GG918 may also be explained by slight inhibition of metabolite efflux transport into the lumen.

The distribution of midazolam metabolites across rat intestine contrasted with our findings from studies performed in CYP3A4-transfected Caco-2 cells. In the cell culture studies, 1-OH midazolam was preferentially excreted to the apical side of the cell even though inhibition experiments showed P-gp was not responsible for this phenomenon. In the perfusion studies, however, 1-OH midazolam was primarily found in the blood (in the presence and absence of GG918), whereas 4-OH midazolam was detected at similar levels in the intestinal lumen and the blood. Quantitation of 4-OH midazolam was not performed in the in vitro studies because of its low formation rate; therefore, its distribution could not be compared with that observed in vivo. One explanation for the disparity in the excretion patterns for 1-OH midazolam may be the presence of a transporter in rat intestine that is not present in the Caco-2 system. Alternatively, because other metabolites that are not substrates for P-gp have been found at higher levels in the blood after intestinal perfusion (e.g., 2',3'-dideoxyinosine, formed from 6-chloro-2',3'-dideoxyxypurine; Singhal et al., 1998), it is possible that excretion of metabolites from the enterocytes occurs by default into the blood unless the metabolites are substrates for apical efflux transport. The preference to enter the blood could be rationalized based on plasma protein binding and blood flow considerations (Chung et al., 2001). Additional studies looking at other dual P-gp/CYP3A substrates and exclusive CYP3A substrates would be required to determine whether this hypothesis is valid.

The \( K_m \) for metabolism of K77 to N-desmethyl and N-oxide K77 measured in human liver microsomes is 18 to 20 \( \mu M \) (Jacobsen et al., 2000). Preliminary studies testing different concentrations (10, 25, and 50 \( \mu M \)) of K77 in the intestinal perfusion found that the drug was only detectable in mesenteric blood after a 50 \( \mu M \) dose, likely due to the high efflux capacity of P-gp for K77. To ensure that metabolism and transport were not saturated at this concentration, perfusion studies were also performed at 100 \( \mu M \). The nonlinear increase in the appearance of K77 in the blood indicated that P-gp was still actively effluxing the drug from the enterocytes, and the 1.8-fold increase in metabolism indicated that CYP3A was not yet saturated under the control condition (50 \( \mu M \) K77; Table 1).

To determine whether P-gp was influencing the extent of intestinal metabolism in this in vivo model, the fraction metabolized was estimated from the permeability values obtained from blood and perfusate data. For the dual P-gp/CYP3A substrate K77, we found a significant decrease in the fraction metabolized when P-gp was inhibited (from 95 to 85%). In addition, no change in the fraction metabolized was observed for midazolam (our negative control) because it was not a P-gp substrate. These data support the hypothesis that P-gp was increasing the extent of metabolism of the drug when it was active, through repeated cycles of absorption and efflux. An alternate explanation for the decrease in the fraction metabolized for K77 is inhibition of CYP3A by GG918. This is unlikely, however, because GG918 has been shown to be considerably more selective for P-gp (\( K_i = 35 \text{nM} \); Wallstab et al., 1999) compared with CYP3A4 (\( K_i = 10 \text{\mu M} \); see Salphati et al., 2000) at www.aapspharmsci.org/scientificjournals/pharmsci/am_abstracts/2000/153.html]. Furthermore, in our studies, the fraction metabolized for midazolam actually increased slightly (although not significantly) in the presence of GG918, ruling out the possibility that GG918 was directly inhibiting CYP3A4 under these experimental conditions. When the extent of metabolism was calculated using the known CYP3A-derived metabolites incorporated into an extraction ratio, we found that for K77 a similar trend was observed as seen for the fraction metabolized, i.e., the ER decreased from 49 to 37% when P-gp was inhibited. No change in the midazolam ER was observed with P-gp inhibition, indicating that GG918 was not inhibiting CYP3A under the conditions tested. These data further support the role of P-gp in enhancing metabolism for dual CYP3A/P-gp substrates.

Single-pass intestinal perfusion experiments are often performed in the absence of mesenteric cannulation as a screening tool to estimate whether a new drug candidate will exhibit low or high permeability in vivo (Salphati et al., 2001; Sutton et al., 2002). From our in situ perfusion data, we found that the luminal permeabilities (\( P_{\text{lumum}} \)) for K77 and midazolam were very similar and relatively high (Table 1), suggesting that both compounds are highly absorbed. However, from the \( P_{\text{blood}} \) data we discovered that K77 actually had a very low permeability compared with midazolam, due to extensive metabolism and drug efflux. This difference between the two values occurs because permeability calculations based only on the disappearance of drug from the lumen cannot distinguish drug losses from absorption to those from metabolism (Dackson et al., 1992).

The fraction metabolized calculated for K77 was very high (95%) and this result assumes that all drug not accounted for by absorption is lost through metabolism. Although this may be the case, we did not obtain complete recovery for K77 by measuring only the known CYP3A metabolites (N-desmethyl and N-oxide). The recovery was 71% (control) and 75% (with GG918) and included the measurement of K77 bound to the intestine collected at the end of the experiment (~1.5% of the dose, control and with GG918). Other possible routes of loss include absorption of drug in the lymphatic system and metabolism to unidentified products. Without the use of radio-labeled drug, it would be very difficult to account for all drug lost; therefore, the actual value for fraction metabolized must be interpreted with caution. However, we believe the finding that the extent of CYP3A metabolism was decreased for K77 when P-gp was inhibited is valid, because the extent of metabolism with GG918 was compared against a control condition for the same compound and the extent of nonspecific tissue binding was unchanged with GG918. In addition, the same trend was observed for the K77 ER, which directly incorporates CYP3A metabolites.

In summary, these results demonstrated in an in vivo model that P-gp increased intestinal metabolism by CYP3A, because the extraction ratio of K77 (CYP3A/P-gp substrate) decreased when P-gp was inhibited but was unchanged for midazolam (exclusive CYP3A substrate). These are the first studies performed in an isolated in vivo system using specific substrates and inhibitors of P-gp and CYP3A to demonstrate this phenomenon. These data are also consistent with our in vitro studies using CYP3A4-transfected Caco-2 cells in which the K77 extraction ratio was decreased with P-gp inhibition.
Based on our combined in vitro and in vivo findings we conclude that intestinal drug metabolism is dependent not only on the activity of CYP3A in the intestine but also on the activity of P-gp, which modulates the access of the drug to the metabolizing enzyme.

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References


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