Unmasking the Dynamic Interplay between Intestinal P-Glycoprotein and CYP3A4

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Received September 6, 2001; accepted November 13, 2001 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Drug efflux by intestinal P-glycoprotein (P-gp) is known to decrease the oral bioavailability of many CYP3A4 substrates. We hypothesized that the interplay occurring between P-gp and CYP3A4 at the apical membrane would increase the opportunity for drug metabolism. To define the roles of P-glycoprotein (P-gp) and CYP3A4 in controlling the extent of intestinal absorption and metabolism, two substrates were tested. The transport, metabolism, and intracellular levels of N-methyl piperazine-Phe-homoPhe-vinylsulfone phenyl (K77, a cysteine protease inhibitor; P-gp and CYP3A4 substrate) and felodipine (CYP3A4 substrate only) were measured across CYP3A4-transfected Caco-2 cells in the presence of an inhibitor of CYP3A4 and P-gp, cyclosporine (CsA), or an inhibitor of P-gp and not CYP3A4, GG918 (N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine). The extent of metabolism was measured by calculating the extraction ratio (ER) across the cells, while accounting for intracellular changes occurring with P-gp inhibition. The (A)pical to (B)asolateral and B→A ERs for K77 were 0.33 and 0.06, respectively. These changed with GG918 to 0.14 and 0.12 and with CsA to 0.06 and 0.04. Felodipine ERs were similar in both directions, 0.26 and 0.24 (A→B and B→A), and were unchanged in the presence of GG918 but decreased with CsA (0.14 and 0.11). The K77 absorption rate was increased 5 and 4.2-fold in the presence of CsA and GG918, respectively, whereas no change was observed for felodipine absorption. The decreased A→B ER and increased absorption of K77 with GG918 suggest that P-gp influences the extent of drug metabolism in the intestine via prolonging the access of drugs to CYP3A4 near the apical membrane and decreasing transport across the cells.

Intestinal drug metabolism by cytochrome P450 3A (CYP3A) is increasingly being recognized as an important determinant in limiting drug bioavailability. CYP3A4 is the most prominent oxidative cytochrome P450 enzyme present in the human intestine (Watkins et al., 1987; Zhang et al., 1999), where it is localized to the columnar epithelial cells lining the intestinal lumen (Kolars et al., 1994). Despite the lower CYP3A4 content in the intestine relative to the liver, first-pass metabolism in the intestine by CYP3A has conclusively been shown to be important in the disposition of midazolam (Paine et al., 1996) and cyclosporine (CsA) (Kolars et al., 1991) from studies with anhepatic patients. Drug interaction studies performed with grapefruit juice [a suicide inhibitor of intestinal CYP3A (Schmiedlin-Ren et al., 1997a)] have also shown significant increases in the oral bioavailability of many CYP3A4 substrates including felodipine (Edgar et al., 1992).

Drug absorption can also be hindered by efflux transporters in the intestine. P-glycoprotein (P-gp) is a plasma membrane-bound drug efflux protein found primarily in drug-eliminating organs and presumably functions as a detoxifying transporter because it actively extrudes xenobiotics from the body (Ambudkar et al., 1999). In the small intestine, P-gp has been localized to the apical membrane of the intestinal epithelial cells (Thiebaut et al., 1987), consistent with a role in effluxing compounds back into the intestinal lumen. Pharmacokinetic studies of paclitaxel, digoxin, and CsA in mdr1a (−/−) knockout mice have revealed the importance of intestinal P-gp in limiting the oral bioavailability of these drugs (Schinkel et al., 1995; Sparreboom et al., 1997). Furthermore, in humans, intestinal P-gp was found to contribute to the variability in the pharmacokinetic

Financial support was provided by National Institutes of Health Grant CA72006 (to L.Z.B.) and Affymax Research Institute (to C.C.), as well as by an unrestricted gift from Amgen, Inc. This work was presented in part as an oral presentation at the American Society for Pharmacology and Experimental Therapeutics Meeting in Orlando, FL, March 31, 2001.

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ABBREVIATIONS: P-gp, P-glycoprotein; CsA, cyclosporine; TPA, 12-O-tetradecanoylphorbol 13-acetate; GG918, 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 carboxamine; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; ER, extraction ratio; K77, K11777; N-methyl piperazine-Phe-homoPhe-vinylsulfone phenyl; LC/MS, liquid chromatography/mass spectrometry.
properties of CsA (Lown et al., 1997b) and tacrolimus (Hashida et al., 2001) in organ transplant patients. Interestingly, most substrates of CYP3A4 are also substrates of P-gp, demonstrating the mutually broad selectivity of these proteins (Wacher et al., 1995). Coinduction of CYP3A and P-gp by rifampin was shown in human LS180 colon carcinoma cells (Schuetz et al., 1996) and human intestine (Kolars et al., 1992; Greiner et al., 1999) but not in rat liver (Salphati and Benet, 1998). Resolution of these findings is now possible with the knowledge that the nuclear receptor SXR/PXR, which can activate both CYP3A4 and P-gp, has species specific patterns of induction (Lehmann et al., 1998; LeCluyse, 2001; Synold et al., 2001).

The considerable overlap in the substrate selectivity, tissue localization, and coinducibility of CYP3A4 and P-gp has led to the hypothesis that these two proteins work together to coordinate an absorption barrier against xenobiotics (Benet et al., 1996; Wacher et al., 1998; Hall et al., 1999; Zhang and Benet, 2001). In the enterocyte, the spatial separation of P-gp, located on the apical plasma membrane, and that of CYP3A4, located on the endoplasmic reticulum, supports the idea that P-gp may control the access of drugs to intracellular metabolism by CYP3A4. Drugs absorbed into the intestinal epithelium can interact with P-gp and be actively extruded back into the intestinal lumen. If this process of diffusion and active transport occurred repeatedly, the circulation of the drug from the lumen to the intracellular compartment would potentially prolong the intracellular residence time of the drug, decrease the rate of absorption, and result in increased drug metabolism by CYP3A4 relative to the parent drug crossing the intestine. Support for this hypothesis was obtained from a simulation model (Ito et al., 1999) as well as experimental studies examining the metabolism and transport of indinavir in vitamin D₃-induced Caco-2 cells (Hochman et al., 2000).

The goal of this study was to identify the contribution of P-gp in determining the extent of CYP3A4 drug metabolism using reasonably selective CYP3A4/P-gp substrates and inhibitors. Two compounds were tested: one a dual P-gp and CYP3A4 substrate (K77: N-methyl pipерази-ne-Phe-homoPhe-vinylsulfonyl phenyl) and the other only a CYP3A4 substrate (felodipine). K77 was shown to be metabolized by CYP3A4 (Jacobsen et al., 2000) and is an excellent substrate for P-gp. Felodipine is metabolized by CYP3A4 and is known to undergo extensive gut metabolism (Lown et al., 1997a) but is not a substrate for P-gp efflux (Soldner et al., 1999). The substrates were administered with the inhibitors CsA (dual inhibitor of P-gp and CYP3A4) or GG918 (inhibitor of P-gp and not CYP3A4). As P-gp can drastically affect intracellular drug levels, the drugs were dosed at or below their respective CYP3A4 Kₘ values. With these relatively selective substrate/inhibitor studies we discovered that when P-gp alone was inhibited, the extent of metabolism of the dual CYP3A4 and P-gp substrate was decreased, but there was no change in the extent of metabolism for the exclusive CYP3A4 substrate. These data indicate that P-gp, when active, can work in concert with CYP3A4 to increase metabolism.

**Experimental Procedures**

**Materials.** K77 (K1177; Axys Pharmaceuticals, South San Francisco, CA), felodipine (Interchem Corp., Paramus, NJ), and GG918 (GF120918; GlaxoSmithKline, Research Triangle Park, NC) were kind gifts of the manufacturers. Falcon polyethylene terephthalate cell culture inserts (diameter 4.2 cm²) and Costar six-well plates were obtained from Fisher Scientific (Pittsburgh, PA). Sodium butyrate, 12-O-tetradecanoylphorbol 13-acetate (TPA), NADPH, and CsA were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade and were obtained from Fisher Scientific.

**Cell Culture Growth Conditions.** CYP3A4-transfected Caco-2 cells (Gentest, Woburn, MA; passages 4–5) were grown using Dulbecco’s modified Eagle’s medium containing 8.5 g/liter glucose, 25 mM HEPES, and 2.2 g/liter NaHCO₃, and nonessential amino acids (custom-made from the University of California San Francisco, Cell Culture Facility, San Francisco, CA) containing 15% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) and 100 μg/ml hygromycin B (Invitrogen, Carlsbad, CA). Cells were seeded onto polyethylene terephthalate cell culture inserts at a density of 300,000 cells/insert and grown to confluence for 13 to 14 days. Twenty-four hours prior to an experiment, the cell culture medium was replaced with growth medium containing 4 mM sodium butyrate and 100 nM TPA for CYP3A4 protein induction (Cummins et al., 2001).

**Metabolism Studies Using CYP3A4-Transfected Caco-2 Cell Homogenates.** Cells from 36 inserts were rinsed in phosphate-buffered saline (PBS) and scraped from the insert into a small volume of PBS using a cell scraper until they were visibly detached from the membrane. The suspended cells were collected in a 15-ml falcon tube and washed with PBS. The cells were pelleted by centrifugation at 1500g, resuspended in PBS, and washed two more times. After the final wash step, the cell pellet was resuspended in 900 μl of lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 20% glycerol, pH 7.4) and sonicated on ice for 3 × 5 seconds. The cell homogenates were aliquoted and frozen at −80°C until use. The protein concentration of the CYP3A4-transfected Caco-2 homogenates was determined using the Bradford assay with bovine serum albumin as the standard (Bio-Rad, Hercules, CA). Cell homogenates were thawed on ice to which 0.1 M Na+/K⁺ phosphate buffer and 1 mM NADPH was added (to give a final protein concentration of 0.25 mg/ml) along with the inhibitors CsA (final concentration 10 μM), GG918 (200 nM), or DMSO (solvent control). The mixture was preincubated for 3 min at 37°C, and the reaction was initiated by the addition of the substrate (K77 or felodipine in DMSO to a final concentration of 10 μM). The final volume of the incubation samples was 1 ml with all reactions containing 1.5% DMSO. The reactions were stopped at 10 min (K77) or 20 min (felodipine) with 400 μl of MeOH:0.2 M ZnSO₄ (7.3, v/v). Samples were vortexed and centrifuged at 4°C and 10,900g for 10 min. Supernatants were transferred to HPLC vials (Agilent Technologies, Palo Alto, CA) for analysis.

**Transport and Metabolism Studies across Intact CYP3A4-Transfected Caco-2 Monolayers.** Cell monolayers were preincubated in transport buffer (Hanks’ buffered salt solution containing 25 mM HEPES and 1% fetal bovine serum, pH 7.4) for 30 min at 37°C. Transepithelial electrical resistance values were measured across the monolayers using the Millipore Millicell (Bedford, MA) equipped with chopstick electrodes. The range of transepithelial electrical resistance values obtained over the course of the experiments was 252 to 575 ohm-cm² with an average value of 347 ± 30 ohm-cm² (n = 180). The study was initiated by adding the test compound (10 μM K77 or felodipine) to the donor compartment and transport buffer to the receiver compartment. In the experiments in which inhibitors were present, the same concentration of inhibitor was added to both sides of the monolayer (either 10 μM CsA or 200 nM GG918). All solutions contained 1% DMSO, and the final volume in each of the chambers was 1.5 ml on the apical side and 2.5 ml on the basolateral side. Samples were obtained from both sides of the monolayer at 1, 2, and 3 h (K77 and felodipine) or 12, 24, and 36 min (for felodipine early time points). At the first two time points, 150-μl samples were taken and then replaced with fresh donor or receiver solution to maintain the original starting volumes. After the last time point, the apical solutions were removed by suction and each filter was dipped.
two times each in three different beakers containing large volumes of ice-cold PBS. The inserts were inverted to remove residual liquid and intracellular measurements of parent drugs and metabolites were obtained by solubilizing each cell culture insert with 1 ml of ice-cold MeOH:H2O (7:3, v/v) and sonicing (in an ultrasonic bath) for 10 min. The homogenate was centrifuged for 5 min at 10,900g, and the resulting supernatant was analyzed by LC/MS. Each experiment was conducted in triplicate on two separate occasions but due to slight interday cell variations the data were analyzed separately from each experiment. The felodipine transport studies were performed under conditions of reduced light to minimize nonenzymatic oxidation to the metabolite. The felodipine data were corrected for the low level of felodipine metabolite present in the donor solutions at the start of the experiment.

**LC/MS Analysis of K77, Felodipine, and Their Respective Metabolites.** All transport and metabolite samples were analyzed by HPLC/electrospray-MS in combination with an on-line column-switching extraction step using an HP1100 HPLC connected to a 5989B mass spectrometer through a 59987A electrospray interface (all Agilent Technologies). The solvents for the on-line column extraction step were delivered by a PerkinElmer binary HPLC pump (Norwalk, CT) controlled by the external contacts of the HP1100 HPLC system. Analysis of K77 and its metabolites was performed as previously described (Jacobsen et al., 2000) with minor modifications. Briefly, the sample was loaded onto the precolumn (Hypersil ODS, 2 × 10 mm, 10 μm; Keystone Scientific, Bellafore, 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 following gradient was run: 0 min, 60% methanol; 6 min, 90% methanol. Using selective ion monitoring, signals for [M + H]⁺ ions of K77 (m/z = 575, retention time 5.3 min), N-oxide K77 (m/z = 591, retention time 4.1 min), and N-desmethyl K77 (m/z = 561, retention time 7.8 min) were obtained. As metabolite standards were not available for N-oxide K77 and N-desmethyl K77 (Fig. 1A), 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.

Felodipine and its main metabolite, dehydrofelodipine (Fig. 1C), were analyzed with the same system but were separated on a ZORBAX Eclipse XDB-C8 column (4.6 × 50 mm, 3.5 μm; Agilent Technologies) using an isocratic gradient of 2 mM ammonium acetate/methanol 25:75. The most abundant ion observed for felodipine was the acyl cation [M-46]⁺ (m/z = 338, retention time 6.6 min), resulting from α-cleavage at the ethyl ester. For the dehydrofelodipine metabolite, the positive ion [M + H]⁺ was most abundant (m/z = 382, retention time 5.8 min). As the metabolite of felodipine generates an aromatic ring, it was not surprising that the fragmentation patterns between parent and metabolite were different. Because of the additional aromaticity of the metabolite, the UV spectra of the parent and

Fig. 1. Metabolic pathways for CYP3A4-mediated metabolism of K77 (A) and felodipine (C). The inhibitors CsA (10 μM) and GG918 (200 nM) were tested in CYP3A4-transfected Caco-2 cell homogenates for their ability to inhibit the metabolism of 10 μM K77 (B) and 10 μM felodipine (D). Data shown represent the mean ± S.D. (n = 3).
metabolite were very different, and therefore, a response factor could not be calculated to quantitate the metabolite. As only the relative levels of metabolites were required for these studies, the felodipine MS calibration curve was used to quantitate the metabolite levels.

Data Analysis. Various equations were used to compare the extent of drug metabolism across CYP3A4-transfected Caco-2 cells. Equation 1 (Hochman et al., 2000) incorporates the sum of the amount of metabolites formed in all compartments divided by the amount of parent drug crossing into the monolayer.

\[
\text{Metabolite to parent ratio} = \frac{\sum \text{metabolites}_{\text{donor, receiver, intracellular}}}{\sum \text{parent}_{\text{receiver}}} \tag{1}
\]

Equation 2 (Fisher et al., 1999) is an ER because it includes in the denominator the sum of metabolites formed since they were originally derived from the parent drug. Overall, the equation yields the fraction of drug that was metabolized relative to the amount of drug that crossed into the receiver chamber.

\[
\text{ER} = \frac{\sum \text{metabolites}_{\text{donor, receiver, intracellular}}}{\sum \text{parent}_{\text{receiver}} + \sum \text{metabolites}_{\text{donor, receiver, intracellular}}} \tag{2}
\]

Equation 3 (Cummins et al., 2001) is a modified ER that includes the amount of intracellular parent drug levels in the denominator since the drug inside the cell could interact with the enzyme to produce metabolites.

\[
\text{ER} = \frac{\sum \text{metabolites}_{\text{donor, receiver, intracellular}}}{\sum \text{parent}_{\text{receiver}} + \sum \text{metabolites}_{\text{donor, receiver, intracellular}} + \sum \text{metabolites}_{\text{donor, receiver, intracellular}}} \tag{3}
\]

Analysis of variance followed by multiple pairwise comparisons using the Bonferroni t test were used to determine significance of data. The prior level of significance was set at \( p < 0.05 \).

Results

Specificity of CsA and GG918 for Inhibition in CYP3A4-Transfected Caco-2 Cell Homogenates. Due to the extensive overlap in the substrate specificity of CYP3A4 and P-gp, the inhibitors used in this study were tested to determine whether they would act as anticipated. GG918 is a known potent inhibitor of P-gp, and CsA is a known inhibitor of both P-gp and CYP3A4 (\( K_i \) for CYP3A4 = 4.9 \( \mu \)M; Wandel et al., 1999). Recently, Salphati et al. (2000) reported that GG918 was also capable of inhibiting 1-OH midazolam formation with a \( K_i \) of 10 \( \mu \)M. To ensure the specificity of GG918 for P-gp relative to CYP3A4, we tested the relative potency of CsA (10 \( \mu \)M) and GG918 (200 nM) for inhibiting CYP3A4-mediated K77 and felodipine metabolism in CYP3A4-transfected Caco-2 cell homogenates (Fig. 1, B and D). The inhibitors were tested at the same concentrations as those used in the transport studies. CsA decreased K77 and felodipine metabolite formation to 30 ± 1% and 37.2 ± 0.7% of control, respectively, whereas GG918 had no effect on the extent of metabolism for either K77 (105 ± 3%) or felodipine (98 ± 5%). The extent of inhibition obtained using the homogenates should be greater than that obtained from intact cells since the plasma membrane and transporter barriers are removed, allowing the inhibitors direct access to the enzyme.

Effect of P-gp and CYP3A4 on Parent Drug Transport and Intracellular Levels. Transport of 10 \( \mu \)M K77 across the CYP3A4-transfected Caco-2 cell monolayer was 9-fold greater in the basolateral to apical direction (B→A = efflux) compared with the apical to basolateral (A→B = absorptive) direction (Fig. 2A). This polarized transport was completely abolished in the presence of 10 \( \mu \)M CsA or 200 nM GG918, resulting in a decreased B→A transport and increased A→B transport relative to the control. As CsA is also known to inhibit the multidrug resistance-associated transporter MRP2 and GG918 can inhibit breast cancer resistance protein, the complete inhibition of bidirectional flux observed for K77 with both compounds (Fig. 2B) suggests that neither of these other transporters was involved in K77 excretion. Intracellular K77 was slightly greater from an apical dose compared with a basolateral dose (1.4-fold) and markedly influenced by P-gp inhibition (Fig. 2B). CsA and GG918 caused a 5- and 4.5-fold increase in intracellular K77 after an apical dose, and a 2.6-fold increase after a basolateral dose. This illustrates the pivotal role for P-gp in maintaining lower intracellular levels of drug. The differential amounts of intracellular drug after an apical versus a basolateral dose (under P-gp inhibition conditions) may indicate an inherent preference for drugs to penetrate from the apical side possibly due to the greater surface area of the apical microvilli.

The transport of 10 \( \mu \)M felodipine across CYP3A4-transfected Caco-2 cell homogenates is shown in Fig. 2B. CsA decreased K77 and felodipine metabolism in CYP3A4-transfected Caco-2 cells in the presence of the P-gp inhibitors 10 \( \mu \)M CsA (\( \square \)) and 200 nM GG918 (\( \bigcirc \)). Basolateral to apical transport is represented with solid symbols and apical to basolateral is represented with open symbols. B, intracellular levels of K77 after an apical or basolateral dose (measured at 3 h) in the presence of the inhibitors CsA and GG918. All data are presented as the mean ± S.D. (\( n = 3 \)).
20% donor drug lost) the transport of felodipine was also tested over 36 min (Fig. 3A, inset). No difference in the net transport of felodipine was observed between GG918 and CsA inhibition conditions, suggesting that a 36-min time frame was too short to see the effect of inhibiting CYP3A4 on net transport. The intracellular levels of felodipine were much higher compared with those observed for K77 (Fig. 3B, 36-min data). There was no effect of GG918 on the intracellular levels of felodipine from either direction, which is consistent with felodipine not being a P-gp substrate. A 7% increase in the intracellular felodipine level after an apical dose was observed with CsA at 36 min that became a 49% increase in intracellular felodipine after 3 h (data not shown), indicating that this higher steady state could be a result of inhibiting CYP3A4. The intracellular levels after an apical dose were at least 3.5-fold higher than the intracellular levels after a basolateral dose, again indicating a preference for the drug to penetrate from the apical side.

**Metabolite Exit Profiles from CYP3A4-Transfected Caco-2 Cells.** The metabolites formed after incubation of K77 with CYP3A4 (Fig. 1A) have been extensively studied using human liver microsomes and cDNA-expressed super-somes (Jacobsen et al., 2000). The two main metabolites, N-oxide K77 and N-desmethyl K77, were observed after incubation of K77 with CYP3A4-transfected Caco-2 cells as detected by LC/MS. As CYP3A4 is located inside the cells, metabolites are formed intracellularly; therefore, samples were obtained from both the apical and basolateral compartments at various times to determine the excretion pattern of the metabolites from the cells. The intracellular level of metabolites was determined for the last time point, allowing for a true measure of the total metabolites formed in that time frame. Metabolites of K77 showed very similar excretion profiles, and therefore, the sum of the two were plotted in Fig. 4. The metabolites were preferentially excreted toward the apical side regardless of whether the drug was dosed apically (Fig. 4A) or basolaterally (Fig. 4B). The exit profiles of the metabolites were linear with time but did not always intersect the origin after an apical dose. We have previously shown that CYP3A4 appears to be polarized within the intracellular space toward the apical side (Cummins et al., 2001), and this factor may be contributing to the burst in metabolism occurring immediately after an apical dose. In the presence of CsA or GG918 there was a significant decrease in the preferential excretion of metabolites toward the apical side. The preference for apical metabolite exit was almost completely abolished with both GG918 and CsA when K77 was dosed basolaterally (Fig. 4B). These data strongly suggest that P-gp is involved in mediating the directionality of K77 metabolite exit. The lower metabolite levels observed in the presence of CsA reflect its additional inhibitory role on CYP3A4. Although the metabolite preference to exit apical was still present with GG918 and CsA after an apical dose of K77 (Fig. 4A), the relative metabolite efflux ratio (exit A/exit B) decreased from 6.2-fold in the control to 2.3-fold in the presence of the P-gp inhibitors. The bias that still existed from an apical dose may again reflect the proximity of the enzyme to the apical membrane facilitating metabolite exit in this direction. In addition, although we have shown that other transporters such as MRP2 and breast cancer resistance protein are not affecting parent K77, we cannot rule out that these transporters could have a minor role in metabolite excretion. No N-desmethyl K77 was observed in the intracellular samples. The profile of intracellular N-oxide K77 in the presence of the inhibitors paralleled the profiles observed for the parent drug (Fig. 4C), in that higher levels of N-oxide K77 were observed with inhibition. The increased intracellular metabolites found in the presence of CsA were unexpected (considering CYP3A4 was partially inhibited), although this result may be due to the concurrent inhibition of P-gp allowing higher metabolite levels to accumulate in the cell.

The exit profiles of dehydrofelodipine (Fig. 5) were found to be linear with time. After an apical dose of felodipine, the metabolites were preferentially excreted toward the apical
but this apical secretion was not substantially altered by the presence of the P-gp inhibitors CsA (2.4-fold) and GG918 (3-fold) (Fig. 5A). Dehydrofelodipine that formed after a basolateral dose, however, was found almost at equal levels on both the apical and basolateral sides (Fig. 5B). Lower levels of metabolites were observed from both dosing locations when coincubated with CsA due to inhibition of CYP3A4. Because of the significant amount of metabolites formed during a 3-h incubation and the formation of secondary felodipine metabolites, the same experiment was run over a shorter time period (36 min) to attempt to maintain sink conditions throughout the experiment. The insets in Fig. 5, A and B, show the data obtained for metabolites formed at 36 min. There was a 6-fold preference for metabolite excretion toward the apical side after an apical dose, whereas this decreased to 2.8-fold from a basolateral dose. These data show that the differences in metabolite excretion are greater at the earlier time points and furthermore suggest the importance of CYP3A4 near the apical membrane when intracellular levels have not yet reached pseudo steady state. In both the 3-h and 36-min studies there was no effect of the inhibitors on the excretion of the metabolites, indicating no P-gp involvement (Fig. 5, A and B, insets). Intracellular dehydrofelodipine showed the expected trends for a compound metabolized by CYP3A4 and unaffected by P-gp (Fig. 5C). In the presence of CsA, significantly lower intracellular levels of metabolite were observed, consistent with inhibition of CYP3A4, and only minor differences were seen with GG918.

**Role of P-gp in Influencing CYP3A4-Mediated Drug Metabolism: Extraction Ratio Calculations.** Figure 6 depicts the extraction ratios obtained for K77 and felodipine calculated using eq. 3. The extraction ratio yields a measure of the extent of metabolism obtained relative to the amount of drug that was accessible to the metabolizing enzyme (i.e., drug that crossed the cell monolayer, drug inside the cell, and drug that was metabolized). A 33% extraction of K77 was obtained after an apical dose versus 6% from a basolateral dose (Fig. 6). That is, relative to the amount of drug that entered or crossed the cell, 5.5 times more was metabolized from an apical dose. This points to a role for P-gp in contributing to this process at the apical membrane by slowing absorption through the cell. In the presence of GG918 the apical extraction was decreased to 14% indicating that the extent of metabolism was lower when P-gp was not active. The opposite trend was observed from a basolateral dose (ER increased to 12%), indicating that decreased P-gp clearance resulted in an increase in the extent of metabolism. Coincubation with CsA also had the effect of decreasing the K77 extraction ratios from both sides but this effect was due in part to inhibition of CYP3A4. In contrast, felodipine extraction ratios (Fig. 6) were similar from both an apical and basolateral dose (26% versus 24%). These ERs were unchanged with GG918 but significantly decreased in the pres-
The presence of CsA, indicating that felodipine served as a negative control for P-gp activity. To highlight the importance of intracellular levels on determining the extraction ratio, several different equations were tested in calculating the extent of drug metabolism across CYP3A4-transfected Caco-2 cells (Table 1). Equation 1 is strictly a metabolite to parent ratio and yielded values illustrating that higher metabolite levels were generated after an apical dose compared with a basolateral dose. Equation 2 is an extraction ratio that gives the ratio of metabolites to the parent drug in the receiver as a fraction of the total amount of drug that crossed the cell, whereas eq. 3 incorporates the intracellular levels of parent drug in the calculation. The inclusion of intracellular levels was found to be important when comparing the directional dependence of the extraction ratios.

**Linearity of Metabolism with Increasing Parent Drug Concentrations in Intact Cells.** To determine whether the changes in the ERs observed upon inhibition of drug transport with GG918 could have been attributed to saturation of the enzyme (due to increased levels of intracellular drug with inhibition), we tested the linearity of metabolism in the cells with increasing concentrations of externally applied drug. Linear formation of metabolites was observed for K77 (Fig. 7A) between 5 and 50 μM (A→B, r² = 0.973; B→A, r² = 0.999) but appeared nonlinear above this range. Felodipine metabolism was linear over the concentration range tested (Fig. 7B) between 5 and 50 μM (A→B, r² = 0.994; B→A, r² = 0.997). More metabolites were formed after an apical dose compared with a basolateral dose for both K77 and felodipine. This difference may be due to the higher intracellular drug levels achieved after an apical dose and the proximity of the drug to the metabolizing enzyme.

**Fig. 5.** Felodipine metabolite (dehydrofelodipine) exit profiles after a 10 μM felodipine apical (A) or basolateral (B) dose. Felodipine was dosed alone (■, □) or in combination with the inhibitors 10 μM CsA (▲, △) or 200 nM GG918 (●, ○). Metabolites were found exiting both the apical (solid symbols) and basolateral (open symbols) membranes. The metabolites formed after dosing felodipine for 36 min are shown in the insets. In the insets of A and B, the black bars represent the metabolites exiting the apical side and the gray bars represent metabolites found on the basolateral side. Dehydrofelodipine found inside the cells at 36 min is shown in C. The level of metabolites found intracellularly after an apical dose was as much as 4-fold greater than after a basolateral dose. All data are shown as the mean ± S.D. (n = 3).

**Fig. 6.** Extraction ratios of K77 and felodipine after an apical or basolateral dose calculated using eq. 3. The effect of inhibiting P-gp on the ER is unmasked by incubating the substrate with GG918, whereas the dual effect of inhibiting CYP3A4 and P-gp on the ER is elucidated by incubation with CsA. Data are presented as the mean ± S.D. (n = 3).
TABLE 1
Comparison of methods to evaluate the extent of metabolism of K77 and felodipine across CYP3A4-transfected Caco-2 cells
K77 values were calculated from 3-h data, whereas felodipine measures were obtained from 36-min data. Data shown are the mean (and S.D.) from n = 3.

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<tr>
<td>Control</td>
<td>1.1 (0.2)</td>
<td>0.07 (0.01)</td>
<td>0.52 (0.05)</td>
</tr>
<tr>
<td>With CsA</td>
<td>0.13 (0.01)*</td>
<td>0.06 (0.01)</td>
<td>0.110 (0.007)*</td>
</tr>
<tr>
<td>With GG</td>
<td>0.36 (0.02)*</td>
<td>0.19 (0.02)</td>
<td>0.26 (0.02)*</td>
</tr>
<tr>
<td>Felodipine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17 (2)</td>
<td>3.7 (0.5)*</td>
<td>0.94 (0.04)</td>
</tr>
<tr>
<td>With CsA</td>
<td>7.0 (0.9)*</td>
<td>1.5 (0.3)*</td>
<td>0.87 (0.09)</td>
</tr>
<tr>
<td>With GG</td>
<td>17 (4)</td>
<td>4.2 (0.5)*</td>
<td>0.94 (0.09)</td>
</tr>
</tbody>
</table>

* Significantly different from control for that direction (A → B or B → A) p < 0.05.

A. 

B. 

Fig. 7. Concentration dependence of the CYP3A4-mediated metabolism of K77 (A) and felodipine (B) in intact CYP3A4-transfected Caco-2 cell monolayers. Nonlinearity was observed for K77 metabolism above a 50 μM dose. Data are presented as the mean ± S.D. (n = 3).

Discussion

Drug transporters have become increasingly important in furthering our understanding of the pharmacokinetic properties (including absorption, distribution, and excretion) and interactions of drugs. In this study, we delineate how P-gp can also affect intestinal drug metabolism by controlling the access of the drug to the intracellular metabolizing enzyme. The CYP3A4-expressing Caco-2 cell line expressing both P-glycoprotein and CYP3A4 served as the investigational tool to model this interplay.

Previous studies characterizing the CYP3A4-expressing Caco-2 cells found that P-gp was expressed on the apical membrane and was capable of mediating digoxin efflux (Cummins et al., 2001). In the same study, CYP3A4 was shown to be localized inside the cells, polarized toward the intracellular apical side consistent with the location of this protein in the human intestinal epithelium (Watkins et al., 1987). In addition, the cells were shown to metabolize the CYP3A4 substrates testosterone and midazolam (Crespi et al., 1996; Cummins et al., 2001). In the present study, there was preferential transport of K77 in the basolateral to apical direction, and this polarized efflux was completely inhibited by P-gp inhibitors. The absorptive flux was increased 4.2-fold in the presence of GG918 and only 5-fold with CsA, suggesting that the most important factor limiting K77 absorption is P-gp. Felodipine absorptive flux was only modestly increased by inhibiting CYP3A4 (1.5-fold increase after 3 h) and not affected by P-gp inhibition.

The metabolite exit profiles for K77 and felodipine metabolites were monitored over time in the CYP3A4-transfected Caco-2 cells. Data were also collected for metabolites in the presence of the inhibitors CsA and GG918, allowing a thorough analysis of the cell monolayer characteristics. The preferential efflux of metabolites toward the apical side did not guarantee that the metabolites themselves were substrates for efflux transport. This polarized secretion of metabolites toward the apical side of Caco-2 cells has been noted previously for a number of CYP3A4 metabolites including those of CsA (Gan et al., 1996), midazolam (Schmiedlin-Ren et al., 1997b), testosterone (Hu et al., 1999), terfenadine (Raesi et al., 1999), and indinavir (Hochman et al., 2001). However, the observation that net secretion of metabolites is not necessarily mediated by P-gp was first realized by Schmiedlin-Ren et al. (1997b) using vitamin D3-induced Caco-2 cells and studying the efflux patterns of 1-OH midazolam, for which the ratio of the apical to basolateral secretion was not affected by coincubation with the P-gp inhibitor verapamil. In the current study, although the metabolites of both K77 and felodipine appeared preferentially on the apical side after an apical dose, it appeared that only the K77 metabolites were substrates of P-gp. The inhibition studies monitoring the exit profiles of the metabolites in the presence of CsA and GG918 showed changes in the exit profiles of the K77 metabolites but not of the felodipine metabolite. In addition, there...
seemed to be a bias that was present at the apical membrane that facilitated the exit of metabolites toward the apical side. This may be due to CYP3A4 being closer to the apical side as well as the greater surface area of the apical plasma membrane. Drugs dosed apically would have an advantage for interacting with the enzyme due to the proximity of the CYP3A4 to the apical membrane. Overall, there appeared to be less bias in the direction of metabolite efflux when the drugs were dosed basolaterally. After a basolateral dose, the drug must travel through the cell before it interacts with CYP3A4; therefore, the diffusional proximity of the metabolite to exit the apical membrane and the greater surface area of the apical membrane could still be contributing to discrepancies after a basolateral dose. This was observed for dehydrofodelipine where even after a basolateral dose there was considerable preference for apical secretion (especially at the early time points). This phenomenon will likely be influenced by the diffusion rate of the compound through the cell. We estimate that the ratio of metabolites exiting the apical versus basolateral sides must be greater than 3-fold to be considered due to an efflux mechanism. However, to truly determine whether metabolites are substrates of P-gp, studies using P-gp inhibitors should be done to see whether changes in the metabolite patterns of excretion are observed.

Having demonstrated that the experiments were performed in the linear range for K77 metabolism (Fig. 7A), the extraction ratios for K77 in the presence of GG918 clearly show the importance of P-gp in enhancing the metabolism by CYP3A4 after an apical dose. When the transporter was functional, there was a 33% extraction of K77 across the membrane that decreased to 14% when the transporter was inhibited (Fig. 6). This phenomenon is most likely due to a P-gp effect, as no change in the extraction ratios was observed with felodipine when incubated with GG918. These data are consistent with a role for P-gp in allowing the drug to cross the monolayer (eq. 2). Equation 2 is reasonable if the intracellular drug levels are included (eq. 3) and the other that does not take into account the intracellular levels but only includes the drug that crossed the monolayer (eq. 2). Equation 2 is reasonable if the intracellular amount of drug is negligible compared with the drug that is crossing the cell. However, in the case of felodipine, the influence of inhibiting CYP3A4 was greatly underestimated by omitting the intracellular drug (since it made up the majority of the drug that was metabolized). Furthermore, it appeared that by using eq. 2, the extraction ratios across the cells were different in the A→B and B→A directions when only P-glycoprotein transport was abolished. When intracellular drug levels were included, the extraction ratios across the cells became the same in both directions (for both K77 and felodipine), as would be expected when no P-gp transport was occurring. We believe that eq. 3, because it includes a correction factor for intracellular drug levels that can be dependent on transporter interactions and dosing compartments, depicts a more accurate representation of the ER and should be used when calculating ERs from in vitro systems in which intracellular levels are measurable.

The decreased extraction ratio of K77 after specific inhibition of P-gp showed the importance of the transporter in influencing CYP3A4 metabolism, which further supported the proposed interplay between these proteins in the intestine. The CYP3A4-transfected Caco-2 cells provided a good model for determining what biochemical factors influence the absorption of a drug across the cell. The importance of monitoring intracellular drug levels was also demonstrated, especially when looking at drug-drug interactions with P-gp inhibitors. Studies are ongoing in an in situ rat intestinal perfusion system to determine whether a similar phenomenon will be observed under physiological conditions.

References


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