P-glycoprotein-Mediated Efflux of Indinavir Metabolites in Caco-2 Cells Expressing Cytochrome P450 3A4

JEROME H. HOCHMAN, MASATO CHIBA, MASAYO YAMAZAKI, CUYUE TANG, and JIUNN H. LIN

Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania

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P-glycoprotein (Pgp) is an ATP-driven efflux pump capable of transporting a wide variety of structurally diverse compounds from the cell interior into the extracellular space (Gatmaitan and Arias, 1993; Schinkel, 1997). Initially identified by its overexpression in multidrug-resistant tumor cells, Pgp has since been shown to be constitutively expressed on normal cells, including cells that constitute the barrier and metabolic functions in the intestine, kidney, liver, and brain microvascular endothelia (Cordon-Cardo et al., 1990). The expression of Pgp in these tissues suggests a role of Pgp in protecting the body from xenobiotics. Consistent with this premise, Pgp expression in the intestine, liver, kidney, placenta, and blood-brain endothelial cells is polarized such that its activity would prevent absorption and aid elimination of xenobiotics or prevent exposure of sensitive tissues to xenobiotics.

In the intestine, Pgp is expressed on the brush-border membrane of enterocytes where it pumps compounds out of the cytosol into the lumen of the intestine. This activity runs countercurrent to the absorptive transport of drugs and has been proposed as a barrier to oral absorption of drugs (Leu and Huang, 1995; Fricker et al., 1996; Lown et al., 1997; Kim et al., 1998; Salphati and Benet, 1998). Recently, it has been proposed that Pgp not only functions as a transport barrier to oral absorption but also acts in concert with CYP3A4 to increase presystemic metabolism of drugs (Gan et al., 1996; Watkins, 1997; Wacher et al., 1998; Ito et al., 1999). Two mechanisms have been proposed for how Pgp may enhance the extent of intestinal metabolism. According to one mechanism, Pgp activity results in repeated cycles of absorption and secretion into the intestinal lumen, increasing the residence time of a drug in the intestine and its exposure to intestinal CYP3A4 prior to systemic absorption. A second mechanism that has been proposed suggests that Pgp may facilitate the removal of primary metabolites from the cell interior, thus minimizing the potential for product inhibition of CYP3A4.

In recent studies, we addressed the potential for synergy between CYP3A4 and Pgp by studying transport and metabolism of indinavir in Caco-2 cells induced to express CYP3A4 by culturing the cells with di-OH vit D₃ (Hochman et al., 2000). The results showed that reduction of absorptive trans-
Mochan et al.

port by Pgp leads to more metabolite being formed for every mole of indinavir transported across the Caco-2 cell monolayers. Thus, for every mole of drug that is transported from the luminal side of the monolayer to the serosal side, more of the parent drug is subject to intestinal metabolism. These results are consistent with a synergistic mechanism in which Pgp activity increases the exposure of substrates to CYP3A4 prior to absorption into the systemic circulation.

In addition to showing the effects of Pgp on metabolite production relative to drug transport, we also observed that the metabolites formed intracellularly by CYP3A4 were almost exclusively secreted into the apical (luminal) compartment. Extrapolating these results to an in vivo situation, this active secretion could have the effects of preventing intracellular accumulation of high concentrations of metabolites and could result in direct elimination of metabolites. Although the apical efflux of indinavir metabolites was inhibited by the Pgp inhibitor CsA, the sensitivity of metabolite efflux to CsA was reduced relative to that for Pgp-mediated directional transport of known Pgp substrates. Consequently, another transporter with lower sensitivity to CsA may be mediating efflux of indinavir metabolites. In this article, we report on a series of studies evaluating interactions of indinavir metabolites with Pgp. The results indicate that apical efflux of N-dealkylated- and hydroxylated-metabolites of indinavir in Caco-2 cells is primarily mediated by Pgp.

Materials and Methods

EHS cell attachment matrix was purchased from Promega (Madison, WI). Testosterone, 6b-OH-testosterone, di-OH vit D3, and CsA were purchased from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum, glutamine, trypsin-EDTA solution, penicillin-streptomycin solution, nonessential amino acids, Hanks’ balanced-salt solution (HBSS), and Hepes buffer were purchased from Invitrogen/Life Technologies (Grand Island, NY), and Dulbecco’s modified Eagle’s medium with pyruvate and 4.5 g of glucose/liter was prepared by Mediatech (Ronanoke, VA) and obtained from Fisher Scientific (Pittsburgh, PA). The di-OH vit D3 stock solutions were prepared at 0.1 mg/ml in ethanol and stored at –70°C. Indinavir and M6 metabolite standard were prepared at Merck Research Labs (West Point, PA and Rahway, NJ). The monoclonal antibody C219 was purchased from Signet Laboratories (Dedham, MA) and was dialyzed against 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl prior to use with Pgp-enriched membranes.

Cell Culture and Preparation of mAb UIC2. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and were used at passage 21 to 29. The cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with glutamine, nonessential amino acids, penicillin-streptomycin, and 5% fetal calf serum at 37°C in a humidified 5% CO2/95% air environment. Hybridomas producing the monoclonal antibody UIC2 were obtained from American Type Culture Collection and were maintained at 37°C in a humidified 5% CO2/95% air environment in Dulbecco’s modified Eagle’s medium supplemented with glutamine, nonessential amino acids, penicillin-streptomycin, and 10% fetal calf serum. Spent culture medium was pooled, and the secreted monoclonal antibody UIC2 was isolated by chromatography on a 5-ml protein G plus/protein A-agarose column. The column was washed with 0.1 M sodium phosphate containing 0.15 M sodium chloride and 5 mM EDTA, and the antibody was eluted with 10 mM glycine HCl (pH 3.0). Antibody-containing fractions were detected by absorbance at 280 nm, and the pH was neutralized by the addition of an equal volume of 0.5 M Tris. Pooled antibody-containing fractions were then dialyzed against phosphate-buffered saline (pH 7.4) and sterile filtered.

Caco-2 Transport Studies. Directional transport studies were performed on Caco-2 cells grown on filters using the Biocat HTS Caco-2 assay system (Becton Dickinson, Bedford, MA) in accordance with the manufacturer’s instructions with the modification that cells were maintained in basal-seeding media for an extended period (3–5 days) prior to inducing differentiation with Enterostim media. After 2 days in Enterostim media, filters were rinsed one time with Hanks’ balanced-salt solution with 10 mM Hepes (pH 7.4) prior to performing transport studies. HBSS was added to the receiver compartments, and HBSS containing 10 μM M6 was added to donor compartments. In experiments in which CsA inhibited Pgp efflux, 10 μM CsA was added to both the donor and receiver solutions. Samples of the donor and receiver solutions were collected after 2 h, and the total drug in each compartment was determined. Directional transport studies on the Pgp substrate VBL (100 nM containing 1 Ci/ml [3H]VBL) were run in parallel to assess the functional activity of Pgp in the Caco-2 cells.

UIC2 Binding and Inhibition Studies. Directional transport studies of vinblastine to evaluate UIC2 inhibition of Pgp were performed as described above with the exception that after 1 day in enterostim media, the apical media were replaced with fresh enterostim containing 1 μM CsA, 10 μg/ml UIC2, or 1 μg/ml CsA and 10 μg/ml UIC2 and incubated overnight. The filters were then rinsed with HBSS, and 100 nM vinblastine with 0.05 μCi/ml [3H]VBL in HBSS, containing UIC2, CsA, or both UIC2 and CsA, was added to the apical compartment for A-to-B transport. For B-to-A transport 100 nM vinblastine with 0.05 μCi/ml [3H]VBL was added to the basolateral side and HBSS containing UIC2, CsA, or both UIC2 and CsA was added to the apical side.

Afflux efflux of calcine formed by intracellular hydrolysis of calcine acetoxyethyl ester (calcine AM; Molecular Probes, Eugene, OR) was used to evaluate the effects of UIC2 on MRP-2 activity in Caco-2 cells (Feller et al., 1995; Fujita et al., 1997). Caco-2 cells were incubated overnight with UIC2 or UIC2 and CsA as indicated above. HBSS containing 0.5 μg/ml calcine AM was added to the apical and basolateral compartments with the apical solutions containing UIC2 or UIC2 and CsA. After 1-h incubation at 37°C, the apical and basolateral solutions were collected, and the filters were washed one time with fresh HBSS. The cells were then lysed by the addition of 0.1 ml of 50% ethanol to the apical compartment, 0.4 ml of HBSS was added, and the total sample was collected. Calcine fluorescence was measured with a TECAN SPECTRAFlor Plus microplate fluorometer (Tecan U.S., Inc., Durham, NC) at 485 nm excitation and 535 nm emission.

UIC2 binding was assessed by fluorescence microscopy using a Zeiss Axiovert microscope (Hitchcöfel Optical Inst. Inc., St. Louis, MO) equipped for fluorescent fluorescence. Caco-2 cells grown 2 to 3 weeks on EHS cell attachment matrix enhanced chemiluminescence-coated coverslips were incubated overnight with UIC2, or UIC2 and CsA under the conditions used for inhibition studies. The cells were then washed four times with HBSS, incubated 1 h on ice with Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes), and washed four times to remove residual fluorescent antibody. Fluorescence micrographs were then taken and printed using a set exposure time for comparison of the two conditions.

Efflux of Indinavir Metabolites by CYP3A4 Expressing Caco-2 Cells. Caco-2 cells were plated at 2 × 104 cells/cm2 on 12-mm-diameter polycarbonate filters (Costar transwell, 0.2-μm pore size; Corning Inc., Corning, NY) coated with 2 μg/cm2 of EHS cell attachment matrix and Di-OH vit D3 induction of CYP3A4 was performed as described by Schmiedlin-Ren et al. (1997) as modified by Hochman et al. (2000). Prior to metabolism studies, culture media were removed and the filter-grown Caco-2 cells were pre-equilibrated in Hepes-buffered HBSS, pH 7.4. The HBSS was then replaced with 0.7 ml of fresh HBSS containing 10 μM indinavir on both sides. After incubation for 2 to 4 h at 37°C, the apical and basolateral solutions were collected. The apical side of the filters was rinsed with HBSS and the intracellular contents were collected by lysis of the cells.
with 0.7 ml of 50% ethanol. In cases where Pgp was inhibited with CsA, both the receiver and donor solutions contained the indicated concentration of CsA. For inhibition of Pgp by UIC2, the cells were preincubated overnight by changing the culture media in the apical compartment to media containing 1 μM CsA, 10 μg/ml UIC2, or 1 μM CsA and 10 μg/ml UIC2. The filters were rinsed one time with HBSS, after which 10 μM indinavir in HBSS containing CsA, UIC2, or both CsA and UIC2 was added to the apical compartment and 10 μM indinavir in HBSS was added to the basolateral compartment.

**LC/MS Analysis of Indinavir and Its Metabolites.** Indinavir and indinavir metabolites were separated on a 5-μm betasil C18 reverse phase column (50 × 3 mm) (Keystone Analytical, Bellefonte, PA) and detected by LC/MS with a Sciex API 150 (Applied Biosystems, Foster City, CA) using APCI as described previously (Hochman et al., 2000). In some cases, separation of indinavir and metabolites was performed using a generic gradient for 10 to 90% acetonitrile/ammonium acetate (pH 4.5) over 10 min at a flow rate of 1.5 ml/min. Ions were monitored at m/z of 523, 529, 614, 630, and 613 for M6, M5, indinavir, addition of oxygen to indinavir, and the internal standard, respectively.

**ATPase Activity in Pgp-Containing Membranes.** Membranes from baculovirus-infected insect cells expressing recombinant human Pgp were purchased from GENTEST (Woburn, MA) and used in accordance with the manufacturer’s recommended protocol. M6 (20 μl) in Tris-MES buffer (50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol) was incubated with Pgp membranes (20 μl containing 40 μg of protein) in the presence and absence of 100 μM sodium orthovanadate in 96-well plates. After 5 min, Pgp-ATPase activity was initiated by addition of 20 μl of 10 mM MgATP. ATP hydrolysis was allowed to proceed for 30 min after which the reaction was terminated by addition of 30 μl of 10% SDS. Inorganic phosphate released from ATP was measured as an ammonium molybdate complex at 650 nm in a microplate reader according to Druekes et al. (1995). Pgp-mediated ATPase activity was determined from the difference in inorganic phosphate released in the presence and absence of sodium orthovanadate.

### Results

**Secretion of Indinavir Metabolites in CYP3A4 Expressing Caco-2 Cells.** In previous studies (Hochman et al., 2000), we demonstrated that metabolism of indinavir by Caco-2 cells grown in the presence of di-OH vit D₃ was consistent with metabolism by CYP3A4 (Balani et al., 1996; Lin et al., 1996; Chiba et al., 1996). The primary metabolites identified by LC/MS/MS following incubation of indinavir with di-OH vit D₃-treated, but not untreated Caco-2 cells, were products of N-dealkylation (M6), hydroxylation of M6 (M5), and hydroxylation of the indan (M3) and the phenyl moiety (M4b) (Fig. 1). The N-oxidation of the pyridine ring was also detected but to a lesser extent, and the N-oxide metabolite was not followed in the transport experiments. Under the assay conditions used for quantitation of metabolites, M4b and M3 eluted together and were collectively quantified as OH-indinavir.

Following incubation of CYP3A4 expressing Caco-2 cell with 10 μM indinavir in both the apical and basolateral compartments, M5, M6, and OH-indinavir metabolites were found to be selectively secreted into the apical compartment with over 80% of M5 and M6 and 75% of OH-indinavir appearing in the apical compartment. The extensive secretion into the apical compartment suggests that the metabolites produced inside the cell are being actively transported across the apical membrane. To determine whether the three metabolites share a common transport mechanism, the sensitivity of metabolite efflux to CsA was evaluated at concentrations up to 30 μM CsA (Fig. 2). CsA concentrations ranging from 0 to 30 μM had little effect on the total amount of metabolite formed (data not shown). However, the distribution of all three metabolites was strongly influenced by cyclosporin, with the proportion of M5, M6, and OH-indinavir released in the apical compartment decreasing from 85, 90, and 75%, respectively, in the absence of CsA to 20, 35, and 40% at 20 μM CsA (Fig. 2A). Concomitant with the decrease in the apical secretion of metabolites increased amounts of metabolite were released into the basolateral compartment (Fig. 2B) and retained in the cells. Apical efflux of all three metabolites showed virtually identical sensitivity to CsA with approximately 50% inhibition of the active apical efflux observed at 5 to 10 μM CsA. The similarity in sensitivity to CsA strongly suggests that efflux of all three metabolites occurs by a common transport mechanism.

**Interactions of M6 with Pgp.** Although inhibition by CsA is consistent with the metabolites being transported by Pgp, metabolite efflux was generally less sensitive to CsA than typically observed for CsA inhibition of Pgp in directional transport and substrate accumulation experiments.
This raises the possibility that another transporter may be mediating metabolite efflux. Therefore, further experiments were performed to study interactions of M6 with Pgp. Initial attempts to study vectorial transport of extracellular M6 in Caco-2 cells failed to demonstrate directional transport. Instead, the permeability coefficients for both A-to-B and B-to-A transport were low (Table 1) and were comparable with the values we observe with the hydrophilic paracellular transport marker Lucifer yellow. Given the low permeability coefficients for M6 and its relatively high polarity (Log P = 0.89 with five hydrogen bond donors), it is likely that the majority of the transport observed is via the paracellular rather than the transcellular route. Consequently, the failure to observe Pgp efflux in directional transport experiments probably reflects poor penetration of M6 into the cells, such that M6 is not accessible to Pgp for efflux.

As opposed to the restricted access of M6 to Pgp in directional transport experiments, M6 formed intracellularly by CYP3A4 would have unrestricted access to the substrate binding site of Pgp. To evaluate interactions of M6 with Pgp that could occur from the cell interior, stimulation of Pgp-ATPase activity by M6 was measured using insect cell membranes expressing a high level of human Pgp. Since a portion of the Pgp-enriched membranes is in an inside-out orientation, both M6 and ATP are accessible to the cytoplasmic domain of Pgp. Thus, stimulation of Pgp-mediated ATP hydrolysis can be used to detect interaction of M6 with Pgp that could occur inside the cell. M6 stimulated vanadate-sensitive ATPase activity in a concentration-dependent manner with approximately half-maximal stimulation at 6 μM M6 (Fig. 3A). Stimulation of vanadate-sensitive ATPase activity reflects increased Pgp activity in as much as it was completely inhibited by preincubation of the Pgp membranes with C219, an inhibitory monoclonal antibody that binds to the nucleotide binding site on Pgp (Fig. 3B). The maximum stimulation of ATPase activity observed with M6 was approximately half that observed with 20 μM vinblastine or indinavir (data not shown). These results indicate that when presented to Pgp from the cytoplasmic surface, M6 stimulates ATPase activity in a manner consistent with it being a Pgp substrate.

### Inhibition of Pgp and Metabolite Efflux with UIC2.

Although stimulation of Pgp-ATPase activity is consistent with M6 being a Pgp substrate, the data are not sufficient to conclude that a significant proportion of the metabolite efflux observed in Caco-2 cells is mediated by Pgp. Consequently, conditions were established to specifically inhibit Pgp in Caco-2 cells with a monoclonal antibody that recognizes an extracellular epitope on Pgp. UIC2 is an inhibitory monoclonal antibody that binds to an extracellular epitope on Pgp (Mechetner and Roninson, 1993). The epitope that is recognized by UIC2 is a conformational epitope that corresponds to a transient conformational state present during catalytic cycle for substrate transport (Mechetner et al., 1997). ATP depletion and some Pgp substrates have been shown to increase UIC2 binding, suggesting that the epitope for UIC2 binding corresponds to an ATP-depleted state that exists during the drug transport process. Incubation of Caco-2 cells with UIC2 in the presence of 1 μM CsA resulted in 80% inhibition of Pgp-mediated efflux of vinblastine (Fig. 4A). In repeated experiments, the extent of inhibition of Pgp activity following incubation of Caco-2 cells with UIC2 and 1 μM CsA ranged from 50 to 80%. No significant inhibition of Pgp activity is observed when the cells are incubated with UIC2 alone or with 1 μM CsA alone. Consistent with the Pgp inhibition results, fluorescence microscopy demonstrated significant binding of UIC2 when Caco-2 cells are incubated with UIC2 and 1 μM CsA but very little antibody binding when cells

### Table 1

<table>
<thead>
<tr>
<th>Transport Conditions</th>
<th>Permeability Coefficient cm/s</th>
<th>M6</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A to B</td>
<td>Ave. 1.2E-06, S.D. 5.5E-07</td>
<td>2.7E-06</td>
<td>2.7E-07</td>
</tr>
<tr>
<td>A to B + CsA</td>
<td>Ave. 2.1E-06, S.D. 4.5E-07</td>
<td>4.5E-07</td>
<td>4.5E-07</td>
</tr>
<tr>
<td>B to A</td>
<td>Ave. 6.1E-07, S.D. 6.2E-08</td>
<td>1.5E-05</td>
<td>2.4E-06</td>
</tr>
<tr>
<td>B to A + CsA</td>
<td>Ave. 6.8E-07, S.D. 2.9E-07</td>
<td>2.9E-07</td>
<td>2.9E-07</td>
</tr>
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A: apical; B: basolateral. 

n = 4.
were incubated in the absence of CsA (Fig. 5). Thus, CsA promotes UIC2 binding by stabilizing the conformational epitope, whereas the UIC2 inhibits substrate transport by Pgp.

To establish whether UIC2 inhibition is specific to Pgp as opposed to having a more generalized effect on the transport properties of the cell, the effects of UIC2 binding on MRP-2 (Hirohashi et al., 2000) activity in Caco-2 cells was evaluated. The fluorescent MRP-1, 2 substrate calcine (Feller et al., 1995; Fujita et al., 1997), formed by intracellular esterase hydrolysis of the nonfluorescent compound calcien AM, is primarily secreted into the apical compartment and retained in the cytosol in filter-grown Caco-2 cells (Fig. 4B). In the presence of the MRP inhibitor MK571, the proportion of calcine fluorescence detected in the apical compartment decreased, and the amount retained in the cells increased relative to untreated cells. Similar results have been observed with other MRP inhibitors (data not shown). Binding of UIC2 to Caco-2 cells had no significant influence on the distribution of calcine compared with untreated cells. Thus, UIC2 binding did not inhibit MRP-2 activity. Under these conditions, UIC2 binding inhibited 70% of Pgp-mediated vinblastine efflux, suggesting that UIC2 binding specifically inhibits Pgp and does not have a more global effect on the viability of and transport activity in Caco-2 cells.

The effects of UIC2 binding on the distribution of indinavir metabolites generated intracellularly in Caco-2 cells expressing CYP3A4 are shown in Fig. 6. The results largely parallel those observed for inhibition of Pgp-mediated vinblastine transport. UIC2 or 1 μM CsA alone had no effect on the distribution of M5, M6, or OH-indinavir. In contrast, UIC2 in conjunction with CsA, conditions that produce significant Pgp inhibition, resulted in a decrease in the proportion of M5, M6, and OH-indinavir secreted into the apical compartment and an increase in the basolateral secretion and intracellular accumulation of the metabolites. If we assume that under conditions where active efflux is completely inhibited (i.e., 30 μM CsA), 30 to 40% of the total M6 and OH-indinavir formed would be released into the apical compartment by passive diffusion, then inhibition by UIC2 results in approximately 50% inhibition of active efflux of the metabolites. This inhibition of metabolite efflux is in agreement with the extent of Pgp inhibition observed for vinblastine efflux (approximately 70% inhibition) under the same conditions, indicating that Pgp is the primary mediator of apical efflux of indinavir metabolites.

**Discussion**

In recent studies exploring the interactions between Pgp and CYP3A4 in intestinal epithelial cells, we and others observed that metabolites generated inside the cell by CYP3A4 or by carboxyl esterase activity were preferentially secreted onto the luminal side of the epithelium (Gan et al., 1996; Schmiedlin-Ren et al., 1997; Raaessi et al., 1999; Hochman et al., 2000; Molden et al., 2000). In some studies, apical secretion of metabolites was observed to be partially inhibited by Pgp inhibitors, suggesting that Pgp may have a role in direct elimination of intracellular metabolites (Raaessi et al., 1999; Hochman et al., 2000; Molden et al., 2000). However, since other transporters expressed on the epithelial cells may also show sensitivity to conventional Pgp inhibitors, it is possible that other efflux transporters may account for apical secretion of metabolites. In the present study, we used baculovirus-expressed Pgp, inhibitory antibodies to Pgp, and sensitivity to CsA to assess the role of Pgp in apical secretion of indinavir metabolites in Caco-2 cells expressing CYP3A4.

Following incubation of indinavir with di-OH vit D₃-treated Caco-2 cells, CYP3A4 metabolites formed by hydroxylation of indinavir, N-dealkylation of the methyl pyridine, and both N-dealkylation and hydroxylation of the indan moiety were subject to apical secretion with approximately 85% of each metabolite appearing in the apical compartment. Apical secretion of all three metabolites was inhibited by the Pgp inhibitor CsA with similar sensitivity, suggesting that all three metabolites are secreted by a common transporter. While this is consistent with Pgp-mediated efflux of the metabolites, metabolite efflux is less sensitive to CsA than is observed with exogenously added Pgp substrates. Under conditions...
where CsA inhibited over 80% of indinavir directional transport, apical efflux of the indinavir metabolites was only decreased 20 to 30% (Hochman et al., 2000). The decreased sensitivity to CsA raises the possibility that metabolite efflux is mediated by another transporter. Alternatively, the lower sensitivity to CsA may indicate that the rate-limiting step in metabolite secretion is the rate of metabolite formation, instead of the rate of Pgp efflux. Thus, inhibition of Pgp would not result in a proportionate decrease in metabolite efflux until the rate of Pgp efflux was lower than the rate of metabolite formation.

The reduced sensitivity to cyclosporin A relative to other Pgp substrates and the potential that CsA may be inhibiting transport proteins other than Pgp leaves some question whether Pgp is responsible for metabolite efflux. The N-dealkylated-metabolite M6 stimulated vanadate-sensitive ATPase activity in baculovirus-expressed Pgp membranes, indicating that M6 interacts with Pgp in a manner consistent with it being a Pgp substrate. However, this does not resolve whether Pgp is responsible for a significant portion of the apical efflux of indinavir metabolites. To assess Pgp’s contribution to the metabolite efflux, Pgp in CYP3A4 expressing Caco-2 cells was inhibited using the monoclonal antibody UIC2. UIC2 has previously been shown to inhibit Pgp by binding to a transient conformational epitope on the extracellular portion of Pgp (Mechetner and Roninson, 1993; Mechetner et al., 1997), which is stabilized by some Pgp substrates or by ATP depletion. In our studies, UIC2 by itself did not significantly bind to or inhibit Pgp in Caco-2 cell monolayers. However, CsA at concentrations too low to cause significant inhibition of Pgp promoted UIC2 binding, resulting in 50 to 80% inhibition of Pgp activity. The influence of UIC2 and 1 μM CsA on indinavir metabolite efflux in CYP3A4 expressing Caco-2 cells largely parallels the pattern observed for UIC2 binding and Pgp inhibition. UIC2 or CsA by themselves do not alter the distribution of indinavir metabolites, but UIC2 in combination with 1 μM CsA decreased

Fig. 4. Influence of UIC2 binding on Pgp (A) and MRP-2 (B) activity in Caco-2 cells. A, inhibition of Pgp-mediated vinblastine transport in Caco-2 cells incubated with fresh culture media or culture media containing 1 μM CsA, mAb UIC2, or 1 μM CsA and mAb UIC2 in the apical compartment overnight. Directional transport of vinblastine was then measured in the presence and absence of CsA, mAb UIC2, or both CsA and mAb UIC2 in the apical compartment and the percentage of remaining Pgp activity was determined from the difference between B-to-A and A-to-B transport relative to untreated Caco-2 monolayers. B, efflux of calcine in untreated Caco-2 cells and Caco-2 cells treated with 1 μM CsA and mAb UIC2 or with the MRP inhibitor MK571 (20 μM). The fraction of calcine fluorescence in the apical (■) and cellular extracts (□) was determined from the total relative fluorescence units after adjusting the cell extracts and apical solution to equal volumes. No significant calcine fluorescence was detected in the basolateral compartment. Filters run in parallel showed 70% inhibition of Pgp activity by UIC2 binding.
apical secretion and increased the intracellular retention and basolateral secretion of M5, M6, and OH-indinavir. If we assume that under conditions where active efflux is completely inhibited, 30 to 40% of the metabolite would passively diffuse out the apical membrane, then UIC2 binding inhibited 50% of the active efflux of the metabolites compared with 70% inhibition of vinblastine efflux in the same experiment. Thus, it can be concluded that Pgp is responsible for a major proportion if not all of the active efflux of the three indinavir metabolites that we studied.

It is generally believed that interactions between Pgp and its substrates occur within the inner leaflet of the plasma membrane (Shapiro and Ling, 1997, 1998). Although some polar Pgp substrates, such as colchicine (Debenham et al., 1982) and cimetidine (Pan et al., 1994), have been identified, they are generally considered to be exceptions. In this regard, it is surprising that the more polar indinavir metabolites are subject to such extensive Pgp-mediated secretion. This is particularly true for M6 and M5, which show very little membrane partitioning as indicated by significant accumulation inside the cells when Pgp is inhibited. Moreover, directional transport experiments on M6 showed very little transcellular permeability of M6 in either A-to-B or B-to-A directions, indicative of poor penetration of M6 across the plasma membrane. Based on the poor membrane permeability we would not expect M6 and M5 (hydroxy-M6) to be good Pgp substrates. However, M5 and M6 show extensive Pgp-mediated apical secretion. This apparent discrepancy can be addressed by considering that the extent of apical efflux of metabolites will be dependent on the balance between active efflux of the metabolites and passive permeability across the apical and basolateral membranes. Thus, if increasing polarity has a disproportionately greater influence on passive permeation of the metabolites across the plasma membrane than on Pgp-mediated efflux, the contribution of active efflux will be more pronounced. Given that passive diffusion across
the plasma membrane requires partitioning of the metabolites deep in the membrane interior, whereas Pgp-substrate interactions probably occur in a more shallow position within the membranes inner leaflet, increasing polarity would seem to have a greater effect on passive permeability than on Pgp-substrate interactions. Thus, metabolites could show enhanced apical efflux relative to the less polar parent compounds without showing improved kinetics for Pgp. Clearly, validation of this model requires more mechanistic studies on Pgp substrate-metabolite interactions.

Given that the ultimate goal of metabolism is to detoxify and eliminate xenobiotics, it is intriguing to speculate that Pgp has a more general role in elimination of phase I metabolites. Metabolites of CsA, tenofovir, and diltiazem (Gan et al., 1996; Raeiissi et al., 1999; Molden et al., 2000) have been shown apical efflux in intestinal cells consistent with Pgp-mediated efflux. Similarly, phase I metabolites of verapamil have shown Pgp-mediated directed transport and/or inhibition of Pgp (Pauli-Magnus et al., 2000). Pgp-mediated elimination could serve two functions: 1) direct elimination of metabolites such that systemic exposure to metabolites is minimized, and 2) removal of metabolites from the cytosol, preventing accumulation of high concentrations of metabolites within the cells. In the intestine, Pgp-mediated efflux would result in direct secretion of the metabolites into the lumen of the intestine thus minimizing systemic exposure to the metabolites. By analogy, Pgp in the canalicular membrane of the liver could efflux metabolites directly into the bile. If this activity is significant in vivo, one would expect that extensive inhibition of Pgp would result in higher plasma levels of metabolites. In our studies, the intracellular accumulation of the more polar metabolites M5 and M6 increased 8- and 4-fold, respectively, upon inhibition of Pgp by UIC2. As suggested by Watkins (1997), removal of metabolite by Pgp could serve to minimize potential product inhibition of cytochrome P450 enzymes. In the studies reported in this article, accumulation of metabolites as a result of Pgp inhibition did not inhibit indinavir metabolism. The failure to observe inhibition of indinavir metabolism by increasing intracellular metabolites may indicate poor affinity of the metabolites for CYP3A4 relative to the high-affinity interactions between indinavir and CYP3A4 (Chiba et al., 1997). Removal of metabolites by Pgp could also have a role in minimizing cellular toxicity by preventing the accumulation of intracellular concentrations of metabolites. While evaluation of the functional implications of Pgp-mediated metabolite efflux is beyond the scope of this article, animal studies using gene knockout mice or potent Pgp inhibitors, in conjuction with in vitro analysis, should shed light on the role of Pgp-mediated metabolite efflux in drug metabolism and related toxicity.

In summary, the results presented in this article show that indinavir metabolites, including highly polar metabolites, generated intracellularly by CYP3A4, are subject to extensive efflux by Pgp. Further in vivo and in vitro studies will help to resolve the full implications of Pgp-mediated metabolite efflux on drug disposition and metabolism-related drug toxicity.

References


Address correspondence to: Jerome H. Hochman, Department of Drug Metabolism, WP 75-200, West Point, PA 19486. E-mail: jerome_hochman@merck.com