Evaluation of P-glycoprotein–Mediated Renal Drug Interactions in an MDR1-MDCK Model

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Study Objective. To evaluate P-glycoprotein (P-gp)–mediated renal drug interactions in an in vitro model of tubular secretion.

Design. In vitro experiment.
Setting. University-affiliated pharmacokinetics laboratory.
Cell Lines. Madin-Darby canine kidney (MDCK), multidrug-resistant-1 (MDR1)-MDCK, and human colon carcinoma (Caco-2) cells.
Intervention. Transepithelial transport (basolateral-to-apical and apical-tobasolateral) of cimetidine was assessed in the absence and presence of various concentrations of the P-gp inhibitors itraconazole and PSC-833 in a renal P-gp cell culture model (MDR1-MDCK).
Measurements and Main Results. Apparent permeability of cimetidine was characterized, and level of P-gp expression was determined by Western blot analysis, in MDCK (wild type), MDR1-MDCK, and Caco-2 cells (for relative comparison). In the presence of PSC-833, cimetidine's apparent permeability value for basolateral-to-apical transport decreased from 2.96 to 1.15 x 10^{-6} cm/second, coupled with a decrease in efflux ratio from 2.36 to 1.80. The effect of itraconazole was concentration dependent, with cimetidine's apparent permeability value for basolateral-to-apical transport decreasing from 3.96 to 1.92 x 10^{-6} cm/second ($p<0.05$), resulting in a 50% decrease in efflux ratio. Expression of P-gp was negligible in MDCK (wildtype) cells, but high-level expression was confirmed in both MDR1-MDCK and Caco-2 cells.
Conclusion. P-glycoprotein plays a significant role in the renal tubular secretion of organic cations such as cimetidine, and the high level of P-gp expression in MDR1-MDCK cells makes this a well-suited model for evaluating mechanisms of renal drug interactions.
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Evaluation of drug transport mechanisms is becoming increasingly important in drug-delivery and pharmacokinetics research. P-glycoprotein, a plasma membrane–bound efflux protein, initially was identified in cancer cells resistant to a wide variety of chemotherapeutic agents, including anthracyclines, anthracenediones, and vinca alkaloids.[1,2] Overexpression of P-gp has been associated with drug resistance in various cancers, including solid tumors and hematologic malignancies. During the past decade, Pgp– modulating agents such as PSC-833, verapamil, and cyclosporine have been evaluated clinically as adjunct therapy for multidrugresistant cancers, with little success.[3,4] More recently, P-gp has been identified in healthy human tissue such as intestine, liver, brain, and kidney, suggesting that P-gp function may contribute to drug absorption, distribution, and elimination.[5–7]

The human kidney expresses several basolateral and apical drug transport proteins, including the organic anionic transporter-1, organic cationic transporter (OCT)-1, multidrug P-GLYCOPROTEIN RENAL DRUG INTERACTIONS IN MDR1-MDCK CELLS *Karyekar et al* resistance–associated protein-2, and P-gp.[7,8] Renal P-gp is located primarily in glomerular mesangium and the apical membrane of proximal tubule epithelia, where it is likely involved in the elimination of endogenous and exogenous compounds from the systemic circulation.[2,7,9] Pglycoprotein– mediated tubular secretion involves transport of substrates

across the apical membrane into the tubular lumen. However, the extent of P-gp involvement in the renal elimination of drugs and the effects of drugs and disease states on P-gp function are not clearly understood.

Cimetidine is a histamine2-receptor antagonist that is widely used to treat gastrointestinal ulcers.[10,11] It is predominantly excreted unchanged by the kidney, with renal clearance values approximately 4-fold greater than creatinine clearance, indicating extensive tubular secretion. Classified as an organic cation, cimetidine also has been identified as a P-gp substrate based on drug transport studies conducted in multidrugresistant- 1 (MDR1)–Madin-Darby canine kidney (MDCK) cell lines and P-gp–enriched renal brush border membrane vesicles.[12,13] In an isolated perfused rat kidney model, pretreatment with cimetidine reduced the clearance of rhodamine 123, suggesting that competitive inhibition among P-gp substrates for renal tubular secretion may occur.[14] Recent studies have demonstrated that P-gp function is inhibited by itraconazole and PSC- 833 in vitro. Itraconazole was shown to reverse drug resistance to the P-gp substrates daunorubicin, Adriamycin, and etoposide in P388/ADR cells at concentrations of 0.1–2.8 μg/ml.[15,16] PSC-833, a nonimmunosuppressive analog of cyclosporine, is one of the most potent P-gp inhibitors in development as an adjunct to cancer chemotherapy. It was shown to inhibit tubular excretion of ivermectin in isolated proximal tubules and significantly reduced the renal clearance of vincristine in a rat model.[17,18] Taken together, these in vitro and in vivo data suggest that itraconazole and PSC-833 may inhibit renal P-gp at concentrations achieved clinically, resulting in decreased renal tubular secretion. We sought to establish an in vitro model for screening Pgp– mediated renal drug interactions and to evaluate the effects of itraconazole and PSC-833 on the transepithelial transport of cimetidine in MDR1-MDCK cells.

Methods

The MDR1-MDCK cells were obtained from the National Institutes of Health (Dr. Michael Gottesman, Bethesda, MD). Human colon carcinoma cells (Caco-2) and MDCK (wild-type) cells were purchased from American Type Culture Collection (Rockville, MD). Cells used in this study were between passages 30 and 35. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum, L-glutamine, trypsin (0.25%)- ethylenediaminetetraacetic acid ([EDTA] 1 mmol/L), and penicillin G–streptomycin mixture were purchased from Gibco Laboratories (Lenexa, KS). The T-75 flasks were obtained from Becton Dickinson Labware (Franklin Lakes, NJ), and Transwell clusters (24-mm diameter, 0.4-μm pore size) were obtained from Corning Costar (Cambridge, MA). [^{14}C]mannitol (46.6 mCi/mmol), [^{3}H]propranolol (29.0 Ci/mmol), [^{14}C]cimetidine (21.0 Ci/mmol), and unlabeled cimetidine were purchased from Sigma Chemical Co. (St. Louis, MO). PSC-833 was obtained from Novartis (Basel, Switzerland), and itraconazole was obtained from Research Diagnostics, Inc. (Flanders, NJ). All other analytic-grade chemicals were obtained from Sigma Chemical Co. or Fischer Scientific (Fair Lawn, NJ).

Cell Culture Methods and Drug Transport Studies

All cell lines were grown in T-75 flasks at 37^{o}C with 5% CO_2. The MDCK and MDR1-MDCK cells were grown in DMEM containing glucose 4.5 g/L, sodium pyruvate 1 mmol/L supplemented with 10% fetal bovine serum, L-glutamine 5 mmol/L, penicillin 50 units/ml, and streptomycin 50 μg/ml, as previously described.[12,19] The MDR1-MDCK cells were maintained in the presence of colchicine 80 ng/ml. The Caco-2 cells were grown in DMEM supplemented with 2% L-glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum. Cells were grown to confluence and subcultured by trypsinization, and MDR1-MDCK cells were seeded on polypropylene filters in the Transwell at a density of 2 x 10^6 cells/well. The culture medium (1.5 ml in the apical chamber and 2.6 ml in the basolateral

chamber) was replaced every other day, and transport studies were performed in triplicate on day 6 after seeding.

Before each cimetidine interaction study, the integrity of cell monolayers was measured by means of transepithelial electrical resistance with use of a Millicell-ERS (Millipore Corp., Bedford, MA). In addition, [^{14}C]mannitol and [^{3}H]propranolol transport studies were conducted as markers of paracellular and transcellular transport, respectively.[19]

Transport inhibition studies were performed on MDR1-MDCK cell monolayers. Apical-tobasolateral transport and basolateral-to-apical transport of cimetidine were assessed in the absence and presence of PSC-833 0.5 μmol/L and itraconazole 0.1, 0.5, and 2.0 μg/ml. After preincubation with inhibitor for 30 minutes, both cimetidine and inhibitor were added to the apical compartment in the apical-to-basolateral studies and to the basolateral compartment in the basolateral-to-apical studies. Samples of DPBS from the opposite compartments were obtained at 10, 20, 30, 40, 50, 60, 75, 90, and 120 minutes. The monolayers were maintained at 37°C and were orbitally agitated at 50–60 rpm throughout the length of the experiment.

Immunoblotting Methods

The expression of P-gp was confirmed in each cell line by means of Western blot analysis. Cell lysis was accomplished by adding buffer containing Tris 20 mmol/L at pH 7.4, NaCl 137 mmol/L, EDTA 2 mmol/L at pH 7.4, 1% Triton X- 100, 10% glycerol, phenylmethylsulfonylfluoride 1 mmol/L, sodium vanadate 0.2 mmol/L, and benzamidine 1 mmol/L. The cell lysates were centrifuged at 14,000 rpm at 4°C, the supernatant was diluted 1:1 with 2 times protein sample buffer, and 25 μg of protein from each cell line was loaded onto a 15% sodium dodecylsulfate– polyacrylamide gel and separated on a polyvinylidene defluoride membrane (Millipore Corp.) by using wet electrophoretic transfer. The membrane was washed in Tris-buffered saline containing Tris 50 mmol/L pH 7.5, NaCl 0.15 mol/L, and 0.1% Tween 20, and nonspecific binding sites were blocked with 5% (weight: volume ratio) nonfat dry milk in Tris-buffered saline. The membrane was then incubated overnight in 1:1000 dilution of the specific monoclonal anti–P-gp antibody MRK-16 (Kamiya Biomedical Co., Seattle, WA), followed by antimouse secondary antibody for 1 hour. After washing, the proteins were visualized by using electrochemiluminescence reagent (Amersham Pharmacia Biotech, Buckinghamshire, England).

Analytic Methods

Concentrations of [^{14}C]mannitol, [^{3}H]propranolol, and [^{14}C]cimetidine in DPBS samples were determined by using a Becton-Dickinson scintillation counter. Unlabeled cimetidine concentrations were determined by highperformance liquid chromatography (HPLC) with minor modifications, as previously reported.[20] Briefly, the HPLC system consisted of a Waters 2690 Separations Module equipped with a Waters UV detector set at 228 nm. Separation was achieved on a reversed phase C_{18} column with a mobile phase consisting of acetonitrile and heptanesulfonic acid in 20 mmol/L sodium acetate buffer (23:77) delivered at a flow rate of 1.0 ml/minute.

Data Analysis

The amount of cimetidine transported across each cell layer was quantified for each collection interval. The linear portion of the cumulative amount versus time profile yielded the appearance rate in the

receiver chamber (dQ/dt). Apparent permeability coefficients (Papp) for cimetidine were determined as follows:

$$Papp = (dQ/dt)/(A \times D_0)$$

where (dQ/dt) is the linear appearance rate of mass in the receiver solution, A is the crosssectional area of the Transwell insert, and D_0 is the initial concentration of the compound in the donor compartment. The efflux ratios (R_E) for basolateral-to-apical (BA) and apical-to-basolateral (AB) transport were calculated as follows:

$$R_E = Papp_{BA}:Papp_{AB}$$

Coefficients of determination (r^2) were obtained by linear regression analysis. Apparent permeability coefficients were statistically compared by using one-way analysis of variance, with Dunnett's post hoc

Table 1. Apparent Permeability Coefficients for Apical-to-Basolateral Transport for [^{14}C]Mannitol and [^{3}H]Propranolol in the Absence and Presence of PSC-833 or Itraconazole in MDR1-MDCK Cells

Group	Mannitol (x 10^{-7} cm/sec)	Propranolol (x 10^{-5} cm/sec)
Control	6.31 ± 0.9	1.96 ± 0.1
PSC-833 0.5 µmol/L	6.51 ± 0.6	—
Itraconazole		
0.1 µg/ml	6.50 ± 0.9	2.02 ± 0.1
0.5 µg/ml	6.41 ± 0.5	2.05 ± 0.1
2.0 µg/ml	6.09 ± 0.3	2.07 ± 0.08

MDR1 = multidrug-resistant-1 cell line; MDCK = Madin-Darby canine kidney cell line.
Data are mean ± SD (n=3).

test if needed (SPSS Inc., Chicago, IL). A p value less than 0.05 was considered to indicate a statistically significant difference.

Results

Transport of [$_{14}$C]Mannitol and [$_{3}$H]Propranolol in MDR1-MDCK Cells

The paracellular and transcellular integrity of MDR1-MDCK cells was assessed by evaluating the transport of [$_{14}$C]mannitol and [$_{3}$H]propranolol, respectively. The apparent permeability coefficients for each of the two compounds in the absence and presence of PSC-833 0.5 µmol/L and itraconazole 0.1, 0.5, and 2.0 µg/ml are shown in Table 1. The ranges of apparent permeability values were 6.09–6.50 x 10^{-7} cm/second for mannitol and 1.96–2.07 x 10^{-5} cm/second for propranolol. There were no significant differences observed in the apparent permeability values during exposure to itraconazole or PSC-833, indicating a lack of effect on transcellular or paracellular membrane pathways. Typical transepithelial electrical resistance values for MDR1-MDCK and MDCK cells were 1200–1500 V cm^2.

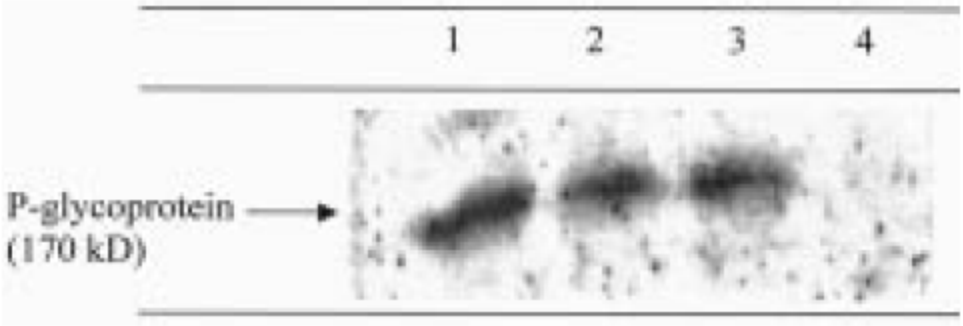

Figure 1. Western blot analysis of Madin-Darby canine kidney (MDCK), mutidrug-resistant-1 (MDR1)-MDCK, and human colon carcinoma-2 (Caco-2) cell lysates. Gels were developed for P-glycoprotein (170 kD) by using the monoclonal anti–P-glycoprotein antibody. Lane 1 = Caco-2; lanes 2 and 3 = MDR1-MDCK; lane 4 = MDCK (wild type).

P-glycoprotein Expression in MDR1-MDCK, MDCK, and Caco-2 Cells

The Western blot results from MDR1-MDCK, MDCK, and Caco-2 cell lysates are shown in Figure 1. A measurable band at molecular weight 170 kD was observed in MDR1-MDCK and Caco-2 cells, indicating expression of P-gp. The relative degree of P-gp expression was similar in both the MDR1-MDCK and Caco-2 cells. P-glycoprotein expression in MDCK cells was negligible.

Cimetidine Transport in MDR1-MDCK Cells

The apparent permeability values (apical-tobasolateral and basolateral-to-apical) of cimetidine in the absence and presence of PSC- 833 0.5 µmol/L and itraconazole 0.1, 0.5, and 2.0 µg/ml, are shown in Figures 2 and 3, respectively. A significant decrease was noted in apparent permeability value for basolateral-to-apical transport in the presence of PSC-833 0.5 µmol/L (from 2.96 to 1.15 x 10^{-6} cm/sec, $p<0.05$). Although the apparent permeability value for apical-to-basolateral transport increased slightly from 1.58 to 1.91 x 10^{-6} cm/second, the change was not statistically significant. Efflux ratio values decreased from 2.36 to 1.80, indicating that net transport of cimetidine was inhibited by PSC-833.

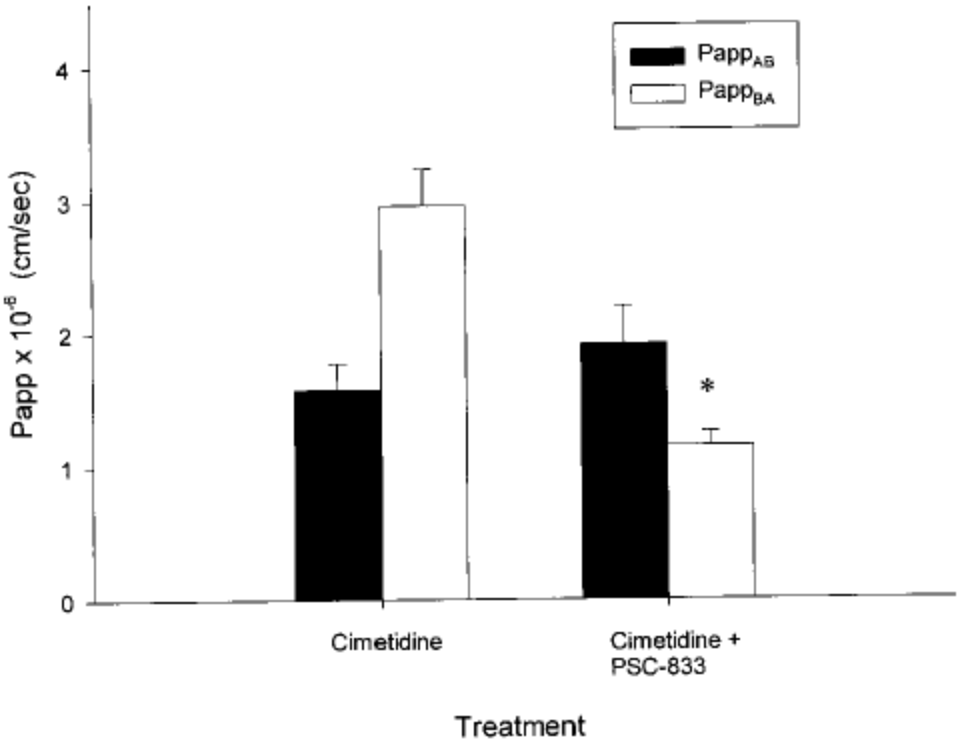

Figure 2. Apparent permeability values for apical-to-basolateral ($Papp_{AB}$) and basolateral-to-apical ($Papp_{BA}$) transport for cimetidine in the absence and presence of PSC-833 0.5 µmol/L in MDR1-MDCK cells. Values reported are mean ± SD (n=3). *$p<0.05$ vs control.

The decrease in apparent permeability value for basolateral-to-apical transport observed in the presence of itraconazole (from 3.96 to 1.92 x 10^{-6} cm/sec) was concentration dependent. Here, efflux ratio values decreased from 2.36 to 1.19 at concentrations up to 2.0 µg/ml. The apparent permeability values for basolateral-to-apical transport were significantly reduced at 0.5 and 2.0 µg/ml ($p<0.05$), whereas those for apical-tobasolateral transport remained unchanged.

Discussion

Results of recent research indicate that multidrug transport proteins, such as P-gp, appear to play an important role in many aspects of drug disposition. For example, P-gp has been implicated in drug interactions at various biologic sites including brain, intestine, liver, and kidney.[9, 21, 22] Recent studies in Caco-2 intestinal cells suggest that apical-to-basolateral transport of P-gp substrates such as cyclosporine and vinblastine is increased in the presence of P-gp inhibitors such as verapamil, nifedipine, daunomycin, and PSC-833.[5, 23] Preclinical and clinical evidence also suggests that P-gp plays a major role in oral bioavailability, especially for anticancer drugs such as paclitaxel.[24, 25] These studies, however, were not designed to evaluate the effect of P-gp inhibitors on hepatic or renal elimination mechanisms, which also may contribute to systemic drug concentrations. Although much research has focused on intestinal P-gp, the role of P-gp in renal drug elimination remains largely unknown.

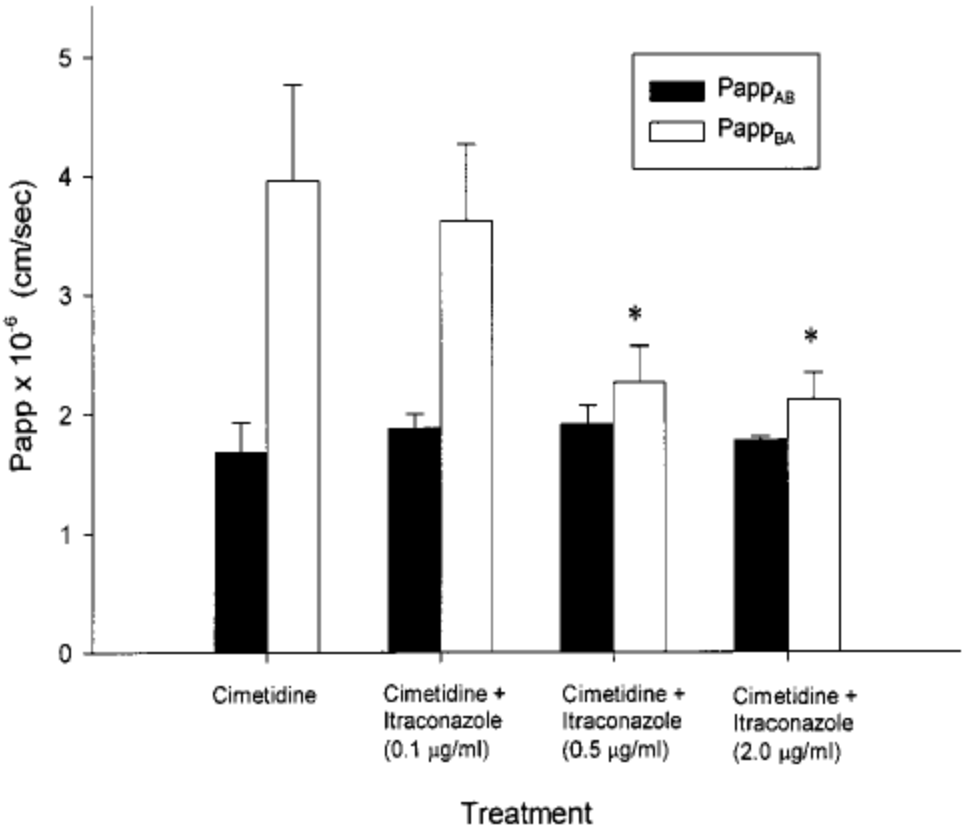

Figure 3. Apparent permeability values for apical-to-basolateral ($Papp_{AB}$) and basolateral-to-apical ($Papp_{BA}$) transport for cimetidine in the absence and presence of itraconazole 0.1, 0.5, and 2 µg/ml in MDR1-MDCK cells. Values reported are mean ± SD (n=3). *$p<0.05$ vs control.

Our study evaluated the effect of known P-gp inhibitors on renal tubular secretion of the organic cation cimetidine. A wide variety of endogenous and exogenous organic anions and cations are known to undergo extensive tubular secretion, including uric acid, penicillin, histamine, and procainamide.[26] Active, energydependent tubular secretion of substrates from basolateral-to-apical membrane occurs by means of membrane-bound transport proteins such as the organic anionic transporter and OCT.[26,27] Traditionally, substrates for these transporters were classified based on chemical properties and ionization of the substrates at physiologic pH. Conversely, it appears that P-gp is less selective for its substrates, with some preference for lipophilic organic cations such as doxorubicin, vinblastine, and fexofenadine.[19,28] In a P-gp knock-out mouse model (–/– for *mdr1*), fexofenadine accumulation in kidney was 4-fold greater than in the wild type (+/+), suggesting a role of P-gp in renal elimination of fexofenadine in vivo.[28]

We used a renal cell monolayer expressing Pgp (MDR1-MDCK) to study interactions with cimetidine, a P-gp substrate that undergoes extensive renal tubular secretion in vivo. A high degree of P-gp expression was confirmed in our MDR1-MDCK model, which was similar to that observed in Caco-2 cells.[29] These results are supported by findings in another study,[30] in which the extent of P-gp expression in the proximal tubules of the kidney was found to be quantitatively similar to that in the smallintestine

epithelial cells. Thus, based on previous evaluations of intestinal drug interactions with P-gp inhibitors, it is likely that such interactions also may be significant in the kidney.

We found that cimetidine was actively transported in MDR1-MDCK cells. This is consistent with results from another study,[12] in which basolateral-to-apical transport of cimetidine was much greater in MDR1-MDCK cells as compared with that in wild-type MDCK cells. The ability of both P-gp and OCT to transport a wide range of organic cations, as demonstrated in experimental models, may explain the high rate of tubular secretion and inability to characterize transport maxima for some cationic drugs in vivo.[31]

In our study, we found that itraconazole and PSC-833, both known inhibitors of P-gp, reduced the basolateral-to-apical transport of cimetidine. The concentrations of PSC-833 (0.5 μmol/L) and itraconazole (0.1–2.0 μg/ml) used in this study were chosen based on concentrations achieved in clinical studies.[24, 32] Interestingly, both agents reduced the basolateral-to-apical transport and efflux ratios of cimetidine at clinically achievable concentrations in the MDR1-MDCK model. Thus, it appears that use of P-gp inhibitors such as itraconazole and PSC-833 may block renal drug excretion, resulting in systemic accumulation and toxicity. This may be especially important for drugs such as cimetidine, here high drug concentrations have been associated with serious neurotoxicity, especially in patients with renal insufficiency.[33–35]

Investigations of renal P-gp function and drug interactions in humans are limited. In healthy volunteers, itraconazole reduced the renal clearance of digoxin and quinidine, known P-pg substrates, by 20–50%.[32, 36] Similarly, cyclosporine caused a 21% decrease in the total dose of doxorubicin excreted in the urine.[4] The authors of one study[37] reported that cyclosporine reduced the renal clearance and nonrenal clearance of etoposide by 38% and 55%, respectively, in patients with cancer. This suggests that administering P-gp inhibitors may significantly alter the renal handling of some drugs that are Pgp substrates. Furthermore, use of P-gp modulators in cancer regimens is becoming increasingly prevalent; thus, the pharmacokinetic and pharmacodynamic implications of renal P-gp inhibition must be evaluated.

We used the MDR1-MDCK monolayer model to investigate a P-gp–mediated drug interaction because it is stably transfected with human MDR1. Although it is reported that other transporters including OCT-2 and multidrug resistance–associated protein-1 may be present in this cell line, the extent of P-gp expressed in this cell line is much greater than the other transporters.[38] Since cimetidine appears to be a substrate for both P-gp and OCT, it is entirely possible that a small amount of cimetidine was transported by OCT-2 present in the MDR1-MDCK. Although we did not determine OCT-2 expression in this P-gp overexpressing system, the mechanism most likely dominating the efflux of cimetidine is this model is P-gp. This is strongly supported by our findings that PSC-833 and itraconazole, both specific inhibitors of P-gp, significantly reduced the transcellular efflux of cimetidine. Thus, the changes in efflux observed for cimetidine in the presence of PSC-833 and itraconazole are most likely due to changes in Pgp–mediated transport. Establishment of in vitro models to evaluate drug interactions in the kidney allows rapid identification of drug candidates and likely drug transport mechanisms. Disadvantages of previously developed models of renal function, such as the intact animal and isolated perfused renal tubules, include high cost of development, need for specialized technical staff, and slow throughput times. The MDR1-MDCK system should be limited to investigation of drugs (P-gp substrates) that are most likely to be susceptible to renal drug interactions. For example, this system can be used to study drugs that are renally cleared (i.e., fraction excreted renally is greater than 30%) and undergo extensive active tubular secretion (i.e., renal clearance greatly exceeds glomerular filtration rate).

In summary, understanding the role of P-gp in renal drug elimination is an important part of identifying renal drug interactions, preventing drug toxicity, and optimizing drug therapy in patients. Use of the MDR1-MDCK cell model is valuable for studying such interactions because of its rapid growth in culture and relatively high level of P-gp expression. Further studies are required to determine in vitro–in vivo correlations and to evaluate the effects of renal disease, drugs, and nephrotoxins on P-gp expression and activity.

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