Can the Enhanced Renal Clearance of Antibiotics in Cystic Fibrosis Patients be Explained by P-Glycoprotein Transport?

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Purpose. To investigate *in vitro* if P-glycoprotein (P-gp) transport can differentiate between antibiotic drugs exhibiting increased active renal clearance (CL_r) in cystic fibrosis (CF) patients (i.e., dicloxacillin, trimethoprim) and drugs that do not exhibit this phenomenon (i.e., cefsulodin, sulfamethoxazole).

Methods. Transport studies were carried out in MDCK (wild type) and MDR1-MDCK (P-gp overexpressing) cells that were grown to confluence on Transwell inserts. [^{14}C]-mannitol transport and transepithelial electrical resistance (TEER) were measured to validate the integrity of the cells. Drug concentrations were analyzed using HPLC.

Results. Dicloxacillin and trimethoprim are substrates of P-gp (B→A/A→B ratios in MDR1-MDCK cells are 32 and 50, respectively). P-gp inhibitors (i.e., GG918, cyclosporine, ketoconazole, vinblastine) decreased the B→A transport of dicloxacillin and trimethoprim and increased the A→B transport of trimethoprim while non-P-gp inhibitors (e.g., PAH) had no effect. In contrast, cefsulodin and sulfamethoxazole are not substrates of P-gp (B→A/A→B values in MDCK and MDR1-MDCK cells are ~1).

Conclusions. Our *in vitro* studies suggest that P-glycoprotein may play a role in increasing renal clearance of drug substrates in CF patients. Dicloxacillin and trimethoprim, which are both substrates of P-gp, show increased active renal clearance in CF patients while cefsulodin and sulfamethoxazole, which are not P-gp substrates, do not show increased active renal clearance in CF patients.

KEY WORDS: P-glycoprotein; cystic fibrosis; renal clearance; dicloxacillin; trimethoprim; cefsulodin; sulfamethoxazole.

INTRODUCTION

Cystic fibrosis (CF) is one of the most common lethal genetic disorders affecting Caucasian populations. It is an autosomal recessive disorder, which occurs in approximately 1 in 2500 live births. The disease is due to a defect in a gene called CFTR (cystic fibrosis transmembrane conductance regulator). The gene encodes a protein that functions as a cAMP-regulated chloride channel that is located on the apical membrane of epithelial cells. The absence of the channel causes abnormal electrolyte transport in the epithelial cells from sweat glands, airways, pancreas, and intestines, causing salty sweat, chronic obstructive pulmonary disease, pancreatic insufficiency and meconium ileus (1).

CF patients suffer from recurrent and chronic respiratory tract infections, which produce marked morbidity and mortality. To treat the infections, the patients take many antibiotics and it was observed as early as 1975 that the renal clearance (CL_r) of many antibiotics is higher in CF patients (e.g., dicloxacillin (2), methicillin (3), cloxacillin (4), trimethoprim (5), ticarcillin (6,7)). Even though there are a few conflicting studies in the literature, the enhanced renal clearance of antibiotics in CF patients has become a recognized pattern and many review articles have been written on this topic (8–13).

To determine what causes the enhanced renal clearance of drugs in CF, we need to examine the components of renal clearance. Three factors govern renal clearance: filtration, tubular secretion and tubular reabsorption. Enhanced CL_r could be due to an increase in filtration rate, an increase in tubular secretion or a decrease in tubular reabsorption. Filtration rate is governed by glomerular filtration rate (GFR) and protein binding and is equal to fu x GFR (fu = fraction unbound). There is no difference in protein binding of many proteins (e.g., dicloxacillin, cefsulodin, trimethoprim, sulfamethoxazole, cloxacillin, ceftazidime, gentamicin) between control and CF patients (2,4,5,14–16). GFR in CF patients has been studied with conflicting results, but most studies have shown that it is not altered in CF patients (3,14,16–18). The Karolinska group has reported slightly increased GFR in their CF patients and it is thought to be due to the essential fatty acid status of their patients (19,20). Thus in most cases enhanced CL_r is not due to an increase in filtration rate but is due to either an increase in tubular secretion or a decrease in tubular reabsorption or both.

Presently it is not possible to determine the exact contribution of tubular secretion or tubular reabsorption to CL_r, one can only determine the net tubular secretion or reabsorption. If the CL_r is greater than fu x GFR then there is a net secretion while if it is less than fu x GFR then there is a net reabsorption. Tubular secretion is an active process that involves transporters such as P-glycoprotein, organic anion and organic cation transporters. Tubular reabsorption is composed of an active process involving transporters and a passive diffusion process that is dependent on the molecular weight, lipophilicity and the pKa of the drug, urine flow rate and urine pH. Hutabarat *et al.* (5) did not find a significant difference in the urine pH between normal and CF patients.

For most drugs, the actual mechanisms involved in their elimination are often unknown. For example, a drug that shows CL_r equal to GFR could imply that tubular secretion and reabsorption are not involved or it could imply that secretion and reabsorption occur to the same extent. As a general rule, elimination of most aminoglycosides is thought to involve tubular reabsorption. For cephalosporins both tubular reabsorption and tubular secretion are present while elimination of penicillins derivatives is thought to involve tubular secretion (10). Dicloxacillin, ticarcillin (penicillin derivatives) and trimethoprim (a weak base) are thought to have net tubular secretion and based on their data, Jusko *et al.* (2), de Groot *et al.* (6), Hutabarat *et al.* (5) and Wang *et al.* (7) hypothesized that enhanced tubular secretion is responsible for the enhanced renal clearance of their test drugs but to date, the exact mechanisms that cause the enhanced CL_r of those drugs have not been elucidated.

We hypothesize that the enhanced CL_r of some drugs in CF patients is due to an increase in tubular secretion, which is

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caused by increased P-gp expression in those patients that occurs due to a defect in CFTR. P-gp is a known transporter that can eliminate drugs from the body. In the kidney, P-gp is located in the apical membrane of proximal tubules and it effluxes drugs from the blood to the lumen. Increased P-gp expression could increase the tubular secretion of drugs that are substrates of P-gp hence increasing their CL_r.

Some evidence in the literature supports the hypothesis that there may be a coordinate regulation of the CFTR and MDR1 (P-gp) genes. CFTR and P-gp have been shown to have inverse patterns of epithelial expression in certain tissues both *in vitro* and *in vivo*. Breuer *et al.* (21) have shown in the human colon epithelial cell line HT-29 that increased protein expression of P-gp is associated with a corresponding decrease in CFTR protein expression and vice versa. Trezise *et al.* (22) have shown in mouse cells (e.g., intestine, uterus, pancreas) that express cftr that mdr1 mRNA expression cannot be detected and vice versa. They also observed a switch from cftr to mdr1 mRNA expression in the rat uterine epithelium on pregnancy. Trezise *et al.* (23) have also shown that in cftr neonatal and 3-4 week old knockout mice, cftr mRNA expression in the intestines was reduced 4-fold while there is a corresponding 4-fold increase in mdr1 mRNA expression and an intermediate level of mdr1 mRNA in heterozygous mice.

The structural and functional similarities between CFTR and P-gp further support the hypothesis that P-gp expression might be upregulated in CF patients. Both CFTR and P-gp are members of the ABC transporter superfamily, they have similar structures and molecular weights and both proteins are located in the apical membrane of epithelial cells. The genes that encode the proteins are located in chromosome 7q (CFTR 7q.31, MDR1 7q.21) and have similar promoters (24). CFTR is a chloride channel while P-gp, besides acting as an efflux pump for xenobiotics, is also involved in modulating the function of cell-swelling activated chloride channels (25). Thus CFTR and P-gp are functionally related.

The coordinate regulation of P-gp and CFTR, the many similarities between the two proteins and their similar roles as regulator of chloride channels provide circumstantial evidence that these two proteins might have complementary roles. We hypothesize that the absence of CFTR in CF patients might be compensated for by upregulation of P-gp that in turn causes the enhanced CL_r of drugs that are substrates of P-gp.

To determine whether P-gp may play a role in enhancing renal clearance of some drugs in CF patients, we investigated *in vitro* if dicloxacillin and trimethoprim, both of which showed enhanced active CL_r in CF patients, are substrates of P-gp. For controls, we examined if cefsulodin and sulfamethoxazole, drugs that do not show enhanced active CL_r in CF patients, are not substrates of P-gp.

MATERIALS AND METHODS

Materials

The MDCK and MDR1-MDCK cell lines were generously provided by Dr. Ira Pastan of the National Cancer Institute (26). GG918 (GF120918) was a gift from Glaxo-Wellcome. All cell culture media were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Transwell inserts and six-well plates were from Costar Corp. (Cambridge, MA). [^{14}C]-mannitol and [^{3}H]-digoxin were purchased from New England Nuclear (Boston, MA). All other chemicals (e.g., dicloxacillin, trimethoprim, sulfamethoxazole, cefsulodin) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

MDCK and MDR1-MDCK cells were seeded onto Transwell inserts of a 6-well plate system in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at a density of roughly 300,000 cells/insert. For MDR1-MDCK cells, 80 ng/ml colchicine was added to select for the transfecting cells. The cells were grown as a monolayer for 5–7 days at 37°C and 5% humidified CO_2-atmosphere. Cell integrity and monolayer confluence were tested by microscopy and transepithelial electrical resistance (TEER) measurements (Millipore Millicell-ERS system). The TEER values were 90–250 Ω-cm^2 for MDCK and 3800-6300 Ω-cm^2 for MDR1-MDCK cells.

Transport Experiments

The transport experiments were adapted with modifications from Zhang *et al.* (27). Briefly, the cells were washed once and preincubated for ~30 min at 37°C in 5% CO_2 in Hank's Balanced Salt Solution with 22.5 mM HEPES (HBSS-H). For measuring drug secretion (B→A), 2.5 ml of HBSS-H solution containing the drug was put into the basal (B) side and 1.5 ml of HBSS-H was put into the apical (A) side. At selected times, 200 μl samples were taken from the A side and replaced with fresh HBSS-H. For measuring drug absorption (A→B), the drug solution was put into the A side and samples were taken from the B side. For inhibition studies, the inhibitor was put in both the A and B sides. During the studies, the cells were incubated in a shaking incubator (Boekel Scientific, Feasterville, PA). To establish cell integrity, [^{14}C]-mannitol (a paracellular marker) transport was measured for 1 h at the end of the experiments. Transport of [^{3}H]-digoxin, a known P-gp substrate, was measured as the positive control.

HPLC Analysis

Samples were stored at −20°C until analysis by high performance liquid chromatography (HPLC) using methods adapted from Jehl *et al.* (28). Cefsulodin, dicloxacillin and trimethoprim were analyzed using an Ultrasphere ODS 150 × 2 mm (Phenomenex, Torrance, CA) column while sulfamethoxazole was analyzed with an Ultracarb ODS 150 × 4.6 mm column. The flow rate was 0.25 ml/min for cefsulodin, dicloxacillin and trimethoprim, 0.5 ml/min for sulfamethoxazole. The organic mobile phase for all compounds was acetonitrile (5 to 70% in 14.5 min for cefsulodin and dicloxacillin, 5 to 70% in 7 min for sulfamethoxazole and 20% isocratic for trimethoprim). The aqueous mobile phases for cefsulodin, dicloxacillin, trimethoprim and sulfamethoxazole were 5 mM TBAB (pH 3.7), 20 mM ammonium acetate (pH 5.8), 100 mM KH_2PO_4 (pH 2.7), and 0.1% triethylamine (pH 5.9), respectively. The limit of detection for dicloxacillin and cefsulodin was 0.025 μg/ml, for trimethoprim and sulfamethoxazole it was 0.1 μg/ml. All compounds were detected at 214 nm.

Table I. Published Renal Clearance Values for Four Antibiotics in Control and CF Patients

Drug (reference)	Renal clearance (CL_r)	
	Control	CF
Dicloxacillin (2)	95 ± 28 ml/min/1.73m^2	282 ± 135 ml/min/1.73m^{2a}
Trimethoprim (5)	82 ± 19 ml/min/1.73m^2	126 ± 30 ml/min/1.73m^{2a,b}
Sulfamethoxazole (5)	3.1 ± 1.3 ml/min/1.73m^2	2.2 ± 1.7 ml/min/1.73m^{2b}
Cefsulodin (14)	141 ± 36 ml/min/1.73m^2	140 ± 34 ml/min/1.73m^2
Cefsulodin (19)	92 ± 18 ml/min/1.73m^2	122 ± 19 ml/min/1.73m^{2a,c}
Cefsulodin (19)	89 ± 5 ml/min/1.73m^2	118 ± 16 ml/min/1.73m^{2a,c}

[a] Statistically significant difference from control, $P < 0.05$.
[b] Converted from L/h/kg.
[c] Increased CLr in CF is due to increased GFR.

RESULTS

Cell Monolayer Integrity

The calculated apparent permeability values (P_{app}) for [^{14}C]-mannitol in MDCK and MDR1-MDCK cells (~5×10^{-7} cm/s) agrees with the literature values, validating the integrity of the tight junction. The transport of [^{3}H]-digoxin (positive control P-gp substrate) was as expected with the B→A/A→B ratio equal to 6 in MDCK cells and equal to 18 in MDR1-MDCK cells.

Bidirectional Transport in MDCK and MDR1-MDCK Cells

Trimethoprim P_{app} in the B→A direction was larger than the A→B direction in MDR1-MDCK cells (B→A/A→B ratio = 50) (Table II). The B→A and A→B P_{app} were not different in MDCK cells (B→A/A→B ratio = 1). MDR1-MDCK cells showed a larger B→A P_{app} and a smaller A→B P_{app} than MDCK cells. These results were first presented in abstract form (29) and recently Polli *et al.* (30) also reported trimethoprim to be a P-gp substrate.

Dicloxacillin, like trimethoprim, showed a larger B→A than A→B P_{app} in MDR1-MDCK cells but showed no difference in P_{app} in MDCK cells (Table II). MDR1-MDCK cells showed a larger B→A P_{app} and a smaller A→B P_{app} than MDCK cells, which means that dicloxacillin is also likely to be a P-gp substrate.

Sulfamethoxazole did not show a difference in B→A and A→B P_{app} in both MDCK and MDR1-MDCK cells (B→A/A→B ratio in both cell lines ~1) and there was no statistically significant difference in P_{app} between MDCK and MDR1-MDCK cells (Table II). Cefsulodin showed similar results (Table II). This indicates that sulfamethoxazole and cefsulodin are likely not substrates of P-gp.

Effect of Inhibitors on Trimethoprim and Dicloxacillin Fluxes in MDR1-MDCK Cells

Cyclosporine, ketoconazole, vinblastine, verapamil (typical P-gp inhibitors) and GG918 (a specific P-gp inhibitor) significantly decreased trimethoprim B→A flux and increased the A→B flux while sulfamethoxazole, tetra-ethyl ammonium chloride (TEA, an organic cation inhibitor) and para-amino hippurate (PAH, an organic anion inhibitor) had no statistically significant effects on either the B→A or the A→B flux (Fig. 1a and 1b). Similar results on dicloxacillin B→A fluxes were seen with cyclosporine, ketoconazole, vinblastine, GG918 and PAH (Fig. 2a). However, the inhibitors had no statistically significant effect on dicloxacillin A→B fluxes (Fig. 2b).

DISCUSSION

It has been shown clinically that the CL_r of many (but not all) drugs in CF patients is enhanced and to date, it is not known what causes the enhanced CL_r. We hypothesize that the enhanced CL_r is due to increased P-gp expression in CF patients resulting in increased tubular secretion of drugs that are substrates of P-gp. In this study we tested *in vitro* whether trimethoprim, dicloxacillin, sulfamethoxazole and cefsulodin are substrates of P-gp. Trimethoprim and dicloxacillin

Table II. Dicloxacillin, Trimethoprim, Sulfamethoxazole, and Cefsulodin Transport in MDCK and MDR1-MDCK Cells

Cell type	Drug name	Drug concentration (μM)	$P_{app} \times 10^7$ (avg ± se, n = 3, cm/s)		B → A/ A → B
			B → A	A → B	
MDCK	dicloxacillin	250	9.9 (0.4)	10.9 (1.1)	0.9
MDR1-MDCK			29.0 (1.2)	0.91 (0.06)	32
MDCK	trimethoprim	100	78.3 (9.1)	78.5 (10.6)	1
MDR1-MDCK			128.7 (2.2)	2.6 (0.3)	50
MDCK	sulfamethoxazole	10	94.2 (14.8)	106.5 (9.1)	0.9
MDR1-MDCK			72.6 (11.3)	59.7 (15.5)	1.2
MDCK	cefsulodin	50	0.88 (0.04)	1.1 (0.12)	0.8
MDR1-MDCK			1.0 (0.08)	1.18 (0.09)	0.9

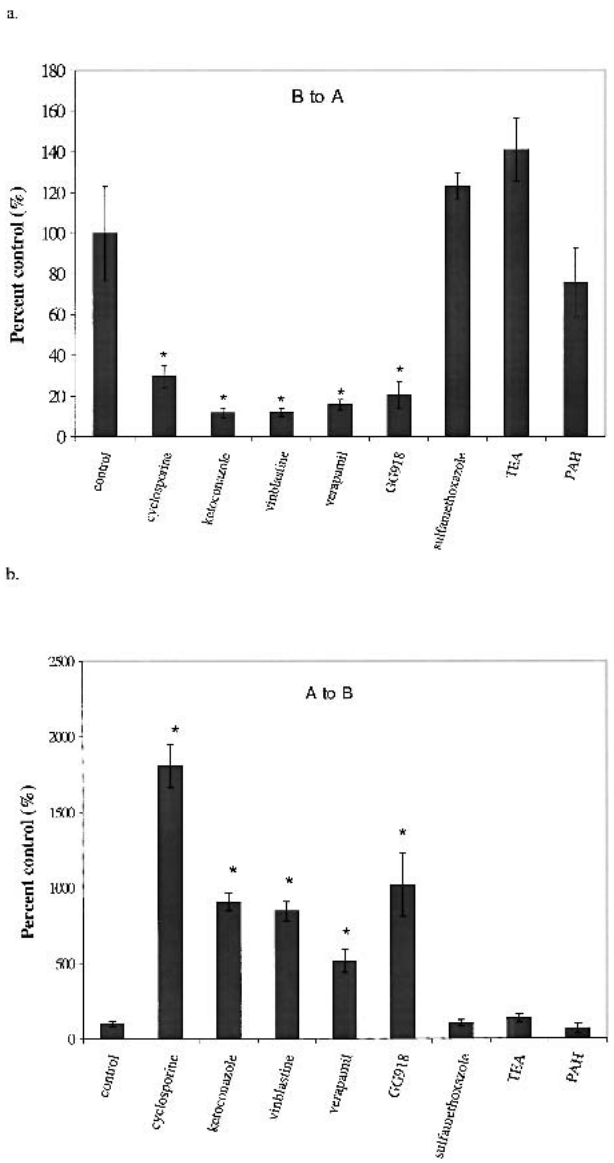

Fig. 1. Effect of various inhibitors on 100 μM trimethoprim flux in MDR1-MDCK cells. *a.* (B→A) and *b.* (A→B) The inhibitors are cyclosporine (20 μM), ketoconazole (100 μM), vinblastine (100 μM), verapamil (50 μM), GG918 (2.5 μM), sulfamethoxazole (500 μM), TEA (500 μM) and PAH (500 μM). *$P < 0.01$

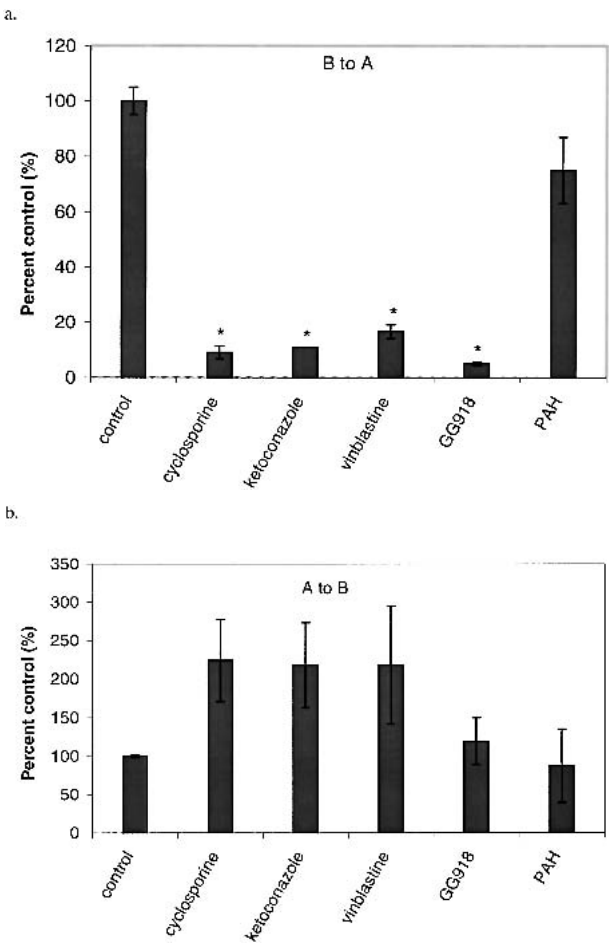

Fig. 2. Effect of various inhibitors on 250 μM (100 μM for cyclosporine and ketoconazole) dicloxacillin flux in MDR1-MDCK cells. *a.* (B→A) and *b.* (A→B) The inhibitors are cyclosporine (20 μM), ketoconazole (100 μM), vinblastine (100 μM), GG918 (2.5 μM), and PAH (1000 μM). *$P < 0.01$

showed enhanced active CL_r while sulfamethoxazole and cefsulodin did not show enhanced active CL_r in CF patients (Table I).

Table I compares CL_r for several drugs between normal and CF patients. Dicloxacillin was the first drug shown to have enhanced CL_r in CF patients, with a 3-fold increase in CL_r compared to normal (2). However, because a microbial assay was used to measure dicloxacillin concentration and the drug is known to have an active metabolite, some of the increase might have been due to enhanced metabolic clearance. For trimethoprim and sulfamethoxazole, HPLC assays were used to measure their concentration but GFR was not studied in those patients thus we could not conclusively rule out the role of GFR on trimethoprim's enhanced CL_r (5). For cefsulodin, a study done in 1983 by the Karolinska group showed no increase in CL_r of cefsulodin in CF patients (14). However, another study done by the same group in 1990 showed that the CL_r was increased in CF patients but the increase was accounted for by an increase in GFR, therefore it was not due to an enhanced active secretion (19). The increased GFR is believed to be due to the patients' essential fatty acid status (20).

The enhanced CL_r of dicloxacillin and trimethoprim were not due to changes in protein binding but were believed to be due to increased tubular secretion. Studies have shown that there is no difference in protein binding of trimethoprim, dicloxacillin, cefsulodin and sulfamethoxazole between control and CF patients, with fu values of 0.63, 0.05, 0.83, and 0.53, respectively (2,5,14). Arvidsson *et al.* (14) and Hedman *et al.* (19) showed that the CL_r of cefsulodin is very similar to inulin CL_r, therefore cefsulodin does not show net tubular secretion or reabsorption. Dicloxacillin, however, clearly showed net tubular secretion (CL_r >> fu x GFR) (2). GFR was not measured by Hutabarat *et al.* (5) for their study with trimethoprim and sulfamethoxazole. By comparing fu x GFR (assuming GFR 100–120 ml/min) with the observed CL_r values, trimethoprim showed a net tubular secretion while sulfamethoxazole showed a net tubular reabsorption.

In CF patients, trimethoprim and dicloxacillin have en-

hanced active CL_r while sulfamethoxazole and cefsulodin do not show enhanced active CL_r and we hypothesize that the increased CL_r is due to increased tubular secretion caused by increased P-gp expression in CF patients. Therefore, according to our hypothesis, we would expect trimethoprim and dicloxacillin to be substrates of P-gp and sulfamethoxazole and cefsulodin not to be substrates of P-gp.

Our bidirectional transport studies showed that the B→A/A→B ratios in MDR1-MDCK cells for trimethoprim and dicloxacillin were 50 and 32, respectively, values that were very different from the ratios in MDCK cells (~1). Sulfamethoxazole and cefsulodin, on the other hand, showed no difference in B→A/A→B ratios (all ~1). This suggests that trimethoprim and dicloxacillin are substrates of P-gp while sulfamethoxazole and cefsulodin are not. Inhibition studies with P-gp and non-P-gp inhibitors further reinforce this conclusion. P-gp inhibitors (cyclosporine, ketoconazole, vinblastine, GG918) decreased the B→A flux of trimethoprim and dicloxacillin and increased the A→B flux of trimethoprim in MDR1-MDCK cells, bringing the B→A/A→B ratios to about 1. On the other hand, PAH, an organic anion transporter inhibitor and not a P-gp inhibitor, had no effect on either trimethoprim or dicloxacillin transport in MDR1-MDCK cells. The same negative result was seen for trimethoprim with TEA, an organic cation transporter inhibitor. Clinically sulfamethoxazole is dosed concomitantly with trimethoprim and we have shown that sulfamethoxazole is not a P-gp substrate and it does not affect trimethoprim transport in MDR1-MDCK cells. Hence, we do not believe that coadministration of sulfamethoxazole in CF patients would affect the CL_r of trimethoprim.

We hypothesize that increased CL_r of some drugs observed in CF patients is due to increased P-gp expression in those patients. In accord with our hypothesis, our studies have shown that trimethoprim and dicloxacillin, drugs that exhibit increased active CL_r in CF patients, are substrates of P-gp while sulfamethoxazole and cefsulodin, which do not show increased active CL_r in CF patients, are not substrates of P-gp. We hope that the hypothesis and results presented here will allow others with access to human kidney tissue to investigate differences in P-gp and CFTR expression between CF and normal patients, and aid in predicting whether or not a compound will have altered renal clearance in CF.

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