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Vectorial Transport of Fexofenadine across Caco-2 Cells: Involvement of Apical Uptake and Basolateral Efflux Transporters

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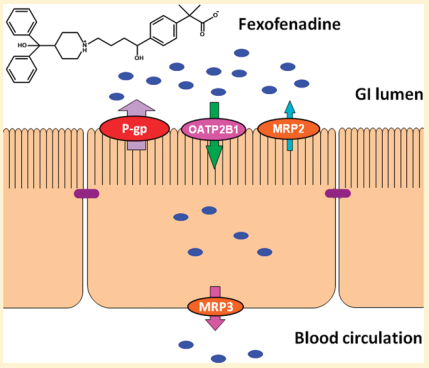
ABSTRACT: Fexofenadine is a nonsedative antihistamine that exhibits good oral bioavailability despite its zwitterionic chemical structure and efflux by P-gp. Evidence exists that multiple uptake and efflux transporters play a role in hepatic disposition of fexofenadine. However, the roles of specific transporters and their interrelationship in intestinal absorption of this drug are unclear. This study was designed to elucidate vectorial absorptive transport of fexofenadine across Caco-2 cells involving specific apical uptake and efflux transporters as well as basolateral efflux transporters. Studies with cellular models expressing single transporters showed that OATP2B1 expression stimulated uptake of fexofenadine at pH 6.0. Apical uptake of fexofenadine into Caco-2 cells was decreased by 45% by pretreatment with estrone 3-sulfate, an OATP inhibitor, at pH 6.0 but not at pH 7.4, indicating that OATP2B1 mediates apical uptake of fexofenadine into these cells. Examination of fexofenadine efflux from preloaded Caco-2 cells in the presence or absence of (i) the MRP inhibitor MK-571 and (ii) the P-gp inhibitor GW918 showed that apical efflux is predominantly mediated by P-gp, with a small contribution by MRP2, whereas basolateral efflux is predominantly mediated by MRP3. These results also showed that while OST$\alpha\beta$ is functionally active in the basolateral membrane of Caco-2 cells, it does not play a role in the export of fexofenadine. MK-571 decreased the absorptive transport of fexofenadine by 17%. However, the decrease in absorptive transport by MK-571 was 42% when P-gp was inhibited by GW918. The results provide a novel insight into a vectorial transport system mainly consisting of apical OATP2B1 and basolateral MRP3 that may play an important role in delivering hydrophilic anionic and zwitterionic drugs such as pravastatin and fexofenadine into systemic circulation upon oral administration.



KEYWORDS: fexofenadine, intestinal absorption, OATP2B1, MRP3, basolateral efflux, Caco-2 cells

INTRODUCTION

After withdrawal of the nonsedative antihistamine drug terfenadine (Seldane) due to serious adverse effects caused by drug−drug interactions associated with coadministered CYP3A4 substrates or inhibitors,[1] its active metabolite fexofenadine (Allegra) was introduced into the market for the same indication. Fexofenadine is charged at physiological pH because it is a zwitterion with two pK_a's of 4.25 and 9.53.[2] Therefore fexofenadine must have poor membrane permeability, and yet it has 33% oral bioavailability in humans.[3] It follows therefore that fexofenadine must depend on one or more intestinal transporter(s) for oral absorption. However, the vectorial transport system involved in the intestinal absorption of fexofenadine remains undefined. Fexofenadine is known to be a substrate for several transporters, which play an important role in the disposition of this drug. Hepatic disposition of fexofenadine has been studied extensively, and multiple transporters have been found to be involved. After uptake by organic anion transporting polypeptide 1B3 (OATP1B3) and OATP1B1 into hepatocytes, fexofenadine is excreted into the bile by multiple efflux transporters in the canalicular membrane including multidrug resistance-associated protein 2 (Mrp2)[4] and bile salt export pump (BSEP).[5] In addition, efflux by Mrp3 in the sinusoidal membrane attenuates hepatic excretion of fexofenadine.[5,6] Surprisingly, P-glycoprotein (P-gp) does not seem to contribute to the biliary excretion of fexofenadine, at least in mice, although it plays a major role in the efflux of fexofenadine in the intestine and blood−brain barrier.[7] P-gp-mediated efflux of fexofenadine in the blood−brain barrier, coupled with its low intrinsic membrane permeability, appears to limit its brain penetration.[8−11] Fexofenadine is also a substrate for renal organic anion transporter 3 (OAT3), which contributes to its renal elimination.[12]

Fexofenadine was initially used as an in vivo probe for assessing the role of P-gp in limiting intestinal drug absorption.[2,8,13] Interestingly, using a human in vivo jejunal perfusion technique, concurrent administration of verapamil or ketoconazole, P-gp inhibitors, failed to increase the effective jejunal permeability of fexofenadine.[14,15] This observation may be explained when one considers that verapamil and ketoconazole not only inhibit P-gp but also other transporters that facilitate absorptive transport of fexofenadine in the intestine, for example, OATPs.[8] This notion was supported by a clinical study, which showed that fruit juices decreased oral absorption of fexofenadine,

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presumably by inhibiting the uptake transporter in apical membrane of enterocytes.[16] Fruit juices inhibit multiple OATP isoforms, including OATP1A2 (SLCO1A2, formerly OATP-A)[16] and OATP2B1 (SLCO2B1, formerly OATP-B).[17] Fexofenadine is a substrate for OATP1A2;[8] however, conflicting results have been reported in the literature about the substrate activity of fexofenadine toward OATP2B1.[18,19] SLCO1A2 gene does not appear to be expressed in human jejunum,[20−22] the major absorption site of fexofenadine,[23] whereas the SLCO2B1 gene (OATP2B1) is highly expressed in this region[21,22] and has been localized in the apical membrane of human small intestine.[24] In addition, human organic cation transporter 3 (hOCT3, SLC22A3)[25] and human novel organic cation transporter 2 (hOCTN2, SLC22A5)[26] are also present in the apical membrane of enterocytes. These transporters transport cations and zwitterions with 1-methyl-4-phenylpyridinium (MPP^+) and L-carnitine as their prototypical substrates, respectively.[27] It is conceivable that these transporters, particularly hOCTN2, may play a role in the absorptive transport of fexofenadine because of its zwitterionic functionality.

Intestinal absorptive transport involves entry into the enterocytes across the apical membrane from gastrointestinal lumen, followed by intracellular translocation and exit from the cells across the basolateral membrane. Depending on their relative efficiency and capacity, an uptake transporter in the apical membrane or an efflux transporter in the basolateral membrane can become rate-limiting for transepithelial movement.[28] However, it is still unclear whether basolateral transporters play an important role in intestinal transport of fexofenadine, and if so, which one. Limited information is available about transporters that are expressed in the basolateral membrane of enterocytes. Among the known basolateral transporters in the intestinal epithelium, two classes of transporters may contribute to basolateral efflux of fexofenadine: heteromeric organic solute transporter $\alpha\beta$ (OST$\alpha\beta$) and MRP3. OST$\alpha\beta$ is highly expressed in human ileum as evidenced by the presence of mRNA levels in this tissue; the expression appears to be largely restricted to the lateral and basal membranes of ileal enterocytes.[29] Although OST$\alpha\beta$ seems to have a major physiologic role in the reabsorption of bile acids by facilitating their egress through the ileal basolateral membrane,[30] this transporter is capable of transporting other compounds, such as estrone 3-sulfate (E1S), digoxin, and prostaglandin E2.[29,31,32] MRPs, the other transporter family that is considered likely to play a role in basolateral efflux of fexofenadine, predominantly transport anionic substances.[33] MRP3 is the most abundantly expressed isoform in the human intestinal epithelium, followed by MRP2.[34] In Caco-2 cells, a well accepted model of intestinal epithelium, MRP2 is the most highly expressed MRP isoform, followed by MRP3 and MRP4.[35] Mrp2 was localized to apical membranes in rat small intestine.[36] Basolateral localization was assumed for other MRPs;[33] however, only Mrp3 showed clear basolateral localization in rat small intestine and colon.[37] MRP4 shows tissue-specific localization, with expression in apical membrane of human kidney tubules[38] and in basolateral membrane of human hepatocytes.[39] Recently, it was localized to the basolateral membrane in Caco-2 cells.[40]

In this study, the role of specific apical uptake and efflux transporters as well as basolateral efflux transporters in the vectorial absorptive transport of fexofenadine was examined using Caco-2 cell monolayers. The results show that OATP2B1 mediates apical uptake of fexofenadine and that P-gp and, to a lesser extent MRP2, attenuate the uptake across this membrane. The results also show that MRP3 mediates fexofenadine efflux across the basolateral membrane and may play a critical role in the intestinal absorption of fexofenadine under certain circumstances.

EXPERIMENTAL SECTION

Materials. MDCKII, CHO, and Caco-2 cells were obtained from ATCC (Manassas, VA). Caco-2 cell clone P27.7 was a kind gift from Dr. Mary Paine (University of North Carolina at Chapel Hill), which was isolated from the parent Caco-2 cell line (ATCC).[41] Dulbecco's Modified Eagle Medium (DMEM), F-12 nutrient mixture, and Eagle's Minimum Essential Medium (EMEM) with Earle's salts and L-glutamate, Dulbecco's Modified Eagle's Medium with L-glutamine but without sodium pyruvate (DMEM), penicillin−streptomycin−amphotericin B solution (100×), nonessential amino acids (NEAA, 100×), *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES, 1M), and penicillin−streptomycin−amphotericin B solution (100×) were obtained from Gibco Laboratories (Grand Island, NY). 1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) was obtained from Biomol (Plymouth Meeting, PA, USA), and mouse laminin was purchased from BD Biosciences (San Jose, CA, USA). Fetal bovine serum (FBS) and trypsin-EDTA solution (1×) were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's Balanced Salt Solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). Geneticin and hygromycin B were obtained from Invitrogen Co. (Carlsbad, CA). [^{3}H]E1S, [^{3}H]L-carnitine, and [^{3}H]MPP^+ were obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]Fexofenadine was obtained as a gift from GlaxoSmithKline (Research Triangle Park, NC) and originally custom synthesized by Amersham Life Sciences (Piscataway, NJ). GW918 was obtained as a gift from GlaxoSmithKline (Research Triangle Park, NC). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

OCTN2 and OATP2B1 cDNAs in pcDNA3.1/hygro vector were provided by Professor Heyo K. Kroemer (Ernst Moritz Arndt University, Germany) and Professor Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany), respectively.

Cell Culture and Transfection. Construction of CHO cells, stably transfected with hOCT3, and mock-transfected cells were described in the previous study.[42] MDCKII cells were transfected with pcDNA3.1 empty vector or the vectors containing the full-length hOCTN2 and OATP2B1 cDNAs using the Nucleofector system (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Transfectants were selected with 0.2 mg/mL hygromycin B for 10 days. A clone with the highest uptake activity with respect to the probe substrate, [^{3}H]L-carnitine and [^{3}H]E1S for hOCTN2 and OATP2B1, respectively, was chosen as a stably transfected cell line for further studies. The stably transfected MDCKII cells were cultured in DMEM with 10% FBS, 10% NEAA, 100 unit/mLpenicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B plus 0.2 mg/mL hygromycin B. Caco-2 cells were seeded at a density of 60000 cells/cm^2 on Transwell filters. Medium was changed the day after seeding and every other day thereafter. The cells were cultured for 21−25 days before use. All cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2.

For metabolic experiment, CYP3A expression was induced in Caco-2 cells according to the following method: 6-well plates containing Biocoat cell culture inserts (4.2 cm^2, 1 μM pore size) were coated with murine laminin (5 μg/cm^2) and then were

seeded with Caco-2 cell clone P27.7 (passages 29−42; obtained from Dr. Mary Paine, UNC-Chapel Hill) at a density of 5×10^5 cells/cm^2. Cultures were grown using growth medium (DMEM containing 20% heat-inactivated FBS, 0.1 mM NEAA, 50 μg/mL gentamicin, and 45 nM vitamin E) until confluence, as assessed by transepithelial electrical resistance (TEER) values $\geq$250 $\Omega \cdot$ cm^2. After that, the cells were treated for 14 days with differentiation medium (DMEM containing 5% heat-inactivated FBS, 0.1 mM NEAA, 50 μg/mL gentamicin, 45 nM vitamin E, 0.1 μM sodium selenite, and 3 μM zinc sulfate) as described by Schmeidlen-Ren et al.[41] and supplemented with 0.5 μM 1α,25-$(OH)_2$-D_3 and 0.2 μM all-*trans*-retinoic acid to induce CYP3A expression.[43]

RT-PCR. Total RNA was isolated from Caco-2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed with 1 μg of total RNA and random hexamer primers. Normalized first-strand cDNA from human ileum was a part of the Human Digestive System MTC panel (BD Clontech, Palo Alto, CA). Gene-specific primers were designed based on the published gene sequences.[32] Multiplex amplification reactions (target gene and β-actin) were performed on a Robocycler 96 PCR system (Stratagene, La Jolla, CA) for 25 cycles. Reactions contained 5 ng of cDNA from tissue mRNA or 150 ng of cDNA from Caco-2 total RNA. The PCR products were analyzed by electrophoresis in agarose gel containing ethidium bromide.

Uptake Studies. Stably transfected MDCKII or CHO cells were grown in 24-well plates. Medium was changed every other day. The cells were used 3−5 days postseeding. Cells were preincubated for 30 min at 37 °C in transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES pH 7.4). Experiments were initiated by replacement of the transport buffer with 400 μL of dose solution. Uptake was determined within the linear uptake region, after which the dose solution was aspirated and cells were washed three times with 4 °C transport buffer. Cells were dissolved in 500 μL of 0.1 N NaOH/0.1% SDS for 4 h with shaking. Radioactivity was determined by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Uptake studies were also conducted in Caco-2 cells grown on Transwell filters. Cell monolayers were preincubated in transport buffer at 37 °C for 30 min. Experiments were initiated by replacement of the apical transport buffer with 400 μL of transport buffer containing test compounds in the absence or presence of inhibitors. Uptake was determined within the linear uptake region, after which the monolayers were washed three times and dissolved in 500 μL of 0.1 N NaOH/0.1% SDS for 4 h with shaking. Radioactivity was determined with the same method as the uptake study in the transfected cells.

Efflux of Fexofenadine Formed from Terfenadine. CYP3A4-expressing Caco-2 cells were used in order to determine the efflux of fexofenadine formed from the prodrug terfenadine. To preload the intracellular space with terfenadine, the parent drug of fexofenadine, 25 μM terfenadine was added to both apical and basolateral compartments and incubated for 1 h. The cell monolayers were washed three times with ice-cold buffer. Then fresh warmed buffer was added, and samples were collected after one minute of efflux, which is within the linear range of efflux for fexofenadine. Cell monolayers were also extracted and analyzed for fexofenadine content by LC-MS/MS as described below.

Accumulation and Efflux of Fexofenadine. Caco-2 cell monolayers were incubated for 1 h with 100 μM fexofenadine in both apical and basolateral sides in the absence or presence of inhibitors. After washing both sides three times with cold transport buffer, efflux was determined over 2 min in the absence or presence of inhibitors. Then monolayers were dissolved in 300 μL of 1% Triton X-100 for 4 h with shaking, and the solution was extracted with 1 mL of ethyl acetate. The organic phase was evaporated to dryness under nitrogen gas, and the residue was reconstituted with 200 μL of 25% methanol. The effluxed and cellular samples were analyzed by LC-MS/MS as described below. The effluxed and cellular amounts together added up to the total loaded amount in the cells, and efflux clearance was calculated based on efflux rates and cellular concentration after loading.

Transport of Fexofenadine. Caco-2 cell monolayers were incubated for 30 min with the transport buffer with or without inhibitors. Transport studies were initiated by replacing the contents of the donor chambers with 10 μM fexofenadine in the absence or presence of inhibitors. Acceptor chambers were sampled at selected times, and analytes were quantified by LC-MS/MS.

LC-MS/MS Analysis of Fexofenadine. LC-MS/MS was performed using API-4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Mobile phases of 0.1% formic acid in water and 0.1% formic acid in methanol were used with a 5−95% methanol linear gradient. The column used was a Phenomenex Synergy Polar RP, 30 mm $\times$ 2 mm (Phenomenex, Torrance, CA). A flow rate of 0.8 mL/min and injection volume of 15 μL were utilized. Samples were ionized using APCI and ions were monitored at the following transitions: 503/466 for fexofenadine and 389/201 for the internal standard, cetirizine. A calibration curve was constructed using the peak area ratio of fexofenadine to the internal standard cetirizine over a concentration range of 1−250 nM ($R^2 > 0.99$). The intraday and interday coefficients of variation obtained for this analysis were typically smaller than 2% and 4%, respectively.

Immunofluorescent Staining. Caco-2 cells were grown on 6.5 mm diameter Transwell inserts for the purpose of immunostaining. Inserts were washed three times in phosphate-buffered saline (PBS); similar washes were included between each of the following stages. Cells were fixed in 1% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific binding sites were blocked by incubation for 30 min with 5% normal sheep serum. Inserts were incubated in primary antibody M3II-9 (Covance, Berkeley, CA) (diluted to 5 μg/mL with PBS) for 60 min. Primary antibody was detected by incubation with Alexa Flour 488 goat antimouse antibody for 60 min. Inserts were washed and mounted in Permount Mounting Medium (Fisher Scientific, Pittsburgh, PA). Staining was viewed using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Data Analysis. Data are expressed as mean $\pm$ SD from three measurements unless otherwise noted. Statistical significance was evaluated using unpaired *t* tests for two-sample comparison or one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA). Uptake clearance (CL_{up}) was determined using eq 1:

$$CL_{up} = \frac{dX/dt}{C_o} \quad (1)$$

where dX/dt is the flux determined from the amount taken up (X) over time (t) during the experiment, and C_o is the initial

concentration in the donor side. Kinetic constants (J_{max}, K_m, and K_d) were obtained by fitting a model incorporating saturable and nonsaturable components to the uptake data at 37 °C. The following model was utilized:

$$J = \frac{J_{max} \cdot C}{K_m + C} + K_d \cdot C \quad (2)$$

where J_{max} is the maximal uptake rate, K_m is the kinetic constant for saturable uptake, K_d is the kinetic constant for nonsaturable uptake, and C is the fexofenadine concentration. Apparent permeability (P_{app}) was determined using eq 3:

$$P_{app} = \frac{dQ/dt}{A \cdot C_o} \quad (3)$$

where dQ/dt is the flux determined from the amount transported (Q) over time (t) during the experiment, A is the surface area of the porous membrane, and C_o is the initial concentration in the donor side. Efflux clearance (CL_{eff}) was calculated using eq 4:

$$CL_{eff} = \frac{dX/dt}{C_o} \quad (4)$$

where dX/dt is the amount effluxed (X) over time (t) determined in the linear region of efflux and C_o is the initial concentration of fexofenadine loaded in the cells. Initial intracellular concentrations were calculated using the amount loaded at $t = 0$ and Caco-2 cellular volume of 3.66 μL/mg protein.[44]

RESULTS

Uptake of Fexofenadine into Cells that are Stably Transfected with OATP2B1, hOCT3, and hOCTN2. The absorptive transport of fexofenadine across Caco-2 cell monolayers requires its uptake across the apical membrane, which is presumably carrier-mediated because of its zwitterionic nature. Among the transporters in the SLC family, hOCT3, hOCTN2, and OATP2B1 are the major apical transporters in the intestine that are likely to facilitate uptake of a zwitterionic compound like fexofenadine.[24−26] Hence, fexofenadine uptake was examined in cells expressing these three transporters individually. These cells were characterized as follows: uptake of [^{3}H]MPP$^+$ (model substrate for hOCT3), [^{3}H]L-carnitine (model substrate for hOCTN2), and 10 nM [^{3}H]E1S (model substrate for OATP2B1) into CHO-hOCT3, MDCKII-hOCTN2, or MDCKII-OATP2B1 cells was 7.7-fold, 89-fold, or 8.7-fold greater, respectively, than uptake into the mock cells and was abolished by high concentrations of the unlabeled model substrates. Uptake of [^{3}H]fexofenadine into MDCKII-OATP2B1 cells was 2.8-fold ($p < 0.01$) greater than that into the mock cells, however, its uptake into CHO-hOCT3 and MDCK-hOCTN2 cells was equivalent to the control and was not affected by the unlabeled model substrates (Figure 1).

pH-Dependent Apical Uptake of Fexofenadine into Caco-2 Monolayers. To confirm the role of OATP2B1 in the intestinal uptake of fexofenadine, apical uptake of this drug in Caco-2 cell monolayers, an accepted model for human intestinal epithelium,[45] was evaluated. As shown in Figure 2A, uptake of 10 nM [^{3}H]E1S across the apical membrane of Caco-2 cells at pH 6.0 was 1.7-fold ($p < 0.05$) greater than that at pH 7.4 and was reduced at pH 6.0 and 7.4 by 65% and 50%, respectively, in the presence of 200 μM unlabeled E1S ($p < 0.01$). As shown in Figure 2B, uptake of 100 nM [^{3}H]fexofenadine across the apical membrane of Caco-2 cells at pH 6.0 was 1.9-fold ($p < 0.01$) greater than that at pH 7.4, and unlabeled E1S (200 μM) reduced the uptake at pH 6.0 by 40% ($p < 0.05$) but not at pH 7.4. Among the three intestinal transporters that were considered as likely playing a role in fexofenadine apical uptake, OATP2B1 is the only transporter that exhibits pH-dependence with increased transporter activity as the pH is lowered from 7.4 to 6.0.[24] Therefore, these results demonstrated that OATP2B1 is playing a significant role in the apical uptake of fexofenadine in Caco-2 cell monolayers.

Transport of Fexofenadine across Caco-2 Cell Monolayers. Because fexofenadine is a substrate for P-gp and MRP-2,

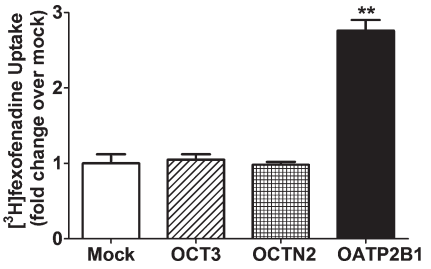

Figure 1. Uptake of [^{3}H]fexofenadine in the CHO or MDCKII cells that are stably transfected with OCT3, OCTN2, and OATP2B1. Cells stably transfected with empty plasmid (Mock), OCT3, OCTN2, or OATP2B1 cDNA were incubated with 100 nM [^{3}H]fexofenadine for 3 min, and uptake clearance of test compounds in these cells was determined and compared to that of mock cells. Data represent mean ± SD of a representative experiment in triplicate. Statistical significance between the uptake clearances for individual transporters was evaluated using one way ANOVA followed by Tukey's test. ** indicates $p < 0.01$ compared with uptake in mock cells.

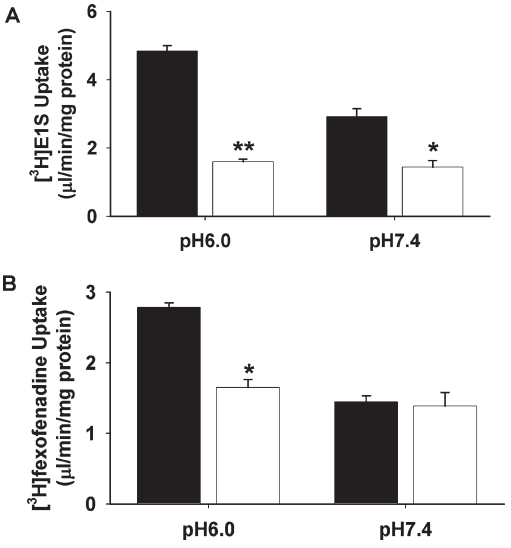

Figure 2. pH-Specific apical uptake of [^{3}H]E1S (A) and [^{3}H]-fexofenadine (B) in Caco-2 cells. Caco-2 cells were incubated at pH 6.0 and pH 7.4 from the apical side with 10 nM [^{3}H]E1S (A) or 100 nM [^{3}H]fexofenadine (B) in the absence (solid bar) or presence (blank bar) of 200 μM unlabeled E1S for 3 min, and uptake clearance of test compounds in these cells was determined. Data represent mean ± SD of a representative experiment in triplicate. Statistical significance between the control and the inhibitor treatment was evaluated using unpaired t tests. ** indicates $p < 0.01$ and * indicates $p < 0.05$ compared with control.

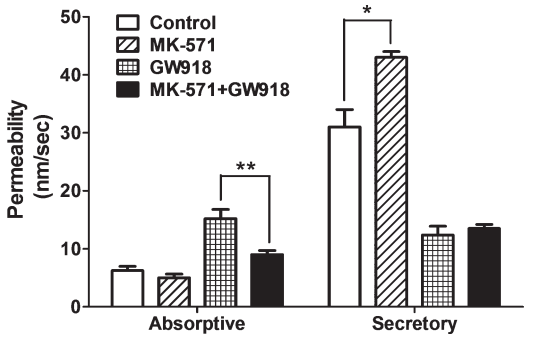

Figure 3. Absorptive and secretory transport of fexofenadine across Caco-2 cells. (A). Absorptive permeability of fexofenadine across Caco-2 monolayers was determined in the absence (control) or presence of 25 μM MK-571, 1 μM GW918, or both. (B). Secretory permeability of fexofenadine across Caco-2 monolayers was determined in the absence (control) or presence of 25 μM MK-571, 1 μM GW918, or both. Data represent mean ± SD of a representative experiment in triplicate. Statistical significance between the permeability in control experiments and upon inhibitor treatments was evaluated using one way ANOVA followed by Tukey's test. ** indicates $p < 0.01$ and * indicates $p < 0.05$ compared between the treatments.

both of which are present on the apical membrane of Caco-2 cells and enterocytes, the role of these efflux transporters in attenuating fexofenadine absorptive transport was evaluated by measuring apparent permeability (P_{app}) in the absence and presence of the MRP inhibitor MK-571 (25 μM) and the P-gp inhibitor GW918 (1 μM). Transport of fexofenadine in the absorptive (apical to basolateral) and secretory (basolateral to apical) directions across Caco-2 cell monolayers is shown in Figure 3 and is expressed as P_{app}. The basolateral to apical P_{app} of fexofenadine was >4 fold higher than its apical to basolateral P_{app} (Figure 3), clearly suggesting that apical efflux transporters, presumably P-gp and MRP-2, are attenuating absorptive transport of fexofenadine. GW918 (1 μM) increased the absorptive P_{app} by 2.3-fold ($p < 0.01$), confirming P-gp's role in attenuating absorptive transport. In contrast, the MRP inhibitor MK-571 (25 μM) slightly (17%) decreased the absorptive transport. Interestingly, when P-gp was inhibited with GW918, further treatment with MK-571 decreased the absorptive P_{app} even more (by 42%, $p < 0.01$). This result provided initial evidence that MRP is involved in facilitating the absorptive transport of fexofenadine instead of attenuating it (by apical MRP-2).

A possible role of MRP in facilitating absorptive transport of fexofenadine, presumably by mediating efflux across the basolateral membrane, was confirmed by results obtained from two different experiments. (i) The secretory transport of fexofenadine increased by 32% ($p < 0.05$) in the presence of MK-571 (25 μM), an MRP inhibitor (Figure 3), suggesting that a member of the MRP family of efflux transporters in the basolateral membrane of Caco-2 cells mediates efflux of fexofenadine across this membrane. (ii) When fexofenadine was formed from its prodrug, terfenadine, by oxidative metabolism in CYP3A-expressing Caco-2 cells (Figure 4A), 66% of intracellularly formed fexofenadine egressed across the apical membrane; however, in the presence of GW918 (1 μM), which shut off P-gp-mediated apical efflux of fexofenadine, almost all of the compound (92%) appeared in the basolateral side (Figure 4B). These results clearly showed that a basolateral efflux transporter, presumably a member of the MRP family, facilitates fexofenadine egress across the basolateral membrane.

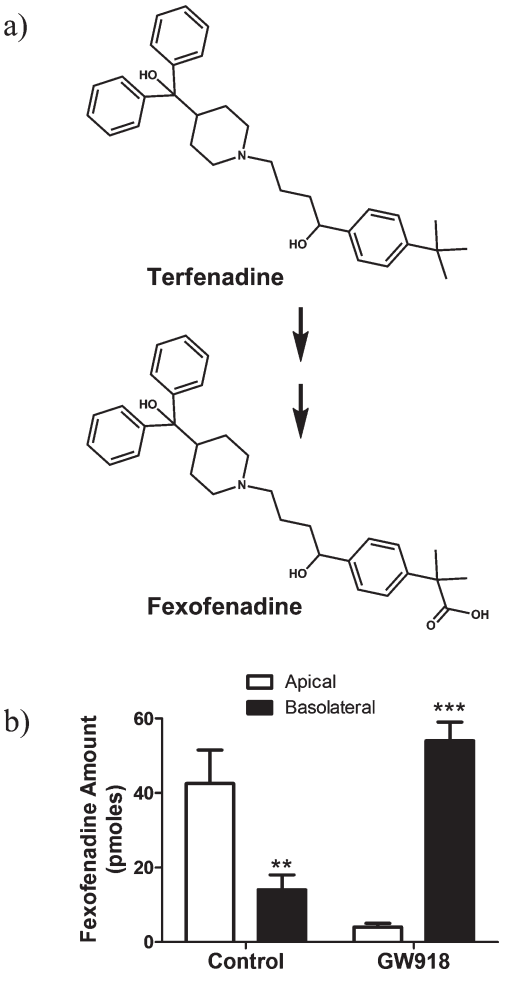

Figure 4. Distribution of fexofenadine formed from terfenadine within Caco-2 cells to the apical and basolateral compartment. Fexofenadine can be formed from its prodrug, terfenadine, by CYP3A-mediated oxidative metabolism (A). The amount of fexofenadine, formed in Caco-2 monolayers from terfenadine, that accumulates into the apical and the basolateral compartment was determined in the absence (Control) or presence of 1 μM GW918 (B). Data represent mean ± SD of a representative experiment in triplicate. Statistical significance between the apical and basolateral efflux was evaluated using unpaired *t* tests. *** indicates $p < 0.001$ and ** indicates $p < 0.01$.

Apical and Basolateral Efflux of Fexofenadine from Preloaded Caco-2 Cells. As shown in Figure 5A, MK-571 (25 μM) and GW918 (1 μM) stimulated the accumulation of fexofenadine into Caco-2 cells by 2-fold ($p < 0.05$) and 4-fold ($p < 0.01$), respectively, when cells were exposed to fexofenadine from both apical and basolateral sides. After preloading, fexofenadine egressed out of the cells on both apical and basolateral sides, thus confirming that it is subject to efflux across both membranes. Interestingly, fexofenadine was preferentially transported out of the cells across the apical membrane over the basolateral membrane (2-fold, $p < 0.01$). Thus the apical efflux transporters, P-gp and MRP-2, are more effective than the basolateral efflux transporters, such as MRP3 and OST$\alpha\beta$, in mediating fexofenadine egress. When P-gp was inhibited with GW918, the preference for fexofenadine egress changed from apical to basolateral side, with the basolateral egress 1.5-fold ($p < 0.01$) higher than the apical egress (Figure 5B,C). MK-571 inhibited

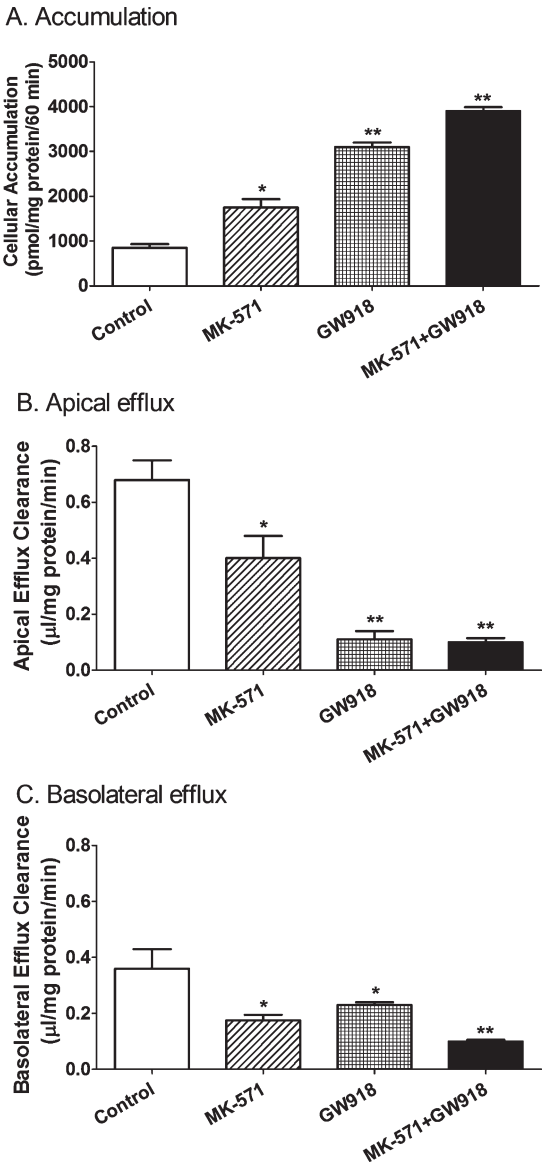

Figure 5. Role of the apical efflux transporter P-gp, and the apical (e.g., MRP2) and basolateral (e.g., MRP3) efflux transporters in the fexofenadine accumulation into Caco-2 cells (A) and fexofenadine apical (B) and basolateral efflux (C) from the preloaded cells. Accumulation of fexofenadine from apical and basolateral sides of Caco-2 monolayers was determined over 1 h in the absence (control) or presence of 25 μM MK-571, 1 μM GW918, or both. (B). Apical efflux clearances of fexofenadine from Caco-2 monolayers was determined over 3 min in the absence (control) or presence of 25 μM MK-571, 1 μM GW918, or both after 1 h preloading in the same treatments. (C). Basolateral efflux clearances of fexofenadine from Caco-2 monolayers was determined over 3 min in the absence (control) or presence of 25 μM MK-571, 1 μM GW918, or both after 1 h preloading in the same treatments. Data represent mean $\pm$ SD of a representative experiment in triplicate. Statistical significance between the control and the inhibitor treatments was evaluated using one way ANOVA followed by Tukey's test. *** indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$ compared with control.

the efflux of cellular fexofenadine across the apical and basolateral membranes by 41% ($p < 0.05$) and 57% ($p < 0.05$), respectively (Figure 5B,C). Both of these results suggested that the basolateral MRP3 is more effective toward fexofenadine than the apical

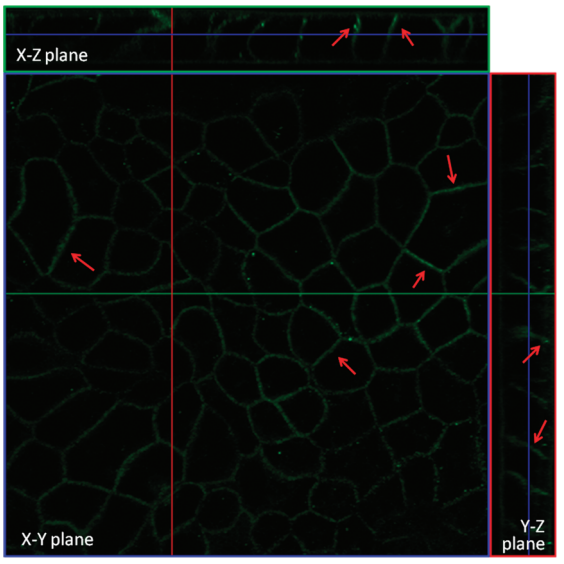

Figure 6. Immunostaining for MRP3 in Caco-2 cell monolayers. Indirect immunofluorescent staining was performed (see Experimental Section) and viewed via confocal laser scanning microscopy. The X−Y image (center) is shown as projections through the whole plane of the cell monolayer viewed en face, and similarly perpendicular X−Z (upper) and Y−Z (right) images are also shown as projections through appropriate axes. The lateral localization of MRP3 is indicated with the arrows in all three images.

MRP2. Results in Figure 5C show that the basolateral efflux for fexofenadine is somewhat inhibited by GW918. A comparison of fexofenadine basolateral efflux in the presence of MK-571 with that in the presence of MK-571 and GW918 also suggests that GW918 decreases basolateral efflux. This may be explained if one assumes that the increased intracellular concentration achieved when P-gp is inhibited may saturate MRP3 on the basolateral membrane and thus decrease the efflux clearance. Alternatively, GW918 may inhibit MRPs, in addition to P-gp, to some extent.

Immunolocalization of MRP3 in the Basolateral Membrane of Caco-2 cells. The cellular localization of MRP3 in Caco-2 was examined by confocal laser scanning microscopy after immunostaining of the cells with MRP-3 antibody M3II-9.[46] As shown in Figure 6, the fluorescent stain in the confocal image is localized in the basolateral membrane of Caco-2 cells, showing that MRP3 is localized in the basolateral membrane of Caco-2 cells, the same cellular localization as in the rat intestine.[37] Thus, fexofenadine efflux across the basolateral membrane of Caco-2 cells is mediated, at least in part, by MRP3.

Basolateral Transport of Fexofenadine into Caco-2 Monolayers. Another basolateral transporter that could play a role in fexofenadine egress is OST$\alpha\beta$ because this transporter has a similar selectivity as OATPs and it is localized in the basolateral membrane in the intestine.[29] As shown in Figure 7, RT-PCR of RNA isolated from Caco-2 cells and human ileum gave bands at 526 and 254 base pairs for OSTα and OSTβ, respectively, and demonstrated the presence of mRNAs of these two subunits in Caco-2 cells. Because OST$\alpha\beta$ is a bidirectional transporter, basolateral uptake studies were used to demonstrate its functional activity to mediate egress of its substrates in Caco-2 cells as these studies are easier to perform. Basolateral uptake of [^{3}H]E1S into Caco-2 cells showed a combination of saturable and passive diffusion mechanisms (K_m = 589 μM, V_{max} = 36

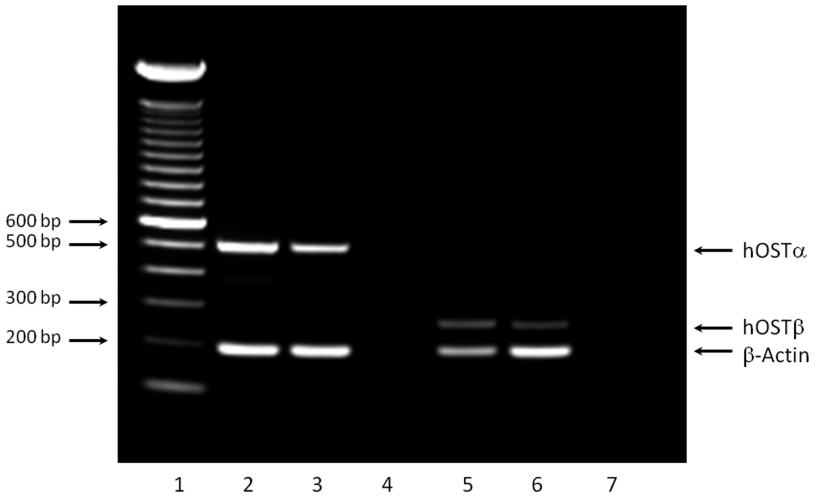

Figure 7. Detection of hOST$\alpha\beta$ mRNA in Caco-2 cells by RT-PCR. Lane 1, 100 bp DNA ladder; lane 2, hOSTα in Caco-2 cells; lane 3, hOSTα in human ileum; lane 4, hOSTα in water; lane 5, hOSTβ in Caco-2 cells; lane 6, hOSTβ in human ileum; lane 7, hOSTβ in water.

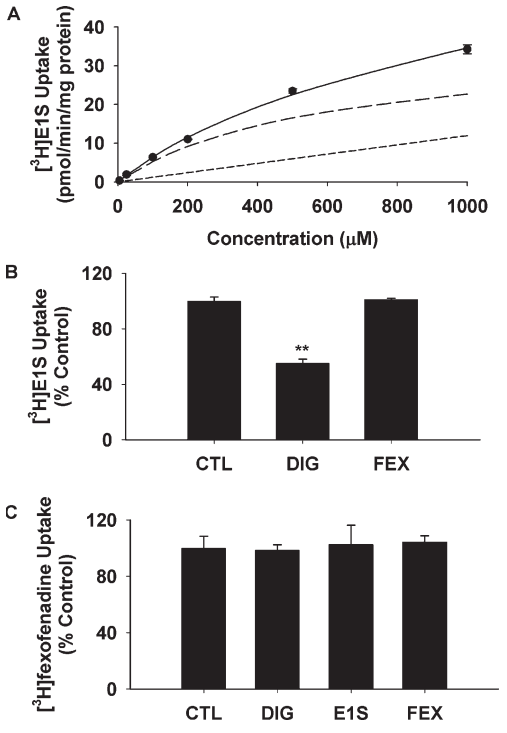

Figure 8. Basolateral uptake of [^{3}H]E1S (A,B) and [^{3}H]fexofenadine (C) in Caco-2 cells. (A). Basolateral uptake of [^{3}H]E1S in Caco-2 cells was determined over 3 min at the indicated concentrations. The solid, dashed, and dotted lines represent the best fit to the [^{3}H]E1S uptake data, and the saturable and passive components of the uptake, respectively. (B). Basolateral uptake (expressed as % of control) of [^{3}H]E1S in Caco-2 cells was determined over 3 min in the absence (CTL) or presence of 500 μM digoxin (DIG) or 500 μM fexofenadine (FEX). (C). Basolateral uptake (expressed as percent of control) of [^{3}H]fexofenadine in Caco-2 cells was determined over 3 min in the absence (CTL) or presence of 500 μM digoxin (DIG), 500 μM E1S (E1S), or 500 μM unlabeled fexofenadine (FEX). Data represent mean ± SD of a representative experiment in triplicate. Statistical significance between the control and the inhibitor treatments was evaluated using one way ANOVA followed by Tukey's test. *** indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$ compared with control.

pmol/mg/min, and K_d = 0.012 μL/mg/min) (Figure 8A). The K_m value was comparable with that in oocytes expressing hOST$\alpha\beta$ (320 μM).[32] Digoxin (500 μM), another hOST$\alpha\beta$ substrate/inhibitor,[32] inhibited basolateral uptake of [^{3}H]E1S (10 nM) by 45% ($p < 0.01$), whereas 500 μM fexofenadine did not reduce it in Caco-2 cells (Figure 8B). In addition, neither E1S nor digoxin at 500 μM reduced the basolateral uptake of 100 nM [^{3}H]fexofenadine in Caco-2 cells (Figure 8C). These results clearly established that OST$\alpha\beta$ is not involved in the egress of fexofenadine across basolateral membrane of Caco-2 cells.

DISCUSSION

Fexofenadine is a zwitterionic compound that must depend on one or more intestinal transporter(s) for oral absorption. The observation that fruit juices decreased oral absorption of fexofenadine in humans, possibly by inhibiting intestinal uptake transporter(s), supported the notion that transporters contribute to intestinal absorption of fexofenadine.[16] However, the identity of the specific uptake transporter involved in the intestinal absorption of fexofenadine remains unclear. OATP1A2 was first suggested to mediate the apical uptake of fexofenadine into enterocytes.[16,47] However, studies from several other groups did not show the presence of OATP1A2 mRNA in human intestine[20−22] or Caco-2 cells,[21] casting doubt on the role of OATP1A2 in the intestinal absorption of fexofenadine. In contrast, OATP2B1 is highly expressed in human jejunum[21,22] and Caco-2 cells[21] and has been localized in the apical membrane of human small intestine.[24]

The results of this study show that among the apical intestinal transporters tested, OATP2B1, but not OCT3 or OCTN2, stimulate uptake of [^{3}H]fexofenadine into transfected cells. In addition, apical uptake of [^{3}H]fexofenadine into Caco-2 cells was decreased (45%) by the OATP substrate/inhibitor E1S (200 μM) at pH 6.0, but not at pH 7.4, providing evidence for the role of OATP2B1 in the apical uptake of fexofenadine into Caco-2 cells because OATP2B1 is the only transporter in the OATP family that exhibits higher transporter activity as the pH is lowered from 7.4 to 6.0.[19,48] OATP2B1-mediated uptake of fexofenadine may be the major transport mechanism in vivo

because of the acidic microclimate in small intestine.[49] These results also explain why OATP2B1 would not play a significant role in hepatic uptake of fexofenadine because of lower activity of this transporter in fexofenadine uptake at the neutral pH of blood.

When fexofenadine was formed from terfenadine metabolism in CYP3A-expressing Caco-2 cells, it was primarily effluxed to the apical side; however, when apical P-gp was abolished by chemical inhibition, most of the compound appeared in the basolateral side (Figure 4B), revealing the presence of an efflux transporter for fexofenadine in the basolateral membrane. This study further showed that basolateral efflux of fexofenadine is mediated by an MK-571-sensitive MRP transporter. However, this result did not rule out the possibility that a part of the fexofenadine basolateral efflux could be mediated by the facilitative bidirectional transporter OST$\alpha\beta$, whose presence in the mouse intestinal epithelium has been reported previously.[50] The presence of OST$\alpha\beta$ mRNAs and its functional activity (E1S transport) in the basolateral side of Caco-2 cells was demonstrated for the first time in this study (Figures 7 and 8), and therefore its role in fexofenadine basolateral efflux was further evaluated. The results in Figure 8 show that fexofenadine does not inhibit the basolateral uptake of E1S in Caco-2 cells, and OST$\alpha\beta$ substrates/inhibitors do not decrease uptake of [^{3}H]fexofenadine into the basolateral membrane of Caco-2 cells. Furthermore, the basolateral uptake of fexofenadine does not show a saturable mechanism. OST$\alpha\beta$ is a bidirectional facilitative transporter mediating both uptake and efflux processes. While a small possibility exists that OST$\alpha\beta$ could mediate fexofenadine egress across the basolateral membrane of Caco-2 cells despite a lack of basolateral uptake of the drug, we conclude that the basolateral egress of fexofenadine does not involve OST$\alpha\beta$ or other facilitative transporters.

The MRP inhibitor MK-571 (25 μM) reduced the basolateral efflux of fexofenadine from preloaded Caco-2 cells, suggesting that basolateral MRPs mediate transport of fexofenadine out of Caco-2 cells. The leukotriene LTD4 receptor antagonist MK-571 specifically modulates MRP associated, but not P-gp related, multidrug resistance.[51] MK-571 is an effective inhibitor of MRP2,[52] MRP3,[53] and MRP4[54] and showed the highest potency ($IC_{50} \approx 5$ μM) for inhibition of MRP3-mediated methotrexate transport among 17 inhibitors tested in that study.[53] MK-571 also inhibits hepatic transporter OATP1B3.[55] However, this transporter is not expressed in human intestine or Caco-2 cells;[21] furthermore, our data with OATP2B1-transfected MDCKII cells showed that MK-571 does not inhibit OATP2B1 at all at the concentration used in this study (25 μM) to evaluate the role of MRP transporters. These results indicate that the modulation of fexofenadine transport by MK-571 in Caco-2 cells is due to inhibition of MRP transporter(s) and not due to inhibition of OATPs. Matsushima et al. have reported that fexofenadine is a substrate for MRP3, but not for MRP4,[5] and that Mrp3 played an important role in sinusoidal efflux of this drug in mouse liver.[5,6] Immunofluorescence studies using laser scanning confocal microscopy have showed that MRP3 was localized to the basolateral membrane of Caco-2 cells (Figure 6). Combination of the results from the literature and this study led to the conclusion that basolateral MRP3 in Caco-2 cells mediates the efflux of fexofenadine out of the cells. The present results also showed that MK-571, in addition to reducing the basolateral efflux, also reduced the apical efflux of fexofenadine from preloaded Caco-2 cells. MRP2 is localized on the apical membrane of enterocytes,[36] indicating that MRP2 also may be mediating apical efflux of fexofenadine in addition to P-gp. A study on hepatic disposition of fexofenadine supported the notion that disposition of fexofenadine could be affected by MRP2 by showing that Mrp2 is primarily responsible for the biliary excretion of fexofenadine in mice.[4]

Inhibition of MRPs reduced the absorptive transport moderately, whereas inhibition of P-gp increased the transport over 2-fold. The results show that when P-gp is functional, modulation of MRPs does not affect the overall fexofenadine transport rate significantly, suggesting that apical entry into the enterocytes, predominantly influenced by P-gp-mediated apical efflux, is rate-limiting in absorptive transport of fexofenadine in Caco-2 cells. However, when P-gp is inhibited, the role of MRP3 in facilitating basolateral efflux and absorptive transport of fexofenadine is more clearly seen because MK-571 treatment (of Caco-2 cells whose P-gp is completely inhibited) causes over 40% inhibition in the overall rate of fexofenadine absorptive transport across Caco-2 cell monolayers. In fact, this result likely underestimated the role of MRP3 in mediating basolateral efflux and absorptive transport of fexofenadine because concurrent inhibition of apical MRP2 by MK-571 must have caused an increase in the absorptive transport rate, counteracting some of the effect of basolateral MRP3 inhibition. Further work is needed in which effective siRNA sequences can be designed to suppress MRP2 or MRP3 specifically in Caco-2 cells in order to fully elucidate the roles of apical MRP2 and basolateral MRP3 in the intestinal absorption of fexofenadine. It is conceivable that basolateral efflux by MRP3 may control the absorption rate of fexofenadine in human small intestine when P-gp is not fully effective in attenuating the cellular entry of this drug across the apical cell membrane.[56]

Studies described in this report provide novel insight into the roles of basolateral transporters in intestinal absorption of a zwitterionic drug like fexofenadine. For a drug with poor membrane permeability to cross the intestinal epithelium efficiently, a basolateral transporter is required for egress of the drug into the blood side after apical transporters mediate uptake into enterocytes. This notion has been established for intestinal absorption of bile acids by studies which showed that knocking out the basolateral transporter Ost$\alpha\beta$ in mice abolished most of the transileal transport of taurocholate.[30] Altered drug absorption of fexofenadine due to modulation of MRP3 activity may have important clinical implications. Pharmacokinetic studies of fexofenadine in Mrp3 knockout mice, and further studies in humans, are required to define the in vivo functions of MRP3 in oral absorption of this and other MRP3 substrates. Collectively, the vectorial transport system consisting of apical OATP2B1 and basolateral MRP3 (and possibly other basolateral MRPs) may be an important pathway to deliver hydrophilic anionic and zwitterionic drugs such as pravastatin and fexofenadine into systemic circulation upon oral administration.

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■ ABBREVIATIONS USED

CHO, Chinese hamster ovary; E1S, estrone 3-sulfate; MDCKII, Madin Darby canine kidney II; MPP^+, 1-methyl-4-phenylpyridinium; MRP, multidrug resistance-associated protein; OAT, organic anion transporters; OATP, organic anion transporting polypeptides; OCT, organic cation transporter; OCTN, novel organic cation transporter; SLC, solute carrier family; TEA, tetraethylammonium

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