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ABSTRACT

Human intestinal epithelium expresses a number of drug efflux and influx transporters that can restrict and/or facilitate intestinal drug uptake during absorption. Organic anion-transporting polypeptide 2B1 (OATP2B1), a multispecific organic anion uptake transporter localized at the brush-border membrane of intestinal epithelial cells, is known to transport many endogenous substrates (e.g., steroid conjugates) and xenobiotics (e.g., statins). At present, limited information is available on the mechanism of HIV protease inhibitor (PIs) intestinal uptake. In this study, we examined the interaction of PIs with the OATP2B1 transport system in Caco-2 cells, an in vitro model of human intestinal epithelium, and Madin-Darby canine kidney II cells stably transfected with OATP2B1. The expression of OATP2B1 transcript and protein was confirmed by reverse transcription-polymerase chain reaction and immunoblot analysis, respectively. Estrone-3-sulfate (E3S) uptake demonstrated biphasic saturation kinetics in Caco-2 cells, with dissociation constants ($K_m$) of $6.2 \mu M$ and $1.5 \pm 0.2 \mu M$. Several PIs potently inhibited OATP2B1-mediated transport in Caco-2 cells at clinically relevant IC$_{50}$ concentrations for ritonavir (0.93 $\mu M$), atazanavir (2.2 $\mu M$), lopinavir (1.7 $\mu M$), tipranavir (0.77 $\mu M$), and neftavir (2.2 $\mu M$). An inwardly directed proton gradient was identified as the driving force of E3S uptake through NH$_4$Cl intracellular acidification studies with a H$^+$:E3S stoichiometry for OATP2B1 of 1:1. In contrast, although atazanavir and ritonavir uptake by Caco-2 cells was stimulated by low extracellular pH, this process was not mediated by OATP2B1 and was not affected by an outwardly directed H$^+$ gradient. Because OATP2B1 exhibits an increasing number of drug substrates, including several statins, alterations of its function by PIs could result in clinically significant drug–drug interactions in the intestine.

Introduction

Carrier-mediated uptake processes by solute carrier (SLC) transporters are being increasingly recognized as a major determinant of drug absorption, tissue distribution, and elimination (Ho and Kim, 2005; Petzinger and Geyer, 2006). Members of the organic anion-transporting polypeptide (OATP) superfamily (SLCO gene family) mediate sodium-independent transport of amphipathic organic compounds including many endobiotics, such as bile acids, steroid conjugates, and thyroid hormones, and several classes of pharmacological agents, in particular HMG-CoA reductase inhibitors (Ho and Kim, 2005). Some OATPs are expressed in multiple human tissues (e.g., OATP1A2, OATP2B1, OATP3A1, and OATP4A1), whereas others have more restricted tissue expression profiles, such as OATP1B1 (liver), OATP1B3 (liver), and OATP1C1 (brain, testis) (Hagenbuch and Meier, 2004). OATP2B1 is expressed ubiquitously in human tissues, including the small intestine, liver, and placenta, and has
tissue-specific physiological and pharmacological functions (König et al., 2006). In placenta and mammary gland, OATP2B1 plays a role in the uptake and recirculation of sulfate conjugates of steroid hormones, such as estrone-3-sulfate (E3S) and dehydroepiandrosterone sulfate (Tamaï et al., 2000a; St-Pierre et al., 2002). In the liver, OATP2B1 mediates the hepatic uptake of many xenobiotics, including bromosulfophthalein (BSP), benzylpenicillin (Tamaï et al., 2000a), fenofoxadine (Nozawa et al., 2004), glibenclamide (Satô et al., 2005), pravastatin (Kobayashi et al., 2003), atorvastatin (Grube et al., 2006b), rosuvastatin (Ho et al., 2006), and fluvastatin (Noë et al., 2007). Furthermore, OATP2B1 exhibits a diverse spectrum of inhibitors, including many organic anions, monocarboxylic and dicarboxylic acids (Kobayashi et al., 2003; Sai et al., 2006), steroid hormones and their derivatives (Grube et al., 2006a), drugs such as rifampycin SV (Vavríčka et al., 2002), pravastatin (Kobayashi et al., 2003), cyclosporin, and gemfibrozil (Tamaï et al., 1997), and constituents of citrus juices (Satô et al., 2005) and herbal extracts (Fuchikami et al., 2006).

Although the intestinal expression of OATP2B1 is higher compared with other OATP isoforms, such as OATP3A1, OATP4A1, and OATP1A2 (Sai et al., 2006; Meier et al., 2007), limited information is available on its role in intestinal drug absorption (Kobayashi et al., 2003). It is noteworthy that most human and rodent OATPs, including OATP2B1, have higher transport activity at acidic extracellular pH, the characteristic microenvironment at the intestinal brush-border membrane (Kobayashi et al., 2003; Nozawa et al., 2004; Leuthold et al., 2009). Intestinal absorption of many organic acids and bases has been shown to involve a pH-dependent carrier-mediated transport mechanism, rather than passive diffusion via pH partitioning (Tamaï et al., 1997, 2000b). Because OATP2B1 has been shown to localize at the apical membrane of intestinal epithelial cells (Kobayashi et al., 2003), it could play an important role in the pH-dependent uptake of amphipathic organic compounds in the small intestine.

Anti-HIV drugs are involved in many clinically important drug–drug and drug–food interactions (de Maat et al., 2003). Highly active antiretroviral therapy involves concurrent administration of three or more antiretroviral drugs, such as nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and HIV protease inhibitors (PIs) (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2009). Antiretrovirals are known to act as substrates, inhibitors, and/or inducers of many drug efflux transporters, such as P-glycoprotein (Pgp), multidrug resistance-associated proteins, breast cancer resistance proteins, and drug-metabolizing cytochrome P450 enzymes (Zastre et al., 2009; Dickinson et al., 2010; Kis et al., 2010). Furthermore, several PIs (i.e., saquinavir, lopinavir, and darunavir) have recently been identified as substrates for human OATP1A2 and OATP1B1 (Su et al., 2004; Kis et al., 2010), and many have been reported to inhibit human OATP1A2, OATP1B1, and OATP1B3 (Cvetkovic et al., 1999; Tirona et al., 2003; Campbell et al., 2004; Kis et al. 2010). These studies were performed in transporter-overexpressing cell lines and cells of hepatic origin, such as Hep G2 cells. However, the role of OATPs in the intestinal absorption of PIs has not been clearly established. Atazanavir (ATV), one of the PIs currently recommended as first-line therapy, has been reported to exhibit a significant decrease in its oral bioavailability when coadministered with acid-reducing agents (de Maat et al., 2003). This effect could potentially be mediated by the alteration in the acidity of the intestinal lumen; however, the exact mechanism of this interaction has not been elucidated.

In the present study, we examined potential interactions of antiretroviral drugs with the intestinal organic anion uptake transporter OATP2B1 in Caco-2 cells, an established in vitro model of human intestinal epithelium. We report a potent inhibitory effect of PIs on OATP2B1-mediated transport and demonstrate that the uptake of atazanavir and ritonavir (RTV) by Caco-2 cells is pH-dependent and stimulated by an acidic extracellular pH.

**Materials and Methods**

**Materials.** [1H]E3S (57.3 Ci/mmol; chemical name: 3-hydroxyestr-1,3,5(10)-trien-17-one hydrogen sulfate) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]Atazanavir (3 Ci/mmol) and [3H]ritonavir (2 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled antiretrovirals drugs were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Reagents, E3S, BSP [chemical name: 3,3’-(4,5,6,7-tetrahydro-3-oxo-1H)-isobenzofuranylidene/bisin-6-hydroxy-disodium salt], rifampycin SV (generic name: rifampin), pravastatin, murine monoclonal antiactin antibody (AC40), and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody were purchased from Sigma-Aldrich (Oakville, ON, Canada). Anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from Cedarlane Laboratories (Burlington, ON, Canada). Madin-Darby canine kidney II (MDCKII) cells stably expressing OATP2B1 (MDCKII/OATP2B1), corresponding wild-type MDCKII cells, and rabbit polyclonal anti-OATP2B1 antibody were kindly provided by Dr. M. Grube, Ernst-Moritz-Arndt-University (Greifswald, Germany). Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA), unless indicated otherwise. All buffers and Triton X-100 were purchased from Sigma-Aldrich.

**Cell Culture.** MDCKII and Caco-2 cells were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1% minimal essential medium nonessential amino acids, and 1% penicillin/streptomycin. MDCKII/OATP2B1 medium was additionally supplemented with 400 µg/ml hygromycin B (Sigma-Aldrich) to provide selection pressure for stably transfected cells. Protein expression of OATP2B1 in MDCKII/OATP2B1 cells was induced by incubating the cells with 2.5 mM sodium butyrate 24 h before transport experiments were performed. Butyrate incubation was found to increase the functional activity of OATP2B1 more than 2-fold, as demonstrated by enhanced uptake of E3S in induced MDCKII/OATP2B1 cells compared with noninduced cells of the same passage (data not shown).

All cells were maintained at 37°C in humidified 5% CO2 with fresh medium replaced every 2 to 3 days. Cells were subcultured with 0.05% trypsin-EDTA upon reaching 95% confluence. For transport experiments, cells were seeded into 48-well plates with a cell density of 2×10⁴ cells/cm² for MDCKII cells and 6×10⁴ cells/cm² for Caco-2 cells. MDCKII cells were used for experiments upon reaching 100% confluence (3–4 days). Caco-2 cells were cultured for 20 to 25 days to allow cell differentiation into a tight intestinal epithelial monolayer with transepithelial electrical resistance of 500 to 700 Ω·cm². The medium in all 48-well plates was replaced every 2 to 3 days.

**Reverse Transcription-Polymerase Chain Reaction Analysis.** Reverse transcription-polymerase chain reaction (RT-PCR) anal-
yis of OATP2B1 expression was performed as described previously (Ronaldson and Bendayan, 2006). Total RNA of MDCKII/OATP2B1 and Caco-2 cells was isolated by using TRIzol reagent, treated with DNase I, and reverse-transcribed into cDNA, followed by gene amplification via PCR. OATP2B1-specific primers were derived from the GenBank accession NM_007256 sequence (Tamai et al., 2001): forward, 5'-CATGGGACCCAGTATGGGCAAGC-3', and reverse, 5'-GGCTGGGCCCACATGCTGATCTG-3'. PCR was run for 34 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min by using a GeneAmp 2400 Thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated by using 2% agarose gel electrophoresis and stained with ethidium bromide.

**Immunoblot Analysis.** Immunoblotting was performed as described previously (Ronaldson and Bendayan, 2006; Zastre et al., 2009) with minor modifications. In brief, cells were harvested, washed twice with PBS, and lysed on ice for 15 min by using lysis buffer containing 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40 (Sigma-Aldrich), and 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic proteases, and aminopeptidases, used to prevent protein degradation. The samples were sonicated for 10 s and centrifuged for 10 min at 20,000 g and 4°C, and the supernatant was analyzed for protein content by using a protein assay kit from Bio-Rad Laboratories at 20,000g and 4°C, and the supernatant was analyzed for proteases, and aminopeptidases, used to prevent protein degradation. The samples were sonicated for 10 s and centrifuged for 10 min at 20,000 g and 4°C, and the supernatant was analyzed for protein content by using a protein assay kit from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin (BSA) as the standard. Samples were incubated with Laemmli buffer (20 mM Tris-HCl, 2 mM EDTA, 2% SDS, 20% glycerol, 0.2% bromphenol blue) and 10% β-mercaptoethanol for 10 min at 37°C. Proteins were separated on a 12% SDS polyacrylamide gel, and then transferred onto polyvinylidene fluoride transfer membranes. The blot was blocked overnight at 4°C in 5% milk Tris-buffered saline containing 0.1% Tween 20 and incubated for 2 h with primary anti-OATP2B1 antibody (1:500), a rabbit polyclonal antibody recognizing the C-terminal epitope of human OATP2B1 (Grube et al., 2006a), or primary mouse antiactin (AC40) antibody (1:500). The blots were incubated for 1 h with corresponding horseradish peroxidase-conjugated anti-rabbit (1:5000) or anti-mouse (1:3000) secondary antibody, respectively. Signals were enhanced by using the chemiluminescence SuperSignal West Pico System (Thermo Fisher Scientific, Waltham, MA) and detected by exposing them onto X-ray film.

**Transport Experiments in MDCKII/OATP2B1 and Caco-2 Cells.** All transport experiments were performed by using Hank's balanced salt solution (HBSS), containing 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, 5.3 mM KCl, 0.44 mM KH2PO4, 138 mM NaCl, 0.34 mM Na2HPO4, and 5.6 mM d-glucose. The buffer was balanced salt solution (HBSS), containing 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, 5.3 mM KCl, 0.44 mM KH2PO4, 138 mM NaCl, 0.34 mM Na2HPO4, and 5.6 mM d-glucose. The buffer was supplemented with 25 mM HEPES, pH 7.4, and 0.01% BSA to reduce the binding of radiolabeled compounds to the glass and plastic. Throughout this work, this HBSS buffer is referred to as transport buffer. Confluent monolayers of Caco-2 cells were preincubated with transport buffer (pH 7.4) at 37°C for 15 min, and then incubated with a similar buffer containing 0.2 μCi/ml [3H]E3S, 0.1 μCi/ml [3H]azetanavir, or ritonavir supplemented with unlabeled compound to reach the desired concentration. To evaluate the effect of an inhibitor, a specific concentration of the inhibitor was added to the preincubation buffer and the radioactive transport buffer. At the desired time interval, the radioactive medium was removed and cells were washed twice with ice-cold PBS and solubilized in 1% Triton X-100 at 37°C for 30 min. The content of each well was collected and mixed with 2 ml of PicoFluor 40 scintillation fluid (PerkinElmer Life and Analytical Sciences), and the total radioactivity was measured by using a Beckman Coulter (Fullerton, CA) LS8500 Scintillation counter. In each experiment, “zero time” uptake (background) correction was applied to correct for the nonspecific binding and variable quench time, estimated by the retention of radiolabeled compound in the cells after a minimum (zero) time of exposure, determined by removing the radiolabeled solution immediately after its introduction into the well, followed by two washes with ice-cold PBS and collection of cells for liquid scintillation counting. Uptake of the radiolabeled probes was normalized to total cellular protein content per well, which was measured in each well by using the Bio-Rad DC Protein Assay kit with BSA as the standard.

To examine the effect of extracellular pH, the uptake of [3H]E3S (or Pls) at pH 7.4 was compared with the uptake measured in transport buffer of pH 5.5, prepared with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES), or pH 8.5, prepared with 25 mM HEPES. Each buffer was supplemented with 0.01% BSA and adjusted to the desired pH at 37°C. The cells were preincubated at pH 7.4, followed by measurement of the uptake of radiolabeled substrate in buffer of pH 5.5, 7.4, or 8.5 to generate a proton gradient. To further demonstrate the role of H⁺ gradient in the observed transport stimulation at pH 5.5, 5 μM monensin, a Na⁺/H⁺ ionophore, was added to the preincubation and the uptake buffers to abolish the proton gradient. A similar experiment was performed at pH 7.4 and demonstrated a lack of interaction of monensin with the uptake of E3S (data not shown). For NH₄⁺ preincubation experiments, to achieve intracellular pH acidification, cells were preincubated for 15 min with 30 mM NH₄Cl before performing the uptake measurements in standard transport buffer (pH 7.4), according to a previously published protocol (Bendayan et al., 1994; Hong et al., 2001). Removal of the external NH₄Cl results in dissociation of intracellular NH₃ into NH₄⁺ and protons. Passive diffusion of NH₃ out of the cell, driven by the concentration gradient, leaves protons trapped inside the cell, resulting in rapid acidification of the intracellular medium to pH 6. An inhibitor of the Na⁺/H⁺ antiporter, amiloride (1 mM), was added to the transport buffer to delay the natural recovery of intracellular pH (Bendayan et al., 1994; Hong et al., 2001). We also examined the effect of 1 mM amiloride at pH 7.4 to demonstrate that it does not directly interact with E3S uptake (data not shown).

To evaluate inhibitory potencies of selected inhibitors and antiretroviral drugs, cellular accumulation of E3S was evaluated in the absence (dimethyl sulfoxide methanol control) or presence of increasing concentrations of inhibitor [I], and IC₅₀ values were estimated by fitting the data to a sigmoidal equation (eq. 4).

**Measurement of Intracellular pH.** Intracellular pH was measured by fluorometric assay as described previously (Bendayan et al., 1994; Hong et al., 2001), using a pH-sensitive carboxyfluorescein derivative, 2′,7′-bis(carboxyethyl)-5(6′-carboxyfluorescein) (BCECF) (Molecular Probes, Eugene, OR). The nonfluorescent membrane-permeable acetoxyethyl ester derivative BCECF-acetoxyethyl ester (BCECF-AM) was allowed to accumulate intracellularly in the presence of 1 μM PSC833 (Pgp inhibitor). Inside the cell, BCECF-AM is cleaved by cytosolic esterases to yield nonpermeable BCECF acid, which exhibits fluorescence properties proportional to the pH level. Confluent Caco-2 monolayers grown in 48-well plates were preincubated for 40 min in transport buffer (pH 7.4) containing 20 μM BCECF-AM at 37°C. Cells were then washed and incubated with 30 mM NH₄Cl for 30 min, which was then replaced with pH 7.4 transport buffer with or without 1 mM amiloride. BCECF fluorescence was detected with dual excitation, at 440 and 495 nm, and emission intensity was measured at 535 nm. A calibration curve relating the 440/495 emission ratios to the pH was generated by using standard HBSS solutions of varying pH (5.5–8.5) containing 100 μM BCECF. The pH was buffered by using 0.1 mM MES (pH 5.5 and 6.1), N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 6.7 and 7.1), HEPES (pH 7.4 and 7.7), or Tris (pH 8.1 and 8.5). Transport buffer was used for baseline measurements.

**Determination of H⁺:E3S Stoichiometry for OATP2B1.** The uptake of E3S by MDCKII/OATP2B1 cells was measured at 2 min and 37°C at different extracellular pH of the transport buffer, ranging between pH 5.3 and 9.0. Buffers of 12 different H⁺ concentrations were prepared by using MES (for pH 5.3, 5.5, 5.6, 5.75, 6.0, and 6.5), HEPES (for pH 7.0 and 7.4), or Tris base (for pH 8.0, 8.25, 8.5 and 9.0) and adjusted at 37°C to the desired pH by using NaOH or HCl. The initial rates of E3S uptake were plotted against the H⁺ concen-
tration and fitted into a Michaelis-Menten model with Hill coefficient (eq. 3) (Bush, 1990; Hong et al., 2000).

**Data Analysis.** Each experiment was repeated at least three times with cells from different passages. In an individual experiment, each data point represents triplicate trials. Results are represented as mean ± S.E.M. All statistical analysis was done with SigmaStat version 2.0 software (SPSS, Inc., Chicago, IL). Statistical significance was assessed by two-tailed Student’s t test for unpaired experimental values or one-way analysis of variance for test of repeated measures, as appropriate. p < 0.05 was considered statistically significant.

Kinetic analysis for the estimation of Michaelis-Menten dissociation constant (Kd) and maximal velocity (Vmax) was applied by using the following equations, which consist of one or two saturable Michaelis-Menten components combined with a nonsaturable component:

\[
V = \frac{V_{\text{max}1} \times [S]}{K_{M1} + [S]} + \frac{V_{\text{max}2} \times [S]}{K_{M2} + [S]} + k_{ns} \times [S] \\
V = \frac{V_{\text{max}} \times [H^+]_c}{K_{H^+} + [H^+]_c} + k_{ns} \times [S] \\
\]

where V is the total rate of uptake, \( V_{\text{max}} \) is the maximum uptake rate, \( K_{H^+} \) is the dissociation constant, [S] is the substrate concentration, and \( k_{ns} \) is the coefficient for nonspecific uptake by diffusion.

The \( H^+:E3S \) stoichiometry of OATP2B1 was evaluated by fitting the initial rates of E3S uptake against the \( H^+ \) concentration into a Michaelis-Menten model with Hill coefficient:

\[
V = \frac{V_{\text{max}} \times [H^+]_c}{K_{H^+} + [H^+]_c} + \frac{V_{\text{max}} \times n [H^+]_c}{K_{H^+} + [H^+]_c} \\
\]

where V is the total rate of uptake, \( V_{\text{max}} \) is the maximum uptake rate, \( K_{H^+} \) is the concentration of \( H^+ \) that gives 50% of the maximum rate, and n is the Hill coefficient, which reflects the \( H^+:E3S \) stoichiometry.

The half-maximal inhibitory (IC50) concentrations values were extrapolated by fitting the data to a sigmoidal equation (Hong et al., 2001):

\[
V_c = \frac{V_i}{1 + (IC50)^n} \\
\]

where \( V_i \) and \( V_c \) represent E3S uptake in the presence or absence (control) of inhibitor, respectively; [I] is the inhibitor concentration; and n is the Hill coefficient (inverse of the slope at 50%).

In all of the uptake experiments performed in Caco-2 cells, the total uptake was corrected for the nonspecific binding (i.e., background radioactivity at zero time) and the nonsaturable or diffusion component. In the MDCKII/OATP2B1 cell system, all uptake data in OATP2B1-overexpressing cells were corrected for the background and uptake measured in the wild-type cell line. The fitting of data into each model was performed by nonlinear least-squares analysis using rabbit polyclonal anti-OATP2B1 antibody recognizing the C-terminal epitope of human OATP2B1. The expression of actin was determined by using murine monoclonal antiactin antibody (AC40) to confirm appropriate loading of each sample. MDCKII/OATP2B1 and Caco-2 cells show a robust band at approximately 84 kDa (Fig. 1B), a molecular mass previously reported for OATP2B1 (St-Pierre et al., 2002). As expected, OATP2B1 expression in wild-type MDCKII cells was very low. As demonstrated previously (Sai et al., 2006; Köck et al., 2010), we confirmed OATP2B1 membrane localization in fully differentiated Caco-2 cell monolayers by immunocytochemical analysis applying laser confocal microscopy (data not shown).

**Results**

**Expression of OATP2B1 in Caco-2 and MDCKII/OATP2B1 Cell Lines.** OATP2B1 mRNA and protein expression was evaluated by RT-PCR and Western blot analysis, respectively. OATP2B1 mRNA transcript was detected in Caco-2 and MDCKII/OATP2B1 cells at the appropriate size (i.e., 250 base pairs) (Fig. 1A). OATP2B1 protein expression in crude membrane fractions obtained from MDCKII/OATP2B1, wild-type MDCKII, and Caco-2 cells was determined by Western blot analysis. Expression of human OATP2B1 was detected by antibody staining using rabbit polyclonal anti-OATP2B1 antibody recognizing the C-terminal epitope of human OATP2B1. The expression of actin was determined by using murine monoclonal antiactin antibody (AC40) to confirm appropriate loading of each sample. MDCKII/OATP2B1 and Caco-2 cells show a robust band at approximately 84 kDa (Fig. 1B), a molecular mass previously reported for OATP2B1 (St-Pierre et al., 2002). As expected, OATP2B1 expression in wild-type MDCKII cells was very low. As demonstrated previously (Sai et al., 2006; Köck et al., 2010), we confirmed OATP2B1 membrane localization in fully differentiated Caco-2 cell monolayers by immunocytochemical analysis applying laser confocal microscopy (data not shown).

**Transport Characteristics for E3S Uptake by Caco-2 and MDCKII/OATP2B1 Cells.** To confirm functional activity of OATP2B1 in Caco-2 and MDCKII/OATP2B1 cells, we determined several transport properties, such as a time profile, specificity, and kinetics of E3S uptake. In Caco-2 cells, the uptake of \( [3H]E3S \), an established OATP2B1 substrate (Tamai et al., 2000a; St-Pierre et al., 2002), was linear over 7 min and displayed a plateau after 45 min (Fig. 2A). In the MDCKII/OATP2B1 cell system, E3S uptake was linear over 2 min, reaching a plateau after 10 min (Fig. 2B). After 30 min, E3S accumulation by the MDCKII/OATP2B1 cells was
Fig. 2. Time course of $[^3]$H$)$E3S uptake by Caco-2, MDCKII/OATP2B1, and wild-type MDCKII cells. A and B, total (●) uptake of $[^3]$H$)$E3S (1 μM) by Caco-2 cells over 60 min (A) or MDCKII/OATP2B1 cells (●) and wild-type MDCKII cells (○) over 30 min (B) were evaluated at pH 7.4 and 37°C. In Caco-2 cells, the total E3S uptake was corrected for the nonspecific binding (background) and diffusion rate of E3S, which were estimated through a mathematical analysis described under Materials and Methods. In MDCKII/OATP2B1 cells, the total accumulation of E3S was corrected for the corresponding values obtained in the wild-type MDCKII cells to estimate the OATP2B1-mediated E3S uptake. The insets represent specific early time uptake in each cell system, depicting linearity of the process. C, the effect of known OATP2B1 inhibitors, BSP (100 μM), rifamycin SV (100 μM), and pravastatin (5 mM), on the uptake of 1 μM $[^3]$H$)$E3S was determined at pH 7.4, 37°C after incubation for 5 min (Caco-2) or 2 min (MDCKII/OATP2B1 and wild type). Data represent the mean ± S.E.M. for n = 3 independent experiments. ***, p < 0.001.
>10 fold higher compared with the wild-type MDCKII cells (Fig. 2B). The uptake was susceptible to inhibition by standard OATP2B1 inhibitors: 100 μM BSP, 100 μM rifamycin SV, and 5 mM pravastatin. In Caco-2 cells, these inhibitors demonstrated 55 to 70% inhibition of E3S uptake, whereas in the MDCKII/OATP2B1 cells, BSP, rifamycin SV, and pravastatin demonstrated potent inhibitory effect (80–90%), resulting in similar uptake levels as observed in the wild-type cells (Fig. 2C).

To evaluate E3S transport kinetics in Caco-2 cells, the initial rates of E3S influx, measured after 2 min, were determined for E3S concentrations ranging from 0.0017 to 1000 μM. The total rate of uptake (Fig. 3, solid line) was corrected for nonspecific binding (i.e., background radioactivity at zero time) and the nonsaturable component (Fig. 3, dashed line), as described under Materials and Methods, to obtain the saturable component (Fig. 3, bold dotted line). Caco-2 cells demonstrated biphasic saturation kinetics for E3S uptake, as reported previously (Sai et al., 2006; Annaert et al., 2010), suggesting the presence of two kinetically distinct saturable components. The dissociation constants ($K_M$) and maximum rates of uptake ($V_{max}$) for these two sites were estimated by nonlinear regression analysis (eq. 1). The kinetic parameters $K_M$ and $V_{max}$ for the high- and low-affinity sites were estimated by least-square nonlinear regression analysis using Prism 5 software. Data represent the mean ± S.E.M. for $n = 3$ independent experiments.

Effect of pH Gradient on E3S Uptake by MDCKII/OATP2B1 and Caco-2 Cells. Specific uptake of [3H]E3S by MDCKII/OATP2B1 and Caco-2 cells was measured at 37°C in the presence of different extracellular pH conditions (5.5, 7.4, and 8.5) (Fig. 5). In both cell lines, the uptake was stimulated by an inwardly directed proton gradient (extracellular pH 5.5) and inhibited by an outwardly directed proton gradient (extracellular pH 8.5). To test whether the inwardly directed proton gradient (under pH 5.5 < pH 7.4 conditions) was responsible for the observed enhancement in E3S uptake, the proton gradient was abolished by using 5 μM monensin, a Na⁺/H⁺ ionophore, while the extracellular pH was kept at 5.5. In the presence of monensin, the uptake of E3S by MDCKII/OATP2B1 cells (Fig. 5A, inset) and Caco-2 cells (Fig. 5B, inset) at pH 5.5 decreased significantly, suggesting that an inwardly directed H⁺ gradient may act as the driving force for OATP2B1-mediated transport of E3S. At pH 7.4, 5 μM monensin did not affect the uptake of E3S by these cells, suggesting that the observed effect is pH-mediated (data not shown).

To further confirm the role of pH gradient as the driving force, an outwardly directed H⁺ gradient (pHₖ 7.4 > pHₖ 6.3) was generated intracellularly by using the NH₄Cl preincubation method (Fig. 6A). The addition of NH₄Cl caused a slight initial alkalinization of intracellular medium to pH 8.0, which returned to normal over the 20-min preincubation period. Removal of NH₄Cl from the extracellular compartment, as described under Materials and Methods, resulted in rapid intracellular acidification from pH 7.4 to 6.0 caused by the diffusion of NH₃ and consequent trapping of protons in the intracellular compartment. In the presence of 1 mM amiloride, a Na⁺/H⁺ antiporter inhibitor, the pH recovered to its initial value after ~20 min (Fig. 6A). For E3S uptake studies, after 20-min preincubation, NH₄Cl buffer was replaced with pH 7.4 buffer containing [3H]E3S (1 μM) and 1 mM amiloride. The presence of an outwardly directed proton gradient resulted in a significant reduction of E3S uptake by MDCKII/OATP2B1 cells (Fig. 6B) and Caco-2 cells (Fig. 6C). At pH 7.4 and in the absence of NH₄Cl preincubation, 1 mM amiloride did not alter the uptake of E3S by these cells (data not shown). These data suggest that OATP2B1-mediated uptake of E3S is driven by an inwardly directed proton gradient.

Stoichiometry of H⁺:E3S Transport by OATP2B1. The stoichiometry of H⁺:E3S uptake by OATP2B1 was evaluated in MDCKII/OATP2B1 cells by measuring the initial rates of E3S uptake (at 2 min) at different extracellular pH, ranging between pH 5.3 and 9.0. The data for the initial rates of E3S uptake at 12 different pH were plotted against the H⁺ driving force, resulting in a significant reduction of E3S uptake by MDCKII/OATP2B1 cells (Fig. 6B) and Caco-2 cells (Fig. 6C). At pH 7.4 and in the absence of NH₄Cl preincubation, 1 mM amiloride did not alter the uptake of E3S by these cells (data not shown). These data suggest that OATP2B1-mediated uptake of E3S is driven by an inwardly directed proton gradient.

Inhibitory Effect of Antiretroviral Drugs on E3S Uptake. The interactions of antiretroviral drugs, including several PI, NNRTI, and NRTI, on OATP2B1-mediated specific E3S uptake were examined in MDCKII/OATP2B1 and Caco-2 cells. In the initial screening, E3S uptake by MDCKII/
Fig. 4. Kinetics of E3S uptake by MDCKII/OATP2B1 cells. A, rates of uptake of [3H]E3S at varying concentrations up to 100 μM were measured at 1 min, pH 7.4, and 37°C in MDCKII/OATP2B1 cells. The total uptake rate in MDCKII/OATP2B1 cells (▼) was corrected for the nonspecific component, estimated by E3S uptake in the wild-type MDCKII cells (▲), to obtain the OATP2B1-specific E3S uptake rate. B, the OATP2B1-specific rate of uptake in MDCKII/OATP2B1 cells was fitted into a single site saturation model (eq. 2), as detailed under Materials and Methods. The kinetic parameters $K_M$ and $V_{max}$ for E3S uptake by MDCKII/OATP2B1 were estimated by least-square nonlinear regression analysis using Prism 5 software. Data represent the mean ± S.E.M. for $n = 3$ independent experiments.

Fig. 5. Effect of extracellular pH on OATP2B1-specific E3S uptake by MDCKII/OATP2B1 and Caco-2 cells. Time-dependent specific uptake of [3H]E3S was evaluated at 37°C and extracellular pH 5.5, 7.4, and 8.5 in MDCKII/OATP2B1 cells (A) and Caco-2 cells (B). Transport buffers of several pH were prepared by using HBSS buffer supplemented with 25 mM MES, pH 5.5, or 25 mM HEPES, adjusted to pH 7.4 or 8.5. Insets, [3H]E3S-specific uptake at pH 5.5 in MDCKII/OATP2B1 cells (A) and Caco-2 cells (B) was measured in the absence (▼) or presence of the Na+/H+ ionophore monensin (▲). Monensin (5 μM) was added to the preincubation buffer (pH 7.4) for 15 min before uptake initiation and to transport medium (pH 5.5) containing [3H]E3S. Data represent mean ± S.E.M. for $n = 3$ independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
OATP2B1 cells at 90 s was potently inhibited by several PIs at 10 μM concentration, including tipranavir (85% reduction in uptake), lopinavir (82%), atazanavir (77%), nelfinavir (75%), and saquinavir (63%), whereas darunavir, a recently marketed PI, and efavirenz, an NNRTI, demonstrated a potent inhibitory effect only at 100 μM concentrations (Table 1). In the wild-type MDCKII cells, these antiretroviral drugs had no significant effect on E3S uptake (data not shown), confirming that the observed interactions with E3S uptake most likely involve inhibition of OATP2B1-mediated transport by these drugs. In Caco-2 cells, the effect of antiretroviral drugs on E3S uptake was similar but of lower magnitude compared with the interactions observed in MDCKII/OATP2B1 cells, suggesting potential involvement of OATP2B1 in these interactions (Table 1).

To further characterize the inhibitory potencies of the identified inhibitors, a concentration-dependent effect of these compounds was evaluated in Caco-2 cells (Fig. 8A) and MDCKII/OATP2B1 cells (Fig. 8B), and the half-maximal inhibition (IC50) concentrations were evaluated by fitting the data into sigmoidal dose–response curve (eq. 4), as described under Materials and Methods (Fig. 8). The IC50 concentrations for standard OATP2B1 inhibitors, BSP and rifamycin SV, were similar in Caco-2 and MDCKII/OATP2B1 cells, and this was also observed for all antiretroviral drugs, with the exception of efavirenz (Table 2). PIs potently inhibited E3S uptake by Caco-2 cells in a concentration-dependent manner (Fig. 8A), with low IC50 concentration observed for several PIs, including ritonavir (0.93 μM), atazanavir (2.2 μM), lopinavir (1.7 μM), tipranavir (0.77 μM), and nelfinavir (2.2 μM) (Table 2). Similar IC50 values, with the exception of efavirenz, were obtained in the MDCKII/OATP2B1 cells (Fig. 8B; Table 2).

**Uptake of [3H]Atazanavir and [3H]Ritonavir by Caco-2 and MDCKII/OATP2B1 Cells.** To examine potential mechanisms involved in pH-dependent atazanavir absorption, we evaluated the effect of pH on atazanavir uptake by Caco-2 cells. Because atazanavir is currently recommended in combination with ritonavir, pH dependence of ritonavir uptake and its potential transport by OATP2B1 were also examined. Uptake of 1 μM [3H]atazanavir by Caco-2 cells was sensitive to the extracellular pH (Fig. 9A).
The initial rate of E3S uptake was plotted against pH from 5.3 to 9.0. The initial rate of E3S uptake by MDCKII/OATP2B1 cells at 37°C was measured at 2 min in transport medium containing different pH concentrations ranging from pH 5.3 to 9.0. The initial rate of E3S uptake was plotted against the H+ concentrations. As described under Materials and Methods, a least-square fit of the data into eq. 3 was applied to estimate the Hill coefficient, using Prism 5 software. Data represent the mean ± S.E.M. for n = 3 independent experiments.

**Fig. 7. Determination of the stoichiometry of H⁺:E3S transport by OATP2B1.** The effect of H⁺ concentration on the initial rate of 1 μM [³H]E3S uptake by MDCKII/OATP2B1 cells at 37°C was measured at 2 min in transport medium containing different H⁺ concentrations (ranging from pH 5.3 to 9.0). The initial rate of E3S uptake was plotted against the H⁺ concentrations. As described under Materials and Methods, a least-square fit of the data into eq. 3 was applied to estimate the Hill coefficient, using Prism 5 software. Data represent the mean ± S.E.M. for n = 3 independent experiments.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>[³H]E3S Uptake (Caco-2 Cells)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSP</td>
<td>100 μM</td>
<td>12 ± 0.4***</td>
<td>15 ± 2***</td>
</tr>
<tr>
<td>Rifamycin SV</td>
<td>100 μM</td>
<td>29 ± 2***</td>
<td>13 ± 2***</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>5 mM</td>
<td>8 ± 2***</td>
<td>17 ± 6***</td>
</tr>
<tr>
<td>PIs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amprenavir</td>
<td>10 μM</td>
<td>71 ± 6*</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>Atazanavir</td>
<td>10 μM</td>
<td>34 ± 8**</td>
<td>35 ± 2***</td>
</tr>
<tr>
<td>Darunavir</td>
<td>100 μM</td>
<td>34 ± 7**</td>
<td>26 ± 1***</td>
</tr>
<tr>
<td>Inclavir</td>
<td>10 μM</td>
<td>85 ± 2</td>
<td>77 ± 7*</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>10 μM</td>
<td>37 ± 8**</td>
<td>18 ± 2***</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>10 μM</td>
<td>38 ± 6**</td>
<td>25 ± 8***</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>10 μM</td>
<td>32 ± 3***</td>
<td>22 ± 2***</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>10 μM</td>
<td>31 ± 3***</td>
<td>37 ± 2***</td>
</tr>
<tr>
<td>Tipranavir</td>
<td>10 μM</td>
<td>9 ± 10***</td>
<td>15 ± 3***</td>
</tr>
<tr>
<td>NNRTIs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100 μM</td>
<td>48 ± 11**</td>
<td>21 ± 1***</td>
</tr>
<tr>
<td>Etravirine</td>
<td>100 μM</td>
<td>83 ± 13</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>NRTIs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abacavir</td>
<td>100 μM</td>
<td>90 ± 10</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>100 μM</td>
<td>104 ± 3</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>100 μM</td>
<td>106 ± 3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>100 μM</td>
<td>107 ± 9</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Tenofovir DF</td>
<td>100 μM</td>
<td>91 ± 5</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>100 μM</td>
<td>94 ± 5</td>
<td>103 ± 5</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001.

Although there was no difference in the uptake when pH 7.4 and 8.5 were compared, the uptake was significantly enhanced when extracellular pH was lowered to 5.5 (P < 0.001). Because an outwardly directed H⁺ gradient (pHᵣ 8.5 > pHᵢ 7.4) did not result in decreased atazanavir uptake, we suspected that the uptake enhancement observed at pHᵣ 5.5 may be caused by changes in atazanavir ionization state. Atazanavir is a weak base with pKᵣ 4.25; hence, it becomes partially ionized at pH 5.5.

To further investigate the role of proton gradient in atazanavir uptake, we used NH₄Cl preincubation to temporarily acidify the intracellular compartment of Caco-2 cells while buffering the extracellular medium to pH 7.4, producing an outwardly directed H⁺ gradient (pHᵣ 7.4 > pHᵢ) (Fig. 9B). In contrast to the E3S uptake, this proton gradient did not alter the accumulation of atazanavir, further confirming that a proton gradient is not the driving force for atazanavir uptake by Caco-2 cells. Therefore, the increase in accumulation of atazanavir under pH 5.5 < pH 7.4 conditions is likely to be mediated by the acidic pH of the extracellular medium, which alters the ionization state of atazanavir, rather than the inwardly directed proton gradient.

The uptake of ritonavir by Caco-2 cells also demonstrated pH dependence. At low extracellular pH 5.5, ritonavir intracellular accumulation increased significantly, whereas no significant difference was observed when its accumulation at pH 7.4 and 8.5 was compared (Fig. 9C). Similarly to atazanavir, ritonavir is a weak base with pKᵣ 2.8. Hence, lowering extracellular pH would lead to increased ionization of ritonavir, which may affect its solubility or transport affinity for membrane transporters. To examine whether a proton gradient acts as a driving force for ritonavir transport, we applied the NH₄Cl preincubation method to reverse the gradient across the membrane of Caco-2 cells. Similarly to atazanavir, the outwardly directed proton gradient had no effect on ritonavir accumulation (Fig. 9D), suggesting that ritonavir uptake is stimulated by the acidic pH of the solution, rather than a proton gradient-dependent transporter.

To explore the potential involvement of OATP2B1 in the pH-dependent uptake of atazanavir, we examined whether OATP2B1 expression in MDCKII cells had an effect on intracellular accumulation of [³H]atazanavir (1 μM) (Fig. 10A). Atazanavir accumulation by the OATP2B1-overexpressing and wild-type MDCKII cells was compared, and the effect of known OATP2B1 inhibitors, E3S (100 μM), BSP (100 μM), and pravastatin (5 mM), on atazanavir uptake by MDCKII/ OATP2B1 cells was determined (Fig. 10B). There was no significant difference in the uptake of atazanavir by the OATP2B1-overexpressing cells versus the wild-type cells. Furthermore, OATP2B1 inhibitors did not reduce atazanavir accumulation by MDCKII/OATP2B1 cells, suggesting that atazanavir is not a substrate for OATP2B1, despite its strong inhibitory effect. Likewise, the role of OATP2B1 in the uptake of [³H]ritonavir (1 μM) was evaluated by comparing its uptake by OATP2B1-overexpressing and wild-type MDCKII cells (Fig. 10C) and evaluating the effect of the OATP2B1 inhibitors, E3S (100 μM), BSP (100 μM), and pravastatin (5 mM) (Fig. 10D). Ritonavir uptake by MDCKII/OATP2B1 cells was not significantly different from the one measured in the wild-type cells, and the OATP2B1 inhibitors did not decrease ritonavir accumulation in the MDCKII/OATP2B1 cells, suggesting that ritonavir is not a substrate for OATP2B1. In addition, we observed that BSP modestly, but significantly, enhanced the uptake of ritonavir by the MDCKII/OATP2B1 cells. Because MDCKII cells are derived from canine renal epithelial cells, this effect may be mediated...
by an efflux transporter endogenously expressed in MDCKII cells.

**Discussion**

Complex pharmacotherapy has introduced new obstacles in the management of HIV infection such as drug-induced toxicities and drug–drug interactions (Fichtenbaum and Gerber, 2002; de Maat et al., 2003). Clinical studies demonstrate that many drug–drug interactions occur as a result of specific combinations of antiretroviral drugs (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2009). In addition to the well established interactions of antiretrovirals with metabolic cytochrome P450 enzymes and drug efflux transporters, recent studies suggest that SLC transporters belonging to the OATPs family play a role in their hepatic uptake and disposition. Epithelial cells of the small intestine are known to express several OATPs (Sai et al., 2006). OATP2B1 transporter has been demonstrated to localize at the apical membrane of enterocytes and is thought to play an important role in the intestinal uptake of organic anions and many drugs (Kobayashi et al., 2003). In this study, we used a well established intestinal in vitro model (i.e., Caco-2 cell system) to further examine OATP2B1 transport activity, mode of transport, and interactions with anti-HIV drugs in the context of intestinal drug absorption. Because Caco-2 cells are known to display high variability in transporter expression and function, it was important to confirm in our own hands, OATP2B1 gene and protein expression and functional activity in this cell system. Uptake of E3S by Caco-2 cells was inhibited by standard OATP2B1 inhibitors, 100 µM BSP, 100 µM rifamycin SV, and 5 mM pravastatin. In OATP2B1-overexpressing MDCKII cells, these compounds demonstrated potent inhibitory effect (80–90%), resulting in E3S uptakes comparable with those observed in the wild-type cell line. In contrast, the inhibition was less potent in Caco-2 cells (50–70%), suggesting that additional E3S uptake mechanisms may exist in these cells.

Caco-2 cells demonstrated biphasic saturation kinetics for E3S uptake, suggesting the presence of two kinetically distinct saturable components. Our results for transport kinetics characteristics (high-affinity $K_M = 6 \pm 2$ µM and $V_{max} = 72 \pm 9$ pmol/mg protein/min and low-affinity $K_M = 1.5 \pm 0.2$ mM and $V_{max} = 2.7 \pm 0.2$ nmol/mg protein/min) are in agreement with values reported in the literature (Sai et al., 2006; Annaert et al., 2010). By comparing the intrinsic clearance values ($V_{max}/K_m$) for the high- and low-affinity sites, we estimate that the contribution of the high-affinity site to overall E3S uptake by Caco-2 cells at low E3S concentrations is approximately six to seven times larger than that of the low-affinity site. In MDCKII/OATP2B1 cells, E3S uptake kinetics demonstrated one saturable component with $K_M$ of 16 µM, similar to previously reported values in this cell system (Grube et al., 2006a). The high-affinity $K_M$ and $V_{max}$ in Caco-2 cells (6 ± 2 µM and 72 ± 9 pmol/mg protein/min, respectively) are similar to the values obtained in MDCKII/OATP2B1 cells (16 ± 2 µM and 210 ± 10 pmol/mg protein/min, respectively), suggesting that this component may cor-

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Concentration-dependent inhibition of OATP2B1-specific E3S uptake by PIs in Caco-2 cells (A) and MDCKII/OATP2B1 cells (B). Specific uptake of [3H]E3S (1 µM), at pH 7.4 and 37°C, was measured in Caco-2 cells for 5 min (A) and in MDCKII/OATP2B1 cells for 2 min in the absence (dimethyl sulfoxide control) or presence of increasing concentrations of PIs added to the uptake medium containing [3H]E3S (B). Concentration-dependent inhibition curves were fitted into a model sigmoidal dose–response equation (eq. 4) using Prism 5 software to determine the IC$_{50}$ concentrations. Data represent mean ± S.E.M. of $n = 3$ independent experiments.

**TABLE 2**

<table>
<thead>
<tr>
<th>IC$_{50}$ Values of standard OATP2B1 inhibitors and antiretroviral drugs on OATP2B1-specific 1 µM [3H]E3S uptake by Caco-2 and MDCKII/OATP2B1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>µM</strong></td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td><strong>Standard Inhibitors</strong></td>
</tr>
<tr>
<td>BSP</td>
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<tr>
<td>Rifamycin SV</td>
</tr>
<tr>
<td>Pravastatin</td>
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<tr>
<td><strong>PIs</strong></td>
</tr>
<tr>
<td>Lopinavir</td>
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<tr>
<td>Tipranavir</td>
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<td>Nelfinavir</td>
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<tr>
<td>Ritonavir</td>
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<tr>
<td>Atazanavir</td>
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<tr>
<td>Saquinavir</td>
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<tr>
<td>Darunavir</td>
</tr>
<tr>
<td><strong>NNRTIs</strong></td>
</tr>
<tr>
<td>Efavirenz</td>
</tr>
</tbody>
</table>

ND, not determined.
respond to the OATP2B1-mediated transport. The low-affinity component may represent E3S uptake by other OATP isoforms expressed in Caco-2 cells, such as OATP1A2, OATP3A1, or OATP4A1, or a different binding site on the OATP2B1 transport protein (Cvetkovic et al., 1999; Sai et al., 2006).

Many OATPs, including OATP2B1, have been shown to mediate pH-dependent transport of E3S and other substrates (Nozawa et al., 2004; Leuthold et al., 2009). However, in Caco-2 cells the pH dependence of OATP2B1 has not been examined previously. In this study, we observed that E3S uptake by Caco-2 cells is stimulated by an inwardly directed proton gradient (pHo 5.5 \( \leq \) pHi 7.4), whereas an outwardly directed proton gradient (pHo 8.5 \( > \) pHi 7.4) leads to a reduced intracellular accumulation of E3S. These results suggest that H\(^{+}\) gradient may act as the driving force for E3S uptake by Caco-2 cells. We further confirmed this finding by demonstrating that transport stimulation observed at pHo 5.5 \( < \) pHi 7.4 can be reversed when the proton gradient is abolished with the addition of the Na\(^{+}/H^{+}\) ionophore, monensin, in both Caco-2 and MDCKII/OATP2B1 cells. In addition, an outwardly directed H\(^{+}\) gradient (pHo 7.4 \( > \) pHi 6.3), generated by NH\(_{4}\)Cl preincubation, resulted in decreased E3S uptake by Caco-2 cells. Overall, these results suggest that an inwardly directed proton gradient acts as the driving force for OATP2B1-mediated E3S uptake by Caco-2 and MDCKII/OATP2B1 cells. We also demonstrated that the method of intracellular acidification by NH\(_{4}\)Cl preincubation, a technique we have described previously in different cell systems (Bendayan et al., 1994; Hong et al., 2001), can be useful in Caco-2 cells for examining the pH dependence of substrate transport. Furthermore, the stoichiometry of H\(^{+}\):E3S uptake by OATP2B1 was evaluated in MDCKII/OATP2B1 cells, and a hyperbolic relationship between E3S initial uptake rate and H\(^{+}\) concentration was established with an estimated Hill coefficient of 0.7 \( \pm \) 0.2, suggesting a 1:1 stoichiometry for proton:E3S uptake by OATP2B1.

Many PIs have been reported previously to inhibit human OATPs, such as OATP1A2, OATP1B1, and OATP1B3 (Cvetkovic et al., 1999; Tirona et al., 2003; Campbell et al., 2004). A recent study by Annaert et al. (2010) also examined the interactions of PIs with OATP transporters in Caco-2 cells, reporting that indinavir, saquinavir, and ritonavir significantly inhibited E3S uptake by Caco-2 cells and suggesting this was caused by the inhibition of OATP2B1-mediated transport by these PIs. In our initial screening for interac-

![Fig. 9. Effect of directional pH gradients on the uptake of atazanavir and ritonavir by Caco-2 cells. Uptake of 1 \( \mu \)M \[^{3}H\]atazanavir (A and B) or ritonavir (C and D) by Caco-2 cells was measured at 37°C and different extracellular pH (5.5, 7.4, and 8.5). Transport buffers were prepared by using HBSS buffer supplemented with 25 mM MES, pH 5.5, or 25 mM HEPES, adjusted to pH 7.4 or 8.5. To evaluate the effect of intracellular acidification on atazanavir (B) or ritonavir (D) uptake by Caco-2 cells, 1 \( \mu \)M \[^{3}H\]atazanavir or \[^{3}H\]ritonavir uptake was measured after 20-min preincubation with 30 mM NH\(_{4}\)Cl in the presence of 1 mM amiloride. Data represent mean \( \pm \) S.E.M. for \( n = 3 \) independent experiments. **, \( p < 0.01; ***, \( p < 0.001 \).
tions in OATP2B1-overexpressing MDCKII cells, we observed potent inhibition of specific E3S uptake by several PIs (i.e., tipranavir, lopinavir, nelfinavir, ritonavir, atazanavir, saquinavir, and darunavir) and one NNRTI (efavirenz), whereas other antiretroviral drugs did not significantly alter E3S uptake. Likewise, in Caco-2 cells these antiretroviral drugs significantly inhibited E3S uptake, although the inhibitory effect was less potent. Together, these results suggest that OATP2B1-mediated transport of E3S is sensitive to inhibition by many PIs.

We further characterized the concentration-dependent effect of the inhibitors on the specific uptake of E3S by the two cell systems. Standard inhibitors, i.e., BSP and rifamycin SV, had similar IC₅₀ concentrations in Caco-2 and MDCKII/OATP2B1 cells. Likewise, the IC₅₀ concentrations for antiretroviral drugs, except for efavirenz, were also very similar in the two cell lines. The inhibitory potencies (IC₅₀ values) in Caco-2 cells were highest for lopinavir (1.7 μM), tipranavir (0.77 μM), ritonavir (0.93 μM), atazanavir (2.2 μM), nelfinavir (2.2 μM), and saquinavir (3.5 μM). Annaert et al. (2010) recently reported that only indinavir, saquinavir, and ritonavir have a significant inhibitory effect on E3S uptake by Caco-2 cells, with IC₅₀ concentrations of 3.9, 5.3, and 6.3 μM, respectively. The differences in our findings could be explained by the differences in the methods applied, such as the source from which the cells were obtained, culture conditions, seeding density, and the level of Caco-2 cells differentiation, which is related to the time in culture after confluence (Hayeshi et al., 2008).

The effect of these interactions during oral drug absorption may have significant clinical relevance compared with other tissues. Although OATP2B1 is expressed ubiquitously, drug concentrations achieved at the brush-border membrane of enterocytes are substantially higher compared with the reported circulating concentrations of these drugs, especially for drugs with low oral bioavailability such as PIs. Hence, the inhibitory effect of PIs on OATP2B1-mediated transport at this site is expected to be more significant compared with their effect on the same transporter in other tissues. Overall, PIs could potentially alter oral bioavailability of OATP2B1 drug substrates.

Atazanavir, a component of first-line highly active antiretroviral therapy, has been reported to be a potent inhibitor of OATP2B1-mediated transport of E3S. Therefore, the potential for drug-drug interactions with OATP2B1 substrates, such as atazanavir, needs to be considered in the clinical setting.
roval therapy regimens, and several other PIs have been found to have lower oral bioavailability when coadministered with acid-reducing agents (de Maat et al., 2003). We hypothesized that this interaction may be caused by the increase in the pH of intestinal lumen by acid-reducing agents, resulting in decreased intestinal uptake of atazanavir by a pH-sensitive uptake transporter, such as OATP2B1. Because ritonavir is recommended in atazanavir-based regimens, we also examined the mechanism of intestinal uptake of ritonavir. Atazanavir uptake by Caco-2 cells was significantly enhanced at pH 5.5 compared with pH 7.4 (P < 0.001). This effect, however, did not seem to be mediated by an inwardly directed proton gradient, because increasing extracellular pH to 8.5 did not alter atazanavir accumulation. Furthermore, the use of NH4Cl preincubation to temporarily acidify the intracellular compartment did not alter atazanavir accumulation, showing that even though the outwardly directed gradient was produced and maintained, atazanavir transport was not affected. Similar pH effects were observed for ritonavir, with significant enhancement in intracellular accumulation in the presence of acidic extracellular pH. Hence, the increase in atazanavir and ritonavir accumulation at pH 5.5 seems to be mediated by the decrease in the pH of the extracellular medium, resulting in increased protonation of these PIs. Atazanavir and ritonavir are weak bases with pKₐ of 4.25 and 2.8, respectively. A change in pH from 7.4 to 5.5 is expected to result in up to 100-fold increase in their charged protonated form. Because large ionized molecules cannot cross membranes easily by diffusion, this suggests that carrier-mediated processes may be involved in the pH dependence of PIs’ intestinal absorption.

Several PIs (i.e., saquinavir, lopinavir, and darunavir) have been recently identified as substrates for human OATP1A2 and OATP1B1 (Su et al., 2004; Ks et al., 2010). A few studies have documented Pgp-mediated efflux of atazanavir (Jaheneh et al., 2009; Zastre et al., 2009; Ks et al., 2010). However, the uptake system for atazanavir is still unknown, and the role of OATP2B1 in its intestinal uptake has not been evaluated. Therefore, we examined whether the observed pH dependence of atazanavir and ritonavir uptake by Caco-2 cells involves OATP2B1-mediated transport. Our data demonstrate that there is no significant difference in the uptake atazanavir or ritonavir uptake by OATP2B1-overexpressing and wild-type MDCCKII cells and that their uptake is not susceptible to inhibition by OATP2B1 inhibitors, ESS, BSP, or pravastatin, suggesting that OATP2B1 is not implicated in the uptake of these PIs. The potential role of other OATP transporters in PIs uptake needs to be further investigated in the intestine. In summary, we have demonstrated a potent inhibitory effect of several PIs on OATP2B1-mediated ESS uptake. Thus, PI-based regimens may interfere with physiological functions of OATP2B1, such as the supply of sulfate-conjugated steroid hormone precursors and reabsorption of steroid degradation products. In addition, PIs may alter the disposition of drugs transported by OATP2B1 (e.g., statins). Further studies are required to elucidate the clinical implications of the identified interactions between PIs and OATP2B1-mediated transport and the nature of PIs’ uptake transporters at the intestinal barrier.

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