molecular pharmaceutics

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pH-Sensitive Interaction of HMG-CoA Reductase Inhibitors (Statins) with Organic Anion Transporting Polypeptide 2B1

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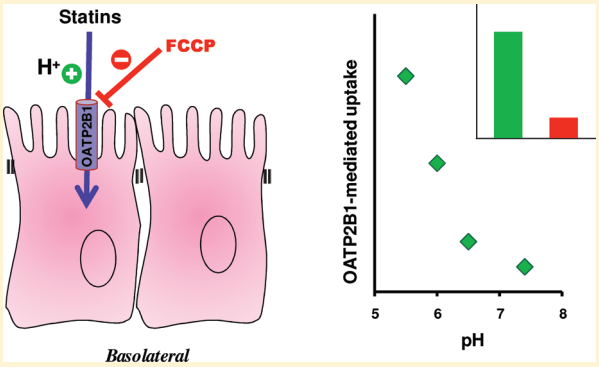
ABSTRACT: The human organic anion transporting polypeptide 2B1 (OATP2B1, *SLCO2B1*) is ubiquitously expressed and may play an important role in the disposition of xenobiotics. The present study aimed to examine the role of OATP2B1 in the intestinal absorption and tissue uptake of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase inhibitors (statins). We first investigated the functional affinity of statins to the transporter as a function of extracellular pH, using OATP2B1-transfeced HEK293 cells. The results indicate that OATP2B1-mediated transport is significant for rosuvastatin, fluvastatin and atorvastatin, at neutral pH. However, OATP2B1 showed broader substrate specificity as well as enhanced transporter activity at acidic pH. Furthermore, uptake at acidic pH was diminished in the presence of proton ionophore, suggesting proton gradient as the driving force for OATP2B1 activity. Notably, passive transport rates are predominant or comparable to active transport rates for statins, except for rosuvastatin and fluvastatin. Second, we studied the effect of OATP modulators on statin uptake. At pH 6.0, OATP2B1-mediated transport of atorvastatin and cerivastatin was not inhibitable, while rosuvastatin transport was inhibited by E-3-S, rifamycin SV and cyclosporine with IC_{50} values of 19.7 ± 3.3 μM, 0.53 ± 0.2 μM and 2.2 ± 0.4 μM, respectively. Rifamycin SV inhibited OATP2B1-mediated transport of E-3-S and rosuvastatin with similar IC_{50} values at pH 6.0 and 7.4, suggesting that the inhibitor affinity is not pH-dependent. Finally, we noted that OATP2B1-mediated transport of E-3-S, but not rosuvastatin, is pH sensitive in intestinal epithelial (Caco-2) cells. However, uptake of E-3-S and rosuvastatin by Caco-2 cells was diminished in the presence of proton ionophore. The present results indicate that OATP2B1 may be involved in the tissue uptake of rosuvastatin and fluvastatin, while OATP2B1 may play a significant role in the intestinal absorption of several statins due to their transporter affinity at acidic pH.



KEYWORDS: HMG CoA reductase inhibitors (statins), OATP2B1, estrone-3-sulfate, Caco-2 permeability, intestinal absorption

INTRODUCTION

The 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase inhibitors (statins) are generally well-tolerated lipid-lowering drugs and represent the first-line therapy for hypercholesterolemia.[1−4] Statins inhibit the synthesis of mevalonate, a rate-limiting step in the biosynthesis of cholesterol, lowering the plasma low density lipoprotein (LDL) cholesterol. Statins are given in the orally active hydroxy acid form, with the exception of lovastatin and simvastatin, which are administered as lactone prodrugs.[5] Hepatic uptake and biliary excretion as well as the metabolism play important roles in the statins disposition. Statins such as atorvastatin, lovastatin and simvastatin are metabolized mainly by CYP3A in humans, while biliary excretion is considered to be the major clearance mechanism for hydrophilic statins.[6−9]

The organic anion transporting polypeptides (OATP, *SLC21A*) are sodium-independent transporters that facilitate transport of amphipathic organic compounds. Tissue expression of OATPs can be selective, OATP1B1 and OATP1B3 in liver and OATP1C1 in brain and testis,[10−12] or ubiquitous, OATP1A2, OATP2B1, OATP3A1 and OATP1A4.[13,14] In liver, OATP1B1, OATP1B3 and OATP2B1 are expressed on the sinusoidal membrane of the hepatocytes and facilitate uptake. It has been shown that OATP1B1 and OATP1B3 facilitate the hepatic uptake of most statins, but its significance seems to be greatest for hydrophilic statins, such as pravastatin and rosuvastatin.[9] Indeed, clinically relevant drug−drug interactions (DDIs) are attributed to inhibition of statin transport mediated by members of the OATP family. For example, coadministration of cyclosporine resulted in about a 6- to 10-fold increase in the plasma levels of pravastatin and rosuvastatin, although these statins are not appreciably metabolized in humans.[9] Furthermore, polymorphisms of *SLCO1B1* (encoding OATP1B1) may lead to low

Received: March 2, 2011
Accepted: June 28, 2011
Revised: June 22, 2011
Published: June 28, 2011

transporter activity and may decrease the cholesterol lowering effect of statins (e.g., pravastatin), presumably due to decreased hepatic drug exposure and concomitant increase in plasma concentrations, which could increase relative peripheral tissue exposure and the risk of muscle toxicity.[15−17] The expression of OATP2B1 is relatively ubiquitous with its localization in intestine, liver, placenta, heart and skeletal muscles, and may play a pivotal role in tissue specific transport of endogenous substances and xenobiotics as part of normal physiological function.[10,13,14] However, OATP2B1 contribution to hepatic statin disposition and tissue uptake is not clear.

The oral bioavailability of simvastatin and lovastatin is low (∼5%), largely as a result of their CYP3A-mediated first-pass metabolism in the intestine and liver. However, the bioavailability of other statins ranges from 12% (atorvastatin) to more than 60% (pitavastatin) with fraction absorbed for all statins more than 30%.[18] The relatively higher fraction absorbed of the statins, especially hydrophilic statins, is likely due to the involvement of transporter systems in the enterocytes. This is consistent with the recent reports on the involvement of OATP1A2 and OATP2B1 in the absorption of drugs such as fexofenadine, talinolol and pravastatin.[19−23] Kobayashi et al. studied the impact of pH on the uptake of both estrone-3-sulfate and pravastatin and suggested the physiological relevance of OATPs in the intestinal absorption.[24] Evidently, intestinal expression of OATP2B1 is higher than that of other OATP isoforms such as OATP1A2, OATP3A1 and OATP4A1; and it is localized on the apical membrane of enterocytes facilitating cellular uptake of substrates.[25,26] However, the interaction of the statins with OATP2B1 and its transport activity at the luminal pH is not fully understood.

In this study, we aim to elucidate the functional role of OATP2B1 in the intestinal absorption and tissue uptake of statins. Transfected HEK293 cells and Caco-2 cells were used as the primary experimental tools to assess statin substrate specificity and the effect of extracellular pH on OATP2B1 activity. Transport of estrone-3-sulfate (E-3-S), a known OATP2B1 substrate, was characterized in parallel with the statins. We demonstrate that OATP2B1 is capable of transporting rosuvastatin, fluvastatin and atorvastatin at neutral pH but has broader substrate transport capacity at acidic pH. Additionally, we characterized the inhibition of the OATP2B1-mediated transport and the pH dependency, which suggest the potential risks of drug−drug interactions when OATP2B1-mediated drug transport is inhibited.

MATERIALS AND METHODS

Materials. [^{3}H]Estrone-3-sulfate (57.3 Ci/mmol) was obtained from Perkin-Elmer (Boston, MA). [^{3}H]Cerivastain (5 Ci/mmol), [^{3}H]pitavastatin (5 Ci/mmol), [^{3}H]pravastatin (5 Ci/mmol) and [^{3}H]rosuvastatin (10 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, pitavastatin, rosuvastatin and simvastatin were obtained from Sequoia Research Products (Pangbourne, U.K.). Amiloride, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Hanks balanced salt solution (HBSS) plus calcium chloride (0.14 g/L) with 20 mM 2-[*N*-morpholino]ethanesulfonic acid (MES, pH 6.5) and 20 mM *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonate (HEPES, pH 7.4) were obtained from Invitrogen (Gibco Laboratories, Grand Island, NY, USA). Ready Safe liquid scintillation cocktail was from Beckman Coulter (Fullerton, CA).

Caco-2 Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture medium [Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% nonessential amino acids (NEAA), 1% GlutaMAX-1, 100 U/mL penicillin and 100 μg/mL streptomycin] was obtained from Invitrogen, Grand Island, NY, USA.

Caco-2 cells were cultured at 37 °C with cell culture medium in an atmosphere of 10% CO_2 and 90% relative humidity (Nuaire Incubator, Plymouth, MN). The cells were passaged upon reaching approximately 90% confluence from T-flasks using 0.25% trypsin-EDTA (Invitrogen, Gibco Laboratories, Grand Island, NY). For Transwell assays, Caco-2 cells were seeded onto polycarbonate membrane inserts of the 24-well Transwell plate (Corning, NY, USA) at a density of ∼2.5 × 10^5 cells per insert. The cell culture medium was changed 2−3 times a week. Caco-2 cell monolayers were used for experimentation 21 days postseeding. Transepithelial electrical resistance (TEER) was measured using EVOM from World Precision Instruments (Sarasota, FL, USA). For Caco-2 uptake assays, cells were seeded onto 24-well BD Purecoat amine plates from BD Biosciences (Franklin Lakes, NJ). Cells were seeded at a density of ∼3.0 × 10^5 cells per well. The cell culture medium was changed 2−3 times a week, and the plates were used for experimentation 16 days postseeding.

HEK293 Cell Culture. Mock-transfected HEK293-*Flip in* cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium (DMEM), 10% v/v heat-inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 100 μg/mL Zeocin (Invitrogen). Stably transfected hOATP2B1-HEK293 cells were obtained from Dr. Dietrich Keppler (Heidelberg, Germany) and were grown in DMEM containing 10% heat-inactivated fetal bovine serum, 50 μg/mL gentamicin, and 400 μg/mL Geneticin. These cells were assessed for their expression and functional activity of OATP2B1.[27] Uptake assays were carried out on 24-well poly-D-lysine coated plates (Biocoat, Horsham, PA). Cells were seeded at a density of ∼3.0 × 10^5 cells per well. The cells were then incubated overnight at 37 °C/10% CO_2/95% RH. On the following day, culture medium was replaced with 500 μL of prewarmed (37 °C) medium to each well to avoided nutrient deficiency, and these samples were used for the uptake experiments, 48−72 h postseeding.

Transwell Caco-2 Permeability Assays. Transport studies were initiated by removing the cell culture medium from the apical (AP) and basolateral (BL) sides of the cell monolayers. Cell monolayers were washed once with HBSS (37 °C), which was then replaced with fresh transport buffer and equilibrated for 15 min. For absorptive (AP-to-BL) transepithelial transport assay, 0.3 mL of compound solution in HBSS was added to the apical compartment and 1 mL of fresh HBSS was added to the basolateral compartment (n = 3 monolayers per condition). Studies were carried out at 37 °C with continuous agitation. After 90 min incubation, 200 μL samples were collected from the basolateral compartment. Permeability across Caco-2 cell monolayers was measured in the absence and presence of OATP2B1 inhibitor (rifamycin SV, 100 μM). Permeability of nadolol, a low permeability marker, was measured to assess the cell monolayer integrity at different apical pH. Transepithelial electrical resistance (TEER) values were measured across the cell membranes prior to beginning the experimentation and at the last sample collection time point to assess the integrity of the Caco-2 monolayers (TEER > 350 $\Omega \cdot cm^2$).

Transporter Uptake and Inhibition Assays Using hOATP2B1-HEK293 Cells. Uptake of statins and E-3-S into OATP2B1-transfected

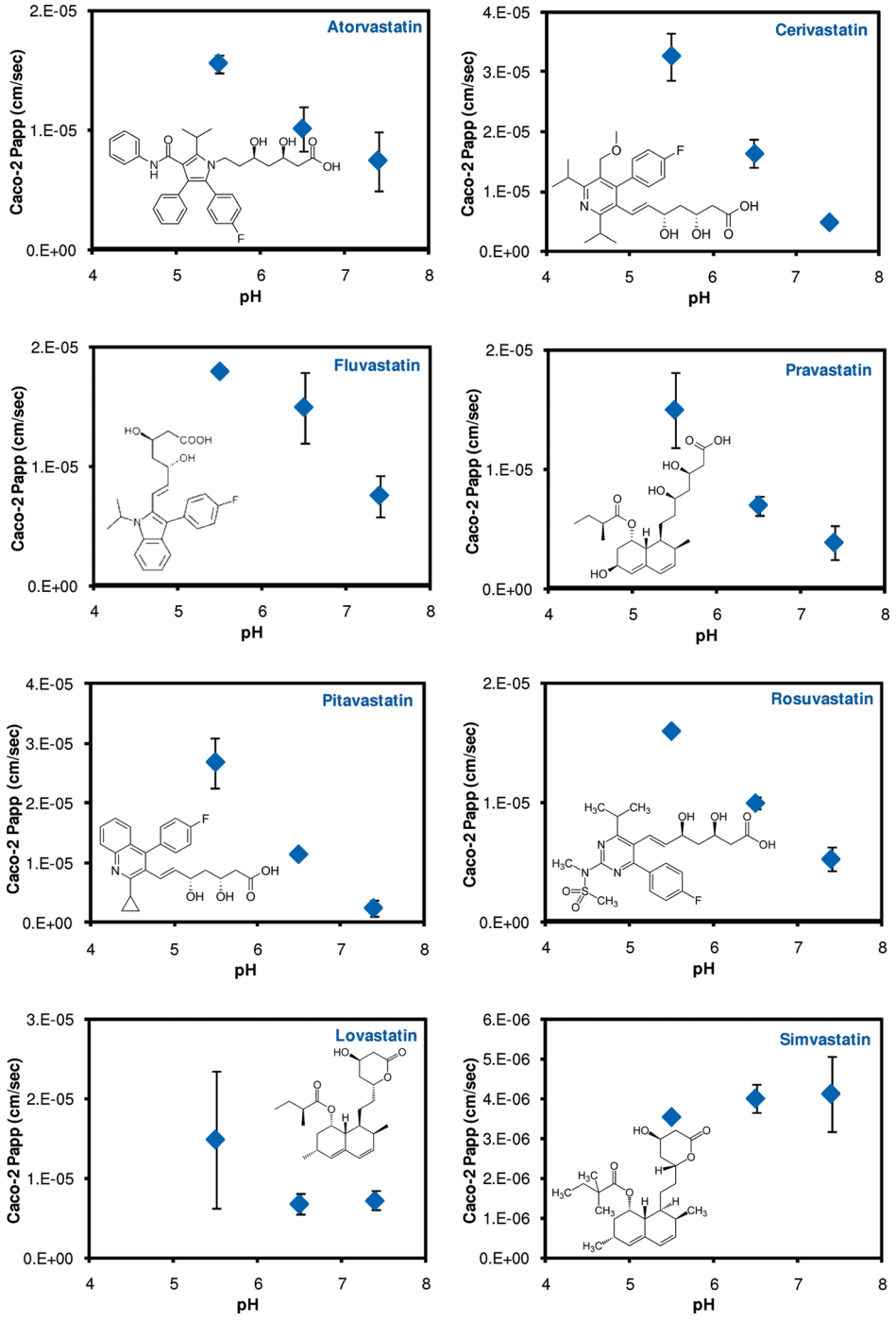


Figure 1. pH-dependent absorptive permeability of statins across Caco-2 cell monolayers. Permeability was measured in apical-to-basolateral direction with the apical pH of 5.5, 6.5, and 7.4 and constant basolateral pH of 7.4. Data points represent mean ± SD (n = 3).

and mock-transfected HEK293 cells was studied at various extracellular pHs. For inhibition assays, uptake of E-3-S and statins was investigated in the absence and presence of OATP2B1 inhibitors over a range of concentrations. Prior to the experiment, cells were placed on a 37 °C heat block and were washed three times with 1 mL of selected pH HBSS (pH 5.5, 6.0, 6.5, or 7.4). After 15 min acclimation incubation, cells were incubated with 300 μL of transport buffer containing test substrate with and without inhibitor (n = 3). The cells were incubated on the orbital heating block with shaking. Two minutes after dosing, cellular uptake was terminated by quickly washing the cells three times with 1 mL of ice-cold Dulbecco's phosphate-buffered saline (DPBS) buffer. The cells were then lysed in 400 μL of 1% sodium dodecyl sulfate (SDS) with 0.1% v/v 1 M NaOH in DPBS for radiolabeled samples or 400 μL of methanol solutions for nonradiolabeled samples. The total cellular protein content was determined by using Pierce BCA Protein Assay kit (Thermo, Rockford, IL) according to the manufacturer's specifications.

Transporter Uptake and Inhibition Assays Using Caco-2 Cells. The uptake of radiolabeled rosuvastatin and E-3-S into

Caco-2 was studied over an extracellular pH range of 5.5 to 7.4. Test compound was investigated in the absence and presence of rifamycin SV over a range of concentrations. Similar methods were as described in the section Transporter Uptake and Inhibition Assays Using hOATP2B1-HEK293 Cells.

Sample Analysis. *Radiolabeled.* When radioactive compounds were used for tracing, radioactivity was quantified with a Packard Tri-Carb 2900TR (Waltham, MA) scintillation counter.

LC−MS/MS Detection. Atorvastatin, simvastatin and lovastatin were quantified in the samples using LC−MS/MS methodology. Samples were prepared for mass spectral analysis by treating each well with 500 μL of methanol solution containing internal standard. Cells were then covered and shaken for 10−15 min. Cell supernatant was transferred to a 96 deep well plate where samples were dried down under nitrogen gas on an evaporator set at 50 °C. Samples were reconstituted in 150 μL of 100% methanol, then subjected to vortex for 2 min, and finally centrifuged for 3 min at 1000*g*.

LC−MS/MS analysis was conducted on a Sciex API400 Triple Quad mass spectrometer (turbospray ionization source) with a Shimadzu LC-10 HPLC system and Gilson 215 autosampler. The mass spectrometer was controlled by Analyst 1.4.2 software. The Gilson autosampler was independently controlled by Gilson 735 software and synchronized to Analyst via contact closure. The HPLC method consisted of a step gradient with 25 μL samples loaded onto a 1.5 × 5 mm Showadenko ODP 13 μm particle size column using 95% 2 mM ammonium acetate, 2.5% methanol and 2.5% acetonitrile. Samples were eluted with 10% 2 mM ammonium acetate, 45% methanol and 45% acetonitrile. MS/MS parameters utilized are positive ionization mode with following mass transitions: atorvastatin (559.2 → 440.2), lovastatin (405.0 → 285.0) and simvastatin (419.4 → 285.0). Peak area counts of analyte compound and internal standard (IS) were integrated using DiscoveryQuant Analyze as an add-on to Analyst 1.4.2. Analytical variability was within 5% coefficient of variance for all the statins.

Data Analysis. For Transwell Caco-2 assays, absorptive (AP-to-BL) transepithelial transport was represented as permeability value ($P_{app,A\rightarrow B} \times 10^{-6}$ cm/s) calculated using the following equation:

$$P_{app} = \frac{1}{\text{area} \times C_D(0)} \times \frac{dM_r}{dt} \quad (1)$$

where area is the surface area of the cell monolayer (0.33 cm^2), $C_D(0)$ the initial concentration of compound applied to the donor chamber, t the time, M_r the mass of compound in the receiver compartment, and dM_r/dt the flux of the compound across the cell monolayer. For cellular uptake assays, the transport facilitated by OATP2B1 was determined using the following equation:

$$\text{uptake}_{OATP2B1} = \text{uptake}_{HEK293/OATP2B1} - \text{uptake}_{HEK293/MOCK}$$

where $\text{uptake}_{HEK293/OATP2B1}$ and $\text{uptake}_{HEK293/Mock}$ are uptake values (nmol/min/mg-protein) obtained in OATP2B1-transfected HEK293 cells and mock-transfected HEK293 cells, respectively.

Statistical Analysis. All experiments were performed in triplicate (3 wells or 3 monolayers per condition), and the results are expressed as mean and standard deviation. Statistical differences among groups were evaluated by unpaired t test (between two groups) or one-way ANOVA (between multiple groups) with Dunnett's multiple comparison test (Prism 5.01; GraphPad Software, Inc., La Jolla, CA). When $p < 0.05$, statistical significance was considered to be achieved.

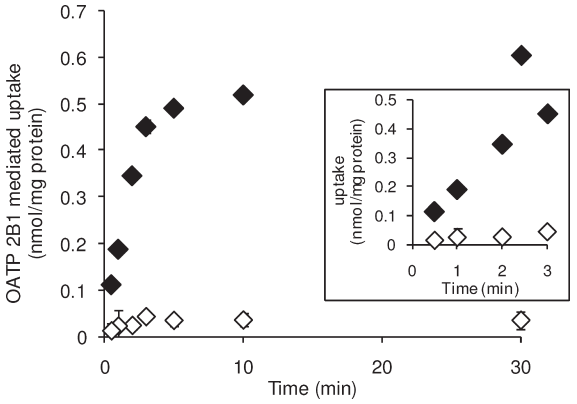


Figure 2. Time course of OATP2B1-mediated uptake of rosuvastatin by transfected cells at extracellular pH 6.0 (closed points) and pH 7.4 (open points). Uptake of rosuvastatin (0.1 μM) by mock-transfected and OATP2B1-tranfected HEK293 cells was measured at 37 °C over the specified time, and the OATP2B1-mediated uptake was obtained by subtracting the uptake in the mock-transfected cells from that by the OATP2B1-transfected cells. Inset shows the values in the lower time range. Each data point represents the mean ± SD (n = 3). OATP2B1-mediated uptake at pH 6.0 was significantly higher than at pH 7.4 at all time points ($p < 0.05$).

RESULTS

pH-Dependent Permeability of Statins Across Caco-2 Monolayers. The effect of apical pH on the absorptive permeability of several statins, including atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, pitavastatin, rosuvastatin and simvastatin, was studied across the Caco-2 monolayers. As shown in Figure 1, lovastatin and simvastatin, which exist in lactone form and have a pK_a value around 13.5, did not show pH-dependent permeability. However, Caco-2 permeability of other statins, which existed in the hydroxy acid form and have acidic pK_a values in the range of 4.2−4.6, showed a significant pH dependence ($p < 0.05$), with higher transport at low apical pH. At pH 7.4, all the statins demonstrated low to moderate permeability with a range of 2.3 ± 1.3 to 7.6 ± 1.7 x10^{-6} cm/s. The absorptive permeability of hydroxy acid statins at pH 5.5 was high and ranged from 15.0 ± 3.2 to 32.6 ± 4.0 × 10^{-6} cm/s, while the lactones (lovastatin and simvastatin) exhibited moderate to high permeability of 3.5 ± 0.9 to 14.9 ± 8.6 × 10^{-6} cm/s. No significant differences in the TEER values and nadolol permeability were observed, suggesting the integrity of Caco-2 monolayer at the pH range studied.

pH-Dependent OATP2B1-Mediated Transport in Transfected Cells. The time course of rosuvastatin uptake by mock-transfected and OATP2B1-transfected HEK293 cells was evaluated at pH 6.0 and 7.4 to determine the incubation time required for initial uptake rate estimates (Figure 2). The uptake of rosuvastatin by OATP2B1-transfected cells was significantly higher ($p < 0.05$) than the uptake by mock-transfected cells, at both the extracellular pH 6.0 and 7.4. Furthermore, active uptake mediated by OATP2B1 increased significantly ($p < 0.05$) at acidic pH, suggesting a pH-sensitive functional transporter activity. Since the uptake rates were linear up to 3 min, uptake at 2 min was measured to obtain the initial uptake rates in

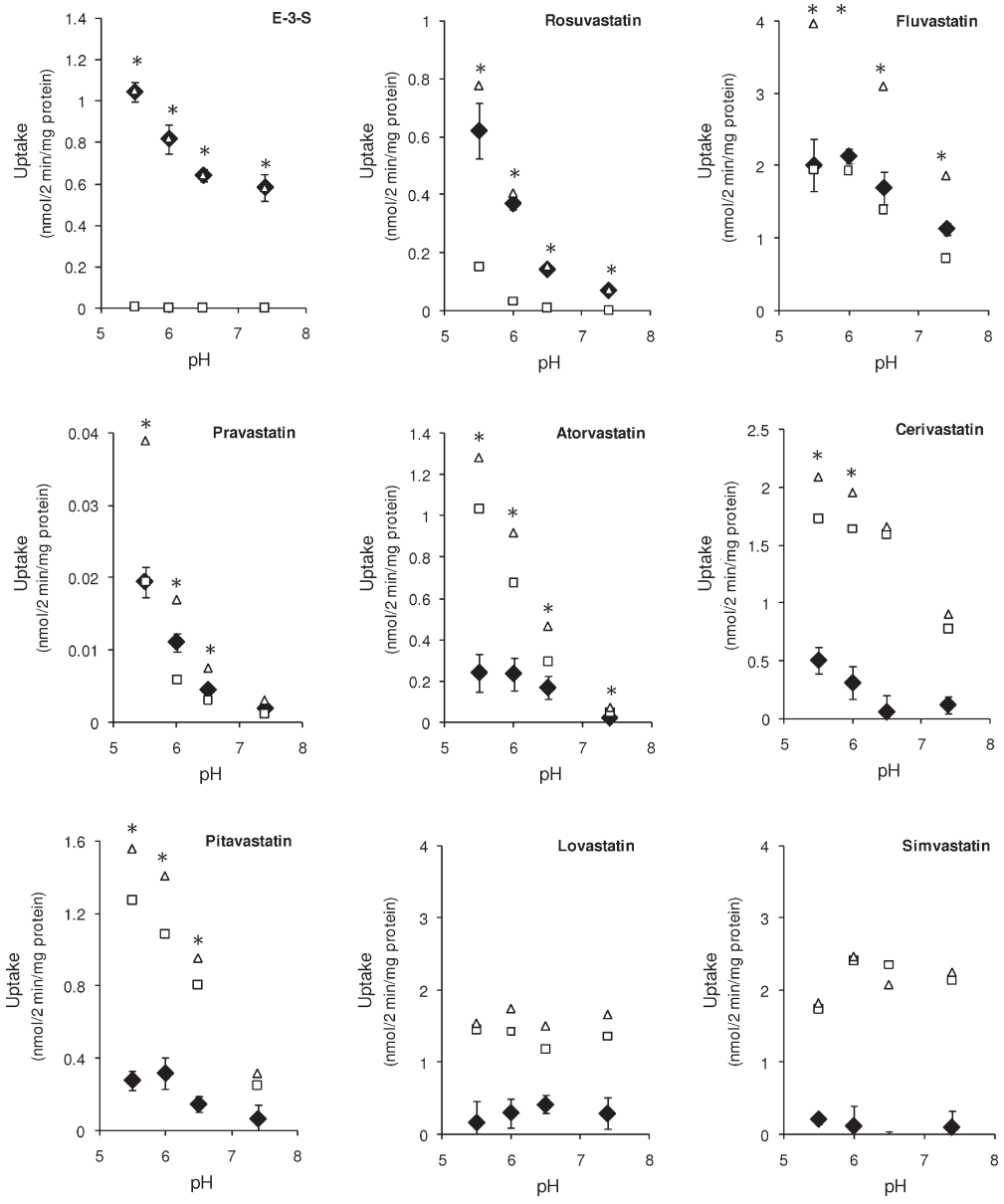


Figure 3. pH-dependent uptake of E-3-S and statins by the mock-transfected and OATP2B1-transfected cells. Uptake by mock-transfected (□) and OATP2B1-transfected (Δ) cells was measured over 2 min, at various extracellular pH values in the range of 5.5 and 7.4. OATP2B1-mediated uptake (◆) was obtained by subtracting the uptake in the mock-transfected cells from that by the OATP2B1-transfected cells. Mean uptake in mock-transfected and OATP2B1-transfected cells is shown, while OATP2B1-mediated uptake data point represents the mean ± SD ($n = 3$). *$p < 0.05$, significant difference between uptake by mock-transfected and OATP2B1-transfected cells at the same extracellular pH.

subsequent studies. Similar results were observed for E-3-S and other statins (data not shown).

We further examined the uptake of E-3-S and eight statins by OATP2B1-transfected and mock-transfected HEK293 cells at various extracellular pHs (Figure 3). Evidently, E-3-S and statins, except lovastatin and simvastatin, showed significantly higher uptake by OATP2B1-transfected cells compared to mock-transfected cells, at the extracellular pH under 6.5 ($p < 0.05$). Moreover, these compounds showed pH-dependent OATP2B1-mediated transport activity, with maximum functional activity at pH 5.5−6.0. For E-3-S, uptake by mock-transfected cells was not significantly influenced by the extracellular pH, however, OATP2B1 activity at pH 5.5 was about 1.8 times higher than at pH 7.4 ($p < 0.05$). In the case of the statins, uptake by mock-transfected cells showed pH dependency with higher transport at lower pH. The OATP2B1-mediated uptake of rosuvastatin, pravastatin and fluvastatin was higher than or comparable to the nonspecific uptake in the pH range studied (OATP2B1-transfected cells/mock-transfected cells uptake ratio ≥2). Furthermore, the OATP2B1-mediated uptake of rosuvastatin and pravastatin at pH 5.5 was more than 8-fold higher compared to that at neutral pH. Although atorvastatin, cerivastatin and pitavastatin demonstrated significant pH dependency in OATP2B1-mediated transport, these statins possessed higher contribution of nonspecific transport to the apparent uptake into OATP2B1-transfected cells (uptake ratio <2). Lovastatin and simvastatin uptake into OATP2B1-transfected cells was not significantly different from the uptake into mock-transfected

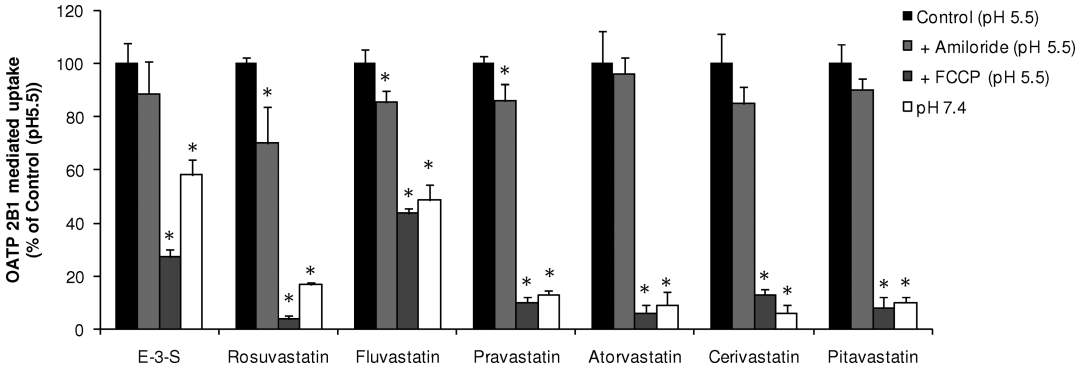


Figure 4. Effect of proton-gradient modulators on the uptake of E-3-S and statins by the OATP2B1-transfected cells. Uptake was measured over 2 min, in the presence of amiloride (1 mM) and FCCP (50 μM) at extracellular pH 5.5. OATP2B1-mediated uptake was obtained by subtracting the uptake in the mock-transfected cells from that by the OATP2B1-transfected cells. Values represent mean ± SD ($n = 3$) . *$p < 0.05$, significant difference compared to uptake at pH 5.5.

cells, in the pH range studied. Also similar to Caco-2 absorptive permeability, lovastatin and simvastatin showed no pH dependency in the uptake by both mock- and OATP2B1-transfected cells.

To further investigate the role of proton gradient as the driving force, OATP2B1-mediated uptake was studied in the presence of Na^+/H^+ exchanger (NHE) inhibitor, amiloride, and proton ionophore, FCCP (Figure 4). FCCP showed a significant inhibition of E-3-S and statin OATP2B1-mediated uptake at the acidic extracellular pH; however, amiloride showed minimal or no effect. Amiloride reduces the intracellular pH by inhibiting NHE and thus lowering the proton gradient across the membrane. However, at the extracellular pH 5.5 used in this study, the proton gradient may still have existed in the presence of amiloride.[19,28] No effects of FCCP and amiloride were apparent in the mock-transfected cells. Overall, these results suggest that OATP2B1-mediated uptake of statins is driven by an inwardly directed proton gradient.

Modulation of OATP2B1-Mediated Transport of Statins in Transfected Cells. To further characterize the OATP2B1-mediated transport of statins, uptake of atorvastatin, cerivastatin, pravastatin and rosuvastatin by OATP2B1-transfected and mock-transfected HEK293 cells was studied, in the presence of various concentrations of an OATP2B1 substrate (E-3-S) and nonselective OATP inhibitors (rifamycin SV and cyclosporine) at pH 6.0. As shown in Figure 5, E-3-S, rifamycin SV and cyclosporine substantially inhibited OATP2B1-mediated transport of rosuvastatin in a concentration-dependent manner with the concentrations of half-maximal inhibitory effect (IC_{50}) of 19.7 ± 3.3 μM, 0.53 ± 0.2 μM and 2.2 ± 0.4 μM respectively. Although pravastatin active transport was significantly inhibited by E-3-S and rifamycin SV, the IC_{50} values could not be estimated in the concentration range studied and are expected to be >3 mM and >0.3 mM, respectively. However, atorvastatin and cerivastatin transport was not significantly inhibited in the presence of E-3-S and rifamycin SV.

OATP2B1-mediated E-3-S and rosuvastatin uptake in the HEK293 cells was inhibited by rifamycin SV in a concentration-dependent manner at extracellular pH 6.0 and 7.4 (Figure 6). The inhibitory profiles and the IC_{50} values of E-3-S uptake inhibition at both pHs are not significantly different (Table 1). However, IC_{50} of rosuvastatin uptake inhibition at pH 6.0 was marginally lower compared to IC_{50} at pH 7.4. Overall, these results indicate that the inhibitor interaction with the transporter is not pH sensitive.

Characterization of OATP2B1-Mediated Transport in Caco-2 Cells. To evaluate the role of OATP2B1 in the intestinal absorption, transport characteristics of E-3-S and rosuvastatin were studied in Caco-2 cells (Figure 7). Uptake of E-3-S by Caco-2 showed significant pH dependency and was inhibitable with rifamycin SV (100 μM). Similarly, rosuvastatin uptake by Caco-2 was inhibitable with rifamycin SV ($P < 0.05$), however, the pH-sensitive active transport was not apparent (Figure 7B). Uptake of E-3-S and rosuvastatin at acidic pH was significantly ($p < 0.05$) inhibited by FCCP, suggesting proton-gradient driven uptake by Caco-2 cells (Figure 7C). Maximum uptake inhibition of E-3-S uptake by rifamycin SV was about 55% with similar IC_{50} values, at both the apical pH of 6.0 and 7.4 (Figure 8A, Table 1). However, uptake of rosuvastatin by Caco-2 cells was inhibited by rifamycin SV to a different extent at different pHs, with larger percentage inhibition achieved at pH 7.4 (Figure 8B). In contrast to the HEK293 transfected cells, where the uptake was inhibited by rifamycin SV up to 90%, the inhibition in Caco-2 cells was less potent, suggesting that additional transport mechanisms may exist in Caco-2 cells. Atorvastatin and pravastatin uptake by Caco-2 showed higher uptake at acidic pH but was not affected by rifamycin SV (data not shown).

We further examined the absorptive permeability of E-3-S and rosuvastatin at pH 6.0 and 7.4, in the absence and presence of 100 μM rifamycin SV (Figure 9). The permeability of E-3-S was higher at pH 6.0. Furthermore, rifamycin SV inhibited the permeability to a larger extent at pH 6.0, compared to that at pH 7.4, suggesting pH-sensitive OATP2B1-mediated transport across Caco-2 cell monolayer. Although rosuvastatin showed higher permeability at pH 6.0, its transport, in contrast to that of E-3-S, was inhibited by rifamycin SV to a larger extent at the apical pH 7.4. Overall, these results are consistent with the extent of rosuvastatin uptake inhibition by rifamycin SV in the Caco-2 cells (Figure 8B).

DISCUSSION

Among the wide variety of SLC transporters, OATPs have been shown to mediate the hepatic uptake and tissue distribution of several statins.[9,29] Furthermore, OATP1A2 and OATP2B1 are known to be localized on the brush-border membrane of the human enterocytes and show functional activity toward anionic substrates.[25,26] In this study, we evaluated the substrate activity of statins to OATP2B1 and investigated the influence of extracellular

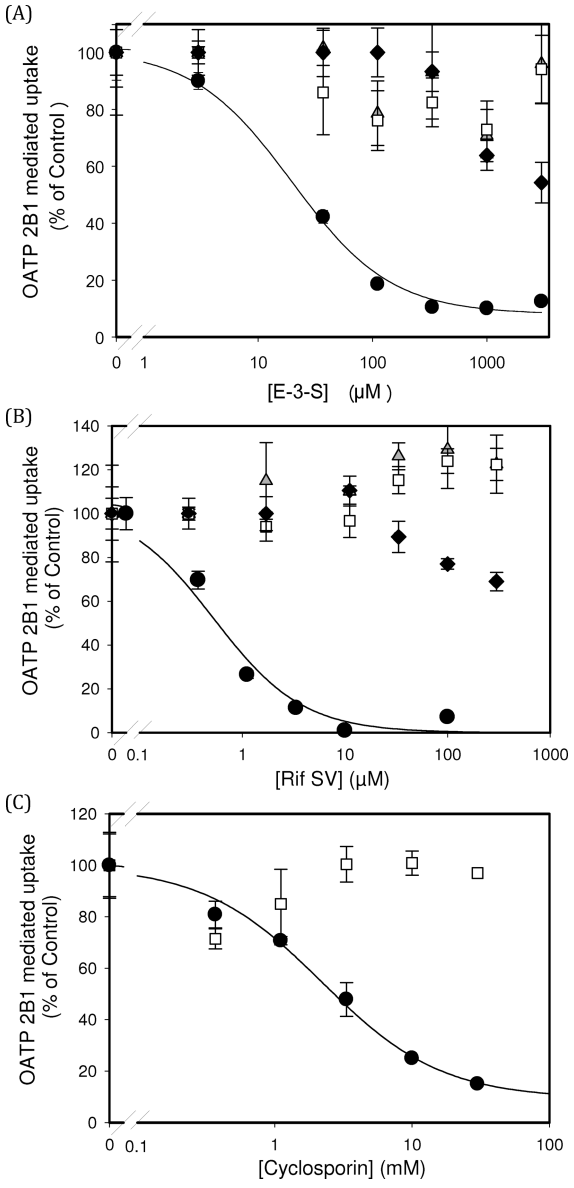


Figure 5. Concentration-dependent effect of E-3-S, rifamycin SV and cyclosporine on the OATP2B1-mediated uptake of rosuvastatin (●), pravastatin (◆), cerivastatin (Δ) and atrovastatin (□) at pH 6.0. Uptake of rosuvastatin (0.1 μM), pravastatin (0.1 μM), cerivastatin (0.1 μM) and atorvastatin (1 μM) was measured in mock-transfected and OATP2B1-transfected cells over 2 min. OATP2B1-mediated uptake was obtained by subtracting the uptake in the mock-transfected cells from that by the OATP2B1-transfected cells. Each data point represents mean ± SD (n = 3) of the % of control.

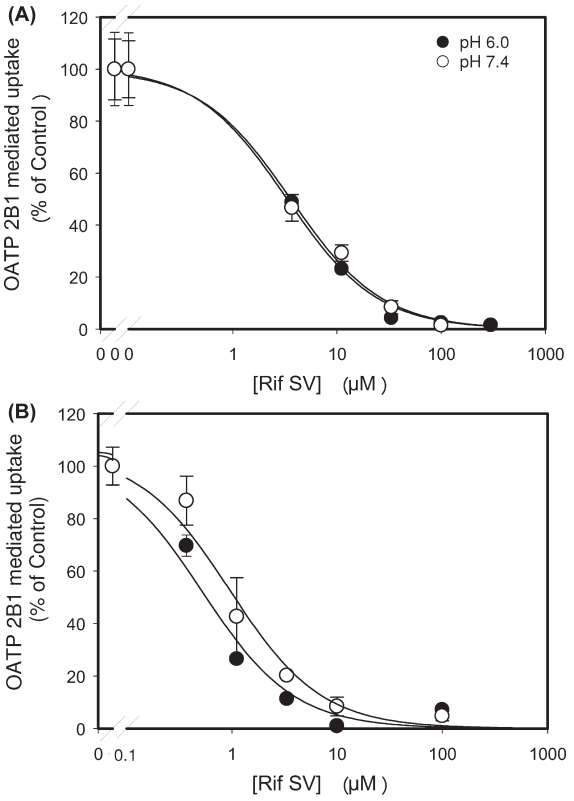


Figure 6. Concentration-dependent inhibitory effect of rifamycin SV on OATP2B1-mediated uptake of E-3-S and rosuvastatin, at extracellular pH of 6.0 and 7.4. Uptake of 0.1 μM E-3-S (A) and 0.1 μM rosuvastatin (B) was measured in mock-transfected and OATP2B1-transfected cells over 2 min. OATP2B1-mediated uptake was obtained by subtracting the uptake in the mock-transfected cells from that by the OATP2B1 transfected cells. Closed and open data points represent mean ± SD of the % of control at pH 6.0 and 7.4, respectively.

Table 1. Rifamycin SV IC_{50} Values for E-3-S and Rosuvastatin OATP2B1-Mediated Uptake Inhibition at Extracellular pH 6.0 and 7.4, in Transfected HEK293 and Caco-2 Cells

	uptake inhibition IC_{50} (μM)			
	transfected HEK293		Caco-2	
	pH 6.0	pH 7.4	pH 6.0	pH 7.4
E-3-S	3.3 ± 0.4	3.6 ± 0.5	3.7 ± 1.7	4.6 ± 2.4
rosuvastatin	0.53 ± 0.3	0.96 ± 0.4	0.34 ± 0.2	0.25 ± 0.1

pH on the transporter activity. We further characterized the uptake of E-3-S and statins in representative human enterocytes using Caco-2 cells and compared it with the OATP2B1-mediated transport assessed using transfected HEK293 cells.

OATP2B1-mediated transport of E-3-S, rosuvastatin, fluvastatin, pravastatin, atorvastatin, cerivastatin and pitavastatin is pH sensitive, with the uptake being increased at acidic extracellular pH compared to pH 7.4. Evidently, OATP2B1-mediated transport of these hydroxy acid statins was variable across the pH range. Rosuvastatin, fluvastatin and atorvastatin showed a significant ($p < 0.05$) OATP2B1 mediated contribution to uptake at all the pHs studied; whereas pravastatin, cerivastatin and pitavastatin were not substrates of OATP2B1 at neutral pH. While OATP2B1 is ubiquitously expressed at the sinusoidal membrane of hepatocytes, endothelial membrane of heart and other muscles, it is apparent from the current results that the transport for rosuvastatin, fluvastatin and atorvastatin could be facilitated by OATP2B1 in the liver, heart, muscle and other tissues due to their affinity at physiological pH.[29,30] However, OATP2B1 show broader substrate specificity at the brush-border membrane of the intestinal epithelial cells due to the presence of acidic (pH 6.0) microenvironment. As the results suggest, hydroxy acid statins studied here showed significantly higher uptake in

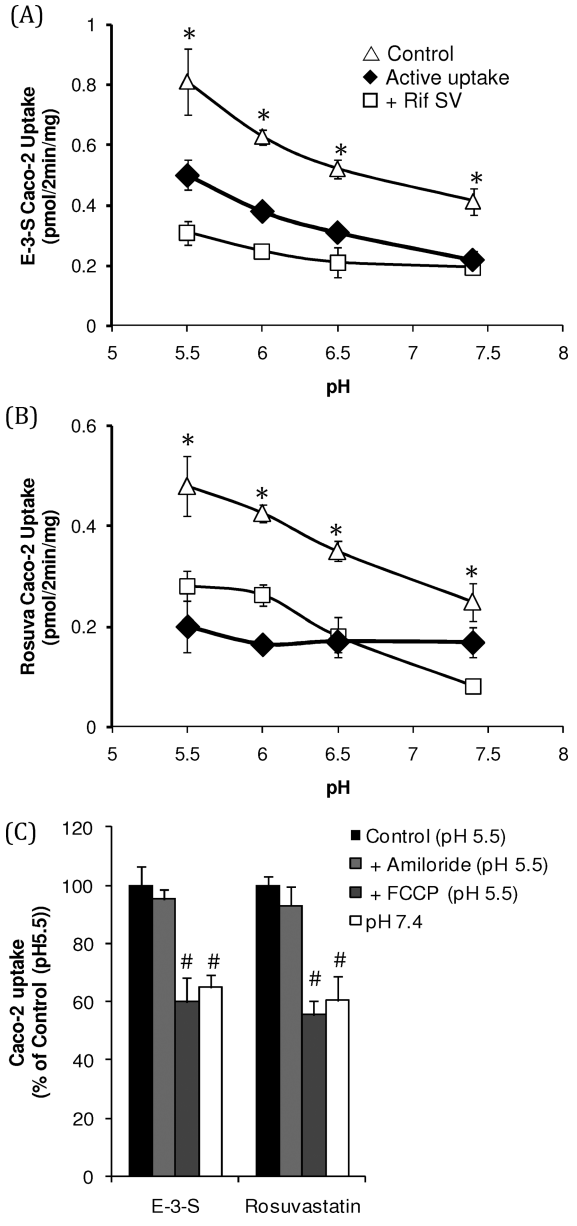


Figure 7. pH-dependent apical uptake of E-3-S (A) and rosuvastatin (B) by the Caco-2 cells, in the absence and presence of OATP inhibition. Uptake of 0.1 μM E-3-S or 0.1 μM rosuvastatin by Caco-2 cells in the absence (Δ) and presence ($\square$) of rifamycin SV (100 μM) was measured at various apical pH in the range of 5.5 and 7.4, over 2 min. Apparent transporter-mediated uptake ($\blacklozenge$) was obtained by subtracting the control uptake from that in the presence of rifamycin SV. Each data point represents the mean $\pm$ SD $^{*}p < 0.05$, compared to the uptake in the absence of rifamycin SV. (C) Effect of proton-gradient modulators on the uptake of E-3-S and statins by the Caco-2 cells. Uptake was measured over 2 min, in the presence of amiloride (1 mM) and FCCP (50 μM) at extracellular pH 5.5. $^{\#}p < 0.05$, significant difference compared to uptake at pH 5.5.

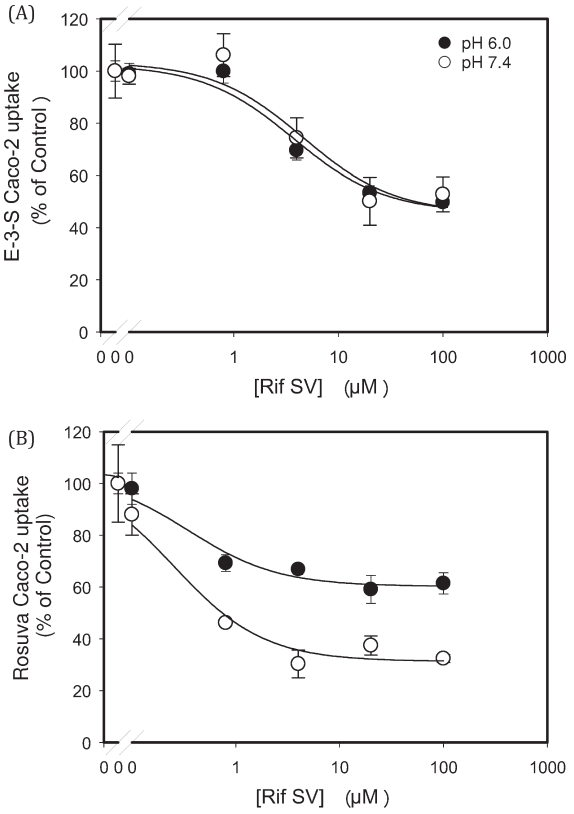


Figure 8. Concentration-dependent inhibitory effect of rifamycin SV on uptake of E-3-S and rosuvastatin by Caco-2 cells, at apical pH of 6.0 and 7.4. Uptake of 0.1 μM E-3-S (A) and 0.1 μM rosuvastatin (B) was measured in Caco-2 cells over 2 min. Closed and open data points represent mean $\pm$ SD of the % of control at pH 6.0 and 7.4, respectively.

OATP2B1-transfected cells compared to mock-transfected cells at the acidic pH, although atorvastatin, cerivastatin and pitavastatin also possess a significant and often predominant passive transport component. The transport of the lactone statins (lovastatin and simvastatin) showed no OATP2B1 specificity, and transport was not influenced by the extracellular pH, suggesting no role of OATP2B1. Nevertheless, the hydroxy acid form of these statins, which could be formed in the systemic circulation, may be transported by OATP2B1.[30]

Evidently, the uptake of E-3-S and hydroxy acid statins by OATP2B1-transfected HEK293 cells and Caco-2 cells is stimulated by an inwardly directed proton gradient (extracellular pH 5.5 to 6.5). This is further confirmed by the inhibition of uptake at acidic extracellular pH by proton ionophore, FCCP (Figure 4). Collectively, these results suggest that an inwardly directed proton gradient acts as the driving force for OATP2B1-mediated cellular uptake of statins. This proton-gradient effect could be favorable for OATP2B1 to play a role in the intestinal absorption of statins. The enhanced transporter activity at acidic extracellular pH could be a result of change in the extent of protonation of the substrate and/or the substrate binding pocket of the transporter. Based on site-directed mutagenesis, Leuthold et al. suggested that the pH dependency may be linked to the highly conserved histidine in the third transmembrane domain of OATP2B1.[31] Similar pH-sensitive activity has also been shown with other OATPs, PEPT1 and MCT1, where lowering the extracellular pH leads to enhanced transport of their substrates.[31−34] Nowaza et al. observed that an acidic extracellular pH increases the transport rate (V_{max}) of OATP2B1-mediated transport, while the apparent affinity (K_m) remains unchanged.[20] In contrast, it was shown that extracellular low pH leads to increase of the apparent affinity (decreased K_m value) with no marked effect on the V_{max}.[31] Our inhibition studies demonstrated that the IC_{50} of rifamycin SV, determined using

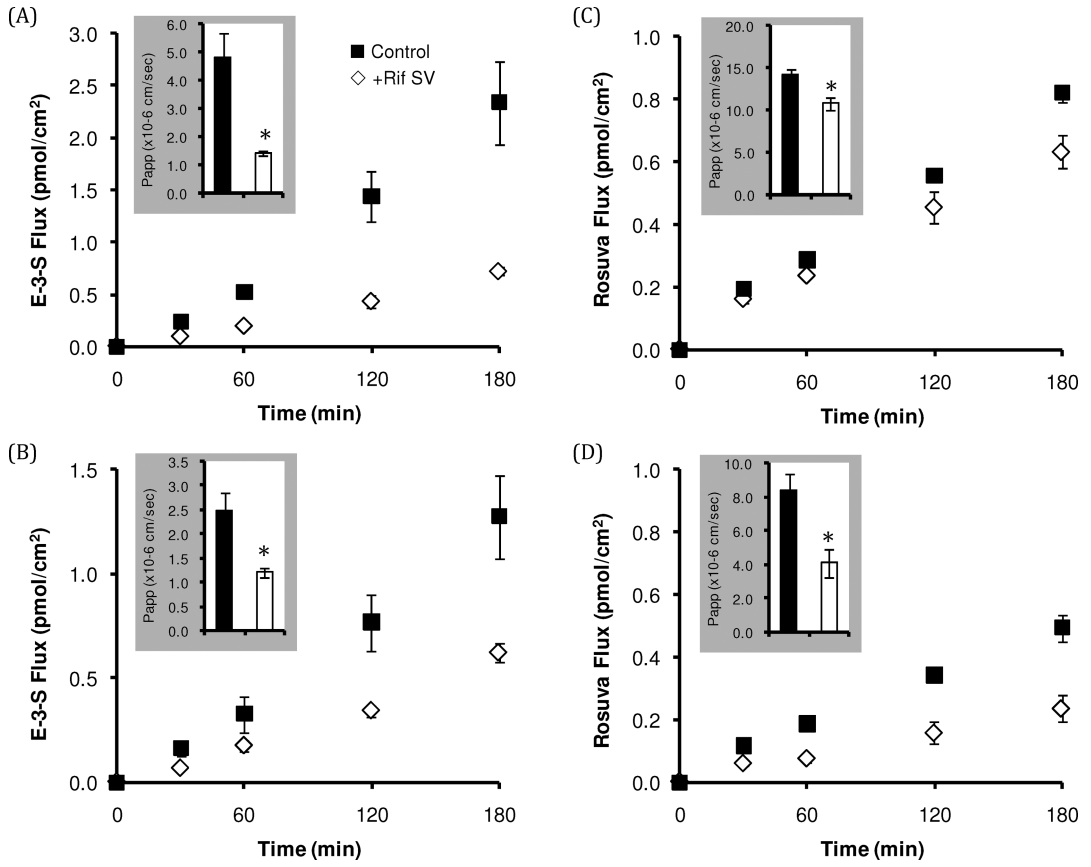


Figure 9. Effect of OATP2B1 inhibition on the pH-dependent transport of E-3-S and rosuvastatin across Caco-2 monolayers. E-3-S (0.5 μM) transport was measured in apical-to-basolateral direction at pH 6.0 (A) and pH 7.4 (B), in the absence and presence of OATP2B1 inhibitor, rifamycin SV. Similarly, rosuvastatin (0.5 μM) transport was measured at pH 6.0 (C) and pH 7.4 (D). Closed and open data points represent mean ± SD (n = 3) of transport in the absence and presence of 100 μM rifamycin SV, respectively. Basolateral pH was always 7.4. Insets show the permeability values of transport in the absence (black bars) and presence (white bars) of 100 μM rifamycin SV. *p < 0.05, compared to permeability in the absence of inhibitor.

E-3-S and rosuvastatin transport inhibition, was not influenced by the extracellular pH (Table 1). Further studies may be warranted to understand the effect of pH on the transporter affinity.

While the OATP2B1 activity increased, the transport of the mock-transfected HEK293 cells also increased significantly at the acidic pH. In accordance with the pH-partition theory, the high nonspecific transport is a result of passive transport of the unionized species of these anionic compounds. For rosuvastatin, the passive transport contribution was lower compared to the OATP2B1 specific transport at all pH values. Our findings suggest that E-3-S, rifamycin SV and cyclosporine inhibited the OATP2B1 specific uptake of rosuvastatin with the estimated IC_{50} values in the μM range (E-3-S, IC_{50} = 19.7 ± 3.3 μM; rifamycin SV, IC_{50} = 0.53 ± 0.2 μM; cyclosporine, IC_{50} = 2.2 ± 0.4 μM). In contrast, statins (atorvastatin and cerivastatin) with a lower OATP2B1 contribution to the overall transport showed minimal or no transport inhibition at pH 6.0. This substrate-dependent inhibitory activity of E-3-S, rifamycin and cyclosporine may be due to involvement of multiple binding sites of OATP2B1. Overall, the role of OATP2B1 in the intestinal absorption of rosuvastatin, fluvastatin and pravastatin could be significant; while, for statins including atorvastatin, cerivastatin and pitavastatin, where passive transport is predominant, the transporter role is likely minimal.[35]

Clinically relevant DDIs for rosuvastatin and pravastatin when coadministered with gemfibrozil or cyclosporine have been documented.[36−38] While OATP2B1 contribution to the liver uptake of statins is considered to be low,[39,40] the transporter was suggested to facilitate statin accumulation in other tissues due to its ubiquitous expression.[29,30,41] Our findings suggest that the hydroxy acid form of rosuvastatin, but not pravastatin, is a substrate to OATP2B1 at the physiological conditions (pH 7.4). In addition, rifamycin SV and cyclosporine are potent inhibitors of rosuvastatin OATP2B1-mediated uptake and therefore, when coadministered, may lead to lowered tissue rosuvastatin exposure while increasing the plasma levels. Our in vitro findings suggest OATP2B1 inhibition may be partially responsible for clinically observed rosuvastatin interactions.[13,37] Nevertheless, potential inhibition of intestinal absorption of these statins may obscure the DDI findings. On the other hand, the contribution of OATP2B1 to the hepatic, muscle and tissue uptake of other statins is likely low, either because they are not substrates for the transporter at physiological pH (7.4) or because they are lipophilic and exhibit relatively high passive diffusion.[35]

The OATP2B1 specific E-3-S uptake by Caco-2 cells (obtained from the difference between uptake in the absence and presence of rifamycin SV) is stimulated in presence of inwardly directed proton gradient, although the pH-sensitivity was relatively lower compared to what were observed using transfected cells. Interestingly, transporter specific uptake of rosuvastatin by Caco-2 cells is not sensitive to the apical pH.

It should be noted that the inhibition of rosuvastatin uptake by Caco-2 in the presence of rifamycin SV was not complete at both pHs (Figure 6B). Furthermore, the extent of inhibition at acidic pH was lower compared to the neutral pH, although the IC_{50} values were similar. These differences in transport characteristics of rosuvastatin between the two cell systems could be due to possible involvement of other transport mechanism(s) across the apical membrane of Caco-2 cells. Several other OATPs including OATP1A2, OATP3A1 and OATP4A1 are also expressed in the human small intestine and Caco-2 cells.[42,43] While OATP1A2 is known to transport rosuvastatin,[41] its involvement may not be attributable to the observed pH insensitivity, as OATP1A2 is also known to show significant pH-dependent transport.[31] Similar pH-dependent transport was also suggested for OATP3A1 and OATP4A1.[31] Another possible explanation is the involvement of Na^+/H^+ exchanger NHE3 (*SLC9A3*), which is functionally expressed on the apical membrane of the Caco-2 cells and maintains the transmembrane proton electrochemical gradient. It is believed that the transport capacity of certain H^+-coupled cotransporters (PepT1 and PAT1) is high when the NHE3 is active.[32−34] The presence of NHE3 in Caco-2 may lead to constant supply of protons onto the apical surface needed for OATP2B1 activity, independent of extracellular pH. Although we observed no significant effect of Na^+ depleted medium on the rosuvastatin uptake by Caco-2 cells (data not shown), further studies are warranted to understand the role of NHE3.

We initially studied the effect of apical pH on the absorptive (APl-to-BL) permeability of statins. Anionic statins demonstrated pH-dependent permeability, while the lactones lovastatin and simvastatin were unaffected. The significant increase in permeability at lower pH may be attributed to a combination of the OATP2B1-mediated uptake and passive transport mechanisms. The role of OATP2B1 in the intestinal absorption is further substantiated by the absorptive permeability of E-3-S and rosuvastatin across Caco-2 cell monolayers (Figure 9). The dependence of transporter activity on luminal pH is of particular relevance because an orally administered drug is absorbed from various segments of intestine, which exhibits a considerable pH gradient.[44,45] Between proximal jejunum and the distal ileum, the pH gradually rises from about 6.0 to 8.0, and can drop to as low as 5.0 in the colon. Yet, the pH in the unstirred water layer or microclimate is about 5.2−6.2 and might be regulated independently of the luminal pH.[44] Considering the luminal pH and the expression of OATP2B1 along the length of intestine,[25] the current results provide evidence for the involvement of OATP2B1 in the intestinal absorption of rosuvastatin, fluvastatin and pravastatin. However, predominant passive transport of other anionic statins (e.g., atorvastatin, cerivastatin and pitavastatin) may mask the active transport component of the overall transcellular transport.[35]

In summary, we evaluated the role of OATP2B1 in the intestinal absorption and tissue uptake of statins. pH-dependent studies using transfected HEK293 cells and Caco-2 cells suggested a potential contribution of OATP2B1-mediated transport in the intestinal absorption and tissue uptake for rosuvastatin and fluvastatin, which may be further inhibitable. However, the other hydrophilic statin, pravastatin, is a substrate only at acidic pH, indicating the potential role of OATP2B1 in its intestinal absorption. Although atorvastatin showed significant OATP2B1-mediated uptake at acidic and neutral pH, the high passive transport contribution driven by its lipophilicity and lack of effect of inhibitors indicate that OATP2B1 is likely to play a minor role in its disposition. Similarly, cerivastatin, pitavastatin, lovastatin and simvastatin demonstrated relatively high passive transport and/or no transporter affinity. The affinity of certain statins toward OATP2B1, in combination with the fact that the transporter shows a wide tissue distribution and is inhibited by certain drugs, implies an important role of OATP2B1 in their disposition and drug−drug interactions.

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ACKNOWLEDGMENT

This study was sponsored by Pfizer Inc. We thank Larry Tremaine, Robert Dow, Jeffrey Pfefferkorn and Steven Hansel for supporting this work and valuable suggestions regarding this research and manuscript. All authors are full-time employees of Pfizer Inc.

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