Atorvastatin Transport in the Caco-2 Cell Model: Contributions of P-Glycoprotein and the Proton-Monocarboxylic Acid Co-Transporter

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Purpose. The purpose of this study was to elucidate the mechanisms by which an HMG-CoA reductase inhibitor, atorvastatin (an organic acid with a pKa of 4.46), was transported in the secretory and absorptive directions across Caco-2 cell monolayers.

Methods. Caco-2 cells were grown on polycarbonate membrane inserts in 6-well Snapwell plates (Costar). The permeability of radiolabeled compounds across Caco-2 cell monolayers was determined using a side-by-side diffusion apparatus (NaviCyte) and an automated liquid handler (Hamilton Microlab 2200). The apical uptake of ^{14}C-atorvastatin was also determined in Caco-2 cells. Cyclosporin A (20 μM) was present in the uptake media to block potential P-glycoprotein-mediated atorvastatin efflux.

Results. Polarized permeation of atorvastatin was observed with the basolateral-to-apical (B-to-A) permeability being 7-fold greater than the A-to-B permeability (35.6×10^{-6} and 4.9×10^{-6} cm/s, respectively). The secretion of atorvastatin was a saturable process with an apparent K_m of 115 μM. The B-to-A permeability of atorvastatin was significantly reduced by cyclosporin A (10 μM), verapamil (100 μM), and a P-glycoprotein specific monoclonal antibody, UIC2(10 μg/ml) (43%, 25%, and 13%, respectively). Furthermore, both CsA and verapamil significantly increased the A-to-B permeability of atorvastatin by 60%; however, UIC2 did not affect the A-to-B permeability of atorvastatin. CsA uncompetitively inhibited the B-to-A flux of atorvastatin with a K_i of 5 μM. In addition, atorvastatin (100 μM) significantly inhibited the B-to-A permeability of vinblastine by 61%. The apical uptake of atorvastatin increased 10.5-fold when the apical pH decreased from pH 7.4 to pH 5.5 while the pH in the basolateral side was fixed at pH 7.4. A proton ionophore, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) significantly decreased atorvastatin uptake. In addition, atorvastatin uptake was significantly inhibited by benzoic acid, nicotinic acid, and acetic acid each at 20 mM (65%, 14%, and 40%, respectively). Benzoic acid competitively inhibited atorvastatin uptake with a K_i of 14 mM. Similarly, benzoic acid, nicotinic acid, and acetic acid significantly, inhibited the A-to-B permeability of atorvastatin by 71%, 21%, and 66%, respectively.

Conclusion. This study demonstrated that atorvastatin was secreted across the apical surface of Caco-2 cell monolayers via P-glycoprotein-mediated efflux and transported across the apical membrane in the absorptive direction via a H^+-monocarboxylic acid cotransporter (MCT). In addition, this study provided the first evidence that negatively charged compounds, such as atorvastatin, can be a substrate for P-glycoprotein.

KEY WORDS: Atorvastatin; Caco-2; P-glycoprotein; monocarboxylic acid transporter.

INTRODUCTION

Atorvastatin (Lipitor®) is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase that is currently used in the treatment of hypercholesterolemia and mixed dyslipidemia. Atorvastatin is a monocarboxylic acid with a pK_a of 4.46 (Fig. 1). Atorvastatin is rapidly absorbed after oral administration with a Tmax of approximately 2 h (1). The absolute bioavailability of atorvastatin was approximately 14%, and the bioavailability of inhibitory activity was 30% due to active metabolites of atorvastatin (2). Atorvastatin was extensively metabolized by CYP 3A4 and predominantly excreted into bile (3).

While the pharmacokinetic characteristics and the metabolite profile of atorvastatin have been studied, the mechanisms by which atorvastatin is transported across biological membranes have not been elucidated. Previous studies in our laboratory have shown that atorvastatin inhibited P-glycoprotein-mediated digoxin secretion in the Caco-2 model (4). Another HMG-CoA reductase inhibitor, lovastatin, has been recently suggested to interact with P-glycoprotein (P-gp) in neuroblastoma cells *in vitro* (5). However, it is unknown if atorvastatin is a substrate for P-gp.

Several other monocarboxylic acids such as acetic acid, benzoic acid, and nicotinic acid are absorbed via a proton-cotransport mechanism termed monocarboxylic acid transporter (MCT) in the intestine as well as in the Caco-2 cells (6 and ref therein). Pravastatin has also been suggested to be a substrate for MCT (7). It was speculated that atorvastatin could be transported by MCT in the intestine given the structural similarity between atorvastatin and pravastatin.

The overall objective of this study was to elucidate the mechanisms by which atorvastatin was transported in secretory and absorptive directions across Caco-2 cell monolayers. In particular, this study examined the potential contributions of P-gp and a MCT in mediating the secretion and absorption of atorvastatin, respectively, in the Caco-2 cell model.

MATERIALS AND METHODS

Materials

Atorvastatin was provided by the Department of Compound Management at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). ^{14}C-Atorvastatin (63 μCi/mg) was provided by the Department of Radiochemistry at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). ^{14}C-Benzoic acid (51 mCi/

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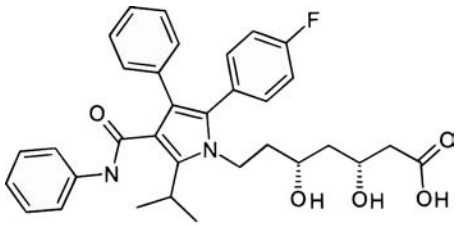
ABBREVIATIONS: Atorva, atorvastatin; BA, benzoic acid; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagles medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FCCP, carbonylcyanide p-trifluoro-methoxyphenylhydrazone; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid; HBSS, Hank's balanced salt solution; MCT, monocarboxylic acid transporter; HOAC, acetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; Nicotinic acid, NA; P-gp, P-glycoprotein; UIC2, a P-glycoprotein specific monoclonal antibody.

mmol) and ^{3}H-vinblastine (7.6 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). ^{3}H-mannitol (19.7 Ci/mmol) and ^{14}C-mannitol (51.5 mCi/mmol) were obtained from NEN Life Science Products (Boston, MA). Dulbecco's modified Eagles medium (DMEM, 4.5 g/L glucose) was purchased from CELOX Laboratories (St. Paul, MN). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals, Inc. (Norcross, GA). MEM nonessential amino acids (10 mM), glutamine (200 mM), and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL Life Technology (Grand Island, NY). Trypsin-versene mixture containing trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) was obtained from BioWhittaker, Inc. (Walkersville, MD). Verapamil hydrochloride (±), cyclosporin A (CsA), benzoic acid sodium salt (BA), nicotinic acid (NA), and sodium acetate were purchased from Sigma (St. Louis, MO). A P-gp specific monoclonal antibody that recognizes an external epitope of human P-gp, UIC2, was obtained from Immunotech (Cedex, France). Snapwell tissue culture plates with polycarbonate filters (12 mm diameter, 0.4 μm pore size, 1.13 cm^2 growth area) and T-75 polystyrene tissue culture flasks with 0.2 μm vented cap were obtained from Corning Costar Corp. (Cambridge, MA). The side-by-side diffusion apparatus was obtained from NaviCyte (Sparks, NV). The automated liquid handler, Microlab 2200, was obtained from Hamilton (Reno, NV). The Caco-2 cell line was obtained from American Type Culture Collection at passage 17 (Rockville, MD). Millicell-ERS used for measuring the transepithelial electrical resistance (TEER) was obtained from Millipore (Bedford, MA). Other chemicals were obtained from either Sigma or Gibco BRL Life Technology.

Cell Culture

Sub-cultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin-versene solution. Cells were maintained in the absence of any antibiotics using DMEM supplemented with 10% FBS, 1% nonessential amino acids, and 1% glutamine. For permeability and uptake experiments, the Caco-2 cells were seeded on Snapwell filters (seeding density: 10^5/filter), and the culture medium was changed every other day and the night before a transport experiment. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells used in this study were at passages 22–49 and days 22–26 in culture. The Caco-2 monolayers formed under these conditions typically gave a TEER value of 300–500 Ω·cm^2.



pKa = 4.46

Fig. 1. Structure of atorvastatin free acid. Atorvastatin (Liptor®) is provided as the calcium salt.

Permeability Experiments

The apparent permeability of radiolabeled compounds across Caco-2 cell monolayers was determined using a side-by-side diffusion apparatus (NaviCyte) and an automated liquid handler (Microlab 2200, Hamilton). For P-gp related permeability experiments, HEPES-HBSS buffer (1.8 mM $CaCl_2$, 5.37 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 136.89 mM NaCl, 4.17 mM $NaHCO_3$, 3.38 mM Na_2HPO_4, 5.55 mM D-glucose, and 5 mM HEPES, pH 7.4) was used. For MCT related permeability experiments, MES buffer (1.8 mM $CaCl_2$, 5 mM KCl, 120 mM NaCl, 10 mM D-glucose, and 10 mM MES, pH 6.0) and HEPES buffer (1.8 mM $CaCl_2$, 5 mM KCl, 120 mM NaCl, 10 mM D-glucose, and 10 mM HEPES, pH 7.4) were used in the apical and basolateral sides, respectively. Prior to experiments, the culture medium was replaced with respective buffers that had been warmed at 37°C, and the TEER was measured. Caco-2 cell monolayers were mounted in a side-by-side diffusion apparatus. The transport experiment was initiated by adding 5 ml drug solution to the donor chamber and 5 ml respective buffer to the receiver chamber. ^{14}C-Mannitol (1.85 μM) or ^{3}H-mannitol (91 nM), used as a cell monolayer integrity marker, was included in the drug solutions. Transport experiments were carried out at 37°C from either the apical-to-basolateral (A-to-B) or the B-to-A directions in the absence or presence of an inhibitor that was present at equal concentrations in both the donor and the receiver chambers (unless otherwise stated in the figure legends). For inhibition studies, ethanol was used as a co-solvent at a final concentration of 2% (v/v). Constant mixing of the solutions in both chambers was achieved by an air-lift system with carbogen (5% CO_2/95% O_2). Fifty microliter aliquots were sampled from both the donor and the receiver chambers at four time points (0, 30, 60, and 90 min, or 0, 40, 80, and 120 min). The radioactivity associated with the samples was determined by liquid scintillation counting on a Packard 2500 TR Tri-Carb counter.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{1}{A \cdot C_D(0)} \cdot \frac{dM_R}{dt}$$

where A is the surface area of the cell monolayer, $C_D(0)$ is the initial drug concentration in the donor chamber, and dM_R/dt is the linear appearance rate of mass in the receiver chamber. The P_{app} of mannitol was typically less than 2.5×10^{-6} cm/s in this study.

For atorvastatin kinetic studies, the B-to-A atorvastatin permeability was determined as a function of atorvastatin concentration ranging from 0.5 to 250 μM at pH 7.4 on both A and B sides. The transepithelial flux ($J_{B\text{-}A}$) was expressed as pmol/cm^2/min, and was fit to the following Michaelis-Menten equation using SigmaPlot™ 4.0,

$$J_{B\text{-}A} = \frac{J_{max} \cdot C}{K_m + C}$$

where $J_{B\text{-}A}$ is the B-to-A flux of atorvastatin at a given concentration, C, of atorvastatin, J_{max} is the maximal B-to-A flux, and K_m is the concentration of atorvastatin at one-half of the maximal B-to-A flux.

For atorvastatin/CsA interaction study, the B-to-A flux of atorvastatin was determined as a function of CsA concentration ranging from 0 to 10 μM, while atorvastatin concentration was fixed at 10, 30, and 60 μM, respectively. This experiment was performed at pH 7.4 on both A and B sides. The reciprocal of the $J_{B\text{-}A}$ of atorvastatin was plotted as a function of CsA concentration, and a linear regression line was obtained for each atorvastatin concentration. The K_i of CsA was derived from the following equation (8):

$$K_i = \frac{\text{Intercept}}{\text{Slope} + \frac{K_m}{C}}$$

where K_m is the affinity constant of atorvastatin for P-gp estimated from atorvastatin kinetic study, C is atorvastatin concentration for each regression line.

Uptake Experiments

Uptake experiments were performed in Caco-2 cells grown on polycarbonate membrane inserts (1.13 cm^2) at 37°C. The MES buffer and HEPES buffer, as specified in the permeability experiments, were used at different pH (pH 5.5–6.5 with MES buffer; pH 7.0 and 7.4 with HEPES buffer). When a monocarboxylic acid was used as an inhibitor at 20 mM in the apical side, the respective buffer had 20 mM less NaCl in order to balance the osmolarity on both sides across the Caco-2 cell monolayer. Prior to experiments, the culture medium was aspirated and rinsed once with the respective buffers that had been warmed at 37°C. For all uptake experiments, 2.5 ml of HEPES buffer (pH 7.4) was added to the basolateral side. Uptake of radiolabeled compounds was initiated by adding 0.4 ml drug solution to the apical side. CsA (20 μM) was included in drug solutions to minimize P-gp-mediated efflux of atorvastatin. For inhibition studies, inhibitors were also included in the drug solutions. Uptake was terminated by rinsing the cell monolayer in ice-cold buffer (pH 7.4). Cells of each monolayer were lysed with 0.1 ml Soluene (Packard) and neutralized with 0.5 ml of a mixture of saturated sodium pyruvate, glacial acetic acid and methanol (4:3:1). Radioactivity associated with the cells was determined by liquid scintillation counting. Uptake data were corrected by non-specific binding which was determined at 0 °C with pH 7.4 in both apical and basolateral sides.

For atorvastatin/BA interaction study, the apical uptake of ^{14}C-atorvastatin was determined as a function of BA concentration ranging from 0 to 15 mM, while atorvastatin concentration was fixed at 2, 10, and 100 μM, respectively. The reciprocal of the uptake of ^{14}C-atorvastatin was plotted as a function of BA concentration, and a linear regression line was obtained for each atorvastatin concentration. The K_i of BA was derived from the following equation (8):

$$K_i = \frac{\text{Intercept}_1 - \text{Intercept}_2}{\text{Slope}_1 - \text{Slope}_2}$$

The mean K_i was obtained from three calculations of three combinations of every two regression lines.

RESULTS

Atorvastatin Flux Across Caco-2 Cell Monolayers

To examine if atorvastatin was transported via a carrier-mediated pathway(s), the transepithelial flux of atorvastatin was first determined in the A-to-B and B-to-A directions as a function of time. Polarized fluxes of ^{14}C-atorvastatin were observed with the B-to-A flux significantly greater than the A-to-B flux (Fig. 2). The apparent permeability coefficient of atorvastatin in the B-to-A direction was 35.6 ($\pm$1.1) $\times$ 10^{-6} cm/s, which was 7-fold greater than that in the A-to-B direction [4.9 ($\pm$0.3) $\times$ 10^{-6} cm/s] at an atorvastatin concentration of 1.4 μM.

To confirm the involvement of a carrier-mediated transport pathway(s), the B-to-A flux of atorvastatin was also determined as a function of atorvastatin concentration ranging from 2 to 250 μM. Data fit better to a simple Michaelis-Menten equation than a kinetic equation that combines a Michaelis-Menten component plus a linear component as judged by the fitting coefficients (data not shown) and visual observation. The B-to-A flux of atorvastatin displayed a saturable process with an apparent K_m of 115 $\pm$ 19 μM and a J_{max} of 141 $\pm$ 11 pmol/cm^2/min (Fig. 3). The concentration dependence phenomenon supported that a carrier-mediated efflux system(s) for atorvastatin was present in Caco-2 cell monolayers.

Effect of P-gp Modulators on Atorvastatin Fluxes Across Caco-2 Cell Monolayers

To determine if P-gp was involved in the secretory transport of atorvastatin across Caco-2 cell monolayers, the permeability of ^{14}C-atorvastatin was examined in the absence and presence of CsA (10 μM), verapamil (100 μM), and a P-gp specific monoclonal antibody, UIC2 (10 μg/ml), respectively. CsA, verapamil, and UIC2 inhibited the B-to-A permeability of atorvastatin by 43%, 25%, and 13%, respectively (Table I). Moreover, both CsA and verapamil stimulated the A-to-B permeability of atorvastatin to the same extent (61% increase); however, UIC2 did not affect the A-to-B permeability of atorvastatin (Table I).

The interaction between atorvastatin and CsA via P-gp-mediated efflux system was further examined. The B-to-A flux of atorvastatin was determined as a function of CsA concentration, while atorvastatin concentration was fixed at 10, 30, and

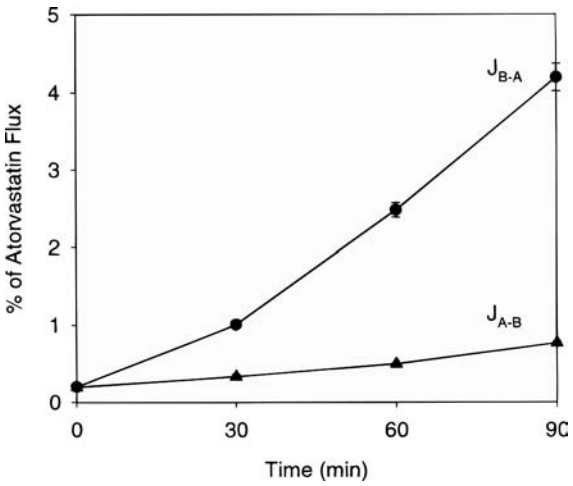


Fig. 2. Transepithelial flux of atorvastatin across Caco-2 cell monolayers. The A-to-B and B-to-A fluxes of ^{14}C-atorvastatin (1.4 μM) were determined at pH 7.4 as a function of time. Each point represents the mean ($\pm$ S.E.M.) of 4–6 determinations (Caco-2 monolayers).

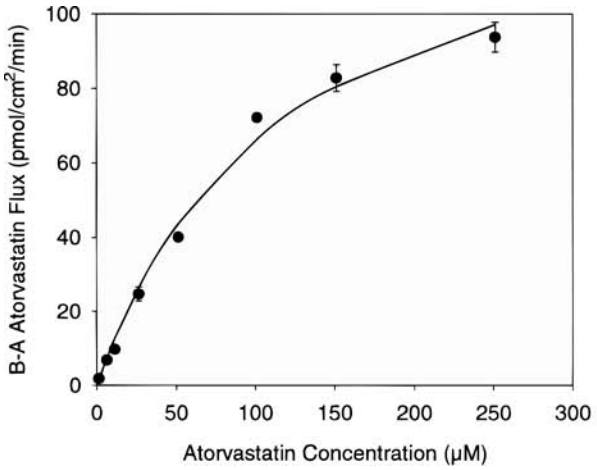


Fig. 3. Secretory transport kinetics of atorvastatin across Caco-2 cell monolayers. The B-to-A flux of atorvastatin was determined at pH 7.4 as a function of atorvastatin concentration. Each point represents the mean (± S.E.M.) of 4 determinations (Caco-2 monolayers). The solid line represents the best fit of the data to the Michaelis-Menten equation.

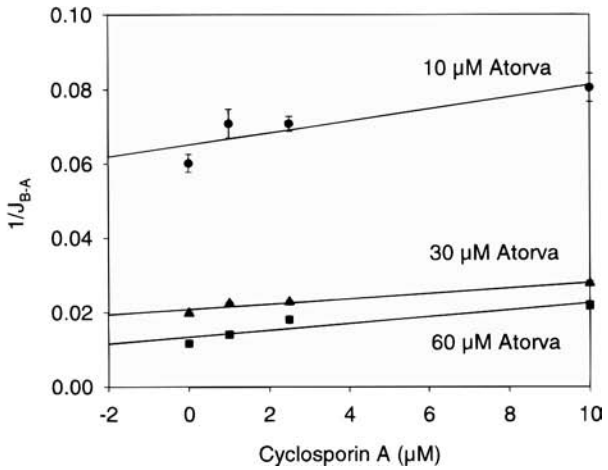


Fig. 4. Dixon-Webb plot of atorvastatin/CsA interaction in the Caco-2 model. The B-to-A flux of ^{14}C-atorvastatin was determined at pH 7.4 as a function of CsA concentration. K_i of Cyclosporine A was derived from the equation K_i = intercept/(slope + K_m/S), where K_m was the affinity constant of atorvastatin for P-gp in the Caco-2 model (115 μM from atorvastatin secretory kinetic study), S was atorvastatin concentration for each regression line. Each data point represents the mean ± S.E.M. of 4 determinations (Caco-2 monolayers).

60 μM, respectively. A Dixon-Webb plot of the data is presented in Figure 4. The parallel pattern of the regression lines suggested that CsA was an uncompetitive inhibitor of P-gp-mediated atorvastatin transport in the Caco-2 cell model (8). The K_i of CsA was calculated as 5.0 ± 0.9 μM (mean ± S.E.M.). This result further suggested that CsA and atorvastatin bind to different sites of P-gp.

Effect of Atorvastatin on the B-to-A Flux of Vinblastine Across Caco-2 Cell Monolayers

Vinblastine is a well-documented P-gp substrate with an apparent K_m of 19 μM in the Caco-2 cell model (9). To examine potential interactions of atorvastatin with vinblastine, the B-to-A permeability of ^{3}H-vinblastine was determined in the presence of atorvastatin (100 μM) under a pH gradient across the Caco-2 cell monolayer (pH 6.5 and 7.4 for A and B sides, respectively). Atorvastatin inhibited the B-to-A ^{3}H-vinblastine permeability by 61% (Table II). Compared to the well-documented P-gp inhibitors, CsA and verapamil, atorvastatin was less potent than CsA but slightly more potent than verapamil in inhibiting P-gp-mediated vinblastine efflux across Caco-2 cell monolayers (Table II). This result was consistent with a previous study which showed that atorvastatin inhibited the B-to-A permeability of ^{3}H-digoxin across Caco-2 cell monolayers by 58% (4).

Effect of Apical pH on the Uptake of Atorvastatin in Caco-2 Cells

The apical uptake of ^{14}C-atorvastatin (1.7 μM) was first determined as a function of time (data not shown). Atorvastatin uptake was linear up to 10 min. As a result, the initial uptake of atorvastatin was determined at time points less than 10 min in subsequent uptake experiments.

Since atorvastatin is an acid (Fig. 1), the effect of the apical pH on the uptake of ^{14}C-atorvastatin was examined. The initial uptake of atorvastatin increased 10.5-fold when the apical pH decreased from 7.4 to pH 5.5 with the pH in the B side fixed at pH 7.4 (Figure 5). Since atorvastatin has a pK_a of 4.46, more than 90% of atorvastatin was ionized in the pH range tested

Table I. Effect of P-glycoprotein Modulators on Atorvastatin Permeability[a]

P-gp modulator	$P_{app,A-B} \times 10^6$, cm/s	$P_{app,B-A} \times 10^6$, cm/s
Control	4.9 ± 0.3	35.6 ± 1.1
Cyclosporin A	7.9 ± 0.2[b]	20.3 ± 0.6[b]
Verapamil	7.9 ± 0.4[b]	26.6 ± 1.0[b]
UIC2	4.5 ± 0.2	30.9 ± 0.5[b]

[a] The apparent permeability of ^{14}C-atorvastatin (1.4 μM) was determined in the absence and presence of cyclosporin A (10 μM), verapamil (100 μM), and a P-gp specific monoclonal antibody, UIC2 (10 μg/ml), respectively. This experiment was performed at pH 7.4 in both the apical and basolateral sides. Data are presented as the mean ± S.E.M. of 3–4 determinations (Caco-2 monolayers).
[b] Significantly different from the respective control ($p < 0.01$).

Table II. Effect of Atorvastatin on Vinblastine Permeability[a]

Inhibitor	$P_{app, B-A} \times 10^6$, cm/s	% Inhibition
Control	32.2 ± 1.6	n.a.[c]
Atorvastatin	12.6 ± 2.0[b]	60.7 ± 6.1[b]
Cyclosporin A	4.5 ± 1.4[b]	89.6 ± 4.6[b]
Verapamil	17.2 ± 0.7[b]	46.7 ± 2.3[b]

[a] The B-to-A apparent permeability of ^{3}H-vinblastine (38 nM) was determined in the absence and presence of atorvastatin (100 μM), cyclosporin A (10 μM), and verapamil (100 μM), respectively. This experiment was performed under a pH gradient (pH_A/pH_B: 6.5/7.4) across Caco-2 cell monolayers. Data are presented as the mean ± S.E.M. of 3–4 determinations (Caco-2 monolayers).
[b] Significantly different from the respective control ($p < 0.01$)
[c] Not available.

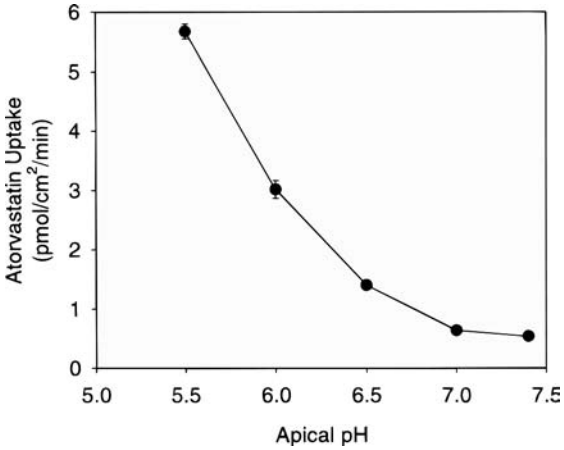


Fig. 5. pH-dependent uptake of atorvastatin in Caco-2 cells. The uptake of ^{14}C-atorvastatin (1.7 μM) was determined at 4 min. The pH in the basolateral side was fixed at pH 7.4. Cyclosporine A (20 μM) was included in the apical solutions to minimize P-gp-mediated atorvastatin efflux. Each data point represents the mean ± S.E.M. of 6 determinations (Caco-2 monolayers).

(91.6% and 99.9% ionized at pH 5.5 and pH 7.4, respectively). Therefore, a 10.5-fold increase in the initial atorvastatin uptake can not be simply explained by the pH partition theory and passive diffusion.

Effect of Proton Gradients on the Uptake of Atorvastatin in Caco-2 Cells

To clarify the nature of the pH-dependent atorvastatin uptake, the uptake of ^{14}C-atorvastatin was determined in the presence of a proton ionophore, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP). FCCP acts as a proton carrier to dissipate the proton gradient across the apical membrane, thereby decreasing the driving force for any proton-cotransport events. In this study, FCCP (50 μM) decreased atorvastatin uptake in the presence of a proton gradient, whereas in the absence of a proton gradient, FCCP did not affect atorvastatin uptake (Table III). These results indicated that atorvastatin uptake was mediated by a proton-cotransporter. Furthermore, the uptake of atorvastatin in the absence of a proton gradient suggested that atorvastatin was likely transported across the apical membrane by passive diffusion to some extent.

Table III. Effect of FCCP on Atorvastatin Uptake[a]

pH_A/pH_B	Ionophore	Uptake (pmol/cm²/min)
7.4/7.4	Control	1.20 ± 0.08
7.4/7.4	+ FCCP	1.11 ± 0.04
5.5/7.4	Control	7.75 ± 0.17
5.5/7.4	+ FCCP	5.51 ± 0.23[b]

[a] The apical uptake of ^{14}C-atorvastatin (1.7 μM) was determined in the presence and absence of FCCP (50 μM) at 37°C for 4 min. CsA (20 μM) was included in the apical solutions to minimize P-gp-mediated atorvastatin efflux. Data are presented as the mean ± S.E.M. of six determinations.
[b] Significantly different from the respective control ($p < 0.01$).

Effect of Monocarboxylic Acids on the Uptake of Atorvastatin in Caco-2 Cells

BA is a well-documented substrate of MCT with a K_m of 4.8 mM in the Caco-2 cell model (6). MCT activity was first confirmed in our Caco-2 cells in a study where the initial uptake of ^{14}C-BA was significantly inhibited by excess unlabeled BA, nicotinic acid (NA), and acetic acid (HOAC) each at 20 mM (100%, 45%, and 87% inhibition, respectively) (Data not shown).

To examine the involvement of MCT in transport of atorvastatin across the apical membrane of Caco-2 cells, uptake of ^{14}C-atorvastatin was determined in the presence of monocarboxylic acids. The initial atorvastatin uptake was inhibited by BA, NA, and HOAC each at 20 mM (65%, 14%, and 40%, respectively) (Table IV). These data suggested that atorvastatin was transported via MCT across the apical membrane of Caco-2 cells. Since 35% of atorvastatin uptake was not inhibited by BA (Table IV), other mechanism(s) such as passive diffusion was likely involved in atorvastatin transport in the absorptive direction.

The interaction of atorvastatin with BA was further examined. The uptake of ^{14}C-atorvastatin was determined as a function of BA concentration, while atorvastatin concentration was fixed at 2, 10, and 100 μM, respectively. A Dixon-Webb plot of the data (Figure 6) suggested that BA was a competitive inhibitor of atorvastatin uptake in the Caco-2 cell model because the three regression lines intersect (8). The K_i of BA was calculated as 14.5 ± 3.0 mM (mean ± S.E.M.) which was in the same range of the K_m of BA for MCT in the Caco-2 cell model (6).

Effect of Monocarboxylic Acids on the A-to-B Flux of Atorvastatin across Caco-2 Cell Monolayers

To examine the potential contribution of MCT in the transpeithelial flux of atorvastatin in the absorptive direction, the A-to-B permeability of ^{14}C-atorvastatin was determined in the presence of the monocarboxylic acids. The A-to-B permeability of atorvastatin decreased in the presence of BA, NA, and HOAC each at 20 mM (71%, 21%, and 66%, respectively) (Table IV). These data suggested that MCT contributed significantly in atorvastatin transport in the absorptive direction across Caco-2 cell monolayers.

Table IV. Effect of Monocarboxylic Acids on Atorvastatin Transport[a]

Inhibitor	$P_{app,A-B} \times 10^6$, cm/s	Uptake, pmol/cm²/min
Control	28.4 ± 0.5	3.25 ± 0.23
Benzoic acid	8.2 ± 0.6[b]	1.14 ± 0.10[b]
Nicotinic acid	22.4 ± 1.4[b]	2.80 ± 0.09[b]
Acetic acid	9.6 ± 0.7[b]	1.94 ± 0.17[b]

[a] The apparent permeability of ^{14}C-atorvastatin (1.3 μM) was determined in the A-to-B direction. This experiment was performed under a pH gradient (pH_A/pH_B: 6.0/7.4) across Caco-2 cell monolayers. The apical uptake of ^{14}C-atorvastatin (1.3 μM) was also determined at pH 6.0/7.4 (pH_A/pH_B) for 2 min. Each monocarboxylic acid was present in the apical side at 20 mM. CsA was added at 20 μM in apical solutions to minimize P-gp-mediated atorvastatin efflux in both permeability and uptake experiments. Data are presented as the mean ± S.E.M. of 4–6 determinations (Caco-2 monolayers).
[b] Significantly different from the respective control ($p < 0.01$).

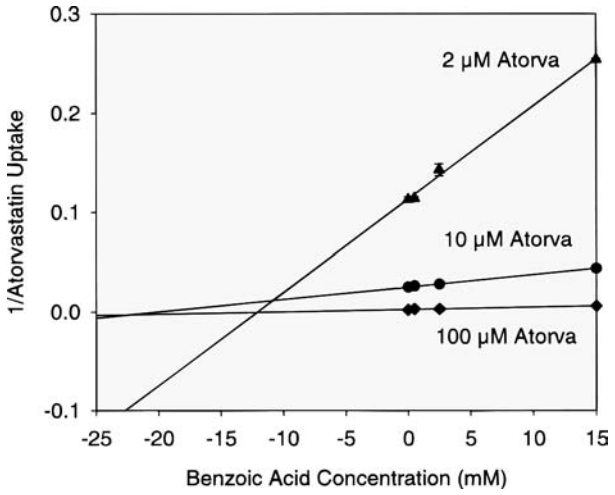


Fig. 6. Dixon-Webb plot of atorvastatin/benzoic acid interaction in the Caco-2 model. The uptake of ^{14}C-atorvastatin was determined as a function of benzoic acid concentration. This experiment was performed at pH 6.0 and pH 7.4 in the apical and basolateral sides, respectively. The K_i of benzoic acid was derived from the equation $K_i = (\text{intercept}_1 - \text{intercept}_2)/(\text{slope}_1 - \text{slope}_2)$. Each data point represents the mean ± S.E.M. of 4 determinations (Caco-2 monolayers).

DISCUSSION

Membrane transport proteins located at the intestinal epithelial cells may be important determinants of intestinal permeability and ultimately intestinal absorption of a large number of therapeutic agents as well as nutrients. In the present study, two transporter proteins, namely, P-glycoprotein (P-gp) and a proton-monocarboxylic acid cotransporter (MCT), were identified as carriers in mediating atorvastatin secretion and absorption, respectively, across the apical membrane of Caco-2 cells.

Polarized permeation of atorvastatin across Caco-2 cell monolayers was first observed in this study, suggesting involvement of a carrier-mediated transport system(s) for atorvastatin (Table I). The 7-fold enhancement in the B-to-A permeability of atorvastatin is a characteristic of efflux system(s) that functions to export atorvastatin from inside the cell back out to the lumen. A saturable B-to-A flux of atorvastatin further supported the presence of an efflux system(s) (Fig. 2). CsA and verapamil inhibited atorvastatin permeation in the B-to-A direction, but stimulated that in the A-to-B direction (Table II). A P-gp specific monoclonal antibody, UIC2, also inhibited atorvastatin permeation in the B-to-A direction but to a lesser extent (Table II). Because CsA and verapamil are well-documented P-gp inhibitors, and UIC2 recognizes an external epitope of human P-gp (10), the interactions of atorvastatin with CsA, verapamil, and UIC2 clearly demonstrated that P-gp was involved in mediating atorvastatin secretion across the apical membrane of Caco-2 cell monolayers. While P-gp has been demonstrated to transport a variety of structurally diversified organic cations and lipophilic compounds, this study provided the first evidence that an organic anion, such as atorvastatin, can be a substrate for P-gp.

The fact that UIC2 was less effective in inhibiting the B-to-A atorvastatin permeation than CsA and verapamil and the lack of stimulation of the A-to-B atorvastatin permeation by UIC2 is still unclear. A possible explanation is that the concentration of UIC2 used in this experiment may not be optimal. Lack of stimulation of the A-to-B vinblastine permeation by P-gp specific monoclonal antibody MRK16 was also observed in the Caco-2 cell model (9). Alternatively, other efflux systems that can not be recognized by UIC2 may also be involved in the secretion of atorvastatin across the Caco-2 cell monolayer. Recent studies suggested that the multidrug resistance-associated protein (MRP1) and one of its homologs, MRP2, were expressed in Caco-2 cells and might be responsible for the efflux of glutathion-methylfluorescein and genistin, respectively (11, 12). Further studies are needed to ascertain if atorvastatin is a substrate of the MRP's.

Atorvastatin is a monocarboxylic acid with a pKa of 4.46 (Fig. 1). The percentage of ionization of atorvastatin was greater than 90% in the pH range used in this study (pH 5.5 to pH 7.4). The unionized atorvastatin was likely to be transported by passive diffusion to some extent. The passively diffused pathway for atorvastatin was also suggested by the fact that atorvastatin was taken up into the cell in the absence of a proton gradient (Table III) and another fact that part of atorvastatin uptake was not inhibited by benzoic acid (Table IV). However, the dramatic effect of the apical pH (Fig. 5) and the influence of a proton-gradient (Table III) indicated that a proton-cotransport system was involved in atorvastatin transport across the apical membrane of Caco-2 cells. The effect of monocarboxylic acids (Table IV) and the competitive interaction of atorvastatin with benzoic acid (Figure 6) demonstrated that the MCT that transports benzoic acid, nicotinic acid, and acetic acid, mediated the transport of atorvastatin in the absorptive direction in the Caco-2 cells. Due to a solubility limit of atorvastatin, the K_m of atorvastatin for MCT was not determined in this study; however, it is reasonable to estimate the K_m of atorvastatin for MCT to be in the mM range. While it is clear from this study that an MCT at the apical membrane of Caco-2 cells is involved in uptake of atorvastatin from the apical side, the mechanisms by which atorvastatin is transported across the basolateral membrane from inside the cells is unknown.

An MCT system was identified in rat and rabbit intestines and in Caco-2 cells using Northern blot hybridization and designated as MCT1 (13). It is interesting to note that the transport activity for pyruvic acid was observed in MCT1-injected Xenopus laevis oocytes (14), whereas pyruvic acid (20 mM) did not affect either the apical uptake or the A-B permeability of atorvastatin in this study (data not shown). Similarly, pyruvic acid did not affect the apical uptake of BA in this study (data not shown). Further studies are needed to clarify if pyruvic acid and BA are transported by different MCT systems or if they interact at different sites of the same MCT system.

The clinical relevance of these data is unclear. Atorvastatin is used clinically as a lipid-lowering agent at 10–80 mg/day. Clinically relevant maximal concentrations in the intestinal lumen are estimated to be in the 70 to 550 μM range, assuming 250 ml water is taken with the dose. Given that the K_m values for P-gp and MCT are in the 115 μM and mM range, respectively, the P-gp-mediated atorvastatin secretion might be saturated when higher doses are given; whereas MCT-mediated transport appears to be a linear process even at the highest dose (80 mg/day). Extent of atorvastatin absorption (AUC) increased in proportion to dose over the 10 to 80 mg dose range (15),

consistent with linear MCT transport. Increases in rate of atorvastatin absorption, as reflected by Cmax, were modestly greater than proportional to dose. The nonlinearity may be related to saturation of P-gp transport, however other explanations were possible (15). If P-gp transport was involved in atorvastatin absorption, its contribution appeared to be minor. Consistent with results from a previous study (4), atorvastatin was a P-gp transport inhibitor in the Caco-2 cell system. Inhibition of digoxin transport by P-gp in vivo may explain, at least in part, the increased absorption of digoxin when atorvastatin and digoxin are coadministered compared to administration of digoxin alone (4).

In summary, this study demonstrated that atorvastatin was a P-gp and MCT substrate. The passive diffusion pathway probably was a minor contributor to atorvastatin absorption in the Caco-2 system. CsA was an uncompetitive inhibitor of P-gp-mediated atorvastatin efflux and benzoic acid competitively inhibited MCT-mediated atorvastatin uptake. In addition, atorvastatin inhibited P-gp-mediated vinblastine efflux. The P-gp-mediated secretion of atorvastatin provided a basis for a potential explanation for the digoxin-atorvastatin drug interaction.

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