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Inhibitory Effect of 5,7-Dimethoxyflavone On Rosuvastatin Uptake From The Apical Membrane Of Caco-2 Cells

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Abstract

5,7-Dimethoxyflavone (5,7-DMF) is a natural polymethoxyflavone, and acts as an inhibitor of ABC efflux transporters (BCRP, MRP2 and/or P-gp), and rosuvastatin is taken up via OATP2B1 and secreted by BCRP and MRP2. In this study, we investigated the effect of 5,7-DMF on the transport of rosuvastatin through the apical membrane and the accumulation in Caco-2 cells. Furthermore, we investigated whether the rosuvastatin accumulation is mediated by monocarboxylate transporter 1 (MCT1), in addition to OATP2B1. Coincubation with 5,7-DMF significantly increased the cellular accumulation of rosuvastatin from the apical membranes of Caco-2 cells cultured on the plastic dish. Coincubation with Ko-143 (a BCRP inhibitor) or MK-571 (an MRP inhibitor) significantly increased the rosuvastatin accumulation, whereas coincubation with verapamil (a P-gp inhibitor) did not. Coincubation with benzoic acid or pravastatin, which are substrates of both OATP2B1 and MCT1, significantly decreased the rosuvastatin accumulation, whereas coincubation with estron-3-sulfate or sulfobromophthalein, which are substrates of both OATP2B1 and ABC efflux transporter, did not decrease and increased the rosuvastatin accumulation, respectively. On the other hand, the transcellular transport of rosuvastatin from basolateral to apical side (B-to-A transport) was markedly higher than that from apical to basolateral side (A-to-B transport). Coincubation with 5,7-DMF from the apical side significantly increased the A-to-B transport and the accumulation of rosuvastatin, whereas that from the basolateral side significantly decreased the B-to-A transport of rosuvastatin with increases in the accumulation. These results suggest that 5,7-DMF may increase the rosuvastatin accumulation as a result of the inhibition of rosuvastatin efflux mediated by BCRP and MRP2, and the rosuvastatin transport through the apical membrane may be mediated by not only OATB2B1 but also MCT1.

Introduction

Most membrane transporters belong to two super-families, ABC (ATP-binding cassette) and SLC (solute-linked carrier). Three major efflux transporters of the ABC family are P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2). These efflux transporters, localized on the brush border membrane of the enterocytes, can limit drug absorption by pumping substrate into the luminal side against a concentration gradient, using ATP as an energy source [1,2].

Organic anion transporting polypeptides (OATPs) belong to the SLC super-family and mediate the cellular uptake of various endogenous and exogenous amphiphilic organic compounds in the brain, liver, lung, kidney, testes, intestine, etc. OATP1B1, OATP1B3, and OATP2B1 are OATPs expressed in the liver, and mediated the uptake of various compounds in the liver [3]. In contrast, OATP2B1 is the main OATP expressed in the human intestine and Caco-2 cells, and the amount of OATP2B1 expression is markedly higher than that of other OATP isomers such as OATP1A2, OATP3A1, OATP4A1, OATP1B1 and OATP1B3 [4-6]. The physiological function of OATP2B1 is distinctly Na^+-independent and pH-gradient dependent with a relatively narrow substrate specificity compared to other OATPs [7]. OATP2B1 could play an important role in oral absorption of various compounds such as sulfobromophthalein (BSP), statins, fexofenadine, glibenclamide, etc. in addition to the physiological sulfate-conjugated steroids and taurocholate [1-3,8].

There is some overlap in apparent substrate specificity between OATP2B1 and monocarboxylate transporter 1 (MCT1). Compounds that are reported to be substrates of both OATP2B1 and MCT1 include nicotinate, benzoic acid, salicylic acid, pravastatin, atorvastatin, etc., and MCT1-mediated transport is enhanced by an increase of H^+ concentration [1,7], as in the case of OATP2B1. Furthermore, OATPs shares with some substrates of ABC efflux transporters [9]; BSP and estron-3-sulfate (E3S), typical substrates of OATP2B1, are likely to act as the substrates of MRP2 and BCRP [10,11], respectively.

Lipid-lowering drugs of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), such as rosuvastatin, pravastatin, atorvastatin, pitavastatin, fluvastatin, simvastatin and lovastatin, are used in the treatment and prevention of atherosclerotic disease [12,13]. Most statins are absorbed from the intestinal lumen mainly by OATP2B1 [9,14-17], and secreted (pumped out) by one or some of BCRP, MRP2 and P-gp [12,18-22]. The statins, except for simvastatin and lovastatin, are monocarboxylic acids, and the absorption of pravastatin and atorvastatin are reported to be mediated by MCT1 [7], in addition to OATP2B1. However, the

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uptake of other statins having monocarboxylic acid by MCT1 has not yet been investigated. Some statins are used as inhibitors of ABC efflux transporter(s), because of their high affinity for ABC efflux transporter(s) [8,11,23].

A methylated flavone, 5,7-dimethoxyflavone (5,7-DMF) is an inhibiter of BCRP, MRP2, and/or P-gp [24-27]. As 5,7-DMF has high oral absorption and bioavailability as compared to non-methylated flavones, with low toxicity and little metabolism [28,29]. 5,7-DMF is promising for use as a chemosensitizing agent for the BCRP- and MRP2-mediated anticancer drug resistance [25,26,30-32]. For instance, 5,7-DMF significantly increased the mitoxantrone accumulation (a BCRP substrate) in BCRP-expressing MDCK cells [32], and 5,7-DMF increased doxorubicin accumulation in A549 cells by the inhibition of MRP-mediated efflux [25]. 5,7-DMF is a major constituent flavonoid of *Kaempferia parviflora*, and the dose of *K parviflora* tincture ingested in herbal medicine (30 mL) is likely to increase the intestinal absorption of drugs which is mediated by MRP2 [25]. However, the mechanism of intestinal absorption of 5,7-DMF has not yet been investigated, and only a few reports focused on the intestinal interaction of 5.7-DMF and drug which is mediated by MRP2, BCRP, and/or P-gp have been reported.

The human colorectal adenocarcinonoma cell line Caco-2 is morphologically and functionally similar to human small intestinal epithelial cells and is widely used to predict intestinal "in vivo" absorption in humans [33]. These cells spontaneously differentiate in culture into polarized cell monolayers with many enterocyte-like properties of transporting epithelia and also remain ABC and SLC transporters. Caco-2 cells are an appropriate model to investigate drug absorption as well as drug-drug and food-drug interactions caused by uptake and efflux transporters [34].

As mentioned above, rosuvastatin and 5,7-DMF are reported to be substrates (inhibitors) of some ABC efflux transporters, BCRP, MRP2, and/or P-gp, and rosuvastatin is reported to be a substrate of uptake transporter of OATP2B1. The aim of this study was to investigate the effect of 5,7-DMF on the intestinal absorption of rosuvastatin using Caco-2 cells. Furthermore, we investigated the uptake and efflux of rosuvastatin using typical substrates (inhibitors) of membrane transporters, in particular, whether rosuvastatin is taken up by MCT1 or not.

Materials & Methods

Materials

Rosuvastatin calcium and pravastatin sodium were obtained from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 5,7-Dimethoxyflavone (5,7-DMF), verapamil hydrochloride, benzoic acid, cyclosporin A (ciclosporin A) and Dulbecco's modified Eagle's medium (DMEM) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MK-571, zosuquidar hydrochloride, sulfobromophthalein (BSP), estron-3-sulfate (E3S), and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). Ko-143 was purchased from Tocris Bioscience (Minneapolis, MN). All other chemicals used were of the highest purity commercially available. Rosuvastatin and some compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the incubation medium at a final concentration of DMSO of 1% or lower.

Cell culture

As reported previously [35,36], Caco-2 cells between passages 55 and 75 were maintained in a culture medium that is DMEM containing 10% fetal bovine serum (FBS), 1% nonessential amino acid, streptomycin (100 μg/mL) and penicillin G (100 U/mL) at 37°C in a humidified atmosphere of 5% CO_2 in the air.

Cellular accumulation of rosuvastatin

Caco-2 cells were grown on 35-mm six-well culture dishes coated with rat tail collagen type I (Corning Incorporated, Tewksbury, MA) at a density of 5×10^4 cells/well in 1.5 mL of culture medium [35,36]. After seeding, confluent Caco-2 cell monolayers cultured for 14-16 days were used in the cellular accumulation study.

The incubation medium used in this study was Hanks' balanced salt solution containing 10 mM MES at pH 6.0 or 10 mM HEPES at pH 7.4. The culture medium was removed, and the cells were preincubated at 37°C for 20 min in 1.5 mL of the incubation medium at pH 7.4. After preincubation, the incubation medium was aspirated, and the cells were incubated in the medium at pH 6.0 or 7.4 containing rosuvastatin for the designated time at 37°C.

To investigate the mechanisms underlying the cellular accumulation of rosuvastatin, the cells were coincubated with 10 μM rosuvastatin and several transporter inhibitors (substrates). To investigate ATP-dependent rosuvastatin efflux, the cellular ATP was depleted by the pretreatment with 10 mM NaN_3 and 10 mM NaF at 37°C for 20 min before the incubation with 10 μM rosuvastatin [37]. Furthermore, to investigate the effects of low temperature on the cellular accumulation of rosuvastatin, the cells were preincubated at 4°C and pH 7.4 for 20 min, and then incubated with 10 μM rosuvastatin at 4°C and pH 6.0 for 30 min.

After incubation with rosuvastatin, the cell surface was quickly washed three times with an ice-cold incubation medium. The cells were suspended in 1.0 mL of extraction solution (1 N H_3PO_4: methanol = 1:1) for 60 min at room temperature, and the cells were scraped off and collected using a cell scraper [35,36]. The cell suspension was centrifuged at 13,000*g* for 10 min, and a 100-μL aliquot of the supernatant was injected into the HPLC system.

Efflux of rosuvastatin

The efflux experiment was performed as described previously [35]. Caco-2 cells were incubated 10 μM rosuvastatin at pH 6.0 and 37°C for 30 min. After incubation, the cell surface was quickly washed three times with an ice-cold incubation medium. The washed cells were then incubated at 37°C and pH 6.0 for the designated time in the incubation medium containing 100 μM 5,7-DMF or an inhibitor of ABC efflux transporter (10 M Ko-143, 50 μM MK-571 or 100 μM verapamil), or the washed cells were incubated in the inhibitor-free medium at 4°C and pH 6.0. After the designated time, the rosuvastatin remaining in the cells was measured as described above.

Transcellular transport of rosuvastatin

Caco-2 cells were seeded on 6-well polyethylene terephthalate Falcon® cell culture inserts (0.4 μm pores, 4.2-cm^2 growth area; Corning Incorporated, Tewksbury, MA) at a density of 5×10^4 cells/well, and then cultured for 21-23 days in DMEM containing 10% FBS, streptomycin (100 μg/mL) and penicillin G (100 U/mL)

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[35]. The monolayers grown on confluency, with a transepithelial electrical resistance of more than 350 Ω cm^2, were used for the transport experiment. The cells were preincubated at 37°C for 20 min in 1.5 and 2.5 mL of the incubation medium at pH 7.4 from the apical and basolateral sides, respectively. After preincubation, the cell monolayers were incubated with 10 μM rosuvastatin or 10 μM rosuvastatin and 100 μM 5,7-DMF for the designated time at 37°C either from the apical side of the monolayers at pH 6.0 (1.5 mL) or from the basolateral side at pH 7.4 (2.5 mL). After the incubation with rosuvastatin, the transcellular transport of rosuvastatin was measured, and the cellular accumulation of rosuvastatin was also measured after the suspension of cells in the extraction solution.

To investigate the effects of several compounds on the transcellular transport and the cellular accumulation of rosuvastatin from the apical side, Caco-2 cells were coincubated with 10 μM rosuvastatin and several transporter inhibitors (10, 100, and 200 μM 5,7-DMF, 100 μM verapamil, 10 μM Ko-143 and 50 μM MK-571).

Apparent permeability coefficient from apical to basolateral side ($P_{app\ A-B}$) and from basolateral to apical side ($P_{app\ B-A}$) were calculated using volume of the incubation medium (1.5 or 2.5 mL), filter surface area (4.2 cm^2), and initial concentration in rosuvastatin (10 μM) [19].

Determination of rosuvastatin and protein concentrations

Determination of rosuvastatin was carried out using an HPLC system consisting of a Shimadzu LC-20Avp pump and SPD-10A UV detector (Kyoto, Japan) equipped with an Inertsil ODS-SP 5 μM column (4.6 mm i.d. × 250 mm; GL Sciences, Tokyo, Japan). The mobile phase consisted of acetonitrile–0.1% phosphoric acid (40 : 60, v/v), and the flow rate was 1.0 mL min^{-1}. The wavelength was 242 nm, and the column was kept at 40°C. The calibration curve of rosuvastatin was linear over the range of 0.01-0.20 nmol/mL (r = 0.99). The protein concentration was determined using a Bio-rad dye reagent (Richmond, CA) with bovine serum albumin as the standard.

Statistical analyses

Data were shown as the mean ± S.E. Data were analyzed by Student's *t*-test, and p value below 0.05 was regarded as statistically significant.

Results

Effects of extracellular pH and 5,7-DMF on the cellular accumulation of rosuvastatin

Caco-2 cells were incubated with 10 μM rosuvastatin at pH 6.0 or 7.4 for up to 120 min in the presence or absence of 100 μM 5,7-DMF (Figure 1).

The cellular accumulation of rosuvastatin after the incubation with 10 μM rosuvastatin at pH 6.0 or 7.4 increased with time, however, the accumulation at pH 6.0 was several-hold higher than that at pH 7.4.

Coincubation with 5,7-DMF significantly increased the rosuvastatin accumulation at pH 6.0 and 7.4, but this increase at pH 6.0 owing to 5.7-DMF was greater than that at pH 7.4: At 30, 60, and 120 min, the accumulation at pH 6.0 in the presence of 5.7-DMF was 3-5-hold higher than that in the absence of 5,7-DMF, whereas the accumulation at pH 7.4 in the presence of 5,7-DMF was only 2-hold higher than that in the absence of 5,7-DMF. Notably, the rosuvastatin accumulation at pH 6.0 in the presence of 5,7-DMF increased greatly and almost linearly up to 30 min.

Figure 2 shows the concentration-dependent effect of 5,7-DMF on the rosuvastatin accumulation. The rosuvastatin accumulation at 5 min and pH 6.0 was apparently increased with the increasing 5,7-DMF concentration (5, 25, 50, 100, and 200 μM). In contrast, the increasing concentration of 5,7-DMF did not increase the rosuvastatin accumulation at pH 7.4.

Effects of 5,7-DMF on the rosuvastatin efflux from Caco-2 cells

Figure 3 shows the effects of 5,7-DMF and ABC efflux inhibitors on the rosuvastatin accumulation in Caco-2 cells. Coincubation with 100 μM 5,7-DMF significantly increased the rosuvastatin accumulation by 2.0-fold. Similarly, coincubation with 10 μM Ko-143 (a BCRP inhibitor) or 50 μM MK-571 (an MRP2 inhibitor) significantly increased the rosuvastatin accumulation by 1.5-hold and 2.1-hold, respectively. In contrast, coincubation with P-gp inhibitor, 1 μM zosuquidar or 100 μM verapamil, did not affect the rosuvastatin accumulation, although these concentrations of P-gp inhibitors are reported to inhibit the P-gp mediated efflux [38,39].

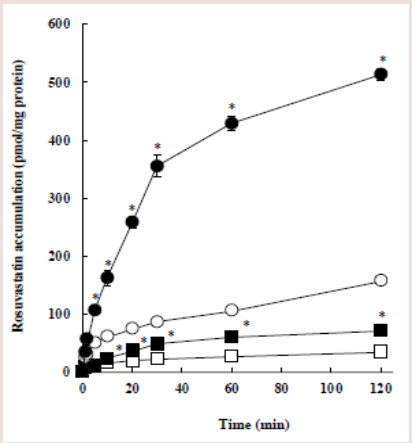

Figure 1: Effects of pH and 5,7-DMF on the cellular accumulation of rosuvastatin in Caco-2 cells. Caco-2 cells were incubated with 10 μM rosuvastatin for the indicated periods in the presence (closed symbols) or absence (open symbols) of 100 μM 5,7-DMF at pH 6.0 (circles) or pH 7.4 (squares). Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control (without 5,7-DMF).

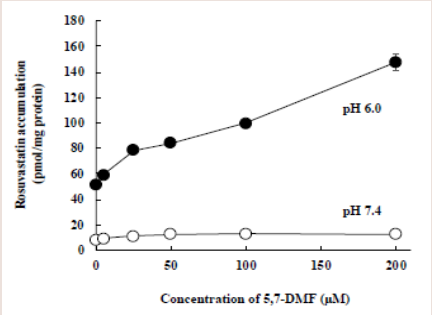

Figure 2: Effect of 5,7-DMF on the cellular accumulation of rosuvastatin in Caco-2 cells. Caco-2 cells were coincubated at pH 6.0 or 7.4 for 5 min with 10 μM rosuvastatin and different concentrations of 5,7-DMF. Each value represents the mean ± S.E. of 4-6 determinations.

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We confirmed whether the increase in rosuvastatin accumulation in the presence of 5,7-DMF was owing to the inhibition of rosuvastatin efflux. Caco-2 cells, preincubated with 10 μM rosuvastatin at 37˚C for 30 min, were further incubated with 5,7-DMF, MK-571, Ko-143, or verapamil, and investigated the change of rosuvastatin efflux (Figure 4).

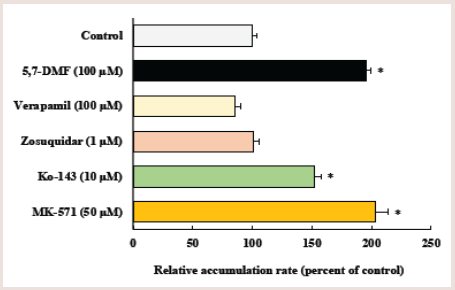

Figure 3: Effects of 5,7-DMF and inhibitors of ABC efflux transporters on the cellular accumulation of rosuvastatin in Caco-2 cells. Caco-2 cells were coincubated with 10 μM rosuvastatin and various compounds at pH 6.0 for 5 min. Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control.

The amount of the rosuvastatin accumulation decreased rapidly with time (control), showing a 93% decrease in the rosuvastatin accumulation at 30 min. 5,7-DMF (100 μM) significantly inhibited the rosuvastatin efflux at 5, 10, 20 and 30 min. At 30 min, 100 μM 5,7-DMF, 10 μM Ko-143 and 50 μM MK-571 significantly inhibited the rosuvastatin efflux by 83, 86 and 65%, respectively. In contrast, 100 μM verapamil did not inhibit the rosuvastatin efflux at 30 min (93%), whereas low temperature (4˚C) markedly decreased the efflux (38%) (Figure 4).

Comparison of rosuvastatin accumulation by various inhibitors

We investigated typical inhibitors of transporters involved in the rosuvastatin accumulation (Figure 5), some of which are known to act as the ABC transporter inhibitors as mentioned in the Introduction.

Coincubation with 100 μM cyclosporin A or 100 μM E3S did not affect the rosuvastatin accumulation. In contrast, coincubation

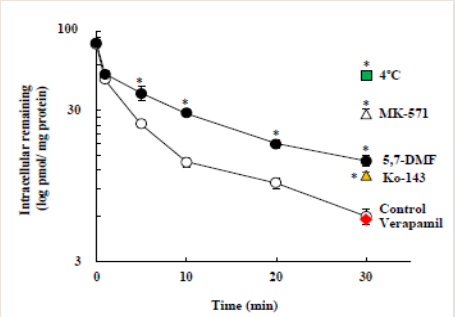

Figure 4: Effects of 5,7-DMF and inhibitors of ABC efflux transporters on the efflux of rosuvastatin from Caco-2 cells. Caco-2 cells, incubated with 10 μM rosuvastatin at 37ºC for 30min, were further incubated at 37ºC for designed time with or without 100 μM 5,7-DMF at pH 6.0. Furthermore, Caco-2 cells, incubated with rosuvastatin, were further incubated at 37ºC for 30 min with MK-571, Ko-143 or verapamil, or further incubated at 4ºC for 30 min with no chemicals. Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control.

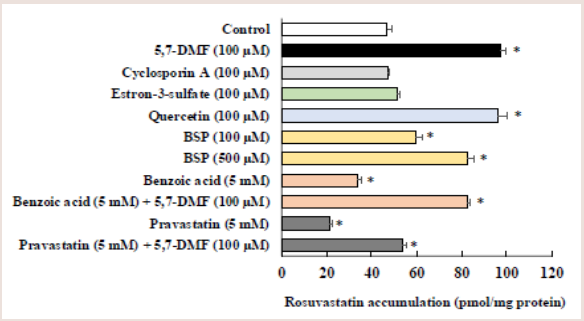

Figure 5: Effects of various compounds on the cellular accumulation of rosuvastatin in Caco-2 cells. Caco-2 cells were incubated with 10 μm rosuvastatin with or without of various compounds at pH 6.0 for 5 min. Each value represents the mean ± S.E. of 3-6 determinations. *Significantly different from the control.

with 100 μM quercetin or 500 μM BSP significantly increased the rosuvastatin accumulation by 2.0-fold and 1.8-fold, respectively, as in the case of 5.7-DMF (2.0-fold), whereas coincubation with a low concentration of 100 μM BSP marginally increased the rosuvastatin accumulation by 1.3-fold. In contrast, coincubation with 5 mM pravastatin or 5 mM benzoic acid significantly decreased the rosuvastatin accumulation by 55 and 29%, respectively, and the decreased accumulation by pravastatin and benzoic acid was greatly enhanced in the presence of 100 μM 5,7-DMF which significantly exceeded the control level.

Effects of low temperature and ATP-depletion on rosuvastatin accumulation

To confirm the involvement of uptake transporter(s) and ATP (energy)-dependent efflux transporters in the rosuvastatin accumulation, Caco-2 cells were incubated with rosuvastatin at 4˚C, or ATP-depleted Caco-2 cells were incubated with rosuvastatin at 37˚C (Figure 6).

The rosuvastatin accumulation was drastically decreased by the incubation at low temperature (4˚C) by 87%. In contrast, the rosuvastatin accumulation was greatly increased by ATP depletion (2.4-fold).

Effects of 5,7-DMF on the transcellular transport and accumulation of rosuvastatin after either apical or basolateral side of incubation

Effects of 5,7-DMF and other efflux transporter inhibitors on the

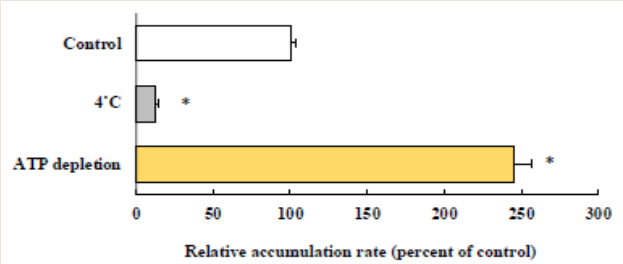

Figure 6: Effects of low temperature and ATP depletion on the cellular accumulation of rosuvastatin in Caco-2 cells. Caco-2 cells were incubated with 10 μM rosuvastatin at 37˚C or 4˚C, or ATP-depleted Caco-2 cells were incubated with 10 μM rosuvastatin at 37˚C. Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control.

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transcellular transport and the accumulation of rosuvastatin were investigated using Caco-2 cell monolayers cultured on permeable membranes. Caco-2 cell monolayers were incubated with 10 μM rosuvastatin at 37°C for the designated time either from the apical side at pH 6.0 or the basolateral side at pH 7.4.

The rosuvastatin transport from the apical to the basolateral side (A-to-B) and from the basolateral to apical side (B-to-A) increased linearly with time (Figure 7a). However, the B-to-A transport at each time was about 20-fold greater than the A-to-B transport: The $P_{app\ A\text{-}B}$ and $P_{app\ B\text{-}A}$ were 0.24 (± 0.02) × 10^{-6} cm/s and 5.13 (± 0.94) × 10^{-6} cm/s, respectively.

Coincubation with 5,7-DMF significantly increased the A-to-B transport of rosuvastatin, whereas it significantly decreased the B-to-A transport: The $P_{app\ A\text{-}B}$ and $P_{app\ B\text{-}A}$ of rosuvastatin in the presence of 5.7-DMF were 2.37 (± 0.25) × 10^{-6} cm/s and 2.48 (± 0.18) × 10^{-6} cm/s, respectively. Thus, a great difference between $P_{app\ B\text{-}A}$ and $P_{app\ A\text{-}B}$ in the absence of 5,7-DMF was diminished by the presence of 5,7-DMF.

On the other hand, the rosuvastatin accumulation at pH 6.0 and 7.4 in the presence of 5,7-DMF was significantly higher than that in the absence of 5,7-DMF, and the difference in the accumulation due to the difference in pH was not observed (Figure 7b). The rosuvastatin accumulation at pH 6.0 and 7.4 after 60 min attained plateau levels, irrespective of 5,7-DMF.

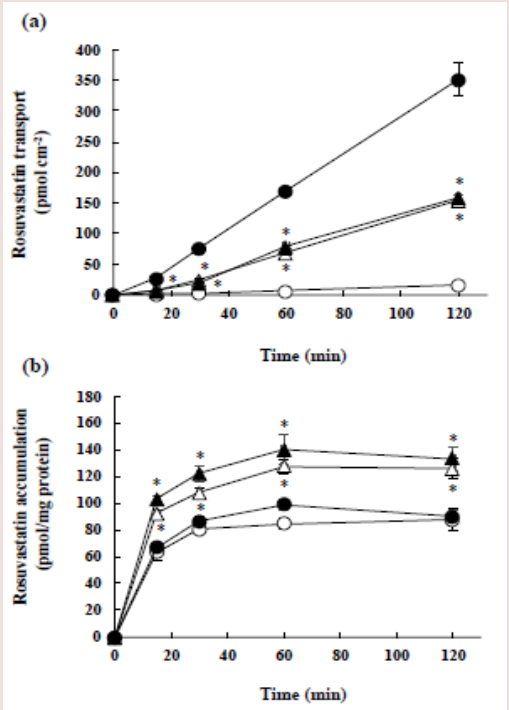

Figure 7: Effect of 5,7-DMF on the transcellular transport (a) and cellular accumulation (b) of rosuvastatin in Caco-2 cell monolayers. Caco-2 cell monolayers were incubated with 10 μM rosuvastatin with (triangles) or without (circles) 100 μM 5,7-DMF from either the apical side at pH 6.0 (open symbols) or basolateral side at pH 7.4 (closed symbols). Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control (without 5,7-DMF).

Table 1 shows the rosuvastatin accumulation in tissue (A), the transcellular transport (T), and the ratio (T/A) in the experiment shown in Figures 7a and b. The ratios (T/A) incubated from the apical side in the absence of 5,7-DMF increased gradually with time (0.04-0.32), whereas the ratios in the reverse direction increased sharply (1.19-10.9), in agreement with the preferential transport of rosuvastatin from the basolateral side (Figure 7a). On the other hand, the ratios from apical side exposure were increased by 5,7-DMF (0.15–2.07), while the ratios from basolateral side exposure were decreased (0.19-3.34). In the presence of 5,7-DMF, no particular differences were found in the ratios between the exposure of rosuvastatin from the apical and basolateral sides.

Effects of efflux transporter inhibitors on transcellular transport and accumulation of rosuvastatin after apical of incubation

The effects of 5,7-DMF, Ko-143, MK-571 and verapamil on the A-to-B transport and the accumulation of rosuvastatin at 60 min were compared (Figure 8). The inhibitors and their concentrations used in this experiment were the same as in the previous experiment (Figure 3). The pH of the apical and basolateral medium was 6.0 and 7.4, respectively.

Coincubation with different concentrations of 5,7-DMF (10, 100 and 200 μM) significantly increased the rosuvastatin transport by 2.9-, 9.1- and 13-fold, respectively, and significantly increased the rosuvastatin accumulation by 1.4-, 1.5- and 2.0-fold, respectively. Coincubation with 10 μM Ko-143 or 50 μM MK-571 significantly increased the rosuvastatin transport by 3.2- and 7.9-fold, respectively, and significantly increased the rosuvastatin accumulation by 1.5- and 1.7-fold, respectively. In contrast, coincubation with 100 μM verapamil did not affect the transport and the accumulation of rosuvastatin.

Discussion

5,7-DMF significantly increased the rosuvastatin accumulation at pH 6.0 (Figure 1), and the A-to-B transport and the accumulation of rosuvastatin from the apical side (Figure 7a and b). Furthermore, 5,7-DMF increased the accumulation and the transport of rosuvastatin at pH 6.0 in a concentration-dependent manner (Figures 2 and 8). As in the case of 5,7-DMF, Ko-143 (a BCRP inhibitor) and MK-571 (an MRP2 inhibitor) significantly increased the transport and

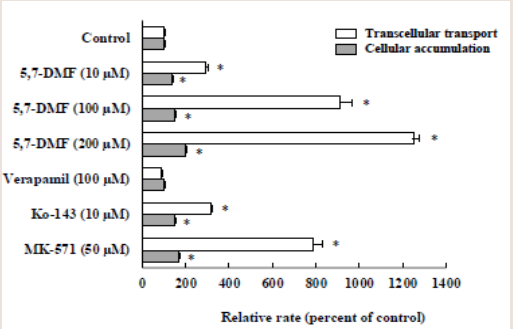

Figure 8: Effects of several compounds on the apical-to-basolateral transport and cellular accumulation of rosuvastatin in Caco-2 cell monolayers. Caco-2 cell monolayers were coincubated for 60 min at pH 6.0 with 10 μM rosuvastatin and various compounds from apical side. Each value represents the mean ± S.E. of 4-6 determinations. *Significantly different from the control.

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Table 1: Ratio of transcellular transport to accumulation of rosuvastatin in Caco-2 cells incubated with rosuvastatin in the presence or absence of 5,7-DMF from the apical compartment or basolateral compartment.

			15 min	30 min	60 min	120 min
Apical Exposure	Control	Accumulation	63.2 ± 5.3	80.7 ± 1.8	84.8 ± 4.3	88.0 ± 8.0
		Transport (A to B)	2.57 ± 0.31	6.32 ± 0.79	11.5 ± 1.9	27.8 ± 0.8
		Ratio (T/A)	0.04 ± 0.01	0.08 ± 0.02	0.14 ± 0.05	0.32 ± 0.02
	with 100 μM 5,7-DMF	Accumulation	92.7 ± 2.2	108 ± 4	128 ± 5	120 ± 8
		Transport (A to B)	13.5 ± 1.6	41.3 ± 4.8	115 ± 4	260 ± 13
		Ratio (T/A)	0.15 ± 0.04	0.38 ± 0.11	0.91 ± 0.09	2.07 ± 0.24
Basolateral Exposure	Control	Accumulation	66.8 ± 2.6	86.3 ± 4.3	98.9 ± 3.5	90.2 ± 4.9
		Transport (B to A)	79.2 ± 3.9	217 ± 2	476 ± 18	987 ± 78
		Ratio (T/A)	1.19 ± 0.12	2.51 ± 0.04	4.81 ± 0.40	10.9 ± 1.74
	with 100 μM 5,7-DMF	Accumulation	103 ± 3	122 ± 6	140 ± 12	133 ± 10
		Transport (B to A)	20.1 ± 1.5	56.2 ± 4.5	222 ± 6	446 ± 15
		Ratio (T/A)	0.19 ± 0.03	0.46 ± 0.07	1.58 ± 0.10	3.34 ± 0.21

See Figure 7. Ratio (T/A) is the ratio of Transport to Accumulation. Data were expressed by pmol/mg or pmol/mL.

the accumulation of rosuvastatin at pH 6.0, whereas verapamil and zosuquidar (P-gp inhibitors) did not (Figures 3 and 8). Thus, 5,7-DMF appears to increase the rosuvastatin accumulation in Caco-2 cells at pH 6.0 as a result of the inhibition of rosuvastatin efflux mediated by BCRP and MRP2, but not by P-gp. The efflux study strengthens the inhibitory effect of 5,7-DMF on the rosuvastatin efflux mediated by BCRP and MRP2 (Figure 4).

The rosuvastatin accumulation at pH 6.0 in the presence of 5,7-DMF increased greatly and almost linearly up to 30 min (Figure 1). A similar increase was reported in talinolol accumulation in cultured cells which was mediated by Oatp and P-gp [40]. The great and linear increase of the rosuvastatin accumulation at pH 6.0 is likely to be explained by the balance of uptake and efflux of rosuvastatin (Figure 1). In contrast, the rosuvastatin accumulation at pH 7.4 in the presence of 5,7-DMF was small (Figure 1). Interestingly, the increasing concentration of 5,7-DMF did not increase the initial accumulation of rosuvastatin at 5 min and pH 7.4 (Figure 2). A possible explanation for this phenomenon is that 5.7-DMF may be a substrate of pH-sensitive transporter as in the case of rosuvastatin, and the increase in 5,7-DMF concentration may strongly inhibit the uptake of rosuvastatin at pH 7.4, because some substrates of ABC efflux transporter are known to act as OATP2B1 substrates [9-11]. Involvement of the transporter in the uptake of 5,7-DMF in Caco-2 cells may be supported by the following that $P_{app\ A\text{-}B}$ of 5,7-DMF in Caco-2 cells (23.3×10^{-6} cm/s, [41]) is higher than that of rosuvastatin (0.56×10^{-6} cm/s, [34]), in addition to the rapid absorption of 5,7-DMF after oral administration [27]. Further study on the uptake mechanism of 5,7-DMF in Caco-2 cells is necessary to explain the little-increasing effect of 5,7-DMF on the rosuvastatin accumulation at pH 7.4 (Figures 1 and 2). As in the case of P-gp [42], the increase in pH is not likely to affect the rosuvastatin efflux mediated by BCRP and MRP2 which use ATP as an energy source.

Varma et al. reported a higher accumulation of rosuvastatin in Caco-2 cells at acidic pH [14]. They also reported that even in the presence of rifamycin SV, an OATP2B1 inhibitor, the rosuvastatin accumulation at acidic pH was higher in Caco-2 cells. This result implies that a pH-sensitive transporter other than OATP2B1 could be involved in rosuvastatin accumulation. Most likely is the involvement of pH-sensitive transporter MCT1, as benzoic acid and pravastatin decreased the rosuvastatin accumulation (Figure 5); benzoic acid and pravastatin are not only OATP2B1 substrates but also MCT1 substrates [7], rosuvastatin is the monocarboxylic acid like pravastatin and atorvastatin, and the uptake of pravastatin and atorvastatin is rereported to be mediated by MCT1 [7]. The involvement of pH-sensitive accumulation of rosuvastatin by simple diffusion is likely to be small as the pKa of rosuvastatin is 4.3 [20,43].

Figure 9 illustrates the membrane transporters related to the transport of statins through brush border and basolateral membranes: The inhibition of BCRP, MRP2 and/or P-gp by 5,7-DMF could increase the A-to-B transport and the accumulation of statins, whereas it could decrease the B-to-A transport with increases in the accumulation [20,23]. The ratio (T/A) of basolateral exposure at 120 min in the absence of 5,7-DMF was very high (10.9) which suggests the strong pumping of rosuvastatin mediated by BCRP and MRP2 using ATP energy (Table 1). The $P_{app\ A\text{-}B}$ value of rosuvastatin in the present study (0.24×10^{-6} cm/s) is similar to that reported previously by Hua et al. [21] (0.261×10^{-6} cm/s), Li et al. [20] (0.25×10^{-6} cm/s) and Fredlund et al. [34] (0.56×10^{-6} cm/s). On the other hand, the $P_{app\ B\text{-}A}$ values reported by Hua et al. [21] (0.594×10^{-6} cm/s), Li et al. [20] (20.66×10^{-6} cm/s), the present study (5.13×10^{-6} cm/s) were all higher than the $P_{apa\ A\text{-}B}$ values although these $P_{app\ B\text{-}A}$ values vary widely.

Pravastatin (5 mM) resulted in a large decrease in the rosuvastatin accumulation by 55% (Figure 5). Pravastatin appears to inhibit the rosuvastatin uptake by OATP2B1 and MCT1, as pravastatin is the substrate of both OATP2B1 and MCT1 [7]. Compared to rosuvastatin, on the other hand, pravastatin may be sparingly secreted by the ABC

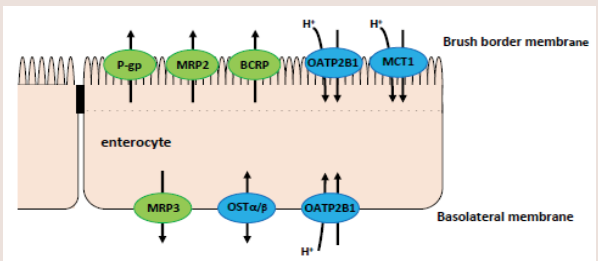

Figure 9: Membrane transporters at brush border and basolateral membranes of intestines involved statins absorption. P-gp: P-glycoprotein, MRP2 and MRP3: multidrug resistance associated protein 2 and 3, BCRP: breast cancer resistance protein, OATP2B1: organic anion transporting polypeptide 2B1, MCT1: monocarboxylate transporter 1, OSTα/β: organic solute transporter α/β.

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efflux transporter(s), as the estimated $P_{app\ B-A}$ value of pravastatin in Caco-2 cells (difference between intrinsic-$P_{app\ A-B}$ and $P_{app\ A-B}$ values) is markedly lower than that of rosuvastatin, whereas the $P_{app\ A-B}$ values of pravastatin and rosuvastatin are similar [34]. Thus, pravastatin may strongly inhibit OATP2B1- and MCT1-mediated uptake of rosuvastatin with a little inhibition of BCRP- and MRP2-mediated efflux of rosuvastatin, eventually, resulting in a marked decrease of the rosuvastatin accumulation in Caco-2 cells by 55% (Figure 5). A great decrease in the rosuvastatin accumulation was found in the incubation at 4°C (about 87%, Figure 6); the incubation at low temperature appears to decrease the uptake of rosuvastatin not only by carrier-mediated process of OATP2B1 and MCT1 but also by a simple diffusion process.

Noteworthy is that the decreased rosuvastatin accumulation by pravastatin and benzoic acid was greatly increased by 5,7-DMF, which exceeded the control level of rosuvastatin accumulation in the absence of these compounds (Figure 5). These phenomena suggest that pravastatin and benzoic acid could act as the inhibitors of OATP2B1 and MCT1, and 5,7-DMF may act as the inhibitor of BCRP and MRP2.

The marked increase of rosuvastatin accumulation owing to ATP-depletion (2.4-fold) is similar to that owing to 5,7-DMF (2.0-fold) (Figure 6), quercetin (2.0-fold) and BSP at 500 μM (1.8-fold) (Figure 5). Marked increases of the rosuvastatin accumulation could be occurred by the depletion of cellular ATP content and the inhibition of ATP-dependent efflux mediated by BCRP and MRP2.

Cyclosporin A is a non-specific inhibitor of OATPs and ABC efflux transporters [44]. However, 100 μM cyclosporin A did not affect the rosuvastatin accumulation in Caco-2 cells (Figure 5), probably by the inhibitory balance between the uptake by OATP and the efflux by BCRP and MRP2. Verma et al. [14] reported the concentration-dependent decreasing effect of cyclosporin A (up to 100 μM) on the rosuvastatin accumulation in OTAP2B1-transfected HEK293 cells. However, the expression of BCRP and MRP2 in these HEK293 cells is unlikely. Contrary to *in vitro* studies, clinically used cyclosporin A increases the rosuvastatin concentration in blood, probably as a result of a decrease in hepatic accumulation of rosuvastatin by the inhibition of OATP1B1 [12,13,43]. However, the exact inhibitory effect of cyclosporin A on transporters is not clear, as cyclosporin A is a potent inhibitor of several transporters including OATP1B1, OATP2B1, OATP1B3, MRP2, BCRP, P-gp, etc. expressed in many tissues.

Quercetin is known to inhibit many transporters such as OATPs and MCT1 as well as the efflux transporters of P-gp, BCRP, and MRP2 [45-47]. In this study, quercetin increased the rosuvastatin accumulation 2.0-fold which is a similar extent of 5,7-DMF (2.0-hold), 500 μM BSP (1.8-fold) and ATP depletion (2.4-fold) (Figures 5 and 6). In this study, quercetin is likely to increase the rosuvastatin accumulation in Caco-2 cells as a result of the inhibition of P-gp and BCRP rather than OATP2B1 and MCT1.

BSP and E3S are well-known substrates of OATP2B1 [1,3,7], but they are reported to act as MRP2 inhibitor and BCRP inhibitor [10,11], respectively. BSP at 100 and 500 μM concentrations in our experimental conditions may act as the inhibitor of MRP2 rather than OATP2B1 [48], and eventually increased the rosuvastatin accumulation (Figure 5). On the other hand, E3S at 100 μM concentration may inhibit both OATP2B1-mediated uptake and BCRP-mediated efflux, resulting in little change of the rosuvastatin accumulation in Caco-2 cells. Ueno et al. (2012) reported that 500 μM BSP and 1000 μM E3S increased the accumulation of SN-38 (a metabolite of irinotecan) in Caco-2 cells because of their inhibitory effects on SN-38 efflux. To our knowledge, the rosuvastatin uptake by OATP2B1 has not been clearly demonstrated in Caco-2 cells, probably due to its strong efflux of rosuvastatin mediated by BCRP and MRP2, and OATP2B1 substrates such as E3S and BSP act as the inhibitors of not only OPTP2B1 but also MRP2 and BCRP. To confirm the OATP2B1-mediated uptake (absorption) of statins, studies have been undertaken using OATP2b1 transfected HEK293 cells and OATP2b1-deficient mice [14,17].

Studies are being undertaken on the transport of statins through the basolateral membrane of enterocytes (Figure 9), and proposed transporters related to statins are MRP3 [49], OSTα/β [20], and OATP2B1 [23]. However, the transport of statins by these transporters has not been confirmed by other researchers, especially the existence of OATP2B1 at the basolateral membrane. In this study, we used MK-571 and E3S as transporter inhibitors through the apical membrane. However, MK-571 is known to activate the inhibitor of MRP3 and E3S is known to be a substrate of OSTα/β [49,50]. Uptake and efflux of statins in enterocytes, especially through the basolateral membrane of rosuvastatin, are not fully understood.

In conclusion, this study showed that 5,7-DMF increased the uptake and the accumulation of rosuvastatin from the apical membrane of Caco-2 cells which seems to be related to the inhibition of rosuvastatin efflux mediated by BCRP and MRP2. The uptake of rosuvastatin from the apical membrane may be mediated by not only OATP2B1 but also MCT1. Some typical substrates of OATP2B1 did not decrease the rosuvastatin accumulation, because they could act as the substrates (inhibitors) of efflux transporters as well.

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