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Research Paper

Characterization of the Uptake Mechanism for a Novel Loop Diuretic, M17055, in Caco-2 Cells: Involvement of Organic Anion Transporting Polypeptide (OATP)-B

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Purpose. M17055 is under development as a novel loop diuretic for oral administration. To investigate the molecular mechanism of its gastrointestinal absorption, we initially aimed to clarify the mechanism of uptake of M17055 by Caco-2 cells, focusing on possible involvement of OATP-B (SLCO2B1), which is localized in the apical membranes of human intestinal epithelial cells.
Materials and Methods. The uptake of [^{14}C]M17055 by Caco-2 cells cultured on multi-well dishes was measured after cultivation for 14 days. Uptake of [^{14}C]M17055 by HEK293 cells stably expressing OATP-B (HEK293/OATP-B cells) was also examined.
Results. M17055 uptake by Caco-2 cells was saturable, and was inhibited by various organic anions, including other loop diuretics, and several bile acids. Uptake of M17055 by HEK293/OATP-B cells was much higher than that by mock cells. The inhibitory profiles of various organic anions and the estimated K_m values for M17055 uptake were similar in Caco-2 and HEK293/OATP-B cells. Moreover, the values of inhibition constants of several inhibitors for M17055 uptake were comparable in the two cell lines.
Conclusion. Our data suggest that OATP-B plays a major role in the uptake of the novel loop diuretic M17055 from apical membranes in Caco-2 cells.

KEY WORDS: Caco-2; intestinal absorption; loop diuretics; membrane transport; OATP-B; transporter.

INTRODUCTION

The small intestine is the first barrier to the gastrointestinal absorption of orally administered drugs. Intestinal drug absorption is generally thought to occur via simple diffusion according to the pH-partition hypothesis, though certain types of therapeutic agents have been proposed to be absorbed via carrier-mediated systems. For example, possible involvement of specific transporter(s) in the uptake of several β-lactam antibiotics in the small intestine was proposed as early as the 1970s (1,2). Oligopeptide transporter 1 (PEPT1, SLC15A1) is expressed on the apical membrane in the intestinal epithelial cells and shows H^+/oligopeptide co-transporting activity for di- and tripeptides, as well as for various β-lactam antibiotics (3–6). Monocarboxylic acid transporter 1 (MCT1, SLC16A1) is another H^+-dependent transporter which is expressed in the intestine and transports various weak organic acids such as L-lactic acid (7–9). Organic anion transporting polypeptide (OATP)-B (OATP2B1) was also recently identified on the apical membrane in human small intestine (10), and transports estrone-3-sulfate, pravastatin, dehydroepiandrosterone sulfate, fexofenedine and taurocholic acid in a pH-dependent manner (11–14). Thus, transporters may be involved in the membrane transport of the anionic drugs, even if pH-dependent membrane transport of weak organic anions can apparently be accounted for by simple diffusion of unionized forms.

Loop diuretics have a potential diuretic effect and are widely used in clinical therapy. After oral administration, they show variable absorption: reported bioavailabilities are 26–65% (furosemide), 68–102% (torasemide), 80% (bumetanide), and 21% (ethacrynic acid) (15,16). The bioavailabilities of furosemide and bumetanide were reduced and unchanged, respectively, in the case of oral administration with food (17). It remains unknown whether carrier-mediated transport is involved in gastrointestinal absorption of these diuretics. Recently, drug–food interaction in humans has been suggested to be linked to carrier-mediated drug uptake from the apical membrane in small intestine (18–20). OATP family members could be candidates for participation in such

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ABBREVIATIONS: BSP, bromosulfophthalein; DIDS, 5-isothiocyanato-2-[2-(4-isothiocyanato-2-sulfo-phenyl)ethenyl]benzenesulfonic acid; MCT1, monocarboxylic transporter 1; PEPT1, oligopeptide transporter 1; SLC, solute carrier.

interactions (18,19). Therefore, it is important to identify small intestinal transporters for loop diuretics to understand the possible mechanisms of the interaction with food.

M17055 has a potent diuretic effect and can be categorized as a loop diuretic, although its structure is different from those of other loop diuretics; M17055, which has a sulfate group in its structure (Fig. 1) (21), is soluble and well absorbed, and its bioavailability in humans is 42–60% (unpublished observation). Considering that the pK_a of M17055 is 2.39 (22), almost of M17055 would be in ionized form at physiological pH in the small intestine. Previously, we showed that multiple organic anion transport systems are involved in renal secretion of M17055, with the organic anion transporter 1 (Oat1, Slc22a6) being partially involved in its tubular secretion in rats (23). Thus, under physiological conditions, the ionized form of M17055 would be transferred across the plasma membrane via transporters, at least in kidney. This seems consistent with the recent conclusion based on studies in *Oat1* gene knock-out mice, that renal secretion of furosemide is predominantly mediated by Oat1 (24). These observations have led us to examine the possible involvement of carrier-mediated transport systems in gastrointestinal absorption of M17055.

In the present study, we aimed to characterize the uptake mechanism of M17055 from apical membranes in Caco-2 cells, one of the widely used model cell lines for human intestinal epithelial cells. Our results confirm the involvement of organic anion-specific transport systems in the uptake of M17055 in Caco-2 cells. Considering that M17055 has a sulfate group in its structure, and that most of the substrates of OATP-B are organic anions with a sulfate group, including steroid sulfates (11,25), we then examined the possible involvement of OATP-B in the uptake of M17055.

MATERIALS AND METHODS

Materials

[^{14}C]M17055 (2.0 GBq/mmol) was synthesized by Amersham International Plc (Bucks, UK) and unlabeled M17055 was supplied by the Pharmaceutical Laboratory of Mochida Pharmaceutical Co., Ltd. (Shizuoka, Japan). Caco-2 cells were supplied by Prof. Per Artursson and used within 95 to 105 passages. Mammalian expression vector pcDNA3 was purchased from Invitrogen (Carlsbad, CA), and OATP-B/pcDNA3 was constructed as described previously (11). All other chemicals were commercial products of analytical grade.

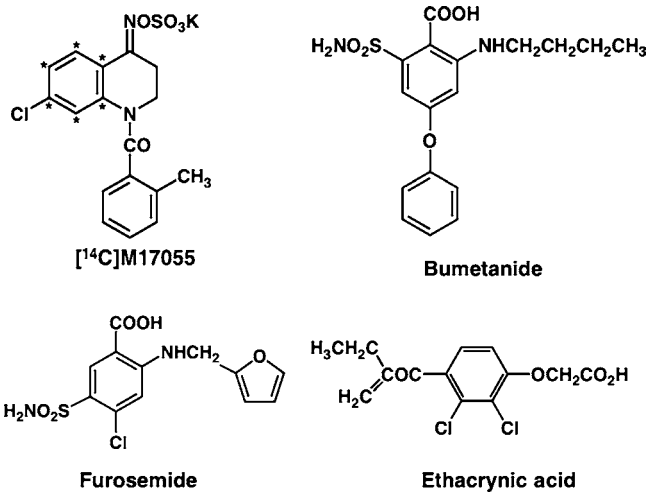

Fig. 1. Chemical structures of M17055 and other loop diuretics. *Radiolabeled positions.

Cell Culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS, Invitrogen), 1% nonessential amino acid (Invitrogen), 109 unit/ml benzylpenicillin and 0.14 mg/ml streptomycin in a humidified incubator at 37°C under 10% CO_2 in air. For uptake study, Caco-2 cells were cultured on multi-well plates and the medium was changed every 2 days for 14 days. HEK293 cell lines stably expressing OATP-B (HEK293/OATP-B cells) were established (14). Briefly, HEK293 cells were transfected with OATP-B cDNA (pcDNA3 vector) by means of the Ca^{2+} phosphate precipitation method. The cells were subcultured in the presence of 1 mg/ml G418 (Sigma-Aldrich, St. Louis, MO). Expression of OATP-B in HEK293/OATP-B cells was confirmed by immunocytochemistry and [^{3}H]estrone-3-sulfate uptake assay. HEK293/OATP-B cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 1% sodium pyruvate, 109 unit/ml benzylpenicillin, 0.14 mg/ml streptomycin and 1 mg/ml G418 in a humidified incubator at 37°C under 5% CO_2 in air.

Uptake Studies in Caco-2 and HEK293 Cells

[^{14}C]M17055 uptake by Caco-2 was measured at 37°C on a warmed hot plate. To equilibrate Caco-2 cells with the transport buffer, the culture medium was replaced with 1 ml of warmed transport buffer (Hanks' balanced salt solution; HBSS, adjusted to pH 7.4) containing 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 137 mM NaCl, 0.385 mM Na_2HPO_4, 25.0 mM D-glucose and 10 mM HEPES. The transport buffer was removed and drug solution containing [^{14}C]M17055 (1.8 μM) and a given inhibitor only in the inhibition study in HBSS (10 mM MES for pH 5.5–6.5 and 10 mM HEPES for pH 7.0 and 7.5) adjusted to the appropriate pH was added. The reaction was terminated by addition of 1 ml of ice-cold HBSS and the cells were washed three times with 1 ml of HBSS. The cells were solubilized by the addition of 5 N NaOH (0.25 ml) with shaking for 2 h. The cell lysate was neutralized and mixed with 3 ml of scintillation cocktail (Clearsol-I, Nacalai Tesque, Kyoto, Japan), then the radioactivity was measured with a liquid scintillation counter (LSC-5100, Aloka Co., Ltd., Tokyo, Japan). Protein determinations were done using the Bradford method (Protein assay kit, BioRad, Hercules, CA) with bovine serum albumin as a standard.

[^{14}C]M17055 uptake by HEK293/OATP-B cells was measured at 37°C. After the cells had reached confluence, they were harvested and suspended in the transport buffer containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose,

1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (adjusted to pH 7.4 with 1 N NaOH). The cell suspension was preincubated at 37°C for 10 min, then centrifuged, and transport was initiated by mixing the solution with drug solution containing [^{14}C]M17055, a given inhibitor only in the inhibition study, 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (adjusted to pH 7.0 or 7.4 with 1 N NaOH) or MES (adjusted to pH 5.5–6.5 with 1 N NaOH).

Data Analysis

Kinetic parameters for M17055 uptake were obtained using nonlinear least-squares regression analysis (MULTI) based on the following equations:

$$v = V_{max} \times s/(K_m + S) + K_{ns} \times s \quad (1)$$

$$v = V_{max1} \times s/(K_{m1} + s) + V_{max2} \times s/(K_{m2} + s) + K_{ns} \times s \quad (2)$$

$$v = V_{max} \times s/(K_m \times (1 + I/K_i) + s) + K_{ns} \times s \quad (3)$$

where v, s, I, V_{max}, K_m, K_i and K_{ns} represent the initial uptake velocity, substrate concentration, inhibitor concentration, maximum uptake velocity, Michaelis constant, inhibitory constant and non-saturable uptake clearance, respectively. The selection of the equation was based on Akaike's Information Criterion. Each kinetic parameter is given as the fitted value ± SD.

Cellular uptake of [^{14}C]M17055 was represented by cell-to-medium ratio, obtained by dividing the uptake amount by the concentration of test compound in the uptake medium. Statistical analysis was performed with Student's *t*-test. The criterion of significance was taken to be $P < 0.05$.

RESULTS

Uptake of M17055 by Caco-2 Cells

[^{14}C]M17055 uptake via the apical membrane into Caco-2 cells at pH 6.0 linearly increased with time (Fig. 2a). This uptake was dramatically reduced in the presence of 1 mM unlabeled M17055 (Fig. 2a). Initial uptake was then determined with an incubation period of 10 min to evaluate the concentration dependence of M17055 uptake in Caco-2 cells. Saturable transport was observed, and kinetic parameters were obtained using Eq. 2 (Fig. 2b, Table I). An Eadie–Hoffstee plot showed two uptake components (high- and low-affinity components), suggesting that M17055 uptake by Caco-2 is mediated by plural transport systems. Kinetic analysis revealed that the contribution of the high-affinity transport system (V_{max1} / K_{m1}) was major, compared with those of the low-affinity transport system (V_{max2} / K_{m2}) and non-saturable transport (K_{ns}) (Table I).

Inhibitory Effect of Various Compounds on M17055 Uptake by Caco-2 Cells

To characterize M17055 uptake by Caco-2 cells, we examined the inhibitory effects of several compounds, including typical inhibitors of organic anion transport systems, substrates of OATP-B, loop diuretics, organic cations and bile acids. We performed the inhibition study for 10 min

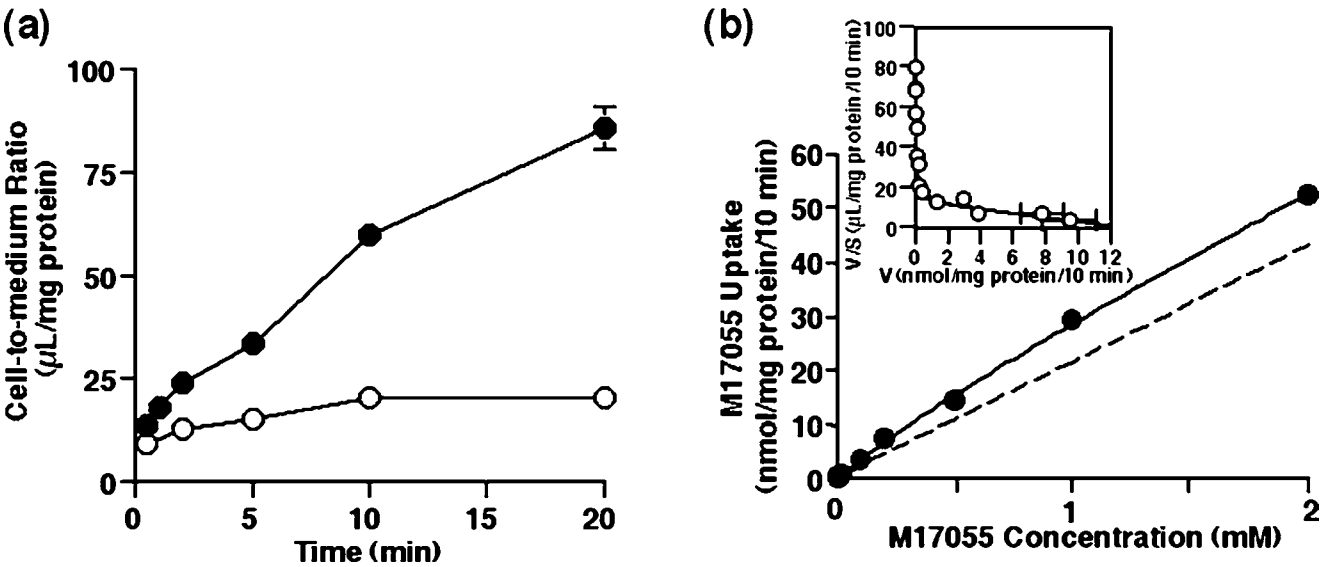

Fig. 2. Time course and concentration dependence of M17055 uptake by Caco-2 cells. (**a**) Uptake of [^{14}C]M17055 (1.8 μM) by Caco-2 cells was measured at pH 6.0 in the absence (*filled circles*) or presence (*empty circles*) of excess unlabeled M17055 (1 mM). (**b**) Uptake of M17055 at various concentrations from 0.10 μM to 2.0 mM was measured for 10 min at 37°C. An Eadie-Hofstee plot of [^{14}C]M17055 uptake after subtraction of the estimated non-saturable component (represented as a *dotted line*) is shown in the inset. Kinetic parameters were obtained by nonlinear least-squares regression analysis (MULTI) and are listed in Table I. The error bar is not shown when the SE value is smaller than the symbol. Each point is the mean ± SE of three or four determinations of two independent experiments.

Table I. Kinetic Parameters for M17055 Uptake by Caco-2 and HEK293/OATP-B Cells

Kinetic Parameters	Caco-2 Cells	HEK293/ OATP-B Cells
K_{m1} (μM)	2.13 ± 0.44	4.48 ± 1.35
K_{m2} (mM)	1.00 ± 0.60	
V_{max1} (pmol/mg protein/min)	13.9 ± 2.6	248 ± 74
V_{max2} (nmol/mg protein/min)	1.31 ± 0.84	
K_{ns}(μl/mg protein/min)	2.19 ± 0.25	12.5 ± 2.6
V_{max1} / K_{m1} (μl/mg protein/min)	6.53	55.2
V_{max2} / K_{m2} (μl/mg protein/min)	1.31	

Estimated kinetic parameters (K_m and V_{max}) represent fitted value ± SD obtained by nonlinear least-squares regression analysis (MULTI).

at 1 mM of inhibitors except the cases of cytotoxicity and/or solubility limitation of the potential inhibitors. As shown in Fig. 3a, several organic anions, including estrone-3-sulfate, pravastatin, BSP, probenecid, loop diuretics and bile acids, significantly inhibited [^{14}C]M17055 uptake by Caco-2 cells, whereas cationic compounds, such as cimetidine and tetraethylammonium, did not, suggesting that the uptake system for M17055 is organic anion-specific.

Sodium Ion-Dependence of M17055 Uptake by Caco-2 Cells

When sodium ion was replaced with choline, the uptake of [^{14}C]M17055 by Caco-2 cells (57.0 ± 1.3 μl/mg protein/10 min) was comparable to the control uptake (60.2 ± 2.4 μl/mg protein/10 min), suggesting that the contribution of sodium-dependent influx transporter(s) to M17055 uptake is minor.

Uptake of M17055 by HEK293/OATP-B Cells

To study whether M17055 is a substrate of OATP-B, an uptake study was performed in HEK293 cells stably expressing OATP-B. The uptake of [^{14}C]M17055 by HEK293/OATP-B cells was much higher than that by mock cells (Fig. 4a). Initial uptake was then determined with an incubation period of 5 min to evaluate the concentration dependence of M17055 uptake in HEK293/OATP-B cells. In the concentration dependence study, saturable uptake of M17055 was observed in HEK293/OATP-B cells, but not in

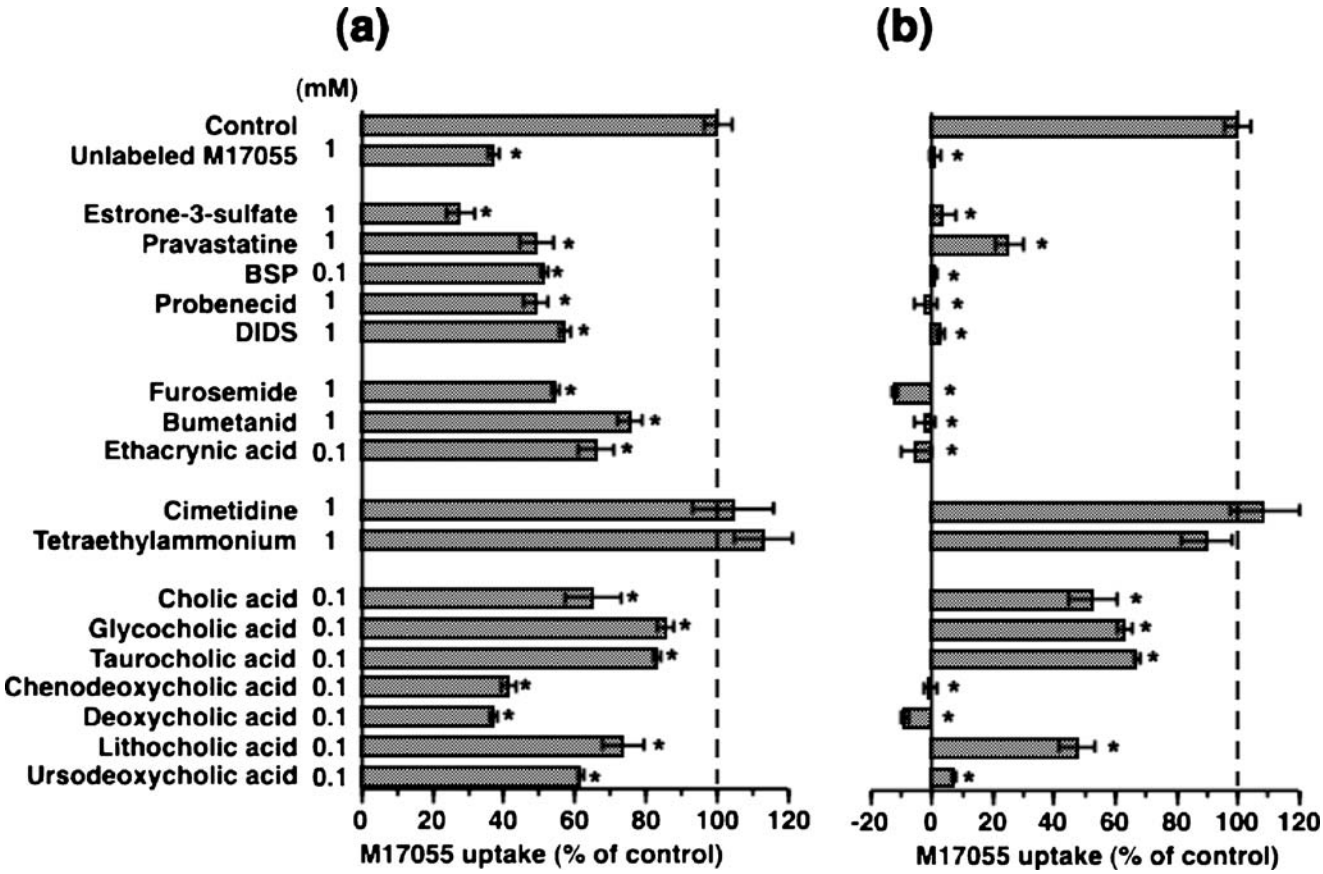

Fig. 3. Inhibitory effects of various compounds on [^{14}C]M17055 uptake by Caco-2 and HEK293/OATP-B cells. Uptake of [^{14}C]M17055 (1.8 μM) by (**a**) Caco-2 and (**b**) HEK293/OATP-B cells in the presence of various compounds was measured at pH 6.0 for 10 and 5 min, respectively. The concentration of each compound was set to be 1 mM, except for BSP, ethacrynic acid and all bile acids (0.1 mM). In panel (**a**), [^{14}C]M17055 uptake by Caco-2 cells is presented after having been divided by the control uptake. In panel (**b**), OATP-B-mediated uptake of [^{14}C]M17055 was calculated by subtraction of the uptake by mock cells from the observed values, and normalized to the control values. The error bar is not shown when the SE value is smaller than the symbol. Each point is the mean ± SE of three or four determinations of one or two independent experiments. *Significant difference from the control experiment.

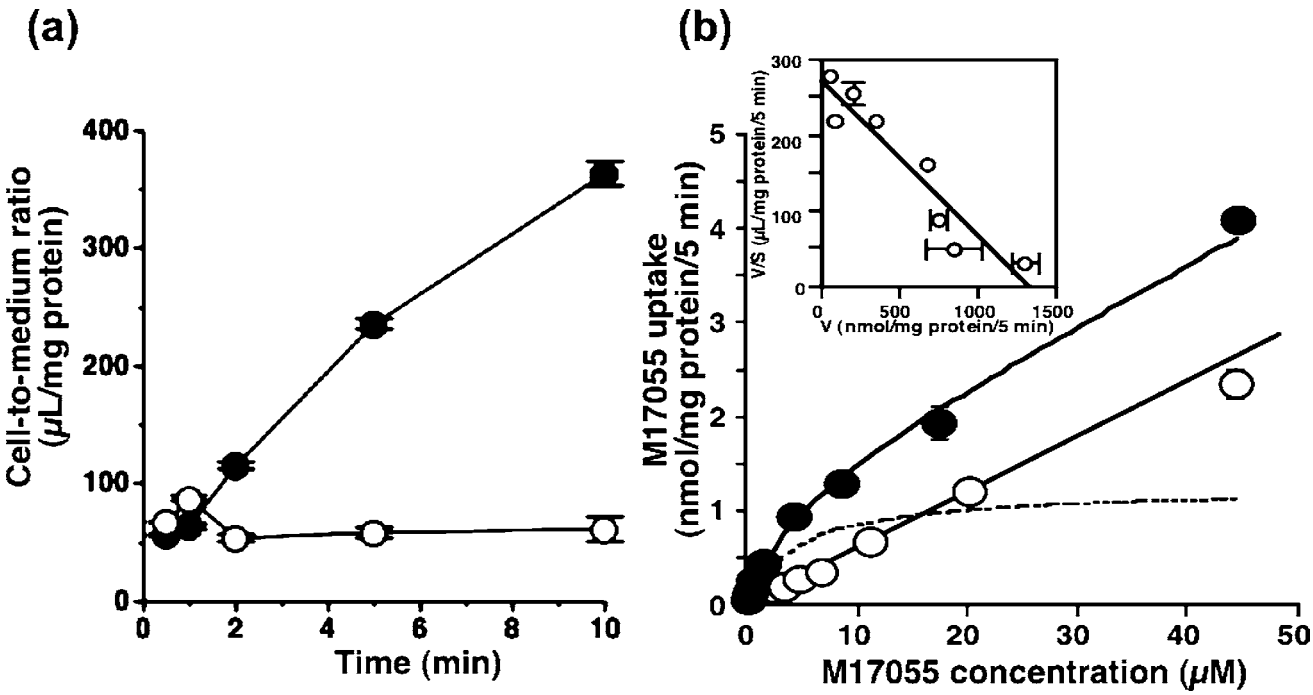

Fig. 4. Time course and concentration dependence of M17055 uptake by HEK293/OATP-B cells. (**a**) Uptake of [^{14}C]M17055 (1.8 μM) by HEK293/OATP-B (*filled circle*) and mock (*empty circle*) cells was measured at pH 6.0. (**b**) Uptake of M17055 at various concentrations from 0.17 to 45 μM was measured for 5 min at 37°C. The *broken line* represents the values obtained by subtraction of the uptake by mock cells from that by HEK293/OATP-B cells. An Eadie-Hofstee plot of [^{14}C]M17055 uptake after subtraction by the uptake by mock cells is shown in the inset. Kinetic parameters were obtained by nonlinear least-squares regression analysis and are listed in Table I. The error bar is not shown when the SE value is smaller than symbol. Each point is the mean ± SE of three determinations of two independent experiments.

mock cells (Fig. 4b). These results indicate that M17055 is a substrate of OATP-B. An Eadie-Hofstee plot for OATP-B-mediated uptake was linear (Fig. 4b), and the estimated K_m value was very similar to that for the high-affinity component in Caco-2 cells (Table I).

Inhibitory Effect of Various Compounds on M17055 Uptake by HEK293/OATP-B Cells

To compare the transport characteristics for M17055 in Caco-2 cells with those of OATP-B, the inhibitory effects of various compounds on initial uptake (5 min) of [^{14}C]M17055 by HEK293/OATP-B cells were examined. OATP-B-mediated transport of [^{14}C]M17055 were estimated by subtracting the uptake by mock cells from that by HEK293/OATP-B cells and shown in Fig. 3b after normalized by control uptake. Organic anions, including estrone-3-sulfate, pravastatin, BSP, probenecid, loop diuretics and bile acids, inhibited [^{14}C]M17055 uptake by HEK293/OATP-B cells again, whereas cationic compounds did not (Fig. 3b). The inhibition profiles in the two cell types were similar (Fig. 3a and b). Several of the inhibitors almost completely blocked the uptake by HEK293/OATP-B cells (Fig. 3b), but not by Caco-2 cells (Fig. 3a), probably because the uptake of M17055 is also partially mediated by low-affinity and/or non-saturable components in the latter (Fig. 2b, Table I). These data suggest that uptake characteristics of M17055 are quite similar both in Caco-2 cells and OATP-B-transfected cells.

Comparison of K_i Values Between Caco-2 and HEK293/OATP-B Cells

To compare in more detail the substrate specificities of the M17055 uptake system in Caco-2 cells and OATP-B-expressing cells, the effects of various concentrations of inhibitors on initial uptakes (10 min in Caco-2 cells and 5 min in HEK293/OATP-B cells) of [^{14}C]M17055 were examined. BSP, estrone-3-sulfate, pravastatin, furosemide and glycocholic acid showed concentration-dependent inhibition in both Caco-2 and HEK293/OATP-B cells (Fig. 5a and b). The K_i values of the inhibitors were estimated using Eq. 3 and are listed in Table II. The K_i values for BSP, pravastatin, furosemide and glycocholic acid are similar in Caco-2 and HEK293/OATP-B cells, while the difference in the case of estrone-3-sulfate was within three-fold (Table II).

pH-Dependence of M17055 Uptake by Caco-2 and HEK293/OATP-B Cells

To further characterize the uptake system in Caco-2 cells, we investigated the initial uptake of [^{14}C]M17055 by Caco-2 cells at various values of extracellular pH. [^{14}C]M17055 uptake by Caco-2 cells showed minimal pH-dependence (Fig. 6a). On the other hand, [^{14}C]M17055 uptake by HEK293/OATP-B cells was maximum at pH 6.0 (Fig. 6b), although the pH dependence was not marked (Fig. 6b).

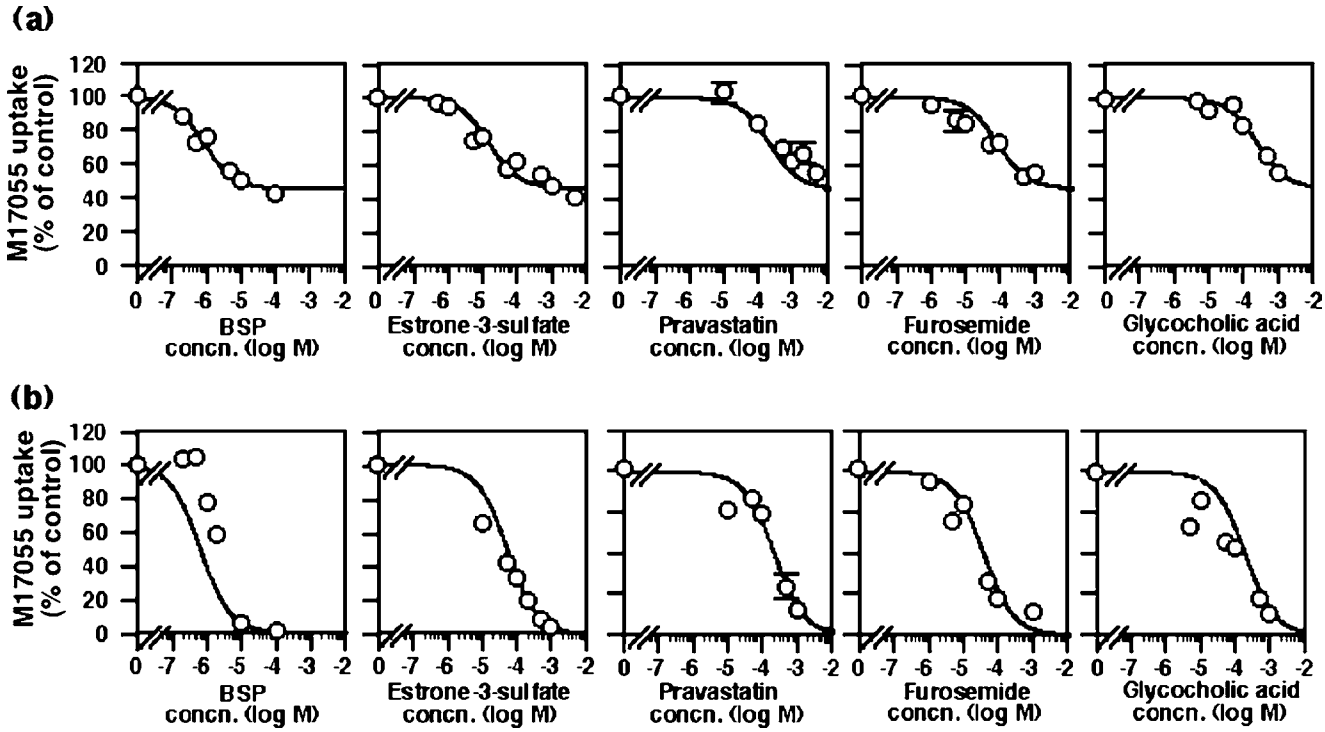

Fig. 5. Concentration-dependent inhibition of [^{14}C]M17055 uptake by Caco-2 and HEK293/OATP-B cells by several compounds. Uptake of [^{14}C]M17055 (1.8 μM) by (**a**) Caco-2 and (**b**) HEK293/OATP-B cells in the presence of various concentrations of each compound was measured at pH 6.0 for 10 and 5 min, respectively. In panel (**a**), M17055 uptake by Caco-2 cells was plotted after having been divided by the corresponding control uptake. In panel (**b**), OATP-B-mediated uptake of [^{14}C]M17055 was calculated after subtraction of the uptake by mock cells from the observed values, and normalized to the control values. Kinetic parameters obtained by nonlinear least-squares regression analysis (MULTI) are shown in Table II. The error bar is not shown when the SE value is smaller than the symbol. Each point is the mean ± SE of three or four determinations.

DISCUSSION

In the present study, we have demonstrated that the novel loop diuretic M17055 is a substrate of OATP-B, which also recognizes other loop diuretics (Figs. 3 and 4). In addition, our results indicate that the uptake of M17055 from the apical membranes in Caco-2 cells is carrier-mediated, with characteristics very similar to those in HEK293/OATP-B cells in terms of the following criteria. (a) The concentration dependence of M17055 transport was almost the same in Caco-2 and HEK293/OATP-B cells, and the K_m value for OATP-B-mediated M17055 uptake was similar to that for high-affinity M17055 uptake in Caco-2 cells (Fig. 2; Table I). (b) The inhibitory effects of various compounds on M17055 uptake were similar in Caco-2 and HEK293/OATP-B cells (Fig. 3). (c) The K_i values of several anionic compounds, including substrates of OATP-B (BSP, estrone-3-sulfate and pravastatin) in Caco-2 cells were similar to those for OATP-B-mediated M17055 uptake (Fig. 5, Table II). Considering our recent observation that OATP-B is localized on the apical membranes in Caco-2 cells (14), these results suggest that OATP-B plays a major role in M17055 uptake in Caco-2 cells.

OATP-B mRNA is expressed in various tissues, including liver, lung, spleen, placenta, mammary gland and small intestine in adult, and ubiquitously in fetal tissues (11,25,26). OATP-B protein is expressed on the apical membrane in human small intestine, as well as in the mammary gland, on the basal surface of the syncytiotrophoblast in the placenta and on the sinusoidal membranes of hepatocytes (10,25–27). OATP-B was first identified as a homolog of OATP-A (OATP1A2) (11) and has been thought to have a narrow range of substrate specificity, compared with other OATPs. The substrates of OATP-B include estrone-3-sulfate, prostaglandin E_2, benzylpenicillin, BSP and dehydroepiandrosterone sulfate (11,25,28). However, Kobayashi *et al.* (10) and Nozawa *et al.* (13) have recently shown that OATP-B transports other substrates, including pravastatin, fexofenadine and taurocholic acid.

It should be noted that the function of OATP-B is enhanced at pH 6–6.5, which is close to the microclimate pH at the surface of apical membranes in the small intestine. Therefore, these previous findings, as well as the present observations, suggest that OATP-B may play an important role in the intestinal absorption of various anionic drugs. It is noteworthy, however, that M17055 uptake by Caco-2 cells showed two saturable components, and the estimated K_m value for OATP-B-mediated M17055 uptake was similar to that of the high-affinity component in Caco-2 cells (Fig. 2b, Table I), indicating that multiple transport systems are involved in M17055 uptake by Caco-2 cells. Regarding transporters that accept M17055 as a substrate, we have previously showed that OAT1 recognizes M17055 and is partially involved in renal secretion of M17055 (23). However, OAT1 mRNA has not been detected in the intestine (29,30). In addition, no inhibitory effect of 1 mM *p*-amino-

Table II. K_i Values of Various Compounds for M17055 Uptake by Caco-2 and HEK293/OATP-B Cells

Inhibitor	Caco-2 cells (μM)	HEK293/OATP-B cells (μM)
BSP	0.982 ± 0.248	1.47 ± 0.48
Estrone-3-sulfate	7.68 ± 3.71	26.7 ± 3.6
Pravastatin	105 ± 47	114 ± 19
Furosemide	41.5 ± 21.1	19.6 ± 10.0
Glycocholic acid	131 ± 47	99.4 ± 16.2

Each value is the fitted K_i value ± SD obtained by nonlinear least-squares regression analysis (MULTI).

hippuric acid, a substrate of OAT1, on M17055 uptake by Caco-2 cells was observed (data not shown). Therefore, involvement of OAT1 in the uptake of M17055 by Caco-2 is likely to be negligible. Dresser *et al.* (18,19) have recently suggested that OATP-A is involved in the intestinal absorption of fexofenadine, and the contribution of OATP-B is minor, even though fexofenadine is a substrate of both OATP-A and OATP-B. OATP-A mRNA was below the limit of detection in human small intestine (11), though its gene product was detected in human small intestine as revealed by Western blot analysis (31). In a preliminarily analysis of mRNA expression of other OATPs, we detected gene expression of OATP-A, D (SLCO3A1) and E (SLCO4A1) in Caco-2 cells by RT-PCR (data not shown). Further studies are necessary to clarify the contribution of each OATP family member to M17055 transport in Caco-2 cells and in intestine *in vivo*.

In the present study, the estimated K_i values of BSP, pravastatin, furosemide and glycocholic acid in HEK293/OATP-B cells differed by no more than two-fold from those obtained in Caco-2 cells, though estrone-3-sulfate was an exception (Table II). In parallel studies using Caco-2 cells, we found that uptake of estrone-3-sulfate also involves multiple transport systems (14). Thus, it is possible that estrone-3-sulfate inhibits not only OATP-B, but also other uptake route(s) of M17055 in Caco-2 cells.

All of the bile acids examined in the present study inhibited M17055 uptake by Caco-2 and HEK293/OATP-B cells (Fig. 3). Deoxycholic acid, chenodeoxycholic acid and ursodeoxycholic acid showed potent inhibitory effects (Fig. 3). Taurocholic acid was transported across Caco-2 cells from the apical membrane side in a sodium-dependent manner, and it has been reported that a sodium-dependent bile acid transport system exists in Caco-2 cells (31–33). Ileal sodium-dependent bile acid transporter (IBAT, SLC10A2) has already been identified as a sodium-dependent transport system. Therefore, it can be hypothesized that M17055 is taken up by Caco-2 cells not only via OATP-B, but also via the sodium-dependent bile acid transporter. However, no significant sodium-dependence was observed in M17055 uptake by Caco-2 cells (see "Results"). Thus, the contribution of sodium-dependent bile acid transporter to M17055 uptake is likely to be minor.

The present study has proposed the relevance of OATP-B as an uptake mechanism from gastrointestinal lumen. However, it should be noted that the results depend on the relationship between the dose of drug and the capacity of the transporter. Thus, when a drug that is a substrate of OATP-B is orally administrated, it is possible that the transporter-mediated absorption is saturated, if the concentration of the drug in the intestinal lumen becomes higher than its own K_m value for OATP-B. Considering the existence of saturable transport system, the present study has also suggested that drug–drug or drug–bile acids interaction may occur in gastrointestinal drug absorption.

Considering the present observation that OATP-B recognizes other loop diuretics than M17055 (Figs. 3 and 5), further studies are required to examine possible involvement of OATP-B in their gastrointestinal absorption, since most of them are orally administered in clinical applications. Transport characteristics of furosemide across Caco-2 cells were previously investigated, and saturable transport was observed only from basolateral to apical side, with minimal saturation in the apical-to-basolateral direction (34). Therefore, although these loop diuretics are recognized by OATP-B, at least as inhibitors, it remains unknown whether carrier-mediated transport system(s), including OATP-B, are com-

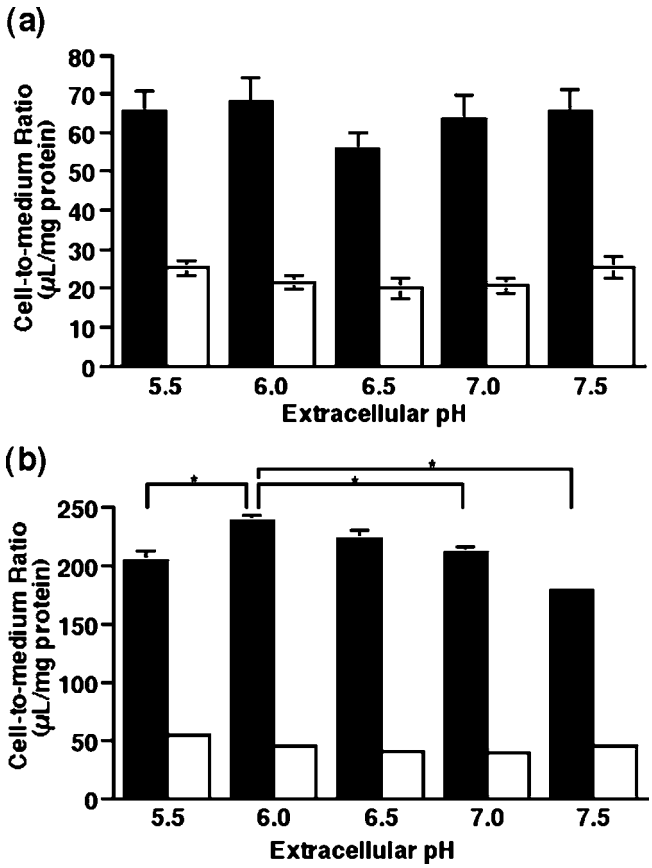

Fig. 6. Effect of extracellular pH on [^{14}C]M17055 uptake by Caco-2 and HEK293/OATP-B cells. (**a**) Uptake of [^{14}C]M17055 (1.8 μM) by Caco-2 cells was measured at pH 5.5–7.5 in the absence (*closed column*) or presence (*open column*) of 1 mM unlabeled M17055. (**b**) Uptake of [^{14}C]M17055 (1.8 μM) was measured at pH 5.5–7.5 in HEK293/OATP-B (*open column*) and mock (*closed column*) cells. Uptake studies were performed for 10 and 5 min in Caco-2 and HEK293/OATP-B cells, respectively. The error bar is not shown when the SE value is smaller than the symbol. Each point is the mean ± SE of three or four determinations of one to three independent experiments.

monly involved in the uptake of these diuretics in Caco-2 cells.

Uptake of M17055 in HEK293/OATP-B cells showed slight pH-dependence, with higher uptake activity being observed under acidic conditions (Fig. 6b). Such pH-dependence is similar to that reported for OATP-B-mediated uptake of estrone-3-sulfate, pravastatin and other acidic compounds (10,13), although the optimum extracellular pH (pH 6.0 for M17055, Fig. 6b) seems to vary according to the substrate (pH 5.5 or less for estrone-3-sulfate, pravastatin and taurocholic acid) (10,13). On the other hand, M17055 uptake by Caco-2 cells showed minimal pH dependence (Fig. 6a). Thus, the pH-dependence of M17055 uptake was not exactly the same in Caco-2 and HEK293/OATP-B cells. One of the possible reasons for this is that, as discussed above, multiple transport systems could be involved in M17055 uptake in Caco-2 cells. Another possible explanation is that proton-supplying sources, such as a sodium–proton exchanger, exist in Caco-2 cells, but not in HEK293 cells. Thwaites *et al.* has proposed that the activity of proton/oligopeptide transporter is controlled by a sodium–proton exchanger functionally expressed in Caco-2 cells, leading to higher transport activity at higher extracellular pH (35). Further studies are needed to clarify the factors that account for the low pH dependence of M17055 uptake by Caco-2 cells.

CONCLUSION

OATP-B is the major contributor to uptake of the novel loop diuretic M17055 by Caco-2 cells. Our results suggest that loop diuretics are recognized by OATP-B in the small intestine and, thus, OATP-B could be a determinant of the bioavailability of orally administered drugs.

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