Pharmaceutical Research, Vol. 25, No. 5, May 2008 (© 2007)
DOI: 10.1007/s11095-007-9472-4

Research Paper

Involvement of Organic Anion Transporting Polypeptide 1a5 (Oatp1a5) in the Intestinal Absorption of Endothelin Receptor Antagonist in Rats

Takeshi Tani,[1] Luise K. Gram,[2,3] Hiroshi Arakawa,[2] Akihiro Kikuchi,[2] Masato Chiba,[1] Yasuyuki Ishii,[1] Bente Steffansen,[3] and Ikumi Tamai[2,4]

Received June 17, 2007; accepted October 4, 2007; published online October 24, 2007

Purpose. To assess the contribution of organic anion transporting polypeptide 1a5 (Oatp1a5/Oatp3) in the intestinal absorption of an orally active endothelin receptor antagonist, (+)-(5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylene-dioxyphenyl)cyclopenteno[1,2-b]pyridine-6-carboxylic acid (compound-A) in rats.
Methods. Uptakes of [^{14}C]compound-A by Oatp1a5-expressing *Xenopus laevis* oocytes and isolated rat enterocytes were evaluated.
Results. The uptake of compound-A by Oatp1a5-expressing oocytes was significantly higher than that by water-injected oocytes and Oatp1a5-mediated uptake was saturable with K_m value of 116 μM. Compound-A was taken up into isolated enterocytes in time- and concentration-dependent manners and the estimated K_m value was 83 μM, which was close to that for the Oatpt1a5-mediated uptake in oocytes. Both uptakes of compound-A by Oatp1a5-expressing oocytes and enterocytes were pH-sensitive with significantly higher uptake at acidic pH than those at neutral pH. Uptakes of compound-A into Oatp1a5-expressing oocytes and enterocytes were significantly decreased in the presence of Oatp1a5 substrates such as bromosulfophthalein and taurocholic acid.
Conclusions. These results consistently suggested that Oatp1a5 is contributing to the intestinal absorption of compound-A at least in part, and the transporter-mediated absorption seems to be maximized at the acidic microenvironment of epithelial cells in the small intestine in rats.

KEY WORDS: absorption; intestine; Oatp; organic anion; transporter.

INTRODUCTION

Intestinal epithelial permeability is one of the important determinants for the bioavailability of orally administered drugs. It has been generally considered that oral absorption of drug molecules is governed by their passive diffusion across the intestinal membrane, which depends on the physicochemical properties of drug molecules such as their lipophilicity and ionization state. However, molecular biological studies have revealed that various transporters are expressed at the apical membrane of intestinal epithelial cells, and these transporters are affecting the oral bioavailability of some drugs (1,2). Organic anion transporting polypeptide (OATP/Oatp) family transporters are expressed in various tissues, and they mediate the transmembrane transport of a wide range of acidic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides and many drug molecules (3,4). OATP/Oatp family transporters are hence expected to play important roles in the absorption, tissue distribution and elimination of acidic drug molecules. We have already suggested that human OATP1A2/OATP-A and OATP2B1/OATP-B and rat Oatp1a5/Oatp3 could be involved in the intestinal absorption of pravastatin, fexofenadine, and quinolone antibacterial agents (5–8). Furthermore, it was demonstrated that OATP1A2 is expressed at the apical membrane of human small intestinal epithelial cells and that it may contribute to intestinal drug absorption (9,10). Accordingly, it is possible that OATPs could contribute to intestinal absorption of clinically used drugs.

(+)-(5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl)cyclopenteno

[1] Drug Metabolism, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., 3 Okubo, Tsukuba, Ibaraki 300-2611, Japan.
[2] Department of Membrane Transport and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641, Yamasaki, Noda, Chiba 278-8510, Japan.
[3] Molecular Biopharmaceutics, Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, 2 Universitetsparken, Dk-2100, Copenhagen Denmark.
[4] To whom correspondence should be addressed. (e-mail: tamai@rs.noda.tus.ac.jp)

ABBREVIATIONS: BSP, bromosulfophthalein; Caco-2, human colon carcinoma cell line; cRNA, complementary ribonucleic acid; ET, endothelin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; K_m, Michaelis–Menten constant; k_{ns}, non-saturable uptake clearance; LLC-PK1, porcine kidney epithelial cell; MES, 2-*N*-morpholinoethanesulfonic acid; OATP/Oatp, organic anion transporting polypeptide; TCA, taurocholic acid; V_{max}, maximum uptake rate.

[1,2-b]pyridine-6-carboxylic acid (compound-A) is a new orally active, nonpeptidic endothelin (ET_A/ET_B) receptor antagonist that is currently under development as a drug for the treatments of hypertension and congestive heart failure (11). It is a hydrophilic compound with a log *D* value of 0.2 at pH 7.4 (in-house data) and it possesses two anionic carboxyl groups. Compound-A exists as an ionized form at physiological pH based on the pKa values of 3.0 and 3.7 of carboxylic acids (in-house data). It would therefore be difficult for compound-A to cross the intestinal epithelial membrane by passive diffusion. In fact, the results from transcellular transport studies in LLC-PK1 and Caco-2 cells indicate that compound-A is poorly permeable by passive diffusion (apparent permeabilities are 3.4×10^{-6} and 1.3×10^{-6} cm/s in LLC-PK1 and Caco-2 cells, respectively, unpublished observation). Nevertheless, more than 65% of dose was absorbed after oral administration of compound-A (unpublished observation) and the oral bioavailability in rats was approximately 34% (11). These data suggested that influx transporter(s) located at the apical membrane of intestinal epithelial cells may be involved in the intestinal absorption of compound-A in rats.

We previously reported that compound-A was extensively excreted into bile as a parent drug, and that active transporter(s) was (were) possibly involved in the hepatobiliary excretion (12). Furthermore, recent preliminary studies using OATP/Oatp transfected cells demonstrated that compound-A is a substrate for OATP/Oatp family transporters in both humans and rats, such as OATP1B1/OATP-C, OATP1B3/OATP8, OATP2B1/OATP-B, Oatp1a1/Oatp1, Oatp1a4/Oatp2, and Oatp1b2/Oatp4 (unpublished observation). In rat intestine, Oatp1a5 is localized at the apical membrane of intestinal epithelial cells and it can mediate the transmembrane transport of various anionic compounds across the intestine (7,13,14). Therefore, we hypothesized that Oatp1a5 located on the apical membrane of intestinal epithelial cells is contributing to the relatively high intestinal absorption of compound-A in rats. The purpose of the present study was to characterize the uptake mechanism for the intestinal absorption of compound-A in rats by using Oatp1a5-expressing *Xenopus laevis* oocytes and isolated rat enterocytes.

MATERIALS AND METHODS

Materials

[^{14}C]compound-A (Fig. 1) was synthesized by Daiichi Pure Chemical Company, Ltd. (Ibaraki, Japan). The labeled compound-A had the specific activity of 3.70 MBq/mg (100 μCi/mg). Nonlabeled compound-A (disodium salt) was synthesized in Banyu Pharmaceutical Co. Ltd. [^{3}H]estrone-3-sulfate (2,120 GBq/mmol) was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). All other reagents were analytical grade and purchased from Sigma-Aldrich (St. Luis, MO) and Wako Pure Chemical Industries Ltd (Osaka, Japan).

Uptake Experiments with *Xenopus* Oocytes Expressing Oatp1a5

All the animal care and experimentations were conducted according to the guidelines of Tokyo University of Science.

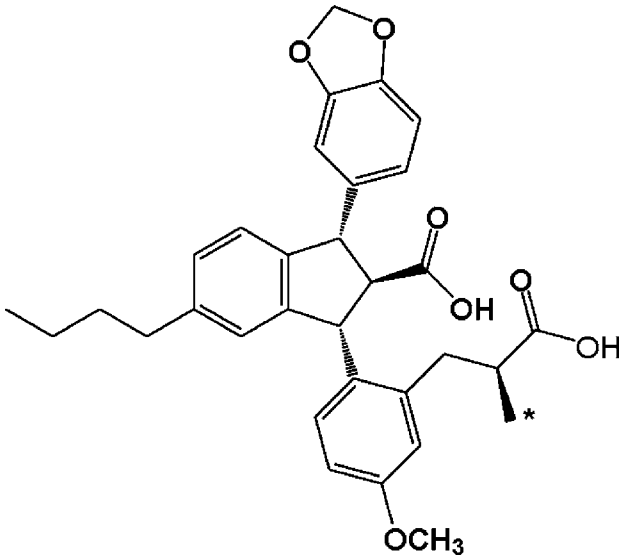

Fig. 1. Chemical structure of compound-A, (+)-(5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl)cyclopenteno[1,2-b]pyridine-6-carboxylic acid. *Asterisk* in the structure indicates location of ^{14}C labeling.

Uptake experiments were conducted with *Xenopus laevis* oocytes (Hamamatsu Creature Materials, Hamamatsu, Japan) microinjected with complementary RNA (cRNA) of Oatp1a5, synthesized *in vitro* by using T7 RNA polymerase (Ambion, Austin, TX), as described previously (7). Transcribed cRNA (50 ng in 50 nL water) was injected into defolliculated *Xenopus laevis* oocytes. Those oocytes were cultured for 3days at 18°C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 10 mM HEPES, adjusted to pH 7.4), and then used for uptake experiments. Uptake was initiated by incubating the oocytes at room temperature in the uptake medium containing [^{14}C]compound-A or [^{3}H]estrone-3-sulfate. Uptake medium contained the same constituents as the modified Barth's solution except that MES for acidic pH (5.5–6.0) was used instead of HEPES. At appropriate time, the uptake was terminated by the addition of ice-cold uptake medium. Oocytes were rinsed three times with ice-cold uptake medium, and placed in individual scintillation vials. After that the oocytes were solubilized in 5% sodium dodecyl sulfate solution, the radioactivity was measured by means of a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan) using Clearsol I (Nakalai tesque, Kyoto, Japan) as liquid scintillation fluid. Oocytes injected with water (50 nL), which were cultured in the same manner as described above, were used as a control.

Uptake Experiments with Isolated Enterocytes

Enterocytes were isolated from a whole small intestine of male Wistar rats (9–11 weeks, Saitama Experimental Animals supply Co., Ltd., Saitama, Japan) by using hyarulonidase (bovine testes Type 1-S, Sigma) as described previously (15). The rats were sacrificed by cervical dislocation under diethyl ether anesthesia. After abdominal incision, the entire small intestine was removed and the luminal contents were flushed out with ice-cold saline. The removed

intestine was slit open and cut into 5–10 mm segments. These segments were gently suspended and incubated at 37°C for 40 min in an isolation medium (125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES at pH 7.0) containing of 1.0 mg/mL bovine serum albumin and 1.5 mg/mL hyarulonidase. To isolate enterocytes, the segment solution was subsequently filtered through a mesh with a pore size of 180 μm. The filtrated cells were washed twice by uptake medium which contained the same constituents as the isolation medium, except that MES for acidic pH (5.5–6.0) was used instead of HEPES. Cells with viability greater than 80% checked by the trypan blue exclusion test were used in the following experiments. After preincubation of enterocytes (1×10^5 cells/tube) in uptake medium at 37°C for 5 min, uptake was initiated by replacing with uptake medium containing [^{14}C]compound-A. The uptake studies were terminated by addition of ice-cold uptake medium, after which the cells were washed twice with ice-cold uptake medium. Then, enterocytes were lysed with 1N NaOH and neutralized with 5N HCl. After addition of liquid scintillation cocktail, radioactivity was measured by a liquid scintillation counter.

Data Analysis

Uptake of [^{14}C]compound-A was expressed as the cell-to-medium ratio (μL/oocyte or μL/10^5 cells), obtained as the amount of radioactivity associated with the oocytes (dpm/oocytes) or the cells (dpm/10^5 cells) divided by concentrations of compound-A in the incubation medium (dpm/μL). To evaluate the kinetic parameters for [^{14}C]compound-A uptake, the initial uptake rates were fitted to Eqs. (1) or (2) by means of nonlinear least-squares regression analysis using the MULTI program (16).

$$v = \frac{V_{\max} \cdot S}{K_m + S} \tag{1}$$

$$v = \frac{V_{\max} \cdot S}{K_m + S} + k_{ns} \cdot S \tag{2}$$

where, v *and* V_{max}, represent the initial uptake rate and maximum uptake rate (pmol/oocyte/h or pmol/10^5 cells/15s), respectively, S *and* K_m represent the concentration of [^{14}C]compound-A in the incubation medium and the Michaelis–Menten constant (μM), respectively, and k_{ns} represents the nonsaturable uptake clearance (μL/10^5 cells/15s). All data were expressed as mean ± SEM, and statistical analysis was performed by the use of Student's t-test with $p < 0.05$ as the criterion of significance.

RESULTS

Characterization of Compound-A Uptake by Oatp1a5 Expressed in *Xenopus laevis* Oocytes

To clarify whether compound-A is a substrate for Oatp1a5, the uptake of [^{14}C]compound-A by Oatp1a5-expresseing *Xenopus* oocytes was examined. Because the physiological pH at the luminal surface in the small intestine is weakly acidic (17), the uptake studies were performed at pH 6.0 to mimic the transport properties of compound-A by Oatp1a5 in the intestinal absorption process. The time course of [^{14}C]compound-A uptake by oocytes injected with Oatp1a5 cRNA or water is shown in Fig. 2. The uptake of [^{14}C]compound-A by Oatpt1a5-expressing oocytes was significantly higher than that by water-injected oocytes ($p < 0.01$) at pH 6.0. Since the Oatp1a5-specific uptake of [^{14}C]compound-A increased linearly up to 60 min, the initial uptake rate was evaluated as the uptake at 60 min in the following studies.

Figure 3(A) shows the pH dependence of [^{14}C]compound-A uptake in the range from pH 5.5 to 7.4. The uptake of [^{14}C]compound-A by Oatp1a5-expressing oocytes was significantly higher than that by water-injected oocytes over the entire pH range tested ($p < 0.01$). The Oatp1a5-specific uptake of [^{14}C]compound-A calculated by subtracting its uptake by water-injected oocytes from that by Oatp1a5-expressing oocytes, exhibited pH dependence, and the uptake at acidic pH was higher than that at pH 7.4. Uptake of [^{14}C]compound-A by water-injected oocytes at acidic pH was also higher than that at neutral pH. Because the pKa values of compound-A are 3.0 and 3.7, the increase of [^{14}C]compound-A uptake by water-injected oocytes at acidic pH seems likely to be due to an increase of the nonionic form of compound-A, leading to increased passive diffusion. To further characterize the pH dependent property of the Oatp1a5 transport activity, the effect of pH on the uptake of [^{3}H]estrone-3-sulfate, which is a typical substrate of Oatp1a5, was also examined in the range from pH 5.5 to 7.4. As shown in Fig. 3(B), the uptake of [^{3}H]estrone-3-sulfate by Oatp1a5-expressing oocytes was increased at acidic pH, whereas the uptake by water-injected oocytes was not affected by pH. Oatp1a5-specific uptake of estrone-3-sulfate obtained by subtracting its uptake by water-injected oocytes from that by Oatp1a5-expressing oocytes, at acidic pH was higher than that at pH 7.4.

The concentration dependence of the Oatp1a5-mediated compound-A uptake was examined at pH 6.0 (Fig. 4). The

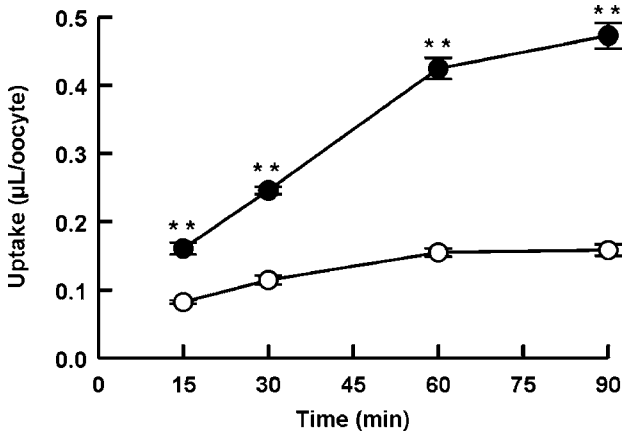

Fig. 2. Time course of compound-A uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of [^{14}C]compound-A (7.2 μM) by *Xenopus* oocytes injected with cRNA of Oatp1a5 (*closed circles*) or water (*open circles*) was measured at pH 6.0 and 25°C. Uptakes are expressed as cell-to-medium ratio. Each result represents the mean±S.E.M. (n=10–13) and ** indicates a significant difference from the uptake by water-injected oocytes (p<0.01).

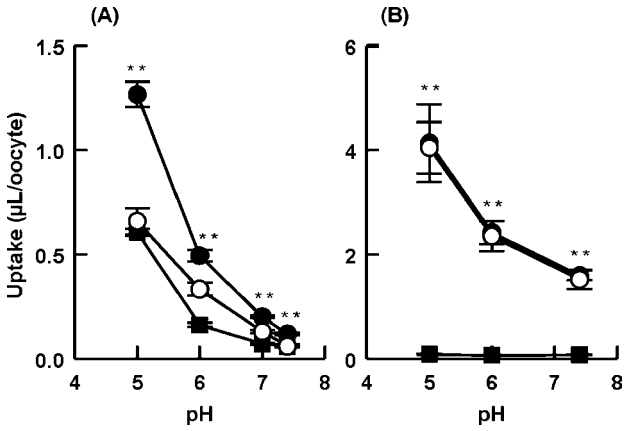

Fig. 3. pH Dependence of uptakes of compound-A (**A**) and estrone-3-sulfate (**B**) by *Xenopus* oocytes expressing Oatp1a5. Uptakes of [^{14}C]compound-A (17 μM) and [^{3}H]estrone-3-sulfate (8 nM) by *Xenopus* oocytes injected with cRNA of Oatp1a5 (*closed circles*) or water (*closed squares*) were measured in the pH range from 5.0 to 7.4 at 60 min and 25°C. *Open circles* represent Oatp1a5-specific uptake obtained by subtraction of the uptake by water-injected oocytes from Oatp1a5-expressing oocytes. Uptakes are expressed as cell-to-medium ratio. Each result represents the mean±S.E.M. (n=8–12) and ** indicates a significant difference from the uptake by water-injected oocyte (p<0.01).

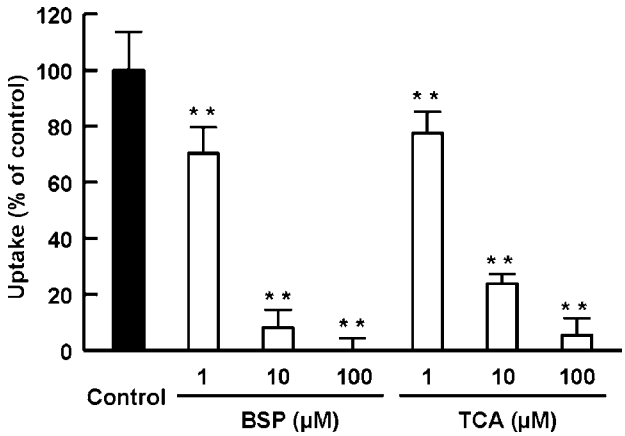

Fig. 5. Inhibitory effects of Oatp1a5 substrates on compound-A uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of [^{14}C]compound-A (9.0 μM) by *Xenopus* oocytes injected with cRNA of oatp1a5 was measured for 60 min at pH 6.0 and 25°C. The results are shown as a percentage of control uptake measured in the absence of Oatp1a5 substrates. Each result represents the mean±S.E.M. (n=9–12). ** indicates a significant difference from the control (p<0.01).

K_m and V_{max} values determined by the nonlinear least-squares regression analysis according to Eq. (1) were 116 ± 44 μM and 74 ± 12 pmol/oocyte/h, respectively.

The inhibitory effects of Oatp1a5 substrates, bromosulfophthalein (BSP) and taurocholic acid (TCA) on Oatp1a5-mediated uptake of [^{14}C]compound-A were evaluated by using Oatp1a5-expressing oocytes. As shown in Fig. 5, BSP and TCA inhibited Oatp1a5-mediated uptake of [^{14}C]compound-A in a concentration dependent manner. At a concentration of 10 μM of BSP and TCA, the uptake was reduced to 8% and 24% of control, respectively.

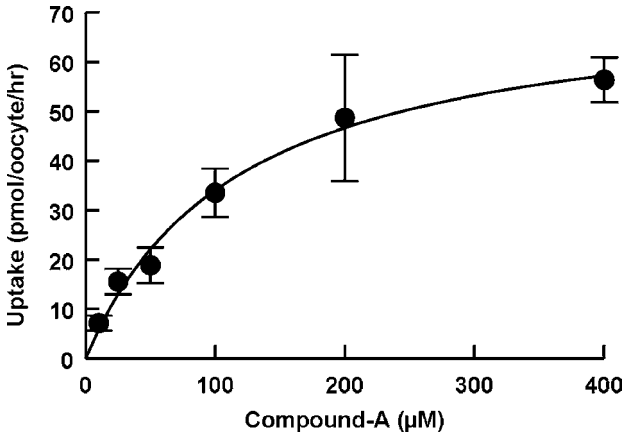

Fig. 4. Concentration dependence of compound-A uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of [^{14}C]compound-A (10–400 μM) was measured for 60 min at pH 6.0 and 25°C. Oatp1a5 specific uptake was obtained by subtraction of the uptake by water-injected oocytes from Oatp1a5-expressing oocytes. Solid line represents the calculated values using the kinetic parameters obtained by nonlinear least-squares analysis. Each result represents the mean±S.E.M (n=5–9).

Characterization of Compound-A Uptake into Enterocytes Isolated from Rats

To elucidate the contribution of Oatp1a5 to uptake of compound-A in enterocytes, the compound-A uptake into isolated enterocytes prepared from rat small intestine was evaluated. Figure 6 shows the time course of [^{14}C]compound-A uptake into enterocytes. The uptake of [^{14}C]compound-A was found to be linear up to 45 s. Therefore, the initial uptake rate was determined at less than 45 s in the following studies.

The effect of pH on [^{14}C]compound-A uptake into enterocytes was evaluated in the range from pH 5.0 to 7.4. As shown in Fig. 7, the uptake of compound-A into isolated

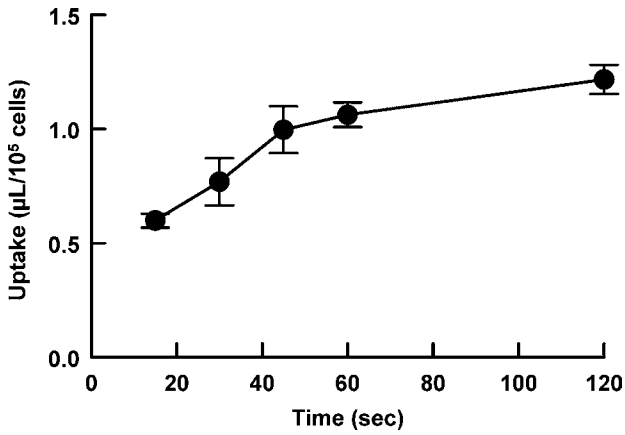

Fig. 6. Time course of compound-A uptake into isolated rat enterocytes. Isolated enterocytes were preincubated for 5 min at 37°C, and uptake of [^{14}C]compound-A (5.6 μM) was then measured at pH 6.0. Data are expressed as cell-to-medium ratio, and each result represents the mean±S.E.M (n=4).

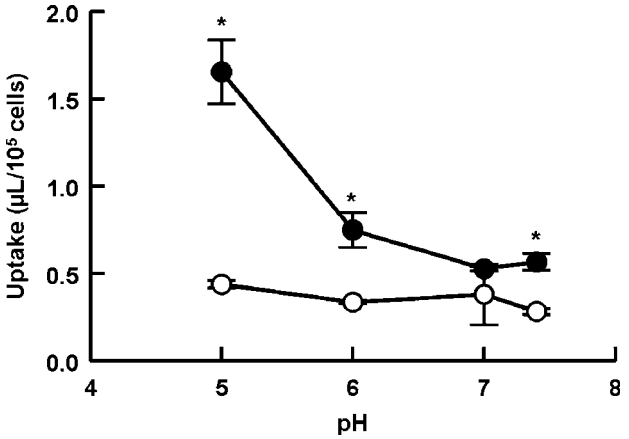

Fig. 7. pH Dependence of compound-A uptake by isolated rat enterocytes. Isolated enterocytes were preincubated for 5 min at 37°C, and uptake of [^{14}C]compound-A (4.6 μM) was then measured for 30 s at pH 5.0–7.4 (*closed circles*). To estimate adsorption and adhesion of compound-A, apparent uptake of [^{14}C]compound-A was measured for 15 s at pH 5.0–7.4 and 4°C in the presence of 800 μM unlabeled compound-A (*open circles*). Data are expressed as cell-to-medium ratio, and each result represents the mean±S.E.M (n=4). * indicates a significant difference from the uptake in the presence of unlabeled compound-A at 4°C (p<0.05).

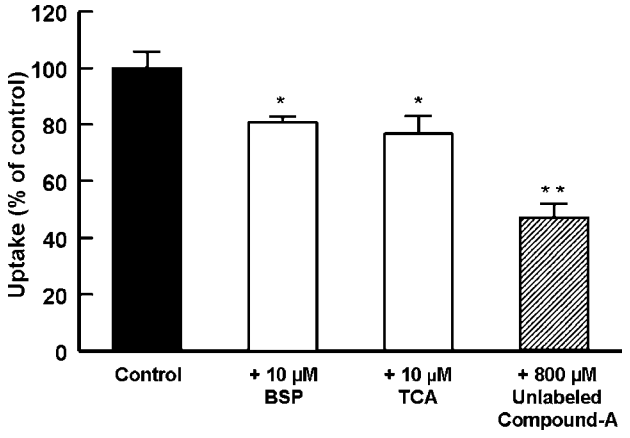

Fig. 9. Inhibitory effects of Oatp1a5 substrates on compound-A uptake into isolated rat enterocytes. Isolated enterocytes were preincubated for 5 min at 37°C, and uptake of [^{14}C]compound-A (4.7 μM) was then measured for 15 s at pH 6.0 in the absence (*control*, *closed column*) or the presence of BSP 10 μM and TCA 10 μM (*open columns*). To estimate adsorption and/or adhesion of compound-A, isolated enterocytes were preincubated for 5 min at 4°C, and then uptake of [^{14}C]compound-A was measured for 15 s at pH 6.0 and 4°C in the presence of 800 μM unlabeled compound-A (*dashed column*). Uptake is expressed as percentage of the control uptake, and each result represents the mean±S.E.M. (n=3). * and ** indicate significant differences from control (p<0.05 and 0.01).

enterocytes tended to be increased at acidic pH, and the uptake at pH 5.0 was significantly higher than that at pH 7.4.

The concentration dependence of [^{14}C]compound-A uptake was evaluated at pH 6.0 in the concentration range from 10 to 400 μM (Fig. 8). The kinetic parameters were determined by nonlinear least-squares regression analysis based on Eq. (2) as follows: K_m, 83 ± 37 μM; V_{max}, 31 ± 15 pmol/10^5 cells/15 s; k_{ns}, 0.31 ± 0.044 μL/10^5 cells/15 s.

To confirm the involvement of Oatp1a5 in the intestinal absorption process of compound-A, the inhibitory effects of BSP and TCA on the compound-A uptake into rat enterocytes were examined. First, to estimate possible adsorption and adhesion of compound-A to cell surface, the uptake of

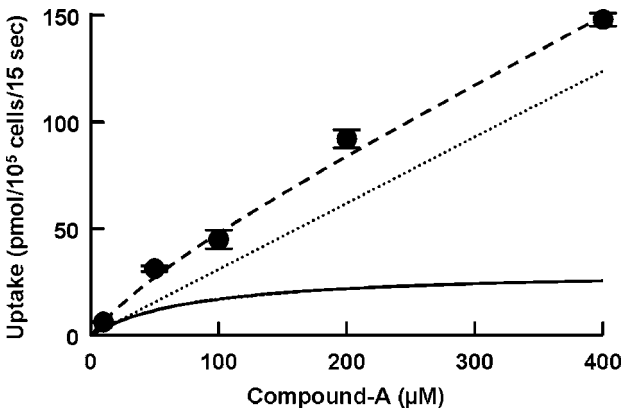

Fig. 8. Concentration dependence of compound-A uptake by isolated rat enterocytes. Isolated enterocytes were preincubated for 5 min at 37°C, and uptake of [^{14}C]compound-A (10–800 μM) was then measured for 15 s at pH 6.0. The dashed and dotted lines represent the total and non-saturable component of the uptake rate calculated from the kinetic parameters obtained by nonlinear last-squares analysis, respectively. Each result represents the mean±S.E.M (n=4).

[^{14}C]compound-A in presence of an excess (800 μM) of unlabeled compound-A at 4°C was evaluated. As a result, the uptake was reduced to approximately 50% of the control, suggesting that nonspecific adsorption to cell surface and passive diffusional uptake account for approximately half of the apparent uptake of compound-A in the experimental method used in the present study (Fig. 9). The affected uptake by an excess of unlabeled compound-A at 4°C was regarded as the carrier-mediated uptake. The carrier-mediated uptake of [^{14}C]compound-A into enterocytes was reduced by BSP and TCA to 58% and 49% of that in absence of inhibitors, respectively, at a concentration of 10 μM that inhibited the Oatp1a5-mediated uptake of compound-A to 8% and 24% of control in oocytes.

DISCUSSION

The present study clearly indicated that Oatp1a5 is involved in the intestinal absorption of compound-A in rats, at least partly, based on the following observations: (1) the uptake of compound-A into isolated enterocytes was greater at acidic pH than at neutral pH, which was consistent with the result obtained from Oatp1a5-expressing oocytes (Figs. 3 and 7); (2) the uptake of compound-A into isolated enterocytes was governed by a saturable component with K_m value which was comparable to that obtained by Oatp1a5-expressing oocytes (Figs. 4 and 8); (3) the uptake of compound-A into isolated enterocytes was inhibited by substrates for Oatp1a5 (BSP and TCA), which was consistent with the results from the study performed in Oatp1a5-expressing oocytes (Figs. 5 and 9).

OATP1A2, OATP2B1 and Oatp1a5 are localized at the apical membrane of epithelial cells in human or rat small intestine, and they could be involved in the intestinal

absorption of acidic compounds (5–10,14). Rat Oatp2b1/Oatp9/moat1 has been regarded as an orthologous to human OATP2B1 and isolated from rat brain (18). However, Oatp2b1 has not been found to be expressed in rat small intestine so far, and Oatp2b1 had little transporting capacity for compound-A (data not shown), suggesting that the contribution of Oatp2b1 to the intestinal absorption of compound-A is unlikely. On the other hand, Oatp1a5 is the only rat Oatp isoform known to be expressed at the apical membrane of intestinal epithelial cells (13), and therefore, we hypothesized in the present study that Oatp1a5 contributes to the uptake of compound-A into enterocytes from small intestinal lumen in rats.

OATP2B1 located at intestinal epithelial apical membrane exhibited pH dependence in its transport activity for acidic compounds (5,6), whereas to our knowledge, such a pH-dependency has not been described for Oatp1a5 mediated uptake. The present study investigated in Oatp1a5-expressing oocytes clearly demonstrated that the uptake by Oatp1a5 is also pH-dependent with an increased uptake activity at acidic pH compared with that at neutral pH (Fig. 3). Because the pKa values of compound-A are 3.0 and 3.7, the increase of compound-A uptake at acidic pH would be partly due to an increase of the nonionic form of compound-A according to the pH partition hypothesis. Indeed the uptake of compound-A by water-injected oocytes increased at acidic pH. However, the Oatp1a5 specific uptake of compound-A also clearly increased at acidic pH (Fig. 3(A)). The Oatp1a5-mediated uptake of estrone-3-sulfate, which is well known as Oatp1a5 substrate, also increased by decreasing pH, whereas the uptake by water-injected oocytes was not affected by pH (Fig. 3(B)). These results suggest that Oatp1a5 optimally plays a role in the acidic condition for these compounds. The physiological pH at the microenvironment in the external surface of small intestinal epithelial cells is acidic, which has been determined to be 5.5–6.0 in human and in laboratory animals (17). The activity of transporters located in the small intestine might be functionally optimal at acidic pH, as shown for other transporters such as OATP2B1, PEPT1, PAT1 or MCT1 (5,6,19–22). In the present study, we demonstrated that the uptake of [^{14}C]compound-A into isolated rat enterocytes increased as the extracellular pH decreased from 7.4 to 5.0 (Fig. 7). Therefore, the transporter(s) including Oatp1a5 is (are) optimally involved in the absorption process of compound-A at the intestinal lumen in rats.

The uptake of [^{14}C]compound-A into isolated rat enterocytes increased with incubation time (Fig. 6), and it was composed of saturable and nonsaturable components (Fig. 8). The K_m value (83 μM) for the uptake of compound-A into enterocytes was similar to that (116 μM) obtained by Oatp1a5-expressing oocytes (Fig. 4), suggesting that Oatp1a5 is contributing to the uptake of compound-A into enterocytes. The relative contributions of the saturable (V_{max}/K_m: 0.37 μL/10^5 cells/15 s) and nonsaturable (k_{ns}: 0.31 μL/10^5 cells/15 s) component were 54% and 46%, respectively. Therefore, Oatp1a5-mediated transport was significantly contributing to the uptake of compound-A into enterocytes at lower substrate concentrations.

To further clarify the contribution of Oatp1a5 to the intestinal absorption of compound-A, the inhibitory effects of BSP and TCA were examined in isolated enterocytes (Fig. 9). The uptake of [^{14}C]compound-A was reduced to approximately 50% of the control in the presence of an excess (800 μM) of unlabeled compound-A at 4°C, suggesting that the carrier-mediated uptake accounts for approximately half of the total uptake of compound-A. The carrier-mediated uptake of compound-A in isolated enterocytes was reduced to 58% and 49% of the control by BSP (10 μM) and TCA (10 μM), respectively, suggesting that BSP and TCA partly share the same transporter(s) as those mediating uptake of compound-A in the enterocytes. BSP and TCA are substrates for Oatp1a5 (13,14), and therefore, the inhibitory effects of BSP and TCA on the uptake of compound-A was likely due to the inhibition of carrier-mediated transport by Oatp1a5. As expected, it was demonstrated that BSP and TCA inhibited the Oatp1a5-mediated uptake of compound-A in a concentration dependent manner in Oatp1a5-expressing oocytes (Fig. 5), and that the Oatp1a5-mediated uptake was reduced to less than 25% in presence of 10 μM BSP or TCA and almost completely reduced in the presence of 100 μM of BSP or TCA.

In conclusion, the uptake studies on compound-A, an orally active nonpeptidic endothelin receptor antagonist, revealed that the uptake of compound-A at acidic pH was higher than that at neutral pH, and that the uptake was inhibited by substrates for Oatp1a5 in isolated rat enterocytes. The characteristics of compound-A uptake observed in isolated rat enterocytes were consistent with those observed in Oatp1a5-expressing oocytes, suggesting that Oatp1a5 is involved in pH dependent carrier-mediated absorption of compound-A in rat small intestine. Further quantitative studies *in vivo* using Oatp1a5-selective inhibitor or Oatp1a5-knockout mouse if they are available, will be required to confirm the significance of Oatp1a5-mediate transport in apparent intestinal absorption of compound-A.

ACKNOWLEDGEMENTS

The Drug Research Academy at University of Copenhagen are acknowledged for financial support, as well as Brorsons travelfund and OTICON foundation are acknowledged for funding external tuition fee and hence enable the collaboration behind this study.

REFERENCES

1. A. Tsuji, and I. Tamai. Carrier-mediated intestinal transport of drugs. *Pharm. Res* **13**:963–977 (1996).
2. T. Katsura, and K. Inui. Intestinal absorption of drugs mediated by drug transporters: mechanisms and regulation. *Drug Metab. Pharmacokinet* **18**:1–15 (2003).
3. I. Tamai, J. Nezu, H. Uchino, Y. Sai, A. Oku, M. Shimane, and A. Tsuji. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem. Biophys. Res. Commun* **273**:251–260 (2000).
4. B. Hagenbuch, and P.J. Meier. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* **447**:653–665 (2004).
5. D. Kobayashi, T. Nozawa, K. Imai, J. Nezu, A. Tsuji, and I. Tamai. Involvement of human organic anion transporting

polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J. Pharmacol. Exp. Ther* **306**:703–708 (2003).
6. T. Nozawa, K. Imai, J. Nezu, A. Tsuji, and I. Tamai. Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J. Pharmacol. Exp. Ther* **308**:438–445 (2004).
7. A. Kikuchi, T. Nozawa, T. Wakasawa, T. Maeda, and I. Tamai. Transporter-mediated intestinal absorption of fexofenadine in rats. *Drug Metab. Pharmacokinet* **21**:308–314 (2006).
8. T. Maeda, K. Takahashi, N. Ohtsu, T. Oguma, T. Ohnishi, R. Atsumi, and I. Tamai. Identification of influx transporter for the quinolone antibacterial agent levofloxacin. *Mol. Pharm* **4**:85–94(2007).
9. G. K. Dresser, D. G. Bailey, B. F. Leake, U. I. Schwarz, P. A. Dawson, D. J. Freeman, and R. B. Kim. Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin. Pharmacol. Ther* **71**:11–20 (2002).
10. H. Glaeser, D. G. Bailey, G. K. Dresser, J. C. Gregor, U. I. Schwarz, J. S. McGrath, E. Jolicoeur, W. Lee, B. F. Leake, R. G. Tirona, and R. B. Kim. Intestinal drug transporter expression and the impact of grapefruit juice in humans. *Clin. Pharmacol. Ther* **81**:362–370 (2007).
11. M. Nishikibe, H. Ohta, M. Okada, K. Ishikawa, T. Hayama, T. Fukuroda, K. Noguchi, M. Saito, T. Kanoh, S. Ozaki, T. Kamei, K. Hara, D. William, S. Kivlighn, S. Krause, R. Gabel, G. Zingaro, N. Nolan, J. O'brien, F. Clayton, J. Lynch, D. Pettibone, and P. Siegl. Pharmacological properties of J-104132 (L-753,037), a potent, orally active, mixed ET_A/ET_B endothelin receptor antagonist. *J. Pharmacol. Exp. Ther* **289**:1262–1270 (1999).
12. N. Kobayashi, T. Tani, A. Hisaka, K. Hara, and T. Yasumori. Hepatobiliary transport of a nonpeptidic endothelin antagonist, (+)-(5S,6R,7R)-2-butyl-7-[2((2S)-2-carboxypropyl)-4-methoxy phenyl]5-(3,4-methylenedioxyphenyl)cyclopentenol[1,2-b]pyridine-6-carboxylic acid: uptake by isolated rat hepatocytes and canalicular membrane vesicles. *Pharm. Res* **20**:89–95 (2003).
13. H. C. Walters, A. L. Craddock, H. Fusegawa, M. C. Willingham, and P. A. Dawson. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol* **279**:G1188–G1200 (2000).
14. V. Cattori, J. E. Van Montfoort, B. Stieger, L. Landmann, D. K. F. Meijer, K. H. Winterhalter, P. J. Meier, and B. Hagenbuch. Localization of organic anion transporting polypeptide 4 (Oatp4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2 and Oatp3. *Pflugers Arch* **443**:188–195 (2001).
15. G. A. Kimmich. Preparation and properties of mucosal epithelial cells isolated from small intestine of the chicken. *Biochemistry* **9**:3659–3669 (1970).
16. K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno. A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**:879–885 (1981).
17. M. Lucus. Determination of acid surface pH *in vivo* in rat proximal jejunum. *Gut* **24**:734–739 (1983).
18. T. Nishio, H. Adachi, R. Nakagomi, T. Tokui, E. Sato, M. Tanemoto, K. Fujiwara, M. Okabe, T. Onogawa, T. Suzuki, D. Nakai, K. Shiiba, M. Suzuki, H. Ohtani, Y. Kondo, M. Unno, S. Ito, K. Iinuma, K. Nunoki, S. Matsuno, and T. Abe. Molecular identification of a rat novel organic anion transporter moat1, which transports prostaglandin D_2, leukotriene C_4, and taurocholate. *Biochem. Biophys. Res. Commun* **275**:831–837 (2000).
19. D. T. Thwaites and C. M. Anderson. H-coupled nutrient, micronutrient and drug transporters in the mammalian small intestine. *Exp. Physiol.* **92**:603–619 (2007).
20. I. Tamai, T. Nakanishi, K. Hayashi, T. Terao, Y. Sai, K. Miyamoto, E. Takeda, H. Higashida, and A. Tsuji. The predominant contribution of oligopeptide transporter PepT1 to intestinal absorption of beta-lactam antibiotics in the rat small intestine. *J. Pharm. Pharmacol* **49**:796–801 (1997).
21. T. Nozawa, S. Sugiura, Y. Hashino, A. Tsuji, and I. Tamai. Role of anion exchange transporter PAT1 (SLC26A6) in intestinal absorption of organic anions. *J. Drug Target* **12**:97–104 (2004).
22. I. Tamai, Y. Sai, A. Ono, Y. Kido, H. Yabuuchi, H. Takanaga, E. Satoh, T. Ogihara, O. Amano, S. Izeki, and A. Tsuji. Immunohistochemical and functional characterization of pH-dependent intestinal absorption of weak organic acids by the monocarboxylic acid transporter MCT1. *J. Pharm. Pharmacol* **51**:1113–1121 (1999).