Functional Characterization of Mouse Organic Anion Transporting Peptide 1a4 in the Uptake and Efflux of Drugs Across the Blood-Brain Barrier

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ABSTRACT:

This study investigated the role of a multispecific organic anion transporter, Oatp1a4/Slco1a4, in drug transport across the blood-brain barrier. In vitro transport studies using human embryonic kidney 293 cells expressing mouse Oatp1a4 identified the following compounds as Oatp1a4 substrates: pitavastatin (Km = 8.3 μM), rosuvastatin (Km = 12 μM), pravastatin, taurocholate (Km = 40 μM), digoxin, ochratoxin A, and [D-penicillamine2,5]-enkephalin. Double immunohistochemical staining of Oatp1a4 with P-glycoprotein (P-gp) or glial fibrillary acidic protein demonstrated that Oatp1a4 signals colocalized with P-gp staining of Oatp1a4 in both the luminal and the abluminal membranes of mouse brain capillary endothelial cells. The brain-to-blood transport of pitavastatin, rosuvastatin, pravastatin, and taurocholate into microinjection into the cerebral cortex was significantly decreased in Oatp1a4−/− mice compared with that in wild-type mice.

The blood-to-brain transport of pitavastatin, rosuvastatin, taurocholate, and ochratoxin A, determined by in situ brain perfusion, was significantly lower in Oatp1a4−/− mice than in wild-type mice, whereas transport of pravastatin and [D-penicillamine2,5]-enkephalin was unchanged. The blood-to-brain transport of digoxin was significantly lower in Oatp1a4−/− mice than in wild-type mice only when P-gp was inhibited by N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl](ethyl)-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918). Taken together, these results show that Oatp1a4 can mediate the brain-to-blood and blood-to-brain transport of its substrate drugs across the blood-brain barrier. The brain-to-plasma ratio of taurocholate, pitavastatin, and rosuvastatin was close to the capillary volume in wild-type mice, and it was not affected by Oatp1a4 dysfunction. Whether Oatp1a4 can deliver drugs from the blood to the brain remains controversial.

Drugs have to cross the blood-brain barrier (BBB) before reaching their target in the central nervous system. The BBB consists of a tight monolayer of brain capillary endothelial cells that are characterized by highly developed tight junctions between adjacent endothelial cells and a paucity of fenestra and pinocytotic vesicles, thereby limiting paracellular transport across the endothelial cells. In addition, xenobiotic transporters act as a barrier by actively effluxing drugs into the blood circulation, thus limiting the transcellular pathway of drugs and other xenobiotic compounds (Kusuhara and Sugiyama, 2005; Hermann and Bassetti, 2007; Ohtsuki and Terasaki, 2007). Indeed, dysfunction of ATP-binding cassette transporters (such as P-gp, BCRP, and multidrug resistance-associated protein 4) results in a significant increase in brain penetration by their substrate drugs (Fomm, 2004; Enokizono et al., 2007, 2008; Ose et al., 2009).

In vivo studies using microinjection into the cerebral cortex have shown that the elimination of diverse amphipathic and hydrophilic organic anions and nucleotide analogs from the cerebral cortex involves saturable mechanisms (Kusuhara and Sugiyama, 2005). Oatp1a4/Slco1a4, a homolog of the hepatic multispecific organic anion transporter Oatp1a1, was cloned from rat brain using homology screening (Noe et al., 1997). Oatp1a4 exhibits broad substrate specificities for amphipathic compounds and has a high affinity for the cardiac glycoside digoxin (Noe et al., 1997; Cattori et al., 2001). Oatp1a4 is abundantly expressed in the brain and liver. Immunohistochemical staining of rat brain has shown that the Oatp1a4 protein is present in both the luminal and the abluminal membranes of the BBB.
(Gao et al., 1999). Because Oatp1a4 mediates cellular uptake, this membrane localization suggests that it may mediate uptake from both the brain and the blood sides. Kinetic analyses of efflux across the BBB in rats after microinjection of Oatp1a4 inhibitors, such as tau-rocholate and digoxin, into the cerebral cortex have indicated that Oatp1a4 plays a major role in the uptake of amphipathic organic anions (including 17β-estradiol glucuronide, pitavastatin, and dehydroepiandrosterone sulfate) across the abluminal membrane and partly accounts for the uptake of pravastatin in the net efflux from the brain to the blood (Asaba et al., 2000; Sugiyama et al., 2001; Kikuchi et al., 2004). Mutual inhibition studies suggest that the uptake of BQ-123 and estrone sulfate is mediated by transporters distinct from Oatp1a4, expressed in the liver, and to a lesser degree in the brain and kidney. This was demonstrated that mOatp1a4 efficiently transports digoxin and BQ-123, although the Km value of digoxin for mOatp1a4 is markedly greater than that for rat Oatp1a4 (Noé et al., 1997; van Montfoort et al., 2002).

Materials and Methods

Chemicals. [3H]Pitavastatin and unlabeled pitavastatin, [3H]rosuvastatin and unlabeled rosuvastatin, and [3H]pravastatin were kindly donated by the Kowa Company Ltd. (Tokyo, Japan), AstraZeneca (Cheshire, UK), and Sankyo (Tokyo, Japan), respectively. [14C]Sucrose and [3H]ochratoxin A were obtained from Moravek Biochemicals (Brea, CA). [1-14C]Carboxyl-inulin, [3H]taurocholate, [3H]dihydroxy- and [3H]PPDFPE were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). GF120918 was a gift from GlaxoSmithKline (Morrisville, NC). GF120918 was a gift from GlaxoSmithKline (Morrisville, NC). A modification of the original binding assay was used for determining the affinity of the receptor for the ligand, where the dissociation constant (Kd) is defined as the concentration of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the medium (micromoles), and Vmax is the maximum uptake rate (picomoles per minute per milligram of protein).


deep-eiandrosterone sulfate), proestrus (E2), and estrone sulfate is mediated by transporters distinct from Oatp1a4, characterized by inhibition by digoxin, verapamil, and fexofenadine (Dagenais et al., 2001).

The role of Oatp1a4 in drug transport at the BBB has only been investigated using Oatp1a4 inhibitors. Thus, the present study was undertaken to investigate the role of Oatp1a4 in drug transport across the BBB using Oatp1a4(−/−) mice. Mouse Oatp1a4 (mOatp1a4) has been cloned (van Montfoort et al., 2002) and found to be abundantly expressed in the liver, and to a lesser degree in the brain and kidney. Functional expression of mOatp1a4 in Xenopus laevis oocytes has demonstrated that mOatp1a4 efficiently transports digoxin and BQ-123, although the Km value of digoxin for mOatp1a4 is markedly greater than that for rat Oatp1a4 (Noé et al., 1997; van Montfoort et al., 2002).

Quantification of mRNA Expression of Various Transporters in the Mouse Cerebral Cortex. The mRNA levels of Oatp1a4, Oatp1c1, Oat3, Mrdr1a, Bcrp, Mrp4, and glyceraldehyde-3-phosphate dehydrogenase were quantified using the real-time PCR method. Total RNA was isolated from the cerebral cortex of each of three mice using ISOGEN (Wako Pure Chemicals, Tokyo, Japan). Real-time PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany). An external standard curve was generated by dilution of the target PCR product, which was purified by agarose gel electrophoresis. The absolute concentration of the external standard was measured using PicoGreen dsDNA Quantification Reagent (Invitrogen).

Isolation of Mouse Brain Capillary-Enriched Fraction. A modification of a method described previously was used for capillary isolation (Ball et al., 2002). In brief, the cortex was homogenized in 0.32 M sucrose (1 g of brain/20 ml of sucrose) using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). The homogenate was centrifuged at 4°C for 10 min at 2200g, and the resulting pellet was suspended in 25% BSA and centrifuged at 4°C for 10 min at 2200g. The supernatant was decanted, and the pellet was washed three times with buffer (10 mM Tris-HCl and 0.5 mM dithiothreitol, pH 7.6). The purity of the brain capillary-enriched fraction was confirmed by α-GTP activity (Goldstein et al., 1975).

Western Blot Analysis. Crude membranes from liver homogenates and brain capillaries were prepared as follows. Liver homogenates and brain capillaries were centrifuged at 4°C for 15 min at 2000g, the supernatant was collected and centrifuged at 4°C for 15 min at 100,000g, and the resulting pellet was resuspended in PBS containing 0.1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and stored at −80°C until used. The protein concentration was measured using the method of Lowry et al. (1951). The

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v = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]
specimens were loaded onto an 8.5% SDS-polyacrylamide gel electrophoresis with a 3.75% stacking gel. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Pall, Port Washington, NY), and the membrane was then blocked for 1 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 2.5% ECL Advance Blocking Agent (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membrane was washed with TTBS and incubated overnight at 4°C with rabbit anti-mOatp1a4 antiseraum, which was raised in rabbits against a synthetic peptide consisting of the 20 carboxyl-terminal amino acids of mOatp1a4 (CTNVHRSPTR-MQNDGERKKTL) keyhole limpet hemocyanin conjugate (1:1000 in TTBS containing 2.5% ECL Advance Blocking Agent). Detection was performed by binding to horseradish peroxidase-labeled anti-rabbit IgG antibody (1:5000 in TTBS containing 2.5% ECL Advance Blocking Agent; GE Healthcare). Immunoreactivity was determined with an ECL Advance Western Blotting Detection Kit (GE Healthcare). Antigen adsorption was performed by incubating rabbit anti-mOatp1a4 antiseraum with mOatp1a4 antigen (50 μg/ml) at 4°C overnight. After the membrane was stripped with Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL), it was incubated with the antigen-adsorbed anti-mOatp1a4 antiseraum.

Identification of Membrane Localization of Oatp1a4 in the BBB by Immunohistochemical Staining. Frozen sections of the brain were prepared from C57BL/6j wild-type and Oatp1a4 (+/−) mice by GenoStaff (Tokyo, Japan). Mouse brain specimens were embedded in OTC compound and frozen by dry ice, and 8-μm-thick sections were sliced by cryostat. Before staining, the sections were pretreated by heat-induced epitope retrieval and fixed to glass slides in methanol (−20°C). The sections were incubated in 1% Triton X-100 for 30 min at room temperature, washed three times with PBS, and then incubated in PBS containing 5% bovine serum albumin (BSA-PBS) to block nonspecific protein binding. The sections were then washed three times with PBS and incubated overnight at 4°C in a 1:50 dilution of rabbit anti-mOatp1a4 antiseraum and a 1:40 dilution of monoclonal anti-P-gp antibody (C219, Signet Laboratories, Dedham, MA), or a 1:20 dilution of rabbit anti-mOatp1a4 antiseraum and a 1:200 dilution of mouse monoclonal anti-GFAP antibody (Millipore, Billerica, MA) in BSA-PBS. After incubation, the sections were washed three times with PBS and then were incubated for 1 h at room temperature in a 1:250 dilution of secondary antibodies [Alexa 594 anti-rabbit IgG and Alexa 488 anti-mouse IgG (Invitrogen); or Alexa 488 anti-rabbit IgG and Alexa 594 anti-mouse IgG] and TO-PRO-3 iodide (Invitrogen) in BSA-PBS. The sections were mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA) and were viewed under a confocal laser microscope [Zeiss LSM 510 apparatus (Carl Zeiss, Thornwood, NY) or Leica TCS-SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany)].

Determination of Blood-to-Brain Transport of Oatp1a4 Substrates Across the BBB Using Microinjection Technique to the Cerebral Cortex. The efflux of test compounds from the brain after microinjection into the cerebral cortex was investigated using the brain efflux index (BEI) method as described previously (Sugiyma et al., 2003). Tritium-labeled compounds (70 nCi/mouse) with the nonpermeable reference compound [14C]carboxyl-inulin (2 nCi/mouse) in 0.5 μl of ECF buffer (122 mM NaCl, 25 mM NaHCO3, 1.0 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, and 10 mM HEPES, pH 7.4) were injected into the S2 region (4.5 mm lateral to the bregma, 2.5 mm in depth). After intracebral microinjection, mice were decapitated, and the radioactivity that remained in the ipsilateral cerebrum was determined. The brain was homogenized with a 4-fold volume of phosphate-buffered saline to obtain a 20% brain homogenate. Plasma specimens (10 μl) were mixed with 40 μl of ethanol, and brain homogenates (100 μl) were mixed with 400 μl of ethanol. All of these mixed solutions were centrifuged at 15,000g for 10 min. The supernatants of the plasma specimens were mixed with an equal volume of water and subjected to LC-MS analysis. The supernatants of the brain specimens were mixed with an equal volume of water and subjected to LC-MS analysis. The supernatants of the brain specimens were centrifuged at 15,000g for 10 min, and an aliquot of the supernatant was subjected to LC-MS analysis.

An LC-MS-2010EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. Samples for pitavastatin were sepa-
rated on an L-column ODS (5 μm, 2.1 × 150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan) in isocratic mode at a flow rate of 0.3 mL/min, and those for rosuvastatin were separated on a CAPCELL PAK C18 MGII column (3 μm, 2 mm × 50 mm; Shiseido, Tokyo, Japan) in binary gradient mode at a flow rate of 0.4 mL/min. The composition of the mobile phase for pitavastatin was acetonitrile-0.05% formic acid (40:60), and the total run time was 5 min. As for rosuvastatin, acetonitrile and ammonium acetate (10 mM) were used for the mobile phase, and the acetonitrile concentration was initially 20%, increased linearly to 50% over 2 min, kept at 50% over 1 min, and finally reequilibrated at 20% for 3 min. Pitavastatin and rosuvastatin were eluted at 3.7 and 3.2 min, respectively.

In the mass analysis, pitavastatin and rosuvastatin were detected at mass/charge ratios of 422.15 and 482.15, respectively, under positive electrospray ionization conditions. The interface voltage was −3.5 kV, and the nebulizer gas (N2) flow was 1.5 L/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

Statistical Analysis. Data are presented as the mean ± S.E. for three to seven animals, unless otherwise specified. A Student’s two-tailed unpaired t test and one-way analysis of variance followed by Tukey’s multiple comparison test were used to identify significant differences among groups, where appropriate. Statistical significance was set at P < 0.05. The mean differences of the elimination rate constant for elimination of the compounds from the brain (k_{e}) between wild-type mice and Oatpl4a(−/−) mice with 95% confidence intervals were used for detection of significant difference. When the 95% confidence interval did not cross 0, the result was regarded as significant.

Results

Time Profiles and Concentration Dependence of the Uptake of Various Compounds by mOatpl4a-Transfected HEK293 Cells. The time profiles of the uptake of pitavastatin, rosuvastatin, pravastatin, taurocholate, digoxin, ochratoxin A, and DPDPE by mock- and mOatpl4a-HEK293 cells are shown in Fig. 1. For each of the compounds the mOatpl4a-HEK293 cells accumulated more than the mock-transfected cells. The specific uptake of pitavastatin and rosuvastatin by mOatpl4a was determined at 0.5 and 2 min, respectively, because the initial velocity was linear during these periods. The concentration dependencies were examined for pitavastatin, rosuvastatin, and taurocholate by mOatpl4a-HEK293 cells. The uptake of [3H]pitavastatin (0.1 μM) (A), [3H]rosuvastatin (0.1 μM) (B), [3H]pravastatin (0.1 μM) (C), [3H]taurocholate (1 μM) (D), [3H]digoxin (0.05 μM) (E), [3H]ochratoxin A (0.02 μM) (F), and [3H]DPDPE (0.005 μM) (G) by mOatpl4a-transfected HEK293 cells at 37°C was examined. ●, ○, ●, ○, ●, ○, and ●, ○, ●, ○ by mOatpl4a-transfected HEK293 cells and vector-control (mock-transfected) cells, respectively. Each point represents the mean ± S.E. n = 3–4.

mRNA Expression of Xenobiotic Transporters in the Cerebral Cortex of Wild-Type and Oatpl4a(−/−) Mice. The mRNA expression levels of transporters in the cerebral cortex of wild-type and Oatpl4a(−/−) mice were determined using real-time PCR to check the adaptive regulation of transporters of Oatpl4a(−/−) mice at the BBB. In Oatpl4a(−/−) mice, the mRNA expression level of Oatpl4a was reduced compared with that of wild-type mice (Table 1). Quantification of mRNA by real-time PCR showed that there were no significant differences between wild-type and Oatpl4a(−/−) mice in the mRNA expression levels of Oatpl1c1, Oat3, Mdr1a, Bcrp, and Mrp4 in the cerebral cortex (Table 1).

Genotyping of Oatpl4a(−/−) Mice. Genotyping was performed by PCR analysis using GS and Neo primers. PCR with GS (E) and GS (T, E) primers produces a fragment of 232 bp in wild-type and heterozygous mice, whereas PCR with GS (T, E) + Neo (T) primers produces a fragment of 476 bp in heterozygous and Oatpl4a(−/−) mice (Fig. 3A). The Oatpl4a(−/−) mice were fertile and exhibited no obvious abnormalities.

Protein Expression and Membrane Localization of Oatpl4a in Mouse Brain Capillaries. The protein expression of Oatpl4a in the mouse brain capillaries, liver, and choroid plexus was determined by Western blot analysis. An antisera against mOatpl4a detected a protein of approximately 83 kDa in the brain capillary-enriched fraction and a protein of approximately 76 kDa in the liver and choroid plexus (Fig. 3B). The difference in the molecular mass was probably accounted for by differences in the degree of glycosylation. These signals were abolished after preabsorption of the mOatpl4a antisera with the antigen, indicating that they were specific for the antigen peptide (Fig. 3B). The membrane localization of Oatpl4a in mouse brain capillary endothelial cells was examined by immunohistochemical staining using frozen sections of mouse brain, with P-gp used as a marker of the luminal side of the brain capillaries and GFAP used as an astrocyte marker. The Oatpl4a signal (Fig. 3C, red) overlapped with the P-gp signal (Fig. 3C, green) on one side of the nucleus (Fig. 3C, blue). Furthermore, the Oatpl4a signals (Fig. 3D, green) were detected between the nucleus (Fig. 3D, blue) and GFAP signals (Fig. 3D, red) in the cross section. These results indicate that Oatpl4a is expressed in both the luminal and the abluminal membranes in mouse brain capillary endothelial cells. However, no signals were detected.

Fig. 1. Time profiles of the uptake of various compounds by mOatpl4a-transfected HEK293 cells. The uptake of [3H]pitavastatin (0.1 μM) (A), [3H]rosuvastatin (0.1 μM) (B), [3H]pravastatin (0.1 μM) (C), [3H]taurocholate (1 μM) (D), [3H]digoxin (0.05 μM) (E), [3H]ochratoxin A (0.02 μM) (F), and [3H]DPDPE (0.005 μM) (G) by mOatpl4a-transfected HEK293 cells at 37°C was examined. ●, ○, ●, ○, ●, ○, and ●, ○, ●, ○ by mOatpl4a-transfected HEK293 cells and vector-control (mock-transfected) cells, respectively. Each point represents the mean ± S.E. n = 3–4.
cerebral cortex are shown in Fig. 4, and the elimination rate constants ($k_d$) are summarized in Table 2. The 95% confidence interval of the mean differences of $k_d$ between wild-type mice and Oatp1a4(-/-) mice did not cross 0 (Table 2); the elimination of these compounds from the cerebral cortex after microinjection was therefore significantly delayed in Oatp1a4(-/-) mice compared with wild-type mice with statistical significance.

**BBB Permeability to Oatp1a4 Substrates Determined by in Situ Brain Perfusion in Wild-Type and Oatp1a4(-/-) Mice.** The brain uptake of pitavastatin (0.1–100 µM), rosuvastatin (0.1 µM), pravastatin (0.1 µM), taurocholate (1 µM), ochratoxin A (0.1 µM), and DPDPE (0.1 µM) was evaluated using an in situ brain perfusion technique in wild-type and Oatp1a4(-/-) mice. The uptake of pitavastatin by the brain was saturable, and this component accounted for the major part of the total uptake in wild-type mice (Fig. 5A). The brain uptake clearances of pitavastatin, rosuvastatin, pravastatin, taurocholate, and ochratoxin A were 57 ± 9, 24 ± 4, 11 ± 2, 11 ± 0, and 24 ± 3 µL/min/g brain, respectively, in wild-type mice (Fig. 5, A–E), whereas clearance of DPDPE was negligible (0.091 ± 0.091 µL/min/g brain). The uptake clearance of pitavastatin, rosuvastatin, taurocholate, and ochratoxin A was significantly reduced in Oatp1a4(-/-) mice (15 ± 4, 7.8 ± 1.6, 0.57 ± 0.37, and 10 ± 2 µL/min/g brain, respectively), whereas that of pravastatin was unchanged (Fig. 5). A similar brain vascular space was observed in wild-type mice (10 ± 2 µL/g brain) and Oatp1a4(-/-) mice (8.9 ± 1.6 µL/g brain) in this section.

**Effect of GF120918 on Brain Uptake of Digoxin in Wild-Type and Oatp1a4(-/-) Mice.** The brain uptake of digoxin (0.1 µM) was determined using an in situ brain perfusion technique in wild-type and Oatp1a4(-/-) mice treated with or without GF120918. The brain uptake clearance of digoxin was very low and similar in wild-type and Oatp1a4(-/-) mice. Wild-type and Oatp1a4(-/-) mice predosed with the P-gp inhibitor GF120918 before brain perfusion showed significantly increased brain uptake of digoxin (Fig. 6). The brain uptake of digoxin was significantly reduced in Oatp1a4(-/-) mice only when the brain efflux function of P-gp was prohibited by GF120918 (Fig. 6). The brain vascular spaces in wild-type mice and Oatp1a4(-/-) mice were 9.7 ± 2.5 and 7.8 ± 1.9 µL/g brain, respectively, and were unchanged by pretreatment with GF120918 (7.8 ± 1.3 and 7.0 ± 2.0 µL/g brain, respectively).

**Brain/Plasma Ratio of [3H]Taurocholate, Pitavastatin, and Rosuvastatin Between Wild-Type and Oatp1a4(-/-) Mice.** [3H]Taurocholate was selected to examine the effect of Oatp1a4 dysfunction on the in vivo uptake by the brain. The brain/plasma ratio of [3H]taurocholate determined at 10 min after the start of the infusion was 7.6 ± 0.6 µg/g brain (n = 7) in wild-type mice and was unchanged in Oatp1a4(-/-) mice (6.2 ± 0.9 µg/g brain, n = 5). Because of its rapid elimination from the systemic circulation, [3H]taurocholate is not a suitable compound to examine the effect of Oatp1a4 dysfunction on the brain concentration after longer exposure. Instead, pitavastatin and rosuvastatin were used as probes. There was no difference in the brain/plasma ratio of pitavastatin and rosuvastatin (microliters per gram of brain) determined at 120 min after the start of the infusion between wild-type and Oatp1a4(-/-) mice: 22 (19 and 26, n = 2) versus 16 (20 and 12, n = 2) and 8.3 (5.6 and 11, n = 2) versus 9.0 (7.9 and 10, n = 2), respectively.

**Discussion**

A number of studies have suggested that the Oatp transporter family mediates the cellular uptake from the brain side and the blood side of the BBB (Dagenais et al., 2001; Sugiyama et al., 2001; Kikuchi et al., 2004; Taogoshi et al., 2005; Ohtsuki et al., 2007). In the

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**TABLE 1**

<table>
<thead>
<tr>
<th>Transporter/Gapdh × 100</th>
<th>Ratio Oatp1a4(-/-)/Wild Type</th>
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<tr>
<td>Wild-Type</td>
<td>Oatp1a4(-/-)</td>
</tr>
<tr>
<td>Oatp1a4</td>
<td>30.3 ± 3.1</td>
</tr>
<tr>
<td>Oatp1c1</td>
<td>1.69 ± 0.10</td>
</tr>
<tr>
<td>Oat3</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>Mdr1a</td>
<td>0.312 ± 0.026</td>
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<tr>
<td>Bcrp</td>
<td>0.906 ± 0.080</td>
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<tr>
<td>Mrp4</td>
<td>4.25 ± 0.08</td>
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*Gapdh, glyceraldehyde-3-phosphate dehydrogenase.*

by mOatp1a4 antisemur in Oatp1a4(-/-) mice brains, showing the specificity of the signals detected by mOatp1a4 antisemur (Fig. 3C).

**Elimination Rates of Pitavastatin, Rosuvastatin, Pravastatin, and Taurocholate from the Brain Across the BBB in Wild-Type and Oatp1a4(-/-) Mice.** The time profiles of the amount of pitavastatin, rosuvastatin, pravastatin, and taurocholate remaining in the brain of wild-type and Oatp1a4(-/-) mice after microinjection into the brain.

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**Fig. 2.** Concentration dependence of the uptake of [3H]pitavastatin (A), [3H]rosuvastatin (B), and [3H]taurocholate (C) by mOatp1a4-transfected HEK293 cells. The uptake of [3H]pitavastatin (A), [3H]rosuvastatin (B), and [3H]taurocholate (C) was determined at various substrate concentrations: pitavastatin, 0.5 to 50 µM; rosuvastatin, 0.5 to 50 µM; and taurocholate, 5 to 500 µM. Mock- and mOatp1a4-HEK293 cells were incubated for 0.5 min (A and B) and 2 min (C) at 37°C to determine the cellular accumulation. Specific uptake was determined by subtracting the uptake by mock-HEK293 cells from that by mOatp1a4-HEK293 cells, fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. n = 3.
present study, the role of Oatp1a4 in the brain-to-blood and blood-
to-brain transport of drugs across the BBB was investigated using
Oatp1a4/−/− mice.

In vitro transport studies using HEK293 cells expressing mOatp1a4
were performed to identify Oatp1a4 substrates. Anionic drugs (includ-
ing pitavastatin, rosuvastatin, pravastatin, and ochratoxin A), a bile
acid (taurocholate), a peptide drug (DPDPE), and a cardiac glycoside
(digoxin) were all Oatp1a4 substrates (Fig. 1). Among the drugs
tested, pitavastatin exhibited the greatest transport activity. The
Km values of pitavastatin, taurocholate, and rosuvastatin were deter-
mined (Fig. 2), and those for the former two were found to be similar to those determined in rOatp1a4 (Abe et al., 1998; Kikuchi et al., 2004).

Western blot analysis elucidated the fact that the molecular mass of
Oatp1a4 was greater in the brain capillary-enriched fraction than that
in the liver and choroid plexus (Fig. 3B). To identify membrane loca-
ization of mOatp1a4 in brain sections from wild-type (+/+ and Oatp1a4(−/−) mice (d). P-gp was used as a marker of luminal expression: red, Oatp1a4;
green, P-gp; blue, nuclei stained with TO-PRO-3. Scale bar, 10 μm (a–c) and 20 μm (d). D, double immunostaining of Oatp1a4 with GFAP in brain sections from wild-type mice. Top panels show the en face image and the bottom panes show the vertical image. The white rigid line in the upper panel indicates the site where the vertical image was obtained. Arrow and arrowhead, luminal and abluminal localization of Oatp1a4, respectively. GFAP was used as a marker of astrocyte: green, Oatp1a4; red, GFAP; blue, nuclei stained with TO-PRO-3. Scale bar, 5 μm.
The elimination rate constant for elimination of the compounds from the brain \( (k_e) \) was obtained by fitting the 100 - BEI (percent) versus time data. The data used for the calculation are shown in Fig. 4. Data represent mean ± computer-calculated S.D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elimination Rate Constant ( (k_e) )</th>
<th>95% Confidence Interval: Wild-Type ( \text{Out}p1a4(-/-) )</th>
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<tr>
<td>Pitavastatin</td>
<td>0.015 ± 0.002</td>
<td>0.0031 ± 0.0011, 0.0060-0.018</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.17 ± 0.02</td>
<td>0.068 ± 0.014, 0.056-0.14</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.019 ± 0.002</td>
<td>0.0086 ± 0.0011, 0.0060-0.014</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>0.021 ± 0.002</td>
<td>0.0042 ± 0.0017, 0.011-0.022</td>
</tr>
</tbody>
</table>

Fig. 4. Time profiles of \[^{3}H\]pitavastatin (A), \[^{3}H\]rosuvastatin (B), \[^{3}H\]pravastatin (C), and \[^{3}H\]taurocholate (D) remaining in the ipsilateral cerebrum after intracerebral microinjection. Remaining radioactive compounds in the cerebral cortex of wild-type (○) and \( \text{Out}p1a4(-/-) \) mice (■) were determined using the BEI method. ——, fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. \( n = 3-4 \). Statistically significant differences between wild-type and \( \text{Out}p1a4(-/-) \) mice: *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

Fig. 5. Comparison of brain uptake of \[^{3}H\]digoxin by the cerebral cortex in wild-type and \( \text{Out}p1a4(-/-) \) mice with or without predosing with GF120918. GF120918 (10 mg/kg and injection volume of 3.3 ml/kg; dissolved in a 3:2 mixture of propylene glycol-water) was injected intravenously into mice 15 min before the perfusion. The uptake clearance was determined by an in situ brain perfusion technique in wild-type mice (○) and \( \text{Out}p1a4(-/-) \) mice (■). The concentration of \[^{3}H\]digoxin in the perfusate was 0.1 µM. Each bar represents the mean ± S.E. \( n = 3 \). Statistical significance was assessed by one-way analysis of variance followed by Tukey’s multiple comparison test. ***, \( P < 0.01 \), significantly different uptake between wild-type and \( \text{Out}p1a4(-/-) \) mice predosed with GF120918. †††, \( P < 0.001 \), significantly different uptake with or without predosing with GF120918.

Fig. 6. Comparison of brain uptake of \[^{3}H\]digoxin by the cerebral cortex in wild-type and \( \text{Out}p1a4(-/-) \) mice with or without predosing with GF120918. There was no marked change in the expression of other xenobiotic transporters in the cerebral cortex, at least at the mRNA level (Table 1). Therefore, it is most likely that the alteration in drug transport across the BBB observed in \( \text{Out}p1a4(-/-) \) mice was due to a lack of \( \text{Out}p1a4 \) in the BBB. The role of \( \text{Out}p1a4 \) in drug transport across the BBB in the brain-to-blood and blood-to-brain directions was investigated using microinjection into the cerebral cortex and in situ brain perfusion, respectively, in wild-type and \( \text{Out}p1a4(-/-) \) mice. Considering the difference in the intensities, we speculate that \( \text{Out}p1a4 \) is more abundantly expressed in the luminal membrane than in the abluminal membrane in mouse brain. This pattern is consistent with human brain in which OATP1A2, corresponding to \( \text{Out}p1a4 \) in humans (72% homology at the amino acid level), was detected only in the luminal membrane by immunohistochemical staining (Bronger et al., 2005) but not with the recent data in which \( \text{Out}p1a4 \) protein was exclusively recovered in the abluminal membrane.
Considering the high plasma protein binding of the compounds tested and short transit time of the blood circulation through the brain, re-uptake of these organic anions effluxed into the blood circulation would be negligible (so-called sink condition). The brain-to-blood transport of the four Oatp1a4 substrates tested across the BBB was significantly decreased in Oatp1a4(-/-) mice compared with that in wild-type mice (Fig. 4). The impact of Oatp1a4 dysfunction was profound for pitavastatin and taurocholate, whereas that for rosu- vastatin and pravastatin was moderate (Table 2). Because the transport activity of rosvastatin by mOat1 is considerably higher than that for taurocholate and pravastatin (Fig. 1), the impact of Oatp1a4 dysfunction on the brain-to-blood transport does not depend solely on Oatp1a4 activity. In a previous study, rOat3 was suggested to mediate the brain-to-blood transport of pravastatin in rats (Kikuchi et al., 2004). mOat3 is also expressed on the abluminal membrane of mouse brain capillaries (Ohtsuki et al., 2004), which may account for the discrepancy.

The impact of Oatp1a4 dysfunction on the blood-to-brain transport was also investigated using Oatp1a4(-/-) mice (Fig. 5). In wild-type mice, saturation was observed in the blood-to-brain transport of pitavastatin (Fig. 5A). The saturable transport of pitavastatin was probably diminished in Oatp1a4(-/-) mice because the blood-to-brain transport of pitavastatin determined at the highest concentration in wild-type mice was similar to that in Oatp1a4(-/-) mice (Fig. 5A). Namely, the saturable mechanisms is ascribed to Oatp1a4-mediated transport. In addition, the blood-to-brain transport of rosu- vastatin, taurocholate, and ocrhrotaxin A was also significantly re-duced in Oatp1a4(-/-) mice, whereas that of pravastatin was not (Fig. 5, B–E). The absence of an effect of Oatp1a4 dysfunction on the brain uptake of pravastatin seems reasonable, considering that pravastatin has the lowest transport activity among the Oatp1a4 substrates tested (Fig. 1). It is worth mentioning that the uptake of pitavastatin, rosvu- statin, ocrhrotaxin A, and pravastatin remaining in Oatp1a4(-/-) mice is greater than that of taurocholate, suggesting involvement of other transporters(s) to the blood-to-brain transport of these compounds. In con-trast, the brain uptake of DPDPE was almost negligible in wild-type mice, whereas DPDPE exhibits moderate transport activity in Oatp1a4-HEK393 cells (Fig. 1G). This discrepancy will be accounted for by P-gp-mediated efflux (Dagenais et al., 2001). Such cooperation of Oatp1a4 and P-gp was observed for digoxin in this study. The blood-to-brain transport of digoxin was significantly lower in Oatp1a4(-/-) mice compared with that in wild-type mice only with GF120918 pretreatment (Fig. 6). GF120918 is a dual inhibitor of P-gp and Bcrp (Jonker et al., 2000; Lentz et al., 2000). Considering the fact that digoxin is not a BCRP substrate (Pavek et al., 2005), the effect of GF120918 is ascribed to the inhibition of P-gp.

Taken together, these results indicate that Oatp1a4 mediates both the blood-to-brain and brain-to-blood transport of amphipathic organic anions across the BBB. Oatp1a4 is an active uptake transporter for organic anions, but it is also known to mediate the efflux down the electrochemical potential (Li et al., 2000). The fact that Oatp1a4 is expressed both in the luminal and abluminal membranes suggests the possibility that Oatp1a4 mediates the uptake and/or subsequent efflux process in the transcellular transport across the BBB. Because the present study determined the overall transport across the BBB, the critical step mediated by Oatp1a4 could be identified.

It was previously shown that administration of lovastatin and prav-astatin reduced total β-amyloid peptides in the brain of TgCRND8 mice expressing mutant human amyloid precursor protein (Chauhan et al., 2004) and that administration of valsartan reduced Alzheimer’s disease-type neuropathology and also attenuated the development of β-amyloid-mediated cognitive deterioration in Tg2576 mice, a mouse model of Alzheimer’s disease (Wang et al., 2007). It is of great concern that Oatp1a4 could deliver anionic drugs to the brain. The brain/plasma ratio of [3H]taurocholate, pitavastatin, and rosvastatin after intravenous infusions, the blood-to-brain transport of which was significantly decreased in Oatp1a4(-/-) mice (Fig. 5), were very low despite Oatp1a4-mediated transport. Their distribution volumes in the brain were very close to those in the capillary space in wild-type mice, and the impact of Oatp1a4 dysfunction was below the limit of detect- 

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**References**


