Multiple Human Isoforms of Drug Transporters Contribute to the Hepatic and Renal Transport of Olmesartan, a Selective Antagonist of the Angiotensin II AT1-Receptor


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ABSTRACT:

Olmesartan, a novel angiotensin II AT1-receptor antagonist, is excreted into both bile and urine, with minimal metabolism. Because olmesartan is a hydrophilic anionic compound, some transporters could be involved in its hepatic and renal clearance. In this study, we characterized the role of human drug transporters in the pharmacokinetics of olmesartan and determined the contribution of each transporter to the overall clearance of olmesartan. Olmesartan was significantly taken up into human embryonic kidney 293 cells expressing organic anion-transporting polypeptide (OATP) 1B1, OATP1B3, organic anion transporter (OAT) 1, and OAT3. We also observed its saturable uptake into human hepatocytes and kidney slices. Estimated from the relative activity factor method and application of specific inhibitors, the relative contributions of OATP1B1 and OATP1B3 to the uptake of olmesartan in human hepatocytes were almost the same, whereas OAT3 was predominantly involved in its uptake in kidney slices. The vectorial transport of olmesartan was observed in OATP1B1/multidrug resistance-associated protein (MRP) 2 double transfectants, but not in OATP1B1/multidrug resistance (MDR) 1 and OATP1B1/ breast cancer resistance protein (BCRP) transfectants. ATP-dependent transport into membrane vesicles expressing human MRP2 and MRP4 was clearly observed, with $K_m$ values of 14.9 and 26.2 μM, respectively, whereas the urinary excretion of olmesartan in Mrp4-knockout mice was not different from that of control mice. We also investigated the transcellular transport of olmesartan medoxomil, a prodrug of olmesartan. Vectorial basol-to-apical transport was observed in OATP1B1/MDR2, OATP1B1/MDR1 double, and OATP1B1/BCRP double transfectants, suggesting the possible involvement of MRP2, MDR1, and BCRP in the limit of intestinal absorption of olmesartan medoxomil. From these results, we suggest that multiple transporters make a significant contribution to the pharmacokinetics of olmesartan and its prodrug.

Olmesartan is a newly developed angiotensin II receptor antagonist for the treatment of hypertension, acting on AT1 receptors expressed in vessel walls, proximal renal tubules, and adrenal glands (Timmermans et al., 1993). Because its pharmacological target, an AT1 receptor, is expressed throughout the whole body, the area under the plasma concentration-time curve should be one of the determinants of its pharmacological effects (Nakagomi-Hagihara et al., 2006). Therefore, it is important to understand the clearance mechanism of olmesartan when considering its pharmacological effects.

Olmesartan is orally administered in the prodrug form, olmesartan medoxomil. Although we do not know exactly how much orally administered olmesartan medoxomil is absorbed via the small intestine as the prodrug, MRP2, MDR1, and BCRP may interfere with the absorption of olmesartan medoxomil and subsequently reduce the bioavailability of olmesartan. Olmesartan medoxomil is thought to be converted rapidly to olmesartan in the gastrointestinal tract after oral administration (Nakagomi-Hagihara et al., 2006). It has been reported that hepatic clearance accounts for about 60% of the total clearance of

ABBREVIATIONS: MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; CCK-8, cholecystokinin octapeptide; E1S, estrone-3-sulfate; E217G, estradiol-17β-glucuronide; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PAH, p-aminohippurate; PCG, benzylpenicillin; RAF, relative activity factor.
olmesartan (Laeis et al., 2001; Schwocho and Masonson, 2001). Olmesartan is not metabolized, and its hepatic clearance is much less than the hepatic blood flow (Laeis et al., 2001; Schwocho and Masonson, 2001), with most of the drug passing into the bile in an unmetabolized form. The rest of the administered olmesartan is recovered in the urine (Laeis et al., 2001; Schwocho and Masonson, 2001). In the renal excretion of olmesartan, some active transport mechanisms may be involved because its renal clearance is greater than the clearance by glomerular filtration. Because olmesartan is a hydrophilic compound with a logD value (at pH 7.0) of ~1.2 (Nakagomi-Hagihara et al., 2006) and has an anionic carboxyl group, it cannot easily penetrate the plasma membrane. Therefore, some drug transporters are probably involved in the pharmacokinetics and disposition of olmesartan.

Up until now, it has been reported that organic anion-transporting polypeptide (OATP) 1B1 and OATP1B3 are involved in the hepatic uptake of olmesartan, whereas MRP2 is mainly involved in its biliary excretion (Nakagomi-Hagihara et al., 2006). However, the relative contribution of each transporter to its hepatic uptake remains to be determined. Information about their contributions is essential to quantitatively estimate the change in the overall clearance when the expression levels and/or transport functions of certain transporters are altered under various circumstances, such as pathophysiological conditions, single-nucleotide polymorphisms, and transporter-mediated drug-drug interactions. Hirano et al. (2004) established methodologies to estimate the quantitative contributions of OATP1B1 and OATP1B3 to the hepatic uptake of various compounds (Hirano et al., 2004). We used one of these methodologies, the relative activity factor (RAF) method, to calculate the contributions of OATP1B1 and OATP1B3 to the hepatic uptake of olmesartan in humans. To investigate the possible involvement of efflux transporters in the biliary excretion of olmesartan, OATP1B1/MRP2, OATP1B1/MDR1, and OATP1B1/BCRP double transfectants can be used to identify the anionic bisubstrates of OATP1B1 and each efflux transporter by measuring the basal-to-apical vectorial transport of the substrates across each monolayer (Matsushima et al., 2005).

In terms of the renal transport of olmesartan, we hypothesized that organic anion transporter 1 (OAT1) and OAT3 play major roles in its uptake on the basolateral membranes of kidney proximal tubule epithelial cells, judging from their substrate specificities. Hasegawa et al. (2010) and have shown that OATP1B1 and OATP1B3 are involved in the hepatic uptake of olmesartan. Using human cryopreserved hepatocytes, human kidney slices, and transporter expression systems, the efflux transporters involved in the biliary and urinary excretion of olmesartan were identified using double transfectants and transporter-expressing membrane vesicles.

**Materials and Methods**

**Materials.** [3H]Olmesartan (79 Ci/mmol), [14C]olmesartan medoxomil (21.6 mCi/mmol), and unlabeled olmesartan and olmesartan medoxomil were kindly donated by Sankyo Co., Ltd. (Tokyo, Japan). [3H]Estradiol-17β-glucuronide (E2-17βG) (45 Ci/mmol), [3H]estrone-3-sulfate (E1S) (46 Ci/mmol), and [3H]anilinophenylalanine (PAH) (4.1 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), and [3H]holocystein in octapeptide (CCK-8) (77 Ci/mmol) and [14C]benzylpenicillin (PCG) (19 mCi/mmol) were purchased from GE Healthcare Bio-Sciences (Waukesha, WI). Unlabeled E2-17βG, E1S, CCK-8, PAH, and PCG were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and commercially available.

**Animals.** Female Mprk knockout and wild-type mice (12–15 weeks old) were used in this study. Mprk knockout mice had been established previously (Leggas et al., 2004). All animals were maintained under standard conditions with a reversed dark-light cycle and were treated humanely. Food and water were available ad libitum. The studies were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

**Cell Culture.** Transporter-expressing or vector-transfected human embryonic kidney 293 (HEK293) cells and MDCKII cells were grown in low-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% CO2 and 95% humidity. LLC-PK1 cells were cultured in Medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Transport Study Using Transporter Expression Systems.** OATP- or OAT-transfected HEK293 cells were previously constructed (Hirano et al., 2004; Tahara et al., 2005). These cells were seeded in 12-well plates at a density of 1.25 × 105 cells/well 72 h before the transport assay. For the transport study, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate 24 h before the transport assay, to induce the expression of transporters.

The transport study was carried out as described previously (Hirano et al., 2004). Uptake was initiated by the addition of Krebs-Henseleit buffer containing radiolabeled and unlabeled substrates, after the cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer consisted of 118 mM NaCl, 23.8 mM NaHCO3, 4.8 mM KCl, 1.0 mM KH2PO4, 1.2 mM MgSO4, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl2 adjusted to pH 7.4. Uptake was terminated at the designated times by the addition of ice-cold Krebs-Henseleit buffer after the removal of the incubation buffer. The cells were then washed three times with 1 ml of ice-cold Krebs-Henseleit buffer, solubilized in 500 μl of 0.2 N NaOH, and stored overnight at 4°C. Aliquots (600 μl) were transferred to scintillation vials after the addition of 250 μl of 0.4 N HCl. The radioactivity associated with the cells and the incubation buffer was measured with a liquid scintillation counter (LS6000SE; Beckman Coulter, Inc., Fullerton, CA) after the addition of 3 ml of scintillation fluid (Clear-sol I; Nacalai Tesque, Kyoto, Japan) to the scintillation vials. The remaining 50 μl of cell lysate was used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard.

**Transport Study Using Human Cryopreserved Hepatocytes.** This experiment was performed as described previously (Hirano et al., 2004). Cryopreserved human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD; lots OCF and ETR) and from the Research Institute for Liver Disease (Shanghai, China; lot 03-013). Immediately before the study, the hepatocytes (1–ml suspension) were thawed at 37°C, then quickly suspended in 10 ml of ice-cold Krebs-Henseleit buffer and centrifuged (50 g) for 2 min at 4°C, followed by the removal of the supernatant. The number of viable cells was determined by trypan blue staining. Before the uptake studies, the cell suspensions were prewarmed in an incubator at 37°C for 3 min. The uptake studies were initiated by the addition of the cell suspension of an equal volume of buffer containing labeled and unlabeled substrates. After incubation at 37°C for 0.5 or 2 min, the reaction was terminated by the separation of the cells from the substrate solution. To do this, an aliquot of 80 μl of incubation mixture was collected and placed in a centrifuge tube (450 μl) containing 50 μl of 2 N NaOH under a layer of 100 μl of oil (density, 1.015; a mixture of silicone oil and mineral oil, 84.4:15.6 w/w; Sigma-Aldrich). The sample tubes were centrifuged for 10 s in a tabletop centrifuge (10,000g; Beckman Microfuge E, Beckman Coulter, Inc.). During this process, the hepatocytes passed through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes, the centrifuge tube was cut and each compartment
was transferred to a scintillation vial. The compartment containing the lysed cells was neutralized with 50 μl of 2 N HCl, mixed with scintillation cocktail, and the radioactivity was measured with a liquid scintillator counter.

Transcellular Transport Study Using Double-Transfected Cells. The protocol has been described previously in detail (Matsushima et al., 2005). In brief, transfected MDCKII cells were seeded in a Transwell membrane insert (6.5-mm diameter, 0.4-μm pore size; Corning Coster, Cambridge, MA) at a density of 1.4 × 10^5 cells per well for 120 h (OATP1B1-expressing, OATP1B1/MDR1-expressing, and vector-transfected control cells) or for 96 h (OATP1B1/MDR1-expressing cells) before the transport study. The recombinant adenovirus containing human BCRP cDNA was transfected into OATP1B1-expressing MDCKII cells 48 h before the transport study. The cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate 48 h (OATP1B1-expressing, OATP1B1/MDR1-expressing, OATP1B1/BCRP-expressing and vector-transfected control cells) or 24 h (OATP1B1/MDR1-expressing cells) before the transport assay. For the uptake studies, the cells were washed three times and preincubated with Krebs-Henseleit buffer. The experiment was initiated by replacing the buffer at either the apical or basal side of the cell layer with buffer containing radiolabeled and unlabeled compounds. The cells were incubated at 37°C and aliquots of the medium were taken from each compartment at several time points. The radioactivity in 100 μl of medium was measured with a liquid scintillation counter after the addition of 3 ml of scintillation fluid. The cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate and solubilized in 400 μl of 0.2 N NaOH. After the addition of 200 μl of 0.4 N HCl, 450-μl aliquots were transferred to scintillation vials. Aliquots (50 μl) of the cell lysates were used to determine the protein concentrations by the method of Lowry et al. (1951) with BSA as the standard.

Determination of the Fraction of Olmesartan Medoxomil in the Medium. After the transcellular transport of olmesartan medoxomil in the OATP1B1/MDR1 and OATP1B1/MDR1 double transfecteds for 120 min, medium samples were collected and evaporated to dryness under reduced pressure in a rotary evaporator, and then dissolved in 20 μl of dimethyl sulfoxide. Then 5-μl aliquots were transferred to scintillation vials to check the recovery ratio. Aliquots (10 μl) were applied to a thin-layer chromatography (TLC) plate (LKD6F; Whatman, Brentford, UK), and unlabeled olmesartan medoxomil and olmesartan were applied to the same plate to determine the locations of spots of the authentic compounds. The TLC plates were developed with n-butanol/acetic acid/water (4:1:1) for 120 min. After development, the TLC plate was attached to an imaging plate for 2 days, and the radioactivity was measured with a liquid scintillation counter. The TLC plates were developed on a Capcell Pak C18 MGII column (3 μm, 2 × 50 mm; Shimadzu, Kyoto, Japan) at 37°C and the radioactivity was measured with a liquid scintillation counter.

Vesicle Transport Assay. The preparation procedure for membrane vesicles expressing human MRPs or MRP4 has been described previously (Hirouchi et al., 2004; Imaoka et al., 2007). The transport medium (10 mM Tris, 250 mM sucrose, and 10 mM MgCl₂, pH 7.4) contained labeled and unlabeled olmesartan, 5 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl creatine phosphokinase). An aliquot of the transport medium (15 μl) was mixed rapidly with the vesicle suspension (5 μg of protein in 5 μl). The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl buffer (pH 7.4). The stopped reaction mixture was passed through a 0.45-μm HA filter (Millipore Corp., Billerica, MA) and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter was measured with a liquid scintillation counter after the addition of scintillation cocktail. Ligand uptake was normalized by the amount of membrane protein.

Preparation of Human Kidney Slices and Uptake of Organic Anions by Human Kidney Slices. This study protocol was approved by the Ethics Review Boards of both the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan) and Tokyo Women’s Medical University (Tokyo, Japan). All participants provided their written informed consent. Samples of human kidneys from the subjects were stored in Dulbecco’s modified Eagle’s medium on ice immediately after kidney excision. After transportation for about 30 min, the kidney slices were prepared as described below. Studies of uptake by human kidney slices were carried out as reported previously (Nozaki et al., 2007). Kidney slices (300 μm thick) from intact human cortical tissue were stored in ice-cold buffer before use. The buffer for the present study consisted of 120 mM NaCl, 16.2 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, and 10 mM NaH₂PO₄/Na₂HPO₄, adjusted to pH 7.5. After preincubation of the slices for 5 min at 37°C, one slice, weighing 2 to 10 mg, was selected and incubated for 15 min at 37°C in a 12-well plate with 1 ml of oxygenated buffer in each well. To estimate the nonspecific binding of olmesartan to the kidney slice, the slice was incubated for 15 min at 4°C after preincubation for 5 min at 4°C. After incubation for 15 min, the slice was rapidly removed from the incubation buffer, washed twice in ice-cold buffer, blotted on filter paper, weighed, and dissolved in 1 ml of Soluene-350 (Packard Instruments, Downers Grove, IL) at 50°C for 12 h. The radioactivity in the specimen was determined in scintillation cocktail (Hionic Fluor, Packard Instruments). In every experiment, the uptake activity of OAT1 and OAT3 in the human kidney slices was checked with PAH and PPG, respectively, as the positive controls.

In Vivo Infusion Study in Mice. Female C57BL/6 J and Mpr4 knockout mice weighing approximately 20 to 30 g were used for these experiments. Under anesthesia induced with pentobarbital (30 mg/kg), the jugular vein was cannulated with a polyethylene-10 catheter for the injection of olmesartan. The mice then received a constant infusion of olmesartan at a dose of 10 nmol/min/kg. Blood samples were collected from the jugular vein and urine was collected in preweighed test tubes at 30-min intervals throughout the experiment. Plasma was prepared by centrifugation of the blood samples at 10,000g for 5 min. After 120 min, the mice were killed and the entire kidney was excised immediately. The kidney was weighed, flash-frozen in liquid nitrogen, and then homogenized in a 3-fold volume of saline. The plasma, urine, and kidney homogenate were treated with methanol, followed by centrifugation at 10,000g for 15 min. The supernatant was analyzed by liquid chromatography/mass spectrometry (LC/MS), as described below.

LC/MS Analysis. An LC/MS-2010EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used to quantify olmesartan. Samples were separated on a Capcell Pak C18 MGII column (3 μm, 2 × 50 mm; Shiseido, Tokyo, Japan) in binary gradient mode. The mobile phase was 0.05% formic acid and acetonitrile. The acetonitrile concentration was initially 18%, and then linearly increased to 70% over 3 min; it was maintained at 70% for a further 1 min. Finally, the column was re-equilibrated with acetonitrile at a concentration of 18% for 3 min. The total run time was 7 min. Olmesartan was eluted at 2.8 min with this method. In the mass analysis, olmesartan was detected at a mass-to-charge ratio of 447.2 under positive electron spray ionization conditions. To quantify creatinine, an Alliance HT 2795 separation module with an autosampler (Waters, Milford, MA) and a Micromass QZ mass spectrometer with an electron ion spray interface (Waters) were used. The samples were separated on a Capcell Pak C18 MGII column (3 μm, 2 × 50 mm; Shiseido) in isocratic flow mode. The mobile phase was 0.1% IPC-M3S (GL Sciences Inc., Torrance, CA)acetonitrile (98:2, v/v). The optimum operating conditions used were as follows: electrospray probe (capillary) voltage 3.5 kV, sample cone voltage 15 V, and source temperature 50°C. In the mass analysis, creatinine was detected at a mass-to-charge ratio of 114.15 under positive electron spray ionization conditions.

Kinetic Analyses of Uptake Transporters. Ligand uptake was expressed as the uptake volume (μl/min/mg protein), given as the amount of radioactivity associated with the cells (pmol/mg protein) divided by its concentration in the incubation medium (pmol/μl). Specific uptake was calculated by subtracting the uptake by the vector-transfected cells from the uptake by the cDNA-transfected cells. Kinetic parameters were calculated using the following equation:

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} + P_{\text{diff}} \cdot S \]  

where \( v \) is the uptake velocity of the substrate (pmol/min/mg protein), \( S \) is the substrate concentration in the medium (μM), \( K_m \) is the Michaelis constant (μM), \( V_{\text{max}} \) is the maximum uptake rate (pmol/min/mg protein), and \( P_{\text{diff}} \) is the nonsaturable uptake clearance (μl/min/mg protein). Fitting was performed with a nonlinear least-squares method using the MULTI program (Yamaoka et al., 1988), and the Damping Gauss-Newton algorithm was used for curve fitting. The input data were weighted as the reciprocals of the observed values.

To determine the saturable hepatic uptake clearance in human hepatocytes, we first determined the hepatic uptake clearance (CLH(2 min–0.5 min))/l/min/10^6
cells) by calculating the slope of the uptake volume \((V_d)\) (μl/10^6 cells) between 0.5 and 2 min (eq. 2). The saturable component of the hepatic uptake clearance \((CL_{hep})\) was determined by subtracting \(CL_{2\,min-0.5\,min}\) in the presence of 100 μM \((E_1/17βG,\,\text{CCK}-8)\) or 300 μM (olmesartan) substrate (excess) from that in the presence of 1 μM \((E_1/17βG,\,\text{CCK}-8)\) or 0.3 μM (olmesartan) substrate (tracer) (eq. 3).

\[
CL_{2\,min-0.5\,min} = \frac{V_d\,1\,min - V_d\,0.5\,min}{2-0.5}
\]  

\[
CL_{hep} = CL_{2\,min-0.5\,min,\text{tracer}} - CL_{2\,min-0.5\,min,\text{excess}}
\]

where \(CL_{2\,min-0.5\,min,\text{tracer}}\) and \(CL_{2\,min-0.5\,min,\text{excess}}\) represent the \(CL_{2\,min-0.5\,min}\) values estimated in the presence of 1 μM or 0.3 μM and 100 μM or 300 μM substrate, respectively.

The saturable component of the renal clearance \((CL_{\text{kidney}})\) was determined by subtracting the uptake volume \((V_d)\) (μl/mg kidney) at 15 min in the presence of 100 μM \((PCG)\) or 60 μM \((PAH,\,\text{olmesartan})\) substrate (excess) from that in the presence of 2.5 μM \((PCG)\) or 1 nM \((PAH,\,\text{olmesartan})\) substrate (tracer) (eq. 4).

\[
CL_{\text{kidney}} = \frac{V_{d,\text{tracer}} - V_{d,\text{excess}}}{15}
\]

**Estimation of the Relative Contribution of Each Transporter to Hepatic and Renal Uptake.** This method for estimating the contribution of OATP1B1 and OATP1B3 to the overall hepatic uptake and the contribution of OAT1 and OAT3 to the overall renal uptake has been used previously (Hasegawa et al., 2003; Hirano et al., 2004). In this analysis, \(E_1/9\) and CCK-8 were chosen as the transporter-selective substrates of OATP1B1 and OAT1B3, respectively, and PAH and PCG were chosen as the transporter-selective substrates of OAT1 and OAT3, respectively. The ratio of the uptake clearance of these reference compounds in human hepatocytes or human kidney slices to that in the expression system was calculated and defined as \(R_{\text{act,OATP}}\) and \(R_{\text{act,OAT}}\), respectively. The uptake clearance mediated by OATPs in human hepatocytes \((CL_{hep,\text{test,OATPs}})\) or by OATs in human kidney slices \((CL_{\text{kidney,\text{test,OATs}}})\) was calculated separately by multiplying the uptake clearance of olmesartan in the double-transfected cells by calculating the concentration ratio in the medium to that in the cells (eq. 10).

\[
C_{\text{med}} = f_{\text{cell}} \cdot C_{\text{cell}}
\]

\[
f_{\text{cell}} = \frac{C_{\text{med}}}{C_{\text{cell}}}
\]

**Kinetic Analysis of the in Vivo Study.** The total clearance \((CL_{\text{tot,p}})\) was calculated by dividing the infusion rate \((I)\) by the mean value of the plasma concentration at 60, 90, and 120 min \((C_{\text{ss},\text{p}})\) (eq. 11). The renal clearance was calculated by dividing the mean value of the urinary excretion velocity at 60 to 90 and 90 to 120 min \((V_{\text{urine}})\) by \(C_{\text{ss},\text{p}}\) (eq. 12).

\[
CL_{\text{tot,p}} = \frac{I}{C_{\text{ss},\text{p}}}
\]

\[
CL_{\text{renal}} = \frac{V_{\text{urine}}}{C_{\text{ss},\text{p}}}
\]

**Results**

**Uptake of Olmesartan by OATP Transporter-Expressing Cells.** Olmesartan was significantly taken up, in a time-dependent manner, into OATP1B1- and OATP1B3-expressing HEK293 cells compared with its uptake into vector-transfected cells (Fig. 1A–C). The saturation kinetics of olmesartan in these cell lines were evaluated for uptake over a period of 2 min, during which the uptake of olmesartan remained linear, and is shown as Eadie-Hofstee plots (Fig. 1, D and E). The concentration dependence of the uptake of olmesartan can be explained by one saturable component in the OATP1B3-expression system, whereas one saturable and one nonsaturable component were observed in the OATP1B1-expression system. The kinetic parameters are summarized in Table 1.

**Uptake of \(E_1/S,\,\text{CCK}-8,\) and Olmesartan by Human Cryopreserved Hepatocytes.** The uptake of \(\text{^{14}H}\)olmesartan by human hepatocytes was inhibited by 300 μM unlabeled olmesartan. The time-dependent uptake of all ligands was observed at 1 μM \((E_1/S,\,\text{CCK}-8)\) or 0.3 μM (olmesartan), and the uptake decreased in the presence of 100 μM \((E_1/S,\,\text{CCK}-8)\) or 300 μM (olmesartan) unlabeled ligands in all batches of hepatocytes examined in this study. The uptake clearance of these substrates in each batch is listed in Table 2. Following a previously described method for estimating the contribution of OATP1B1 and OATP1B3 to the overall hepatic uptake (Hirano et al., 2004) using the data in Table 2, we calculated the clearance of olmesartan mediated by OATP1B1 and OATP1B3 and the relative contributions of these transporters to its hepatic uptake in three batches of human hepatocytes. Our estimation indicates that the relative contributions of OATP1B1 and OATP1B3 depended on the individual batch of human hepatocytes, and the contribution of OATP1B1 ranged from 44.3% to 62.2% (Table 3).
Transcellular Transport of Olmesartan across MDCKII Monolayers. To identify the efflux transporters involved in the biliary excretion of olmesartan, we investigated the transcellular transport of olmesartan across MDCKII monolayers expressing uptake and efflux transporters. We observed no significant vectorial transcellular transport of olmesartan in single-transfected MDCKII cells expressing OATP1B1, MDR1, MRP2, or BCRP, or in vector-transfected control cells (data not shown). As shown in Fig. 2, the basal-to-apical transcellular transport of olmesartan was observed in OATP1B1/MRP2 double-transfected cells (Fig. 2B), but not in OATP1B1 single-transfected cells (Fig. 2A) or OATP1B1/MDR1 (Fig. 2C) and OATP1B1/BCRP (Fig. 2D) double-transfected cells.

![Graphs showing uptake clearance of olmesartan](image)

**TABLE 1**

Kinetic parameters for the uptake of olmesartan by OATP1B1- and OATP1B3-expressing HEK293 cells

Data shown in Fig. 1, D and E, were used to determine these parameters calculated by nonlinear regression analysis as described under Materials and Methods. Each parameter represents the mean ± S.E. (n = 3).

**TABLE 2**

Uptake clearance of reference compounds (E1S and CCK-8) and olmesartan in transporter-expression systems and human hepatocytes

<table>
<thead>
<tr>
<th>Transporter-Expressing Cells</th>
<th>Human Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL&lt;sub&gt;OATP1B1&lt;/sub&gt;</td>
<td>CL&lt;sub&gt;OATP1B3&lt;/sub&gt;</td>
</tr>
<tr>
<td>µmol/min/mg protein</td>
<td>µmol/min/mg protein</td>
</tr>
<tr>
<td>E1S</td>
<td>116</td>
</tr>
<tr>
<td>CCK-8</td>
<td>21.7</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>1.42</td>
</tr>
</tbody>
</table>

**TABLE 3**

Relative contribution of OATP1B1 and OATP1B3 to the hepatic uptake of olmesartan in each batch of human hepatocytes

<table>
<thead>
<tr>
<th>Lot</th>
<th>Ratio of Uptake Clearance</th>
<th>Estimated Clearance of Olmesartan&lt;sup&gt;a&lt;/sup&gt; (µmol/min/10&lt;sup&gt;6&lt;/sup&gt; cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&lt;sub&gt;act,OATP1B1&lt;/sub&gt;</td>
<td>R&lt;sub&gt;act,OATP1B3&lt;/sub&gt;</td>
<td>OATP1B1</td>
</tr>
<tr>
<td>OCF</td>
<td>0.683</td>
<td>0.204</td>
</tr>
<tr>
<td>ETR</td>
<td>0.566</td>
<td>0.121</td>
</tr>
<tr>
<td>03-013</td>
<td>0.447</td>
<td>0.0642</td>
</tr>
</tbody>
</table>

<sup>a</sup>The lower value in the column Estimated Clearance of Olmesartan shows the percentage of OATP1B1- or OATP1B3-mediated uptake clearance relative to the sum of the estimated clearance mediated by OATP1B1 and OATP1B3. The details of this estimation are described under Materials and Methods.

Figure 1. Time profiles of the uptake of [3H]olmesartan (0.1 µM) by OATP1B1-, OATP1B3-, and OATP2B1-expressing HEK293 cells (A–C) and Eadie-Hofstee plots for the uptake of olmesartan by OATP1B1- and OATP1B3-expressing HEK293 cells (D and E). Circles and triangles represent the uptake by transporter-expressing cells and vector-transfected control cells, respectively. The solid lines in D and E are fitted curves calculated by nonlinear regression based on eq. 1, as described under Materials and Methods. Each point represents the mean ± S.E. (n = 3).
Estimation of the Protein-Unbound Fraction in Double-Transfected Cells. To explain the different transport mechanisms should be drastically reduced in the three kinds of double transfectants we tested. The basal-to-apical transport of \( \text{E}_217\beta G \) was 8.6, 3.3 and 7.7 times greater than that in the opposite direction in the OATP1B1/MDR1 (C), or OATP1B1/BCRP (D) was determined. The transcellular transport of \( \text{E}_217\beta G \) (0.1 \( \mu \)M) across MDCKII monolayers expressing OATP1B1 (E), OATP1B1/MDR1 (F), OATP1B1/BCRP (G), and OATP1B1/BCRP (H) was also determined in parallel as positive controls. Open circles and closed circles represent apical-to-basal and basal-to-apical transcellular transport, respectively. Each point represents the mean \( \pm \) S.E. (\( n = 3 \)).

ATP-Dependent Uptake of Olmesartan in Human MRP2-Expressing Membrane Vesicles. To confirm that olmesartan is a substrate of human MRP2, the time-dependent uptake of \( \text{[3H]olmesartan} \) by membrane vesicles prepared from MRP2-expressing LLC-PK1 cells was examined (Fig. 4A). Significant \( \text{[3H]olmesartan} \) was taken up into the membrane vesicles expressing MRP2 in an ATP-dependent manner (Fig. 4A), and this uptake was saturable with \( K_m \), \( V_{\text{max}} \), and \( P_{\text{diff}} \) values of 14.9 \( \pm \) 5.39 \( \mu \)M, 90.6 \( \pm \) 30.8 pmol/min/mg protein, and 0.431 \( \pm \) 0.201 \( \mu \)l/min/mg protein, respectively (Fig. 4B). In parallel, we checked the uptake activity for the typical substrate (\( \text{E}_217\beta G \)) in MRP2-expressing membrane vesicles. The uptake clearance of \( \text{E}_217\beta G \) in MRP2-expressing vesicles was 10.8 \( \pm \) 1.0 \mu l/min/mg protein.

Estimation of the Protein-Unbound Fraction in Double-Transfected Cells. To explain the different transport mechanisms should be drastically reduced and the protein-unbound concentration in the medium should be equal to that in the cells. The concentration ratio was 1.80 \( \pm \) 0.06 at 37°C and 0.420 \( \pm \) 0.084 at 4°C.

Uptake of Olmesartan by OAT Transporter-Expressing Cells. Olmesartan was significantly taken up, in a time-dependent manner, into OAT1- and OAT3-expressing HEK293 cells compared with its
uptake into vector-transfected cells (Fig. 5, A and B). The saturation kinetics of olmesartan in OAT1- and OAT3-expressing cells and vector-transfected HEK293 was evaluated for uptake over a period of 2 min, during which the uptake of olmesartan remained linear, and is shown as an Eadie-Hofstee plot (Fig. 5, C and D). The concentration dependence of the uptake of olmesartan can be explained by one saturable component. The kinetic parameters are summarized in Table 5.

Uptake of PAH, PCG, and Olmesartan by Human Kidney Slices. The concentration dependence of the uptake of olmesartan by human kidney slices is shown as Eadie-Hofstee plots in Fig. 6A. Nonlinear regression analysis revealed that the saturable uptake of olmesartan by human kidney slices consisted of one saturable and one nonsaturable component, with $K_{m}$, $V_{\text{max}}$, and $P_{\text{diff}}$ values of 0.585 ± 0.104 µM, 397 ± 59 mol/min/g kidney, and 72.9 ± 5.7 µL/min/g kidney, respectively. The uptake activities of typical substrates for OAT1 (PAH) and OAT3 (PCG) were also determined. The uptake clearance of PAH and PCG were 214 ± 9 and 169 ± 39 µL/min/g kidney, respectively.

Inhibitory Effects of PAH, PCG, and Probenecid on the Uptake of Olmesartan by Human Kidney Slices. The inhibitory effects of PAH, PCG, and probenecid on the uptake of olmesartan were investigated (Fig. 6, B–D). PCG and probenecid inhibited the uptake of olmesartan by human kidney slices in a concentration-dependent manner, but even in the presence of 1000 µM PAH, no significant inhibition was observed. The uptake of olmesartan at 4°C was reduced to 27.8 ± 3.5% of that at 37°C.

ATP-Dependent Uptake of Olmesartan in Human MRP4-Expressing Membrane Vesicles. To confirm that olmesartan is a substrate of human MRP4, the time-dependent uptake of [3H]olmesartan by membrane vesicles prepared from MRP4-expressing HEK293 cells was examined (Fig. 7A). Significant [3H]olmesartan was taken up into the membrane vesicles expressing MRP4 in an ATP-dependent manner (Fig. 7A), and this uptake was saturable with $K_{m}$ and $V_{\text{max}}$ values of 26.2 ± 4.4 µM and 195 ± 25 pmol/min/mg protein, respectively (Fig. 7B). In parallel, we checked the uptake activity for the typical substrate (dehydroepiandrosterone sulfate) in MRP4-expressing membrane vesicles. The uptake clearance of dehydroepiandrosterone sulfate in MRP4-expressing vesicles was 98.1 ± 11.0 µL/min/g protein.

Urinary Excretion of Olmesartan in Mrp4-Knockout and Wild-Type Mice. To investigate the in vivo function of MRP4 in the urinary excretion of olmesartan, we performed the infusion study using MRP4-knockout mice. The several pharmacokinetic parameters [plasma total clearance, renal clearance, glomerular filtration rate (GFR), and tissue-to-plasma concentration ratio] were calculated and summarized in Table 6. GFR was calculated as renal clearance of creatinine and...
plasma protein unbound fraction (0.045) was cited from the manufacturer’s interview form. The fraction excreted into urine of olmesartan in MRP4-knockout and wild-type mice was 5.3 ± 1.3% and 3.8 ± 1.3%, respectively. Renal clearance of olmesartan in both knockout and wild-type mice was smaller than the product of $f_u$ and GFR (clearance by glomerular filtration). Pharmacokinetics of olmesartan in MRP4-knockout mice was not different from that of wild-type mice.

Transcellular Transport of Olmesartan Medoxomil across MDCKII Monolayers. To investigate which transporters recognize olmesartan medoxomil, we performed a transcellular transport assay using OATP1B1 single-transfected cells and OATP1B1/MDR1, OATP1B1/MRP2, and OATP1B1/BCRP double-transfected cells (Fig. 8). Vectorial transport was observed in the OATP1B1/MDR1 (Fig. 8B), OATP1B1/MRP2 (Fig. 8C), and OATP1B1/BCRP (Fig. 8D) double-transfected cells. In parallel, we also checked the transcellular transport of $E_{17\beta\text{G}}$, the basal-to-apical transport of which had been reported in the three kinds of double transfectants we tested. The basal-to-apical transport of $E_{17\beta\text{G}}$ was 10.8, 6.7, and 13.1 times greater than that in the opposite direction in the OATP1B1/MDR1 (Fig. 8F), OATP1B1/MDR1 (Fig. 8G), and OATP1B1/BCRP (Fig. 8H) double transfectants, respectively. To confirm that the vectorial transport of total radioactivity directly reflected the transport of olmesartan medoxomil, because it is possible that olmesartan medoxomil is easily converted to olmesartan by intracellular esterases, we checked how much of the radioactivity was surely derived from olmesartan medoxomil. The TLC analysis indicated that more than 80% of the radioactivity in each sample originated from olmesartan medoxomil, suggesting that we can regard the total radioactivity as reflecting olmesartan medoxomil in this experiment.

Discussion

Olmesartan is excreted into both bile and urine. It is excreted predominantly in an unchanged form, with minimal metabolism in humans (Laeis et al., 2001; Schwocho and Masonson, 2001). Considering that olmesartan is a hydrophilic anionic compound, a series of transporters for organic anions is thought to play an important role in its membrane transport. Accordingly, we identified the uptake and efflux transporters involved in olmesartan’s hepatic and renal clearance in humans and estimated the relative contribution of each transporter to its overall hepatic and renal uptake.

In the process of hepatic uptake, a recent report demonstrated that olmesartan is taken up by both OATP1B1- and OATP1B3-expressing oocytes (Nakagomi-Hagihara et al., 2006). We confirmed that olmesartan is transported by both OATP1B1- and OATP1B3-expressing HEK293 cells (Fig. 1A and B). OATP2B1 is also expressed in the human liver (Tamao et al., 2000), but no significant uptake of olmesartan was observed in OATP2B1-expressing cells compared with that in vector-transfected control cells (Fig. 1C). To estimate the relative contributions of OATP1B1 and OATP1B3 to the hepatic uptake of olmesartan, we used an approach called the RAF method (Hirano et al., 2004). We used three different batches of human cryopreserved hepatocytes prepared from independent donors because it has been reported that there are large interbatch differences in the uptake activities of human cryopreserved hepatocytes (Shitara et al., 2003). The amount of olmesartan taken up by the human hepatocytes increased for 2 min at 0.3 µM, whereas in the presence of 300 µM olmesartan, its uptake was unchanged between 0.5 and 2 min, indicating that 300 µM olmesartan is sufficient to saturate its transporter-mediated uptake in human hepatocytes. This is consistent with our result indicating that the $K_m$ values of OATP1B1 and OATP1B3 were much lower than 300 µM. After the oral administration of 160 mg of olmesartan medoxomil, the maximum plasma concentration ($C_{\text{max}}$) was 0.49 to 4.7 µM (Warner and Jarvis, 2002) and the plasma unbound fraction was about 1% (Yoshida and Kohzuki, 2004). Therefore, the maximum unbound plasma concentration at the inlet to the liver, calculated by the method of Ito et al. (1998), was about 0.069 µM. $C_{\text{max}}$ and area under the plasma concentration-time curve are directly proportional to the dose (up to 160 mg) in healthy male subjects (Schwocho and Masonson, 2001). This is consistent with our result that the $K_m$ values for the OATP1B1- and OATP1B3-expressing cells were much higher than the maximum unbound
basal-to-apical transcellular transport, respectively. Each point represents the mean 

OATP1B1/MDR1 (G), or OATP1B1/BCRP (H) was also determined in parallel, as positive controls. Open circles and closed circles represent apical-to-basal and (D) was determined. The transcellular transport of [3H]E217 was taken up by both OATP1B1 and OATP1B3 in human 

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valsartan is taken up by both OATP1B1 and OATP1B3 in human 

cells). Previous reports have suggested that OATP1B1 contribute to the uptake of olmesartan in each batch of human hepa-

table function of hepatic clearance. The rank order for the uptake clearance of olmesartan (2.27 μl/min/10⁶ cells), valsartan (3.55 μl/min/10⁶ cells), and telmisartan (866 μl/ min/10⁶ cells) in hepatocytes (lot OCF) was the same as that for in vivo hepatic clearance (olmesartan, 0.387 ml/min/kg; valsartan, 0.658 ml/min/kg; telmisartan, 23.0 ml/min/kg) (Ishiguro et al., 2006; Yamashiro et al., 2006), suggesting that human hepatocytes can be used for the prediction of magnitude correlation of hepatic clearance.

We also estimated that both OATP1B1 and OATP1B3 equally contribute to the uptake of olmesartan in each batch of human hepa-
tocytes (Table 3). Previous reports have suggested that OATP1B1 predominantly accounts for the hepatic uptake of pitavastatin (Hirano et al., 2004), whereas OATP1B3 is mainly involved in the uptake of fexofenadine (Shimizu et al., 2005). Among the angiotensin II receptor antagonists, telmisartan is reported to be transported exclusively by OATP1B3, and not by OATP1B1 (Ishiguro et al., 2006), whereas valsartan is taken up by both OATP1B1 and OATP1B3 in human 

TABLE 6
Pharmacokinetic parameters of olmesartan during constant infusion into wild-type and Mrp4 knockout mice

Data represent the mean ± S.E. [n = 7 (wild-type), 6 (Mrp4-knockout mice)].

<table>
<thead>
<tr>
<th></th>
<th>C_{ss,p}</th>
<th>CL_{ss,p}</th>
<th>CL_{renal,p}</th>
<th>f_p · GFR</th>
<th>K_{p,kidney}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.43 ± 0.09</td>
<td>7.22 ± 0.58</td>
<td>0.384 ± 0.092</td>
<td>1.02 ± 0.12</td>
<td>0.603 ± 0.151</td>
</tr>
<tr>
<td>Knockout</td>
<td>1.58 ± 0.25</td>
<td>7.32 ± 1.32</td>
<td>0.280 ± 0.082</td>
<td>0.857 ± 0.126</td>
<td>0.582 ± 0.144</td>
</tr>
</tbody>
</table>

C_{ss,p}, the mean value of the plasma concentration at 60, 90, and 120 min; CL_{ss,p}, total plasma clearance; CL_{renal,p}, renal clearance with respect to the circulating plasma concentration; f_p · GFR, glomerular filtration rate multiplied with plasma protein unbound fraction; K_{p,kidney}, K_p value obtained by dividing the kidney concentration by C_{ss,p} (at 120 min).

concentration at the inlet to the liver in clinical situations. The rank order for the uptake clearance of olmesartan (2.27 μl/min/10⁶ cells), valsartan (3.55 μl/min/10⁶ cells), and telmisartan (866 μl/ min/10⁶ cells) in hepatocytes (lot OCF) was the same as that for in vivo hepatic clearance (olmesartan, 0.387 ml/min/kg; valsartan, 0.658 ml/min/kg; telmisartan, 23.0 ml/min/kg) (Ishiguro et al., 2006; Yamashiro et al., 2006), suggesting that human hepatocytes can be used for the prediction of magnitude correlation of hepatic clearance.

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hepatocytes (Yamashiro et al., 2006). Thus, the relative contributions of OATP1B1 and OATP1B3 differ, even within the same category of drugs. Therefore, it is necessary to collect information about the contribution of each transporter to the membrane transport of each drug to predict changes in its pharmacokinetics and subsequent pharmacological effects when the functions of certain transporters are changed under a variety of conditions, such as genetic polymorphisms (e.g., OATP1B1*15) (Nishizato et al., 2003).

Hepatobiliary transport is achieved by the coordinate function of uptake and efflux transporters. We investigated the efflux transporters involved in the biliary excretion of olmesartan using MDCKII cells that coexpress OATP1B1/MDR1, OATP1B1/MDR2, and OATP1B1/ 

BCRP. The basal-to-apical vectorial transport of olmesartan was ob-

served in OATP1B1/MDR2 double transfected, but not in OATP1B1/ 

MDR1 and OATP1B1/BCRP transfectants (Fig. 2). A previous study demonstrated that the biliary excretion of olmesartan was drastically decreased in Mrp2-deficient Eisai hyperbilirubinemic rats (Nakagomi-Hagihara et al., 2006), suggesting that it is excreted into the bile mainly
TABLE 7
Relative contribution of OAT1 and OAT3 to the renal uptake of olmesartan in each batch of human kidney slices

<table>
<thead>
<tr>
<th>Lot</th>
<th>Ratio of Uptake Clearance</th>
<th>Estimated Clearance of Olmesartan&lt;sup&gt;a&lt;/sup&gt; (μmol/min/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_{cl, OAT1}$</td>
<td>$R_{cl, OAT3}$</td>
</tr>
<tr>
<td>11 May 06</td>
<td>0.0185</td>
<td>0.0299</td>
</tr>
<tr>
<td>26 Dec 06</td>
<td>0.00291</td>
<td>0.0103</td>
</tr>
<tr>
<td>11 Jan 07</td>
<td>0.00705</td>
<td>0.0127</td>
</tr>
</tbody>
</table>

<sup>a</sup> The lower value shows the percentage of OAT1, or OAT3-mediated uptake clearance relative to the sum of the estimated clearance mediated by OAT1 and OAT3. The details of this estimation are described under Materials and Methods.

Olmesartan is orally administered as the prodrug, olmesartan medoxomil. It is rapidly converted to the active metabolite, olmesartan, by esterase. According to the manufacturer’s interview form, olmesartan medoxomil cannot be detected in human plasma after its administration. However, if olmesartan medoxomil is recognized by efflux transporters in the small intestine, they may affect its intestinal absorption. Therefore, we investigated which efflux transporters might participate in the control of the intestinal absorption of olmesartan medoxomil. A transcellular transport assay using double transfectants indicated that olmesartan medoxomil is a substrate of MDR1, MRP2, and BCRP, which may interfere with its intestinal absorption, although the in vivo relevance of this result is still unknown.

In conclusion, multiple transporters in multiple tissues are involved in the pharmacokinetics of olmesartan and its prodrg. Some reports have shown that certain genetic polymorphisms in transporters affect the pharmacokinetics and pharmacological effects of their substrate drugs. The relative importance of these transporters in humans will be confirmed by clinical studies with genetic tests.

Acknowledgments. We express our great appreciation to Sankyo Co., Ltd. (Tokyo, Japan) for providing the 3H-labeled and unlabeled olmesartan and the 14C-labeled and unlabeled olmesartan medoxomil.

References


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