OATP and MRP2-mediated hepatic uptake and biliary excretion of eprosartan in rat and human

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Introduction

Eprosartan is an angiotensin II receptor antagonist (ARA-II) which has a high affinity for the angiotensin II type 1 receptor (AT1), used in treatment of hypertension and heart failure in clinical patients. Therefore, they have been proposed as an alternative to the traditional angiotensin-converting enzyme inhibitors [25]. Orally administered eprosartan has an absolute bioavailability of 13%, with peak plasma concentrations achieved 1–2 h after administration. The mean terminal elimination half-life of eprosartan is 20 h after multiple-dose administration. Eprosartan is not metabolized by the cytochrome P450 enzyme system and primarily eliminated as unchanged drug via biliary and renal excretion [33].

The hepatobiliary disposition of drugs in the liver is influenced by several processes, such as sinusoidal uptake into the hepatocytes, intracellular translocation and excretion into the bile, which may involve carrier-mediated mechanisms that are saturable and rate limiting. In liver, uptake transporters govern the transport of drugs from the basolateral side of the cell membrane (the blood side) into the hepatocyte, while the exit of drugs from inside hepatocytes was mediated either by a basolateral efflux transporter transported into the blood or by an apical (canalicular) efflux transporter transported into the bile [22,38,39,47]. Both sinusoidal and canalicular membrane transporters are important...
for the hepatic clearance of drugs and may be concerned with potential of drug–drug interactions.

Various transporters are involved in the hepatobiliary transport process. Among them, OATP1B1 (OATP-C/OATP2/LST-1, organic anion–transporting polypeptide) [1] and OATP1B3 (OATP8/LST-2) [2], selectively expressed in the basolateral membrane of human hepatocytes with broad substrate specificities [2,11,12], are thought to be responsible for the hepatic uptake of various endogenous, such as neutral steroids [4,44] and bile salt [43], and xenobiotics, such as statins [13,14,18], sartans [15,29,46] and fexofenadine [36], in a Na+-independent manner. On the other hand, Na+-dependent taurocholate-cotransporting polypeptide (NTCP) is the most relevant Na+-dependent transporter identified as a hepatic uptake transporter and is thought to be responsible for the hepatic uptake of bile salts [43]. Conversely, transporters such as MDR1 (multidrug resistance 1/ABCB1), MRP2 (multidrug resistance-associated protein 2/ABCC2), and BCRP (breast cancer resistance protein/ABCG2) are involved in biliary excretion, which are predominantly expressed on canalicular membrane [5,6]. Moreover, the substrate specificity of each transporter is very broad and the transporters expressed in hepatocytes always share an overlapping substrate spectrum, suggesting that a substrate can be recognized by one transporter or multiple transporters.

However, the transporters involved in the hepatobiliary transport of eprosartan in human and rat have not been fully investigated yet. Therefore, the aim of this study is to show the involvement of transporters in the hepatic uptake and biliary excretion process of eprosartan. Investigation of the transporters involved in the hepatobiliary transport of eprosartan which is eliminated mostly by hepatic uptake and biliary excretion, is important for the prediction of potential eprosartan-related drug–drug interactions and may contribute to our understanding of the mechanisms of hepatobiliary excretion of sartans.

In present study, the perfused rat liver model, an ideal model for examining alterations in the hepatobiliary disposition of substrates without the influence of metabolism/excretion by other organ systems [3], was employed to investigate the possible involvement of hepatic uptake and efflux transporters in the hepatobiliary disposition of eprosartan. Furthermore, rat liver slices [8] and isolated hepatocytes [23] were used to evaluate the transporter-mediated hepatobiliary disposition of eprosartan. Also, bi-directional transport assays were conducted using OATP1B1/ABCG2 double transfectants and OATP1B3 transfected cells [21,24,45] to identify which transporters are involved in the hepatic uptake and biliary excretion of eprosartan in human.

Materials and methods

Materials and animals

Eprosartan was purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China). Tetraethyl ammonium (TEA) and digoxin were purchased from Hubei Saibo Chemical Co., Ltd. and Nanjing ZeLang Medical Technology Co., Ltd., respectively. Cyclosporine A and rifampicin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Probencid was obtained commercially from Alexis Biochemicals (San Diego, CA, USA). Cilostazol (internal standard, IS), p-Aminohippurate (PAH), (RS)-2-4-(2-methylpropyl) phenyl) propanoic acid (ibuprofen), novobiocin and verapamil were purchased from Sigma–Aldrich (St. Louis, MO, USA). The stable transfected OATP1B3-human embryonic kidney cells (HEK) 293 cells, vector-HEK293 cells, OATP1B1-Madin–Darby canine kidney strain (MDCK) II cells, OATP1B1-MRP2-MDCKII cells and vector-MDCKII cells were the generous gift from Professor Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, University of Tokyo (Tokyo, Japan). Cell culture reagents were purchased from Gibco® (Grand Island, NY). All other chemicals were of analytical grade and were commercially available.

Male Wistar rats (220–250 g) obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002) were allowed free access to water and kept under a chow diet but were fasted for 12 h (with water ad libitum) before the experiments. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Media and cell culture

HEK293 cells and MDCKII cells were grown in Dulbecco's modified Eagle medium (low-glucose; Invitrogen, Carlsbad, CA) with 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. All transfected cells were cultured at 37 °C with a 5% (v/v) CO₂ atmosphere and 95% relative humidity during cell culture.

Perfused rat liver experiments

Rat livers were prepared by standard techniques described by previous study [27]. Briefly, the bile duct was cannulated and the liver (n = 3) was perfused in situ through the portal vein with oxygenated Krebs–Henseleit buffer (118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.0 mM glucose, 2.5 mM CaCl₂ adjusted to pH 7.4). After equilibrated with Krebs–Henseleit buffer for approximately 10 min, the liver was allowed to perfuse with oxygenated Krebs–Henseleit buffer containing 20% (v/v) washed bovine erythrocytes at a flow rate of 12 ml/min [20]. In order to collect the effluent perfusate, the superior vena cava was cannulated. Basis of initial bile flow (>2 μl/min) was determined as the standard of normal liver viability. Following equilibration, the liver was perfused for 45 min after addition of eprosartan 1 μM, eprosartan 1 μM + rifampicin 10 μM (final concentration, n = 3). On the other hand, following equilibration, the inhibitor of Mrp2, P-gp or Bcrp (20 μM of probenicid, verapamil or novobiocin; n = 3, respectively) was added to the perfusate buffer 5 min before the addition perfusate of eprosartan. Then the liver was perfused for 45 min after addition of eprosartan (1 μM). Effluent perfusate (500 μl) was collected at 1, 3, 5, 15, 25, 35, 45 min with the superior vena cava cannulation and bile samples were collected in toto at 15 min intervals with the bile duct cannulation. The samples were stored at -20 °C.

Eprosartan uptake in rat liver slices

Rat liver slices were prepared as described by Elferink et al. [8]. In brief, after the animals (n = 3) were anesthetized, livers were excised and immediately immersed in ice-cold buffer, saturated with 95% O₂–5% CO₂ (carbogen; pH 7.4). Then, livers were cut into slices (200–300 μm thickness; 10–14 mg wet weight) with a tissue slicer (ZQP-86; Zhixin Co., Ltd., Shanghai, China) and gassed with carbogen in 6-well culture plates with continuous shaking. After incubation for 3 min at 37 °C, liver slices were transferred to 24-well culture plates and contained with fresh carbogen-saturated eprosartan for further uptake study at 37 °C and 4 °C. Liver uptake of eprosartan (1 μM) was measured at 0, 1, 5, 15 and 30 min. Then, livers were rinsed with ice-cold Hanks' balanced salt solution (pH 7.5) for three times and dried on filter paper. Uptake of eprosartan at different time points was essentially linear, base on this, the overall rate of uptake time was chosen as 15 min. Also, this time was used to examine the effects of inhibitors (detailed specifications
of inhibitors against rat Oatps can be found in Supplemental Table 1), including cyclosporin A (10 μM), ibuprofen (150 μM), digoxin (5 μM), rifampicin (10 μM), TEA (1 mM) and PAH (1 mM) on the uptake of eprosartan. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used to determine the accumulated eprosartan in homogenized liver slices.

**Eprosartan uptake in rat isolated hepatocytes**

Isolated rat hepatocytes (n = 3) were prepared by the collage-nase perfusion method as described by Maeda and Sugiyama [21]. First, the number of viable isolated hepatocytes was determined by trypsin blue staining (viability >80%). Then, performed on ice, isolated hepatocytes were suspended in Krebs–Henseleit buffer and adjusted to 2.0 × 10⁶ cells/ml. Before the uptake study, the cell suspension and Krebs–Henseleit buffer containing the substrate were prewarmed in an incubator at 37 °C for 3 min and then the uptake studies were initiated by adding an equal volume of buffer contained with substrates to the cell suspension. Uptake of doxigoxin (a positive control, substrate concentrations used were 5 μM and 200 μM) and eprosartan (1 μM and 100 μM) by isolated rat hepatocytes were measured over 10 min at 37 °C. After incubation at 37 °C for designated time (0.5, 1, 2, 3, 5 or 10 min), the uptake reaction was terminated by separating the cells from the medium solution by centrifugal filtration. In order to resolve this purpose, a 100 μl sample of incubation mixture was collected and removed to a 450 μl centrifuge tube (Hepatocyte Transporter Suspension Assay Kit, BD Gentest™) contained with sodium acetate (5 M) under an oil mixture layer (100 μl, silicone oil mixed with mineral oil, density 1.015; BD Gentest™). After that, the tube was centrifuged at 10,000 × g for 10 s (ST-16R, Thermo) and the hepatocytes passed through the oil layer into the aequous basic solution. Tubes were frozen in liquid nitrogen immediately after centrifugation and stored at −80 °C until drug measurement by LC–MS/MS. Also, the effects of inhibitors, including cyclosporin A (10 μM), ibuprofen (150 μM), digoxin (5 μM), rifampicin (10 μM), TEA (1 mM) and PAH (1 mM) were performed on the uptake of eprosartan in rat isolated hepatocytes. On the other hand, sodium was replaced by potassium in Krebs–Henseleit buffer to examine the sodium dependence of eprosartan (1 μM) uptake by isolated rat hepatocytes.

**Eprosartan uptake by transporter expression systems**

The transport study was done as described [13]. Briefly, in 24-well culture plates, cells were seeded for 48 h (nearly confluent) before each experiment. Cells were washed twice and incubated with Krebs–Henseleit buffer at 37 °C for 15 min, and then the uptake was started by adding Krebs–Henseleit buffer containing drugs. After removal of the incubation buffer, uptakes were terminated by ice-cold Krebs–Henseleit buffer, added to the culture plates, at designated times. Following that, cells were washed twice with 1 ml ice-cold Krebs–Henseleit buffer, then lysed with 0.3 ml 0.1% (v/v) Triton X-100, and kept at 37 °C for 1 h then took into a polythene tube for quantification by LC–MS/MS. Cell protein was measured by the bicinchoninic acid (BCA) procedure (Solarbio, China) using bovine serum albumin as the standard. The uptake of eprosartan (1 μM) was measured in OATP1B3- and vector-HEK293 cells at designated times (0.5, 1, 2, 3, 5 and 10 min).

**Transcellular transport study**

Modified method of transcellular transport study was done essentially as described [24]. In brief, vector-, OATP1B1-, OATP1B1/ MRP2-transfected MDCK II cells were seeded (1.4 × 10⁶ cells per well) in 24-well Transwell inserts (12 mm diameter, 0.6 cm² growing surface area, 0.4 μm pore size; Corning Costar, Acton, MA). First, MDCKII cells were grown for 3–5 days on Transwell membrane inserts until confluence. In order to estimate the integrity of the cell monolayer, the transepithelial electrical resistance (TEER > 200 Ω cm²) was measured by Millicell-ERS equipment (Millipore, MA, USA). After conforming that, monolayer cells were rinsed gently with transport buffer (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, 1.5 mM CaCl₂, pH 7.4) at 37 °C and the transwell chambers were incubated for 15 min. Subsequently, transepithelial transport experiments were started by changing control incubation buffer to containing eprosartan buffer (1 μM, final concentration) to the apical (400 μl) or basolateral (600 μl) compartment. Cells were incubated at 37 °C under a 5% (v/v) CO₂ atmosphere, and 50 μl aliquots sample were took out of the receiver compartment at 30, 60 and 120 min. After sample retrieval, an equal volume of transport buffer was added immediately to the sampling compartment. Intracellular accumulation (120 min) was measured after washing the monolayer rapidly four times with transport buffer on each side. The inserts with a monolayer were detached from the chambers and cells were lysed by Triton X-100. Protein was measured by the same methods described above. Quantization of eprosartan in this series experiments were determined by LC–MS/MS.

Transcellular transport of eprosartan was calculated as the accumulated amount of substrate in receiver compartment divided by the initial drug concentration in the donor compartment and then normalized to the cell growth surface area (μl/cm²). The apparent permeability (P_app, centimeter/second) from basolateral to apical (P_appB,A) or from apical to basolateral (P_appA,B) was estimated in turn as the slope of area-normalized transcellular transport (microliter per centimeter²) vs. time plot.

**Biological sample preparation**

Liver slice samples were weighed and then homogenized with 300 μl of normal saline in an ice-bath. Eprosartan preparation: first, the homogenate (50 μl) obtained from the liver slice studies was precipitated with 200 μl of acetonitrile following addition of IS (cilostazol, 2 μM, 50 μl). Next, the mixture was vortex-mixed for 30 s and then centrifuged for 10 min at 16,099 × g. Subsequently, the supernatant organic layer was transferred to a polythene tube and dried with nitrogen at 37 °C. Last, the dried residue was redissolved by 200 μl mobile phase. Other preparations were handled as described for the liver slice–homogenized samples of eprosartan. Rat liver hepatocytes in sodium acetate buffer (5 M) were taken from centrifuge tube and placed into fresh tube, then sonicated. After that, this sample (50 μl) was transferred to a polythene tube and then added with IS (cilostazol, 2 μM, 50 μl) and water (500 μl). Following vortex-mixing for 10 s, extraction solvent (acetic ether, 1 ml) was added, vortex-mixed for 3 min and then centrifuged for 10 min at 2795 × g. The supernatant organic layer was transferred to a polythene tube and dried with nitrogen at 37 °C, and then the dry residue was redissolved by 200 μl mobile phase. The uptake and transepithelial transport samples and digoxin (positive control) samples were also handled as described for liver slice–homogenized samples for eprosartan. At last, the biological samples were subjected to LC–MS/MS analysis.

**LC–MS/MS analysis**

In this study, an Agilent LC system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA) was used for LC–MS/MS analysis. Isocratic chromatographic separation column: Hypersil BDS-C18 column (150 mm × 4.6 mm i.d., 5 μm; Dalian Elite Analytical Instruments Co., Ltd., China), maintained at ambient
temperature. Mobile phase: 50% (v/v) acetonitrile, 50% (v/v) water with 0.1% (v/v) formic acid for eprosartan (flow rate: 0.3 ml/min), 70% (v/v) methyl alcohol, 30% (v/v) water with 0.1% formic acid for digoxin (flow rate: 0.5 ml/min). An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, ON, Canada) was operated with a TurboIonSpray interface in positive ion mode for eprosartan and IS (cilostazol), in negative ion mode for digoxin. Analyst 1.4.1 software (Applied Biosystems) and multiple reactions monitoring (MRM) were used to control the equipment, data acquisition and analysis, respectively. MRM fragmentation transitions: m/z 425.1 → m/z 207.1 with a collision energy (CE) of 50 eV for eprosartan, m/z 370.4 → 288.1 with CE of 50 eV for IS (cilostazol), m/z 779.0 → 649.0 with CE of −50 eV for digoxin. Dwell time for each transition was 200 ms.

Data analysis

The hepatic extraction ratio ($E_h$) of eprosartan was calculated as follows:

$$E_h = 1 - \frac{C_{out}}{C_{in}}$$  \hspace{1cm} (1)

where $C_{out}$ and $C_{in}$ are the concentration of eprosartan in effluent perfusate and influent perfusate, respectively.

Cumulative biliary excretion rates were calculated as the infusion rate of eprosartan divided by the biliary excretion rate of eprosartan.

Time courses of eprosartan uptake in rat liver slices, isolated hepatocytes and transporter-expressing transfected cells were expressed as the uptake volume $\mu$l/mg liver, $\mu$l/10$^6$ cells and $\mu$l/mg protein, respectively, given as the eprosartan taken up into cells with 0.1% (v/v) formic acid for eprosartan (flow rate: 0.3 ml/min), 70% (v/v) methyl alcohol, 30% (v/v) water with 0.1% (v/v) formic acid for digoxin (flow rate: 0.5 ml/min). A solution containing 20% (v/v) dimethyl sulfoxide (DMSO), 10% DMSO and 70% water with 0.1% formic acid for IS (cilostazol), 70% (v/v) methyl alcohol, 30% (v/v) water with 0.1% (v/v) formic acid for digoxin.

Specific uptake kinetic parameters of rat isolated hepatocytes were obtained using non-linear, least-squares regression analysis via the Michaelis–Menten equation:

$$v = \frac{V_{max} S}{K_m + S}$$  \hspace{1cm} (2)

where $v$ is the uptake velocity of the substrate (pmol/min per mg protein), $S$ is the substrate concentration in medium ($\mu$mol/l), $K_m$ is the Michaelis constant ($\mu$mol/l), and $V_{max}$ is the maximum uptake rate (pmol/min per mg protein).

Statistical analysis was carried out using SPSS 11.5 package. Data were expressed as mean ± standard error of the mean (SEM). Statistical significance of differences between mean values was calculated using non-paired $t$-test. Differences were considered significantly when values of $p < 0.05$ or $p < 0.01$.

Results

Hepatic uptake and biliary excretion of eprosartan by perfused rat liver studies

To examine the mechanism of hepatic eprosartan uptake, in situ perfused rat liver studies were conducted and the concentrations of eprosartan in the effluent perfusate were determined in the presence or absence of rifampicin (10 $\mu$M), a commonly used inhibitor of OATPs. Based on the value for the hepatic extraction ratio calculated by Eq. (1), the concentration of eprosartan reached less than a plateau after 25 min and the hepatic extraction ratio remained a constant value (35.5 ± 0.1%) until the end of the perfusion (Fig. 1A). When eprosartan was administered simultaneously with rifampicin (10 $\mu$M), the hepatic extraction ratio of eprosartan was reduced to 27.0 ± 0.3% (Fig. 1A).

Moreover, the hepatic extraction ratio of eprosartan was not changed in the presence of the excretion inhibitors (probenecid for Mrp2, verapamil for P-gp and novobiocin for Bcrp, 20 $\mu$M, respectively) when compared to the control (Fig. 1B).

On the other hand, to examine the mechanism of the biliary excretion of eprosartan, the concentrations of eprosartan in the bile were determined in the presence or absence of the inhibitors mentioned above by perfused rat liver in situ. With concomitant perfusion of probenecid, cumulative biliary excretion rates of eprosartan were significantly reduced to 73.3% of the control over 45 min (Fig. 2). While cumulative biliary excretion rates of eprosartan did not change in the presence of verapamil or novobiocin (Fig. 2).

Hepatic uptake of eprosartan in rat liver slices

In order to directly investigate the hepatic uptake of eprosartan from the tissue level, rat liver slice uptake experiments were performed in vitro. The uptake of eprosartan (1 $\mu$M) at 37 °C was linear within the first 15 min and a significant difference could be found between the amount of eprosartan uptake in the liver slice at 37 °C and 4 °C (Fig. 3A). This saturable and temperature-dependent trend was similar to that of digoxin (5 $\mu$M, a positive drug of Oatp1a4) (Fig. 3B). Meanwhile, the inhibitory effects of transporter modulators were investigated on eprosartan uptake to determine...
the mechanism for eprosartan uptake by the liver. Cyclosporine A, ibuprofen, digoxin and rifampicin could significantly inhibit the initial uptake of eprosartan in rat liver slices. In contrast, TEA and PAH did not inhibit the uptake of eprosartan (Fig. 3C).

**Hepatic uptake of eprosartan in isolated rat hepatocytes**

To examine whether eprosartan was taken up into the hepatocytes in cellular level, isolated rat hepatocytes was used to investigate the uptake of eprosartan. The uptake of eprosartan (1 μM) by rat hepatocytes increased linearly up to 3 min (Fig. 4A) and this linear time tendency was similar as that of digoxin (5 μM, linear time: 3 min) (Fig. 4B). Based on the result, we selected 1 min as the linear uptake time to investigate subsequent studies for the uptake of eprosartan. Uptake of eprosartan in rat hepatocytes was a saturable process with $K_m$ $14.25 \pm 0.80$ μM and $V_{max}$ $1606 \pm 25$ pmol/min/10$^6$ cells (Fig. 4C). In addition, the inhibitory effects of transporter modulators were examined, using cyclosporine A (10 μM), ibuprofen (150 μM), digoxin (5 μM), rifampicin (10 μM), TEA (1 mM) and PAH (1 mM) in the presence of eprosartan. Uptake of eprosartan was significantly inhibited by cyclosporine A, ibuprofen, digoxin and rifampicin, but not by TEA and PAH (Fig. 4D). Moreover, the sodium dependent uptake of eprosartan was not found when extracellular sodium ions were replaced with potassium ions at equimolar concentration (Fig. 4D).

**Uptake of eprosartan in OATP1B3-HEK293 cells**

To examine the target transporters involved in eprosartan transport in human, the uptake of eprosartan by HEK293 cells expressing OATP1B3 and vector were measured. However, no significant difference in uptake of eprosartan was found between OATP1B3-HEK293 cells and vector-HEK293 cells.

**Transcellular transport of eprosartan in MDCKII cells**

To investigate which transporters recognize eprosartan, a transcellular transport assay using OATP1B1 single-transfected cells and OATP1B1/MRP2 double-transfected cells were performed in this study. Significant difference was found in the intracellular
accumulations of eprosartan between vector- and OATP1B1-MDCKII cells after 2 h (Fig. 5). Moreover, much higher transcellular flux of eprosartan from basolateral to apical (B → A) was observed in double-transfected OATP1B1/MRP2/MDCKII cells vs. the single-transfected OATP1B1/MDCKII cells and the mock/MDCKII control cells (Fig. 6A). This higher transcellular flux pattern of eprosartan from B → A was similar to that of estrone-3-sulfate (ES) (Fig. 6C) [24] and E217G [21,45], two model substrates of OATP1B1 [19] and MRP2 [35], which were used to verify the double-transfectant system. The flux of eprosartan from A → B in double-transfected OATP1B1/MRP2/MDCKII cells was similar to that in single-transfected OATP1B1/MDCKII cells and the mock/MDCKII control cells, showing that the transport of the compound did not occur (Fig. 6B).

Discussion

Drug uptake followed by metabolism and excretion in hepatocytes is a major determinant of the systemic clearance and exposure of many clinical drugs. While eprosartan is absorbed from blood into hepatocytes and excreted into bile, and it is excreted predominantly in an unchanged form, with minimal metabolism in humans [33]. Therefore, metabolism in hepatocytes is not a major determinant of eprosartan, drug uptake on the basolateral membrane and excretion on the canalicular membrane are thought to be the two key steps in hepatic clearance of eprosartan in liver. Considering several transporters, such as OATP1B1, OATP1B3, MRP2, BCRP and MDR1 are expressed on the basolateral membrane and canalicular membrane of human hepatocytes, and these transporters involved in the hepatobiliary transport of eprosartan have not been fully investigated yet, it is important and necessary to identify these transporters involved in the hepatic clearance of eprosartan in human and rat. Therefore, the pharmacokinetics of eprosartan on hepatic uptake and bile excretion transporters were investigated in detail in this study to understand the transport mechanism of eprosartan.

In the present study, an in situ uptake study (perfused rat livers), two in vitro uptake studies (rat liver slices and isolated rat hepatocytes) and transfected cells (OATP1B3-HEK293 cells and OATP1B1-MDCKII cells) were used to investigate the hepatic
transport mechanisms of eprosartan. The results provide direct evidence that the OATP family (at least OATP1B1) and MRP2 participate in the hepatic transport of eprosartan in human, while multiple Oatps (at least Oatp1a1 and Oatp1a4) and Mrp2 mediate this process in rat.

First, the hepatic extraction ratio of eprosartan was significantly inhibited by rifampicin (the inhibitor of Oatps) \[10\], indicating that the transporters of Oatp family might participate in the uptake of eprosartan in rat liver. Moreover, the uptake of eprosartan in rat liver slices was a saturable process. Compared with 37 °C, the uptake of eprosartan was about 1.5-fold greater than that at 4 °C after incubation for 30 min. The uptake of eprosartan in the liver at 4 °C might be explained by nonspecific membrane binding or intracellular binding after passive diffusion into the cells \[34\]. The difference in drug uptake at 4 °C and at 37 °C indicates that uptake of eprosartan in the liver was partly mediated by a hepatic transporter. These phenomena indicate that the liver may play an un-negligible role in the elimination of eprosartan in rats. Therefore, detailed investigation of hepatic-biliary transport is crucial for understanding the pharmacokinetics of eprosartan in rats and humans. Furthermore, the specific inhibitors of different Oatp isoforms, such as cyclosporin A, ibuprofen, digoxin and rifampicin, were markedly decrease the uptake of eprosartan in rat liver slices at the concentrations which significantly exceeded the respective \( K_c \) values \[10,37\]. These results indicated that multiple Oatp isoforms are involved in the rat liver extraction process of eprosartan. On the other hand, high concentration of TEA and PAH did not inhibit the uptake of eprosartan. Results mentioned above indicate that the Oatp family might be involved in the carrier-mediated transport mechanisms for eprosartan in rat liver slices.

Next, eprosartan transport into rat hepatocytes is time- and concentration-dependent. The apparent \( K_m \) of eprosartan was 14.25 ± 0.80 μM in rat hepatocytes. These results indicate that the process of eprosartan uptake in hepatocytes is partly mediated by transporters. The results of eprosartan uptake in isolated rat hepatocytes are also similar to digoxin, suggesting that eprosartan is a substrate of Oatp1a4. Since Na\(^+\)-dependent and Na\(^+\)-independent hepatic transport systems are known to be responsible for the clearance of various compounds from the systemic circulation \[26,42\], and the transporters of Oatp family are well known as the Na\(^+\)-independent hepatic transport systems expressed on the basolateral membrane \[1,2\]. Therefore, the following uptake study was performed under Na\(^+\)-free conditions and in the presence of various typical inhibitors of hepatic uptake transporters. As expected, no difference of eprosartan uptake was found when sodium was replaced by potassium, suggesting that eprosartan is taken up by rat hepatocytes via a Na\(^+\)-independent transport(s) \[30\]. On the other hand, significant decrease in eprosartan uptake was found in the presence of various typical inhibitors which were used in rat liver slices study. Moreover, neither TEA nor PAH changed the uptake of eprosartan in rat hepatocytes. These results are corresponded well with that in rat liver slices and indicate that the Oatp family (at least Oatp1a1 and Oatp1a4), but not Oct1 or Oat2, might contribute to the uptake of eprosartan in rat liver.

The hepatic extraction ratio is decreased from 35.5% to 27.0% in the presence of rifampicin. In another word, the inhibition rate of rifampicin was nearly 23.9% \([35.5\% - 27.0\%]/35.5\%\) in liver perfusion experiment of eprosartan. This phenomenon can be quantitatively explained by the \( \text{in vitro} \) inhibition assay using rat liver slices or isolated rat hepatocytes. The inhibition effect of hepatic extraction ratio was coincide with rifampicin inhibition effects on rat liver slices and isolated rat hepatocytes experiments (inhibition rate was 27.3% and 23.3%, respectively).

OATP1B1 and OATP1B3 are the main isoforms which are expressed on the basolateral membrane in human hepatocytes and they participate the process of hepatic uptake of various compounds \[1,2\]. In the present study, in order to determine the potential of OATP isoforms for transport of eprosartan, OATP1B3-HEK293 cells and OATP1B1-MDCKII cells were used to evaluate the transport properties of eprosartan. Initially, RT-PCR and Western blot analysis were performed to investigate the transporter expression in HEK293 and MDCKII cells, and the results indicated the expression and function of these transfected cells systems are reliable. However, no significant time-dependent uptake of eprosartan was observed in OATP1B3-HEK293 cells. On the other hand, significant difference was found in the intracellular accumulations of eprosartan between vector- and OATP1B1-MDCKII cells. This result of intracellular accumulations indicates that OATP1B1 is involved in the hepatic uptake of eprosartan. These results mentioned above suggest that eprosartan is transported into hepatocytes by OATP1B1 rather than OATP1B3 in human. Therefore, the hepatic uptake of eprosartan might be influenced if it is administered simultaneously with drugs known as the substrate or inhibitor of OATP1B1.

It is well known that MRP2, BCRP and P-gp play important roles in the efflux of compounds into bile. Some transfected cell lines such as OATP1B1/MRP2 double transfected MDCKII cells \[21,24,45\] and mutant animals \[16\] have been used to estimate the involvement of Mrp2 in vivo or in vitro. However, the transporters involved in the biliary excretion of eprosartan in rat or human has not been fully investigated yet.

\[ \text{Fig. 6. Transcellular transport of eprosartan from the basolateral-to-apical (B–A) direction (A) and from the apical-to-basolateral (A–B) direction (B) in mock/MDCKII (○). OATP1B1/MDCII ( ■ ), and OATP1B1/MDCKII ( ◆ ) cells, respectively.} \]
Probenecid [27,31], but not verapamil [27,32] or novobiocin [27,40], significantly decrease the cumulative biliary excretion rates of eprosartan in perfused rat livers studies. These results suggest that Mrp2 is likely to be involved in biliary excretion of eprosartan, while P-gp and Bcrp have no contribution to this process in rat.

Recently, a double-transfected cells system which expresses both uptake transporter and efflux transporter has been well established and validated as a useful system for the identification of transport substrates or inhibitors [7]. Also, this system has been shown to be more sensitive than membrane vesicles in the study of canalicular transport [35]. According to this system for identification, efflux transporters involved in the biliary excretion of eprosartan were investigated in the OATP1B1/MRP2 double transfected MDCKII cells and the basal-to-apical vectorial transport of eprosartan was observed. The results indicate that eprosartan is transported by both OATP1B1 and MRP2. The transcellular transport of eprosartan exhibited the same trend with ES and E217G, two typical substrates of high affinity to both OATP1B1 and MRP2, which were reported in the results of Liu et al. [21], Matsushima et al. [24] and Yamada et al. [45] in OATP1B1/MRP2 double-transfected system. The ratio of apparent permeability ($P_{app}$) of eprosartan are OATP1B1/MRP2/MDCKII (6.94) > OATP1B1/MDCKII (2.42) > mock/MDCKII (1.26). Taking these results into consideration, it appears that MRP2 plays an important role in the biliary excretion of eprosartan in humans and in rats.

Generally, it is accepted that the major OATP members expressed in hepatocytes, including OATP1B1 and OATP1B3, are involved in sartans (ARA-II) transport. OATP1B1 is responsible for the hepatic uptake of several sartans, such as fimasartan [17], involved in sartans (ARA-II) transport. OATP1B1 and MRP2, which were reported in the results of Liu et al. [21], Matsushima et al. [24] and Yamada et al. [45] in OATP1B1/MRP2 double-transfected system. The ratio of apparent permeability ($P_{app}$) of eprosartan are OATP1B1/MRP2/MDCKII (6.94) > OATP1B1/MDCKII (2.42) > mock/MDCKII (1.26). Taking these results into consideration, it appears that MRP2 plays an important role in the biliary excretion of eprosartan in humans and in rats.

In conclusion, the OATP family (at least OATP1B1) and multiple Oatp isoforms (at least Oatp1a1 and Oatp1a4) are responsible for the hepatic uptake of eprosartan in human and in rat, while the efflux clearance of eprosartan is mainly via MRP2, at least, in human and Mrp2 in rat. These findings may contribute to understand the mechanisms of hepatobiliary transport of sartans and also provide some insight in the use of eprosartan for possible drug interactions at the hepatobiliary disposition, especially when eprosartan is administered simultaneously with drugs which are substrates or inhibitors of OATP1B1 and/or MRP2.

Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

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References


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