Vectorial Transport of Enalapril by Oatp1a1/Mrp2 and OATP1B1 and OATP1B3/MRP2 in Rat and Human Livers

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Running Title: Vectorial transport of enalapril in rat and human livers

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Text: 16 pages

Abstract: 249 words

Introduction: 738 words

Discussion: 1206 words

Table: 1

Figures: 7

References: 40

Abbreviations: Oatp and OATP, rat and human organic anion transporting polypeptide, respectively; Mrp2 and MRP2, rat and human multidrug resistance-associated protein 2, respectively; ACE inhibitor, angiotensin-converting enzyme inhibitor; EHBR, Eisai hyperbilirubinemic rat; TLC, thin layer chromatography; CL_liver,tot, total hepatic clearance; CL_liver,ex, biliary clearance; CL_liver,met, hepatic metabolic clearance; HEK 293, human embryonic kidney 293 cells; MDCK II, Madin-Darby canine kidney II cells; E_217G, estradiol-17β-D-glucuronide; TEER, transepithelial electrical resistance; CMVs, canalicular membrane vesicles

Review Section: Absorption, Distribution, Metabolism, and Excretion
Although Oatp1a1 (rat organic anion transporting polypeptide 1a1) was the transporter found responsible for the hepatocellular entry of enalapril (EN) into the rat liver, the canalicular transporter involved for excretion of EN and the metabolite, enalaprilat (ENA), was unknown. The Eisai hyperbilirubinemic rat (EHBR) that lacks Mrp2 (multidrug resistance-associated protein 2) was used to appraise the role of Mrp2 in the excretion of [3H]EN and its metabolite [3H]ENA in single pass rat liver preparations. Although the total and metabolic clearances and hepatic extraction ratios at steady-state were virtually unaltered for EN in EHBR compared to published values of Sprague Dawley rats, the biliary clearances of EN and ENA were significantly reduced almost to zero \((P < 0.05)\). Involvement of human OATP1B1, OATP1B3 and MRP2 in EN transport was further assessed in single- or double-transfected mammalian cells. Human embryonic kidney (HEK) 293 cells that expressed OATP1B1 or OATP1B3 showed that OATP1B3 transport of EN \((20 \text{ to } 500 \mu M)\) was of low affinity, whereas transport of EN by OATP1B1 was associated with the \(K_m\) of \(262 \pm 35 \mu M\), a value similar to that for Oatp1a1 \((214 \mu M)\). The transcellular transport of EN \(via\) human OATP1B1 and MRP2, investigated with the double-transfected Madin-Darby canine kidney (MDCK) II cells in Transwells® showed that the sinusoidal to canalicular flux of EN in the OATP1B1/MRP2/MDCK cells was significantly higher \((P < 0.05)\) than those of mock/MDCK and OATP1B1/MDCK cells. EN was transported by Oatp1a1 and Mrp2 in rats and OATP1B1/OATP1B3 and MRP2 in humans.
INTRODUCTION

It is increasingly being recognized that drug removal is dependent not only on metabolic enzymes but also transporters. Since both metabolism and biliary excretion occur behind transport barriers, uptake sinusoidal transporters could constitute the rate-limiting step in the hepatic clearance of drugs (Yamazaki et al., 1996). A plethora of sinusoidal transporters, including the organic anion transporting polypeptides (OATPs), sodium-dependent taurocholate cotransporting polypeptide (NTCP), organic anion transporters (OATs), and organic cation transporters (OCTs), and the ATP-driven canalicular transporters: the multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), bile salt export pump (BSEP/ABCB11), and the breast cancer resistance protein (BCRP/ABCG2) facilitate the ultimate excretion of substrates into bile (for review, see Mizuno et al., 2003).

The OATP/MRP combination represents two families of transporters that play important roles in the hepatic transport of organic anions at the sinusoidal and canalicular membranes. In the human liver, OATP1B1 (also known as OATP2 or OATP-C), and OATP1B3 (OATP8) are predominant transporters responsible for the uptake of a variety of organic anions (Mikkaichi et al., 2004). In the rat liver, the transporters, Oatp1a1 (Oatp1), Oatp1a4 (Oatp2), and Oatp1b2 (Oatp4), function similarly as those of OATP1B1 and OATP1B3 in the human liver (Chandra and Brouwer, 2004). Once taken up into hepatocytes, amphiphilic anions and their conjugates may be further excreted into bile by MRP2 (human)/Mrp2 (rat) (Büchler et al., 1996), MDR1/Mdr1, and the BCRP/Bcrp (Breedveld et al., 2004; Hirano et al., 2005; Matsushima et al., 2005). MRP3/Mrp3 competes with MRP2/Mrp2 or BCRP/Bcrp and effluxes the substrate back at the sinusoidal membrane (Kuroda et al., 2004; Manautou et al., 2005). The uptake of anionic drugs via the OATPs/Oatps, followed by excretion via MRP2/Mrp2, constitutes vectorial transport for the hepatobiliary excretion of drugs.
Much of the previous hepatic transport data have been acquired from transport studies with membrane vesicles prepared from basolateral or canalicular membranes (Meier and Boyer, 1990; Ishizuka et al., 1997), intact isolated hepatocytes (Brouwer et al., 1987), sandwich-cultured rat hepatocyte systems (Liu et al., 1999), or liver perfusion studies (de Lannoy et al., 1993). Mutant animals such as the Eisai hyperbilirubinemic rat (EHBR) or TR− Wistar rat that lacks Mrp2 have been utilized to show the involvement of Mrp2 in the excretion of drug substrates or conjugates in vivo or in vitro (Ishizuka et al., 1997). The establishment of various knock-out mice: Mdr1(-/-), Mrp2(-/-), Bcrp(-/-) or Mrp3(-/-), lacking the efflux transporters (Schuetz et al., 2000; Manautou et al., 2005; Nezasa et al., 2006) provides further available tools for transport studies. However, both the mutant and knock-out animals have, besides modulation of the target genes, suffered other alterations in enzymatic activities and/or transporter functions (Schuetz et al., 2000; Newton et al., 2005; Johnson et al., 2005). The targeting of “specific” inhibitors on transporters or enzymes has also shown that the intended inhibition may not be as specific as expected (Hoffmaster et al., 2004).

In vitro gene expression systems in Xenopus laevis oocytes or mammalian cells, such as HeLa cells, human embryonic kidney (HEK) 293 cells, Madin-Darby canine kidney (MDCK) II cells and porcine kidney (LLC-PK1) cells, consisting of the single-transfected (Cui et al., 2001b), double-transfected (Cui et al., 2001a; Sasaki et al., 2002) or even quadruple-transfected (Kopplow et al., 2005) cell systems, are becoming widely utilized in drug transport studies. The methodology provides relevant evidence on the involvement of the transporters examined, but seldom divulges the relative importance of the transporter. Recently, the RNA interference technique is applied to knockdown drug transporters. This approach, if completely effective and specific, could present new insight on the importance of the transport pathway (Tian et al., 2005).
Vectorial transport of the angiotensin-converting enzyme (ACE) inhibitor, temocaprilat, was shown to involve Oatp1a1 and Mrp2 (Ishizuka et al., 1997; 1998). Another popularly used ACE inhibitor prodrug enalapril (EN), but not its hydrolyzed metabolite, enalaprilat (ENA), was found to readily enter the rat liver via Oatp1a1 (Pang et al., 1998). The compound is eliminated primarily via metabolism by the carboxylesterases to ENA in rat liver, and both EN and ENA are excreted into bile (Pang et al., 1984) (Figure 1). Whether Mrp2 is involved in EN and ENA excretion, and whether EN is a substrate of OATP1B1/OATP1B3 and MRP2 in humans is unknown. In this study, we examined the excretion of EN and ENA in EHBR single-pass perfused liver preparations and employed single- and double-transfected cells to study the involvement of OATP1B1/OATP1B3 and MRP2 in the vectorial transport of EN.
MATERIALS AND METHODS

Materials

Bovine blood was a kind gift from Ryding-Regency Meat Packers Ltd. (Toronto, ON, Canada). $[^3]$H Estradiol-17β-D-glucuronide ($[^3]$H$E_2$17G; specific activity 1.7 TBq/mmol) was purchased from the Perkin Elmer Life Sciences (Boston, MA). $[^3]$H Enalapril ($[^3]$H$EN$; specific activity 4.1 TBq/mmol) was synthesized as described previously (Pang et al., 1998). The purities of $[^3]$H$E_2$17G and $[^3]$H$EN$ were > 98.5%, as verified respectively by high performance liquid chromatography (HPLC) for $E_2$17G [gradient of 10 mM NH$_4$Ac and acetonitrile (15% - 50%) at pH 5.0; $E_2$17G retention time of 28 min], and thin layer chromatography (TLC) for EN [solvent systems of n-propanol : acetic acid (1 M) : H$_2$O (10:1:1; v/v/v) and 1-butanol : glacial acetic acid : H$_2$O (4:1:1; v/v/v)]. Unlabeled EN and ENA were kindly supplied by Dr. J.H. Lin, Merck Laboratories (West Point, PA). All other reagents were of the highest available grade.

Single Pass Liver Perfusion with EHBR Rats

Male EHBR rats (240-265 g) were used for single pass liver perfusion according to previously published procedures (Pang et al., 1984; de Lannoy et al., 1993). The EHBR rats were a kind gift from Dr. T. Yoshimura, Eisai Company, Tsukuba City, Japan. Rats were kept under a 12:12 h light:dark cycle and given food and water ad libitum. Studies were conducted in accordance to protocols approved by the Animal Committee of the University of Toronto. Perfusate consisted of 20% washed, fresh bovine red blood cells, 1% bovine serum albumin (Sigma, St. Louis, MO), 5 mM glucose in Krebs-Henseleit Bicarbonate solution (pH = 7.4). $[^3]$H$EN$ was delivered into the liver via the portal vein (10 ml/min for 80 min). Reservoir perfusate was sampled at 30, 50, and 70 min, and the mean of three determinations was taken as the steady-state input concentration, $C_{In}$. Venous outflow samples were collected at 15, 35, 45,
55, 62.5, 67.5, 72.5, and 77.5 min, and the mean of the last four determinations that reflected constancy in output concentrations was taken as the steady-state output concentration, C\text{Out}. Bile was collected at 5- and 10 min intervals during the perfusion, and the mean of the last three or four determinations that reflected constancy in bile concentrations was taken as the steady-state bile concentration, C\text{bile}. [3H]EN and its metabolite, [3H]ENA, in perfusate plasma and bile were separated and analyzed by TLC (Silica Gel HLF; Analtech, Newark, DE) in n-propanol : acetic acid (1 M) : H\text{2}O (10:1:1; v/v/v) solvent system, with unlabeled EN and ENA as authentic markers applied at the origin prior to development of the TLC plate.

For data analysis, the extraction ratio (E) was calculated as \((C_{\text{In}} - C_{\text{Out}})/C_{\text{In}}\). Total hepatic clearance (CL\text{Liver,tot}) was calculated as the product of \(Q_{\text{p}}\), the plasma flow rate, and E, inasmuch as EN was not distributed into red blood cells (Pang et al., 1984; de Lannoy et al., 1993). The biliary clearance (CL\text{Liver,ex}) was estimated as the \(Q_{\text{bile}}C_{\text{bile}}/C_{\text{In}}\), the biliary excretion rate, given by the product of the bile flow rate (\(Q_{\text{bile}}\)) and the concentration of EN in bile (\(C_{\text{bile}}\)), normalized to the arterial EN concentration, \(C_{\text{In}}\). The hepatic metabolic clearance (CL\text{Liver,met}) was given as the difference, CL\text{Liver,tot} - CL\text{Liver,ex}, or directly as the summed appearance rate of ENA in bile and outflow perfusate, divided by the arterial EN concentration, \(C_{\text{In}}\). The apparent extraction ratio of formed metabolite, ENA (E\{mi\}) as \(Q_{\text{bile}}C_{\text{bile}\{mi\}}/[Q_{\text{bile}}C_{\text{bile}\{mi\}} + Q_{\text{p}}C_{\text{p}\{mi\}}]\), in which \(C_{\text{bile}\{mi\}}\) and \(C_{\text{p}\{mi\}}\) were the ENA concentrations in bile and perfusate plasma, respectively.

**EN Uptake by Single-Transfected HEK 293 Cells**

HEK 293 cells that were transfected with OATP1B1 or OATP1B3 or vector (control) were seeded in 6-well plates (coated with 0.1 mg/ml poly-D-lysine) at a density of 1.5-2 \times 10^6 cells per well, and cultured with 10 mM sodium butyrate for 24 h, as previously described (Cui et al., 2001b). Before the uptake experiments, cells were first washed with uptake buffer (142 mM...
NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM glucose, and 12.5 mM HEPES, pH 7.3) and then incubated with 1 ml of uptake buffer containing the [³H]EN. After incubation for 10 min at 37°C, the substrate was immediately removed, and cells were washed three times with cold uptake buffer before they were lysed with 1 ml of 0.1% SDS in water. The cell-associated radioactivity was determined by transferring 250 µl aliquot of the lysate into a scintillation vial for liquid scintillation counting. Protein content was determined according to Lowry et al. (1951) using 100 µl of the lysate. Uptake velocities were measured at the concentrations range from 20 to 500 µM EN (0.3 µCi/ml [³H]EN in buffer) for the determination of Michaelis-Menten constant (Kₘ) and maximal velocity (Vₘₐₓ) subsequent to fitting with SCIENTIST v.2 (MicroMath Scientific Software, Salt Lake City, UT).

**EN Transcellular Transport in Double-Transfected MDCK II Cells**

*Media and cell culture.* The cell construction was previously described (Matsushima et al., 2005). The complete media for mock (vector-control), OATP1B1 and OATP1B1/MRP2 transfected MDCK II cells consisted of Dulbecco’s modified Eagle’s medium (DMEM; low glucose version) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (all from Invitrogen-Gibco, Burlington, ON, Canada). All transfected MDCKII cells were cultured at 37°C with 5% CO₂ with 300 µg/ml of Zeocin (Invitrogen) during cell culture.

*Western blotting analysis.* Cells were homogenized in homogenate buffer (250 mM sucrose, 10 mM HEPES, 10 mM Tris-base, pH 7.4) with a protease inhibitor cocktail (1:100, v/v) (Sigma Canada, Oakville, ON,) to provide crude membrane fractions of the transfected MDCK II cells. The homogenate was first centrifuged at 3,000g for 10 min at 4°C, and then the resultant supernatant was centrifuged at 33,000g for 30 min at 4°C. The formed pellet (crude membrane fraction) was resuspended in buffer (50 mM mannitol, 20 mM HEPES, 20 mM Tris-
base, pH 7.5) with the protease inhibitor cocktail. The protein concentration of the sample was determined by method of Lowry et al. (1951), with BSA as the standard. Tissue samples (50 µg for OATP1B1 and 20 µg for MRP2) in the loading buffer were heated at 95°C for 5 min or 37°C for 15 min. Immunoblotting was conducted with SDS-polyacrylamide gel electrophoresis (7.5% or 12% gel), and proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham, Oakville, ON). Before the blocking step, the ponceau staining was used to confirm the equal amount of protein was loaded. After blocking with TBS-T buffer with 5% nonfat milk, the membrane was incubated first with primary antibody overnight, then with the secondary antibody for 1 h before development for the chemiluminescent detection with ECL (Amersham, Little Chalfont, Buckinghamshire, UK). For the immunodetection of human OATP1B1, 1:500 dilution (v/v) of the primary mouse monoclonal antibody (Abcam Inc., Cambridge, MA) and 1:500 dilution (v/v) of the secondary antibody, HRP-anti mouse IgG (Bio-Rad, Richmond, CA), were used. For human MRP2, 1:750 dilution (v/v) of the primary mouse monoclonal antibody (M2III-6; Alexis Biochemicals, Gru¨nberg, Germany) and 1:2000 dilution (v/v) of the secondary antibody, HRP-anti mouse IgG (Bio-Rad) were used.

Transcellular transport study. The transcellular transport study was conducted as previously reported (Sasaki et al., 2002), with modifications. Briefly, the mock, OATP1B1- and OATP1B1/MRP2-transfected MDCK II cells were seeded in 24-well Transwell® plates (6.5 mm diameter, 0.33 cm² grow surface area, 0.4 µm pore size; Corning Coster, Acton, MA) at a density of 1.4 × 10⁵ cells per well. The MDCK II cells were first grown for 3-5 days on membrane inserts until confluence, and then induced with 5 mM sodium butyrate for 48 h prior to study. Upon confirmation of the integrity of the cell monolayer by transepithelial electrical resistance measurement (TEER; > 200 ohm.cm²) with the Millipore electrical resistance system (Millipore, Bedford, MA), cells were first washed with transport buffer (118 mM NaCl, 23.8 mM NaHCO₃,
4.83 mM KCl, 0.96 mM KH$_2$PO$_4$, 1.20 mM MgSO$_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl$_2$, pH 7.4) at 37°C. Subsequently, radiolabeled substrates, $[^{3}\text{H}]$E$_{217}$G (3.2 ± 0.2 nM or 323,020 ± 17,405 dpm/ml) and $[^{3}\text{H}]$EN (1.6 ± 0.05 nM or 391,215 ± 11,830 dpm/ml) at tracer concentrations, in transport buffer were added separately to the apical (250 µl) or basolateral (1000 µl) compartment. After initiation of the experiment, cells were placed in an incubator at 37°C under an atmosphere of 5% CO$_2$, and 100 µl aliquots in the receiver compartment were sampled at 30, 60 and 120 min. An equal volume of transport buffer was added back to the sampling compartment immediately after sample retrieval. The radioactivities in the samples were measured by the liquid scintillation spectrometry. Transcellular leakage of the transfected MDCK II cells was determined separately by the addition of $[^{3}\text{H}]$inulin to the basolateral compartment and measuring the radioactivity appearing in the apical compartment after an incubation period of 30, 60, and 120 min. The transcellular leakage at these times was 1.6%, 2.3%, and 3.7%, respectively, of the added radioactivity.

Transcellular transport of $[^{3}\text{H}]$E$_{217}$G or $[^{3}\text{H}]$EN was calculated as the accumulated amount of substrate in receiver compartment divided by the initial drug concentration in the donor compartment, then normalized to the cell growth surface area (µl/cm$^2$). The apparent permeability ($P_{\text{app}}$, cm/s) from basolateral to apical ($P_{\text{appB→A}}$) or from apical to basolateral ($P_{\text{appA→B}}$) was estimated in turn as the slope of area-normalized transcellular transport (µl/cm$^2$) versus time plot. The permeability surface area product (PS; µl/min) from basolateral to apical (PS$_{B→A}$) or from apical to basolateral (PS$_{A→B}$) was estimated similarly as the slope of transcellular transport (µl) versus time plot. The PS$_{B→A}$ was divided by the PS$_{A→B}$ in the same type of cells to obtain the flux ratio PS$_{B→A}$/PS$_{A→B}$. 
RESULTS

EN Disposition in EHBR Perfused Rat Liver

The steady-state extraction ratio (E), CL\textsubscript{liver,tot}, and CL\textsubscript{liver,met} for EHBR (n = 5) single pass perfused rat livers were 0.36 ± 0.06, 0.26 ± 0.12 ml/min/g, and 0.26 ± 0.14 ml/min/g, respectively. These values were not significantly changed compared to previously published data on the wild type, Sprague Dawley rats (SDR; n = 4) (de Lannoy et al., 1993), whose values for E, CL\textsubscript{liver,tot}, and CL\textsubscript{liver,met} were 0.51 ± 0.10, 0.36 ± 0.10 ml/min/g, and 0.34 ± 0.05 ml/min/g, respectively (P > 0.05) (Figure 2). However, the bile flow rate (0.26 ± 0.27 µl/min/g) was significantly lower than those for SDR (0.78 ± 0.15 µl/min/g), an observation also made by others (Takikawa et al., 1991). The steady-state biliary clearance of EN, CL\textsubscript{liver,ex} (0.0001 ± 0.0001 ml/min/g) for EHBR was reduced significantly when compared to those of SDR (0.013 ± 0.004 ml/min/g; P < 0.05) (Figure 3A). The apparent extraction ratio of formed ENA (E\{mi\}) was reduced to almost zero (0.02 ± 0.004) in comparison to that noted for SDR (0.35 ± 0.06; P < 0.05) (de Lannoy et al., 1993) (Figure 3A). The biliary excretion of EN and ENA was virtually obliterated for EHBR (Figure 3B).

EN Uptake in Single-Transfected HEK 293 Cells

EN uptake by HEK 293 cells (n=3) that were transfected with OATP1B1 (OATP1B1/HEK) or OATP1B3 (OATP1B3/HEK) were significantly higher compared with that of the vector-control HEK 293 cells (mock/HEK) (P < 0.05) (Figure 4A). The background-subtracted EN uptake rates were obtained as the difference in uptake rates in OATP1B1 or OATP1B3-transfected and vector-control HEK cells at each of the concentrations employed. The results showed that $K_m$ and $V_{max}$ for the transport of EN by OATP1B3 was not certain because of the lower affinity and high background (data not shown). By contrast, the
background-subtracted uptake of EN by OATP1B1 displayed saturable transport, with the $K_m$ of 262 ± 35 µM and $V_{max}$ of 78 ± 5 pmol/min/mg protein upon fitting of the data (Figure 4B).

**Expression of OATP1B1 and MRP2 in Transfected MDCK II Cells**

In mock/MDCK cells, there was no sign of the presence of immunoreactive protein for either OATP1B1 or MRP2, whereas, in OATP1B1/MDCK cells, OATP1B1 but not MRP2 protein was present. In the OATP1B1/MRP2/MDCK, both OATP1B1 and MRP2 proteins were detected, as expected (Figure 5). The molecular weights of the OATP1B1 and MRP2 in Figure 5 were around 70-80 kDa and 170-180 kDa, respectively. A slight split of MRP2 band was observed. The observation is not uncommon (Kopplow et al., 2005), and could be explained by non-glycosylation, partial glycosylation, or full glycosylation of the same protein. All observations are consistent for the presence of the intended immunoreactive proteins in the transfection system.

**EN Transcellular Transport in Double-Transfected MDCK II Cells**

Four sets of experiment were performed for the transport studies, and duplicate analyses were performed for each experiment. E$_{217}$G, the model substrate of high affinity for OATP1B1 (König et al., 2000a) and MRP2 (Sasaki et al., 2002), was used to verify the double-transfectant system. Much higher transcellular flux from basolateral to apical (B to A) of E$_{217}$G was observed in double-transfected OATP1B1/MRP2/MDCK cells vs. the single-transfected OATP1B1/MDCK cells and the mock/MDCK control cells ($P < 0.05$) (Table 1; Figures 6A & 7A). Similarly, the transcellular flux (B to A) of EN by the OATP1B1/MRP2/MDCK cells was significantly higher ($P < 0.05$) than those of the OATP1B1/MDCK and mock/MDCK cells (Table 1; Figures 6C & 7B). The EN transport by OATP1B1/MRP2/MDCK was, however, less than one-tenth that of E$_{217}$G (Table 1; Figures 7A & 7B), evidenced by the pattern for
transcellular flux of EN from B to A presented in the same scale of E217G (Figure 6C). As shown in the inset of Figure 6C, the observations are indicative that EN is a substrate of OATP1B1 and MRP2. The flux of both E217G and EN from A to B was similar to background, showing that the transport of the compounds did not occur (Figures 6B & 6D).
DISCUSSION

The ACE inhibitors are important therapeutic agents for treating patients with hypertension and cardiovascular diseases. Among the three chemical classes of ACE inhibitors, including the sulfhydryl-containing inhibitors exemplified by captopril, the carboxyalkyl dipeptides such as enalapril, and phosphorus-containing inhibitors such as fosinopril (Ondetti, 1988), enalapril is one that is the most prescribed. Being a modified dipeptide, EN was reported to be a substrate of peptide transporters, Pept1 and Pept2, in the rat intestine and kidney (Zhu et al., 2000). For the rat liver, however, EN but not its diacid metabolite, ENA, was transported by Oatp1a1 (Pang et al., 1998). The observation mirrored that shown previously for temocaprilat, another active form of carboxyalkyl dipeptide ACE inhibitors, that was transported by Oatp1a1 (Ishizuka et al., 1998) and Oatp1b2 (Sasaki et al., 2004). Moreover, temocaprilat was excreted into bile by Mrp2 with high affinity (Ishizuka et al., 1997). In addition to the ACE inhibitors, another class of anionic drug, the hydroxymethylglutaryl (HMG) CoA reductase inhibitors, such as pravastatin, also exhibited vectorial transport by Oatp1a1/Mrp2 in the rat liver (Hsiang et al., 1999; Kivisto et al., 2005) as well as OATP1B1/MRP2 in man (Sasaki et al., 2002).

Use of the mutant EHBR or TR^- rat has enhanced the facile identification of Mrp2 substrates and direct exploration of significance of Mrp2 in the hepatobiliary disposition of substrates. In these mutant animals, the expression of enzymes, such as cytochrome P450 (Cyp) and UDP-glucuronosyl transferase 1a (Ugt1a) are known to be changed (Newton et al., 2005; Johnson et al., 2006). However, the lack of change of the metabolic clearance in EHBR perfused livers suggests that EN hydrolysis by the carboxylesterases may have remained unaltered. Changes in transporters such as Mrp3, Oatp1a1 and Oatp1a4 in EHBR rats (Kuroda et al., 2004) could also have affected the uptake of EN. But it may be argued that the influx transport of EN by Oatp1a1 is extremely rapid compared to that of hydrolytic activity (Abu-Zahra and Pang,
2000), and changes in Oatp1a1, if small, would affect EN uptake. However, a dramatic change in EHBR biliary excretion was observed. The almost complete shutdown of the excretion of EN and ENA into bile in this Mrp2-deficient animal model showed unambiguously that EN and ENA are substrates of rat Mrp2. We also attempted to demonstrate EN and ENA transport via Mrp2 with rat canalicular membrane vesicles (CMVs), but failed to directly show EN uptake due to high non-specific binding (data not shown). Nevertheless, it may be suggested that the affinities of EN and ENA for Mrp2 are low. Accordingly, EN failed to affect the transport of temocaprilat, a substrate of Mrp2, into CMVs even at concentration as high as 200 µM (Ishizuka et al., 1997). This is likely due to a much higher $K_m$ for EN.

The discovery that EN was the substrate of Oatp1a1 in rat (Pang et al., 1998) suggests that human OATP1B1 may also transport EN in the liver since common substrate specificities of these transporters have been identified (Chang et al., 2005). This observation exists even though the overlap in sequence homology between Oatp1a1 and human OATP 1B1 is unimpressive. There was good agreement for the pharmacophores containing two hydrogen bond acceptors and two or three hydrophobic features for Oatp1a1 and OATP1B1, and EN is a substrate for both. Our experiments in single-transfected HEK 293 cells demonstrated that EN is a substrate of OATP1B1 and OATP1B3. Moreover, comparable values of $K_m$ for Oapt1a1 (214 µM) (Pang et al., 1998) and OATP1B1 (262 µM) were observed. OATP1B1 and OATP1B3 are remarkably similar at both the amino acid level (80% sequence identity) and the liver specific tissue distribution (liver specific transporter, LST) (König et al., 2000a; 2000b). Thus, it is not surprising to find that EN is a common substrate of OATP1B1 and OATP1B3, as found for other substrates, such as sulfobromophthalein (BSP) and E$_2$17G, albeit with different affinities (König et al., 2000a; 2000b).
Considering the sequence of drug processing in the liver, it is reasonable to utilize an in \textit{vitro} cell system that expresses both transporters - one at the basolateral side for uptake, and an efflux transporter at the apical side for excretion, such as the double-transfected cell system - to mimic hepatobiliary, vectorial transport. In addition, the double transfectant cell system has been shown to be more sensitive than membrane vesicles in the study of canalicular transport, as exemplified in the study of pravastatin (Sasaki et al., 2002). This system has been well established and validated (Cui et al., 2001a; Sasaki et al., 2002). Our Western blotting analysis and the \(E_{217}G\) transcellular transport results further demonstrated that the transfected transporters were expressed and functioning properly. With \(E_{217}G\), a common substrate of high affinity to both OATP1B1 and MRP2, as a positive control, we showed that EN was transported by both OATP1B1 and MRP2. The transcellular transport of both \(E_{217}G\) and EN shared the same trend in the ratio of apparent permeability \((P_{\text{appB}}/P_{\text{appA}})\) (Table 1) or the flux ratio \((PS_{B}/PS_{A})\) (Figure 7), with OATP1B1/MRP2/MDCK \(>\) OATP1B1/MDCK \(\geq\) mock/MDCK. The transcellular transport of EN by OATP1B1/MRP2/MDCK, however, was much lower than that of \(E_{217}G\) (11 times). This phenomenon could be explained by the lower affinity of EN (262 µM) compared with that of \(E_{217}G\) (8.2 µM) to OATP1B1 (König et al., 2000a). Also, it is very possible that a low affinity of EN with MRP2 as with Mrp2 also exists. The transcellular transport (µl) was normalized to the growth surface area (cm²) instead of the cell protein content because the normalization against protein may introduce an additional error due to the small transwell® used and the additional procedure for protein determination (Balakrishnan et al., 2005). Normalizing with respect to the known growth surface area did not add variability and showed good linearity with time. Moreover, the slope of the area-normalized, transcellular transport (µl/cm²) with time directly shows the apparent permeability, \(P_{\text{app}}\). An interesting phenomenon in this transfected cell system is that the different transfected MDCK II
cells (mock-, OATP1B1-, and OATP1B1/MRP2/MDCK) showed the different transcellular transport from apical to basolateral. The observation was independent of the substrate employed, E217G or EN (Figures 6B and 6D), suggesting this to be an effect of transfection treatment on the cell.

In summary, the roles of transporters as important modulators have been recognized in drug research and development. Hence, transporter studies become an integral part of pharmacokinetic screening (Mizuno et al., 2003). Fortunately, tools for transporter study are becoming increasingly available, and facile expression of human transporters with proper function in vitro renders these tools ideal predictors of drug-drug interactions prior to studies in vivo. We took advantage of various transporter study tools to identify the transporters involved in enalapril hepatic transport, although the involvement of other hepatic transporters such as OATP1B3 and OATP2B1 at the basolateral side (Kopplow et al., 2005) or MDR1 and BCRP at the apical side (Matsushima et al., 2005) cannot be ruled out. Furthermore, the data obtained from in vitro systems may be used to predict the in vivo drug disposition (Abu-Zahra and Pang, 2000; Sasaki et al., 2004). The integration of transporter information with metabolism, vascular binding and blood flow provides a profound understanding of drug disposition based on the mechanism (Abu-Zahra and Pang, 2000).
ACKNOWLEDGMENT

We would like to thank Dr. Soichiro Matsushima, Department of Molecular Pharmacokinetics, University of Tokyo, Japan, for his helpful suggestions in the MDCK II cell culture.
REFERENCES


FOOTNOTES

LL was a recipient of the Ontario Graduate Scholarship and University of Toronto Open Fellowship. This work was supported by the CIHR MOP64350 (KSP) and partially presented as an abstract (W4284) at the AAPS Annual Meeting, Toronto, 2002.

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LEGENDS FOR FIGURES

Figure 1 The biological fates of enalapril (EN) and its metabolite, enalaprilat (ENA), in rat and human hepatocytes. EN is taken up by Oatp1a1 (Pang et al., 1998) into rat liver and possibly by OATPs into human liver, then is metabolized by the carboxylesterases to ENA. Both parent drug (EN) and metabolite (ENA) are excreted into bile, possibly via Mrp2 (rat) and MRP2 (human).

Figure 2 The comparison of plasma flow rate, extraction ratio (E), total hepatic clearance (CL\textsubscript{liver,tot}), and hepatic metabolic clearance (CL\textsubscript{liver,met}) between the EHBR (n = 5) and SDR (n = 4) (de Lannoy et al., 1993).

Figure 3 (A) Comparison of bile flow rate, apparent extraction ratio of formed ENA (E\textsubscript{mi}), and biliary clearance (CL\textsubscript{liver,ex}) between the EHBR (n = 5) and SDR (n = 4) (de Lannoy et al., 1993), and (B) comparison of total excretion (EN + ENA) into bile, and EN or ENA excretion rates, as % input rate, into bile between the EHBR (n = 5) and SDR (n = 4) (de Lannoy et al., 1993). *P < 0.05 compared with SD rats.

Figure 4 (A) EN uptake in mock/HEK, OATP1B1/HEK and OATP1B3/HEK. *P < 0.05 compared to mock/HEK (n=3). (B) Background-subtracted EN uptake rates over 10 minutes at concentrations from 20 to 500 µM in OATP1B1/HEK at 37°C showed a single saturable transport system, with the V\textsubscript{max} of 78 ± 5 pmol/min/mg protein and K\textsubscript{m} of 262 ± 35 µM.

Figure 5 Protein expression of OATP1B1 and MRP2 in mock, single-transfected (OATP1B1), and double-transfected (OATP1B1/MRP2) MDCK II cells.

Figure 6 Transcellular transport of \[^{3}\text{H}]\text{E}_2\text{17G} [(A),(B)] and \[^{3}\text{H}]\text{EN} [(C),(D)] from the basolateral to apical (B to A) direction [(A),(C)], and from the apical to
basolateral (A to B) direction [(B),(D)], in mock/MDCK (open circles), OATP1B1/MDCK (solid squares) and OATP1B1/MRP2/MDCK (solid triangles), respectively. The transcellular transport of EN from B to A was shown in C, and an expanded view in the inset. Each data point and vertical bar represents the mean ± SD from four different experiments and each experiment performed in duplicate.

**Figure 7**

The PS$_{B\rightarrow A}$/PS$_{A\rightarrow B}$ ratios of $[^3]$H$E_217G$ (A) and $[^3]$HEN (B) in mock-, OATP1B1- and OATP1B1/MRP2/MDCK, showing transcellular transport of $E_217G$ and EN; * $P < 0.05$ compared to mock/MDCK; ** $P < 0.05$ compared to mock- and OATP1B1/MDCK.
Table 1: The apparent permeability $P_{\text{app}}$ (cm/s) of E217G and EN from basolateral to apical ($P_{\text{appB}\rightarrow A}$) and from apical to basolateral ($P_{\text{appA}\rightarrow B}$) in mock-, OATP1B1- and OATP1B1/MRP2/MDCK, respectively

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{appB}\rightarrow A}$ (cm/s)</th>
<th>$P_{\text{appA}\rightarrow B}$ (cm/s)</th>
<th>$P_{\text{appB}\rightarrow A}/P_{\text{appA}\rightarrow B}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E217G</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mock/MDCK</td>
<td>$1.48\times10^{-7} \pm 0.358\times10^{-7}$</td>
<td>$4.03\times10^{-7} \pm 2.20\times10^{-7}$</td>
<td>$0.423 \pm 0.184$</td>
</tr>
<tr>
<td>OATP1B1/MDCK</td>
<td>$3.42\times10^{-6} \pm 0.667\times10^{-6}$</td>
<td>$1.79\times10^{-6} \pm 0.452\times10^{-6}$</td>
<td>$1.93 \pm 0.145^*$</td>
</tr>
<tr>
<td>OATP1B1/MRP2/MDCK</td>
<td>$1.35\times10^{-5} \pm 0.134\times10^{-5}$</td>
<td>$3.06\times10^{-7} \pm 1.17\times10^{-7}$</td>
<td>$38.8 \pm 10.8^{**}$</td>
</tr>
<tr>
<td><strong>EN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mock/MDCK</td>
<td>$2.77\times10^{-7} \pm 1.57\times10^{-7}$</td>
<td>$4.33\times10^{-7} \pm 0.919\times10^{-7}$</td>
<td>$0.627 \pm 0.334$</td>
</tr>
<tr>
<td>OATP1B1/MDCK</td>
<td>$5.71\times10^{-7} \pm 0.746\times10^{-7}$</td>
<td>$1.83\times10^{-6} \pm 0.770\times10^{-6}$</td>
<td>$0.374 \pm 0.194$</td>
</tr>
<tr>
<td>OATP1B1/MRP2/MDCK</td>
<td>$1.56\times10^{-6} \pm 0.255\times10^{-6}$</td>
<td>$4.56\times10^{-7} \pm 1.46\times10^{-7}$</td>
<td>$3.59 \pm 0.871^{**}$</td>
</tr>
</tbody>
</table>

* $P < 0.05$ compared with mock/MDCK; ** $P < 0.05$ compared with mock- and OATP1B1/MDCK.
Figure 2
Figure 3

(A) Bile Flow Rate (µl/min/g) or E{mi} vs. Excretion in SDR and EHBR.

(B) % Input Rate in Total Excretion and Enalaprilat Excretion.
Figure 4

(A) Enalapril Uptake (pmol/min/mg protein)

- mock/HEK
- OATP1B1/HEK
- OATP1B3/HEK

(B) Uptake Velocity (pmol/min/mg protein)

- $K_m = 262 \pm 35 \mu M$
- $V_{max} = 78 \pm 5 \text{ pmol/min/mg}$

Concentration (µM)

0 100 200 300 400 500 600
Figure 5

[Image of a gel with bands labeled OATP1B1 and MRP2, with conditions mock/MDCK, OATP1B1/MDCK, and OATP1B1/MDCK/MDCK]
Figure 6

(A) B to A

E217G Transcellular Transport ($\mu$L/cm²)

(B) A to B

E217G Transcellular Transport ($\mu$L/cm²)

(C) B to A

EN Transcellular Transport ($\mu$L/cm²)

(D) A to B

EN Transcellular Transport ($\mu$L/cm²)
Figure 7