Prediction of in Vivo Biliary Clearance from the in Vitro Transcellular Transport of Organic Anions across a Double-Transfected Madin-Darby Canine Kidney II Monolayer Expressing Both Rat Organic Anion Transporting Polypeptide 4 and Multidrug Resistance Associated Protein 2

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Received December 8, 2003; accepted June 10, 2004 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

We have proposed previously that the evaluation of transcellular transport across the double-transfected Madin-Darby canine kidney II (MDCK II) monolayer that expresses both human organic anion transporting polypeptide 4 (OATP2/SLC21A6) and multidrug resistance associated protein 2 (MRP2/ABCC2) on the basal and apical membranes, respectively, may be useful in characterizing human biliary excretion (J Biol Chem 277: 6497–6503, 2002). However, to demonstrate that this in vitro system represents in vivo biliary excretion, it is essential to compare in vitro data with in vivo biliary excretion. The problem is that we cannot determine the human biliary excretion for many ligands. In the present study, we have established a double-transfected MDCK II monolayer that expresses both rat Oatp4/Slc21a10 and Mrp2/Abcc2 on the basal and apical membranes, respectively, for the purpose of quantitatively comparing the clearance for transcellular transport with that for in vivo biliary excretion. The basal-to-apical transport of 17β-H9252-estradiol-17β-D-glucuronide, pravastatin, leukotriene C4, cyclo-[D-Asp-Pro-D-Val-Leu-D-Trp] (BQ123), temocaprilat, and tauro-lithocholate 3-sulfate was significantly higher than that in the opposite direction in the double transfectant. Kinetic analysis suggested that that the rate-determining step of these compounds is the uptake process. The extent of the transcellular transport across the rat double-transfectant correlated well with that across the double-transfectant for human OATP2/SLC21A6 and MRP2/ABCC2. Moreover, considering the scaling factor, the clearance values for in vitro transcellular transport correlated well with those for in vivo biliary clearance. The double-transfected MDCK II monolayer may be useful in analyzing the hepatic vectorial transport of organic anions and in predicting in vivo biliary clearance.

One of the major functions of the liver is to eliminate endogenous and exogenous compounds from blood circulation. The vectorial transport of compounds across hepatocytes from blood to bile is supported by the uptake and efflux transporters located on the basolateral and apical membranes, respectively (Keppler and Arias, 1997; Meier et al., 1997; Kullak-Ublick et al., 2000). There has recently been intensive activity involving the molecular cloning and functional analysis of these transporters. Concerning the hepatic disposition of organic anions, it has been established that the organic anion transporting polypeptide (OATP/SLC21A) family of proteins is largely involved in the hepatic uptake (Abe et al., 1999; Hsiang et al., 1999; Cattori et al., 2000; Konig et al., 2000b; Tamai et al., 2000; Cui et al., 2001a; Kullak-Ublick et al., 2001). In humans, OATP2/SLC21A6 and OATP8/SLC21A8 are two major transporters responsible for the uptake of many kinds of organic anions including bile salts, 17β-estradiol-17β-D-glucuronide (E217βG), estrone-3-sulfate, dehydroepiandrosterone sulfate, bilirubin glucuronides, and clinically important drugs such as pravastatin. In rats, Oatp1/Sle21a1, Oatp2/Sle21a5, and Oatp4/Sle21a10 play important roles in the hepatic uptake of previously

This work was supported by Grant-in-Aid 12144201 for Scientific Research on Priority Areas epithelial vectorial transport from the Ministry of Education, Science and Culture of Japan.

ABBREVIATIONS: OATP, organic anion transporting polypeptide; E217βG, 17β-estradiol-17β-D-glucuronide; MRP, multidrug resistance associated protein; MDCK, Madin-Darby canine kidney; LTC4, leukotriene C4; BQ123, cyclo-[D-Asp-Pro-D-Val-Leu-D-Trp]; TLC-S, tauro-lithocholate 3-sulfate; BSA, bovine serum albumin; TBS-T, Tris-buffered saline–Tween 20; PBS, phosphate-buffered saline; bw, body weight.
Materials. [3H]E217G (1.6 TBq/mmol) and [3H]leukotriene C4 (LTC4; 4.81 TBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [3H]BQ123 (1.15 TBq/mmol) and [3H]methotrexate (1.5 TBq/mmol) were purchased from Amersham Bioscience (Little Chalfont, Buckinghamshire, UK). [3H]Taurolithocholate sulfate (TLC-S) was synthesized from lithocholate-3-sulfate using [2-3H]taurine (1.12 TBq/mmol; PerkinElmer Life and Analytical Sciences) as adopted and promulgated as the “Guide for the Care and Use of Laboratory Animals” as adopted and promulgated by the U.S. National Institutes of Health.

Construction of Plasmid Vector. Previously cloned rat Oatp4 cDNA (Cattori et al., 2000), located between the NotI and SalI sites of pSPORT 1 vector, was subcloned into pcDNA3.1/Zeoc mammalian expression vector according to the following method. A full-length of Oatp4 was excised from pSPORT 1 with HindIII and KpnI and was inserted into the HindIII and KpnI site of pcDNA3.1/Zeoc, resulting in the production of pcDNA3.1-Oatp4. The structure of Mrp2 cDNA expression vector is described previously (Kinoshiha et al., 1998).

Cell Culture and Transfection Study. Parental MDCK II cells, kindly provided by Dr. Piet Borst, were cultured in Dulbecco’s modified Eagle medium with 10% FBS at 37°C under 5% CO2. The transfection of plasmids into MDCKII cells was performed using LipofectAMINE (Invitrogen). Transfectants expressing recombinant Oatp4 and/or Mrp2 were selected with 700 μg/ml Zeocin and/or 700 μg/ml G418, respectively. Single colonies were screened for the expression of Oatp4 and Mrp2 by Western blot analysis.

Western Blot Analysis. For Western blot analysis, crude membrane fractions were prepared from cultured MDCK II cells as described previously (Sasaki et al., 2002). The homogenate was prepared from cultured MDCK II cells and rat liver as described below. Cells and rat liver were homogenized in 5 volumes of 0.1 M Tris-HCl buffer, pH 7.4, containing 1 μg/ml leupeptin and pepstatin A and 50 μg/ml phenylmethylsulfonyl fluoride with 20 strokes of a Dounce homogenizer. The membrane and homogenate protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as a standard.

For Western blot analysis, 20 μg of crude membrane, and 50 and 100 μg of homogenate were dissolved in 10 μl of 0.25 M Tris-HCl buffer containing 2% SDS, 30% glycerol, and 0.01% bromphenol blue, pH 6.8, and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel with a 4.4% stacking gel. Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA) using a blotter (Bio-Rad Laboratories, Richmond, CA) at 10 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% BSA overnight at 4°C. After washing with TBS-T, the membrane was incubated for 1 h at room temperature in TBS-T containing 5% BSA and 500-fold diluted anti-Oatp4 rabbit serum, which was raised in rabbits against the 17 amino acids at the carboxyl terminus of the deduced Oatp4 sequence (NGYCVVPDYESQNETPL) or with 500-fold diluted anti-Mrp2 rabbit serum, which was raised against the 17 amino acids at the carboxyl terminus of the deduced Mrp2 sequence (CEAST-KATVKSSDLQEL) (Ogawa et al., 2000).

For the detection of Oatp4 and Mrp2, the membrane was allowed to bind to goat anti-rabbit IgG (H+L, Alexa Fluor680) in PBS-T containing 5% skim milk for 1 h at room temperature. The intensity of the specific band was quantified using an Odyssey infrared imaging system (LI-COR, Inc., Lincoln, NE).

Materials and Methods

Materials. described organic anions (Jacquemin et al., 1994; Eckhardt et al., 1999; Reichel et al., 1999; Cattori et al., 2001).

The organic anions taken up by hepatocytes via these transporters are excreted into the bile across the bile canalicular membrane via the ATP-binding cassette transmembrane transporters. Monovalent bile salts are excreted via bile salt export pump (BSEP/ABCB11) (Gerloff et al., 1998), whereas many kinds of organic anions, including glucuronide and glutathione conjugates formed in the liver (such as bilirubin glucuronides), are excreted via multidrug resistance associated protein 2 (MRP2/ABCC2) (Suzuki and Sugiyama, 1998; Keppler and Konig, 2000). The importance of MRP2/ABCC2 in the disposition of organic anions has been highlighted by the fact that the biliary excretion of substrates is almost completely abolished in MRP2/Abcc2-deficient rats (Bucher et al., 1996; Paulusma et al., 1996). It has also been established that Dubin-Johnson syndrome, characterized by hyperbilirubinemia, results from a hereditary defect in MRP2/ABCC2 expression (Kartenbeck et al., 1996).

Although a series of detailed studies has been performed to characterize the transport properties of these transporters using cells transfected with cDNA and/or injected with cRNA for each transporter, it is necessary to examine the synergistic role of the uptake and efflux transporters to understand the vectorial transport observed under in vivo conditions. Cui et al. (2001b) and we (Sasaki et al., 2002) established a double-transfected MDCK II monolayer that stably expresses human uptake (OATP8 and OATP2) and efflux transporters (MRP2) on the basolateral and apical membranes, respectively, to evaluate the transcellular transport of many kinds of organic anions. It was clearly shown that many substrates are vectorially transported across the double transfectant from the basal to the apical compartments. However, it is essential to compare the in vitro results with those observed under in vivo conditions for the quantitative evaluation of in vitro transcellular transport systems. The problem is that it is difficult to determine the biliary excretion in humans for many kinds of ligands.

In the present study, we have performed a quantitative analysis of the transcellular transport of a series of organic anions across the double transfectant expressing rat uptake and efflux transporters and also determined the biliary excretion of the respective ligands in rats. We found that the extent of the transcellular transport across the double-transfectant expressing rat transporters correlated well with that determined previously in human double transfectant. Moreover, we suggest that in vivo biliary excretion can be predicted from the results of in vitro transcellular transport studies for a series of Oatp4 substrates.

Immunofluorescence Microscopy of Transfected Cells. Transfected MDCK cells were grown on Transwell membrane inserts (pore size of 3 μm; Falcon, Bedford, MA). Sodium butyrate was added to the culture medium 24 h before starting the experiment. After fixation with 4% paraformaldehyde in PBS for 10 min and permeabilization in 1% Triton X-100 in PBS for 5 min, cells were incubated with the previously described polyclonal antibody against Oatp4 (diluted 50-fold in PBS) or the monoclonal antibody against Mrp2 (M2III-6; diluted 40-fold in PBS; Alexis Biochemicals, Laufelfingen, Switzerland) for 60 min at room temperature. Next, cells were washed three times with PBS and incubated with Goat anti-mouse IgG (Alexa 488) or Goat anti-mouse IgG (diluted 250-fold in PBS;
Alexa 546, Molecular Probes, Inc. Eugene, OR) for 30 min at room temperature. Nuclei were stained with SYTO63 (diluted 1000-fold in TBS; Molecular Probes). Membranes were cut from the inserts and mounted on glass slides with Vectashield mounting medium without propidium iodide. Confocal laser-scanning immunofluorescence microscopy (LSM-510 apparatus; Carl Zeiss) was used in the present study.

**Transcellular Transport Studies.** Transfected MDCKII cells were seeded in 24-well plates at a density of 1.4 × 10⁵ cells per well and cultured with 10 mM sodium butyrate for 24 h (Kenig et al., 2000a). For uptake studies, cells were washed three times and pre-incubated with Krebs-Henseleit buffer or Na+-free buffer (the latter was prepared by substituting Na+ with choline) at 37°C for 5 min. Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM Na₂CO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl₂ adjusted to pH 7.3. The experiments were initiated by replacing the medium on either the apical or the basal side of the cell monolayer; the medium contained [³H]E₂₁₇G (1 μM), [³H]pravastatin (1 μM), [³H]LTC₄ (5 nM), [³H]TLC-S (1 μM), [³H]methotrexate (1 μM), [³H]temocaprilat (1 μM), or [³H]BQ123 (1 μM). The cells were incubated at 37°C, and aliquots of medium were taken from each compartment at several time points. Radioactivity in 100 μl of medium was measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA) after addition of 8 ml of scintillation fluid (Hionic flow; PerkinElmer Life and Analytical Sciences). At the end of the experiments, the cells were washed three times with 1.5 ml of ice-cold Krebs-Henseleit buffer and solubilized in 450 μl of 1 N NaOH. After addition of 500 μl of distilled water, 800-μl aliquots were transferred to scintillation vials, and 50-μl aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with BSA as a standard.

**HPLC Analysis.** The extent of metabolism of ligands during the transcellular transport studies was examined using HPLC. At the end of transcellular transport studies, the apical medium was collected and dried up. Mobile phase (100 μl) was added, and 50-μl aliquots were subjected to HPLC. In the analysis, CapcellPAK C18 (5 μm) column (4.6 mm i.d. × 150 mm) (Shiseido, Tokyo, Japan) was used. The mobile phase consisted of 50 μM ammonium acetate, pH 4.5, and methanol. Except for the analysis of metabolism of methotrexate, a linear methanol gradient from 20 to 80% was applied over 30 min. For methotrexate, a linear methanol gradient from 5 to 80% was applied over 30 min. The flow rate was set at 1 ml/min, and the column temperature was kept at 45°C. Eluted mobile phase was collected every 30 s for 40 min. The radioactivity of each fraction was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter) after addition of 4 ml of scintillation fluid (Hionic flow; PerkinElmer Life and Analytical Sciences). Under those conditions, unchanged E₂₁₇G, pravastatin, TLC-S, BQ123, LT₄, methotrexate, and temocaprilat were eluted at 23, 26, 34, 25, 30, 17, and 19 min, respectively.

**Data Analysis.** For the kinetic analysis, the transcellular transport of ligands determined over 2 h was used. The kinetic parameters for transcellular transport of [³H]E₂₁₇G and [³H]pravastatin were estimated from the following equation:

\[
v_{\text{bss}} = \frac{(V_{\text{max}} \times S)(K_m + S) + P_{\text{diff}} \times S}{(V_{\text{max}} \times S)(K_m + S) + P_{\text{diff}} \times S}
\]

where \(v_{\text{bss}}\) is the initial uptake rate of substrates (picomoles per minute per milligram of protein), \(S\) is the substrate concentration in medium (micromolar), \(K_m\) is the Michaelis constant (micromolar), \(V_{\text{max}}\) is the maximum uptake rate (picomoles per minute per milligram of protein), and \(P_{\text{diff}}\) is the nonsaturable transport clearance (microliters per minute per milligram of protein). The uptake data were fitted to this equation by a nonlinear least-squares method with a MULTI program (Yamakawa et al., 1981) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values. Utilization of the Michaelis-Menten type equation in the analysis is justified by the fact that the rate-determining process for the transcellular transport was the uptake process mediated by Oatp4 as discussed under Discussion (Sasaki et al., 2002).

The permeability-surface area product across the apical membrane (PS\(_{\text{apical}}\)) was calculated by dividing the rate for the transcellular transport of [³H]E₂₁₇G or [³H]pravastatin determined over 2 h by the cellular concentration of [³H]E₂₁₇G or [³H]pravastatin determined at the end of the experiments (2 h).

**Infusion Study.** Under light ether anesthesia, both the femoral artery and vein of rats were cannulated with polyethylene catheters (PE-50; Clay Adams, Parsippany, NJ) for blood sampling and drug infusion, respectively. The bile duct was also cannulated with a polyethylene catheter (PE-10; Clay Adams) for bile collection. After being dissolved in saline, [³H]E₂₁₇G, [³H]pravastatin, [³H]methotrexate, and [³H]TLC-S was infused over a period of 120 min at a rate of 7.57 pmol/min/kg of bw, 500 nmol/min/kg of bw, 168 nmol/min/kg of bw, and 666 pmol/min/kg of bw, respectively. Bile was collected in preweighed test tubes at 15-min intervals. The plasma was prepared by centrifugation of the blood specimens (Microfuge E; Beckman Coulter). At the end of the infusion, the liver was excised and weighed. Radioactivity in a 50-μl specimen was measured in a liquid scintillation counter (LS 6000SE; Beckman-Coulter) after addition of 8-ml scintillation fluid (Hionic flow; PerkinElmer Life and Analytical Sciences). Based on the results obtained from the infusion study, pharmacokinetic parameters were calculated according to the following equations

\[
CL_{\text{total}} = IC_{\text{pass}} \quad (2)
\]

\[
CL_{\text{bile}} = IC_{\text{bile}} / IC_{\text{pass}} \quad (3)
\]

where \(I\) represents \(V_{\text{bile}}\), \(C_{\text{bile}}\), \(CL_{\text{total}}\), and \(CL_{\text{bile}}\), \(p\) represent the infusion rate (nanomoles per minute per kilogram), plasma concentration at steady-state (micromolar), biliary excretion rate at steady-state (nanomoles per minute per kilogram), blood concentration at steady-state (micromolar), total body clearance (milliliters per minute per kilogram), biliary excretion clearance (milliliters per minute per kilogram) defined with respect to the plasma concentration, respectively. \(C_{\text{pass}}\) was determined as the mean values of the plasma concentrations between 60 and 120 min. \(V_{\text{bile}}\) was determined as the biliary excretion rate between 60 and 120 min.

**Determination of Plasma Protein Binding.** The plasma protein binding of E₂₁₇G, pravastatin, methotrexate, and TLC-S, was determined by ultrafiltration. Each compound dissolved in phosphate buffer (50 mM, pH 7.4) was diluted 10 times with rat plasma to give a final concentration that was close to the steady-state plasma concentration (3). Plasma protein binding was measured by ultrafiltration (Conbastcone; Aloka, Tokyo, Japan) immediately after incubation. Then, the plasma unbound fraction (\(f_u\)) was calculated by dividing the rate for the transcellular transport of [³H]E₂₁₇G or [³H]pravastatin by the total plasma concentration of E₂₁₇G, 

\[
f_u = \frac{CL_{\text{total}}}{CL_{\text{CI}}}
\]

where \(CL_{\text{total}}\) is the total plasma clearance (milliliters per minute per kilogram), \(CL_{\text{CI}}\) is the clearance of the compound in the plasma, \(f_u\) is the unbound fraction of the compound, and \(C_{\text{CI}}\) is the plasma concentration. The plasma unbound fraction (\(f_u\)) was determined by ultrafiltration (Conbastcone; Aloka, Tokyo, Japan) immediately after incubation.

**Determination of Plasma Protein Binding.** The plasma protein binding of E₂₁₇G, pravastatin, methotrexate, and TLC-S, was determined by ultrafiltration. Each compound dissolved in phosphate buffer (50 mM, pH 7.4) was diluted 10 times with rat plasma to give a final concentration that was close to the steady-state plasma concentration (3). Plasma protein binding was measured by ultrafiltration (Conbastcone; Aloka, Tokyo, Japan) immediately after incubation. Then, the plasma unbound fraction (\(f_u\)) was calculated by dividing the rate for the transcellular transport of [³H]E₂₁₇G or [³H]pravastatin by the total plasma concentration of E₂₁₇G, 

\[
f_u = \frac{CL_{\text{total}}}{CL_{\text{CI}}}
\]

where \(CL_{\text{total}}\) is the total plasma clearance (milliliters per minute per kilogram), \(CL_{\text{CI}}\) is the clearance of the compound in the plasma, \(f_u\) is the unbound fraction of the compound, and \(C_{\text{CI}}\) is the plasma concentration. The plasma unbound fraction (\(f_u\)) was determined by ultrafiltration (Conbastcone; Aloka, Tokyo, Japan) immediately after incubation.

**Determination of Red Blood Cell Distribution.** [³H]TLC-S was dissolved in phosphate buffer (50 mM, pH 7.4) and diluted 10 times with rat whole blood to give the final concentration described above and incubated at 37°C for 30 min. To determine the concentration in whole blood, 50 μl of blood was transferred into paper cups (Conbastcone; Aloka, Tokyo, Japan) immediately after incubation.
The blood was centrifuged at 3000 rpm for 10 min at 4°C to obtain plasma. The specimens were incubated in an oxidizer (Aloka, Japan). The radioactivity was counted in a liquid scintillation counter for 5 min. The blood-to-plasma concentration ratio \( R_b \) was calculated by dividing the concentration in whole blood by the plasma concentration. Based on \( R_b \) values, \( C_{\text{bas}} \) was calculated from \( C_{\text{pas}} \) according to the following equation:

\[
C_{\text{bas}} = C_{\text{pas}} \times R_b
\]

\( CL_{\text{bile, in vivo}} \) is biliary excretion clearance (milliliters per minute per kilogram) defined with respect to the blood concentration, was calculated as follows:

\[
CL_{\text{bile, in vivo}} = \frac{V_{\text{int}}}{C_{\text{bas}}}
\]

The plasma unbound fraction \( (f_u) \) was then calculated according to eq. 6:

\[
f_u = \frac{f_h \times C_{\text{rel}}/C_{\text{bas}}}{f_h \times R_b}
\]

**Comparison of Clearance Values between in Vivo Biliary Excretion and in Vitro Transcellular Transport.** Comparison of clearance values was performed according to the following equation:

\[
CL_{\text{int, in vitro}} = (Q_b \times f_h \times CL_{\text{int, in vitro}}/Q_b + f_h \times CL_{\text{int, in vitro}}) \times \alpha
\]

where \( Q_b \), \( CL_{\text{int, in vitro}} \), and \( \alpha \) represent the hepatic blood flow rate (1.6 ml/min/g of liver), intrinsic clearance (milliliter per minute per gram of liver) which was calculated from the results of the transcellular transport (microliter per minute per milligram of protein) by considering the fact that 1 g of liver contains 160 mg of protein, and the scaling factor, respectively. For this calculation, we have also considered the difference in the expression level of Oatp4 between the double-transfectant and rat liver. The clearance values were fitted to this equation by a nonlinear least-squares method with a MULTI program (Yamaoka et al., 1981) to obtain estimates of the scaling factor. For this calculation, the input data were weighted as the reciprocals of the squares of the observed values.

**Results**

**Expression of Rat Oatp4 and Mrp2 in MDCK II Cells.** The expression of rat Oatp4 and Mrp2 in MDCK II cells was determined by confocal immunofluorescence laser scanning microscopy. As shown in Fig. 1, Oatp4 and Mrp2 were localized on the lateral and apical membranes of transfected MDCK II cells, respectively.

The expression level of these transporters was also confirmed by Western blot analysis (Fig. 2). Semiquantitative analysis of the results of Western blot analysis showed that the expression level of Oatp4 in MDCK II cells expressing Oatp4 (MDCK II/Oatp4) was 1.3 times higher than that in the double-transfectant, whereas that of Mrp2 in MDCK II cells expressing Mrp2 (MDCK II/Mrp2) was the same as that in the double-transfectant. Moreover, we found that the expression levels of Oatp4 and Mrp2 in the homogenate of the double-transfectant were approximately 2 and 5 times higher than those in rat liver homogenate, respectively (Fig. 2).

**Transcellular Transport of Several Organic Anions across MDCK II Monolayers Expressing Rat Oatp4/Mrp2.** The transcellular transport of E217G, pravastatin, LTC4, TLC-S, temocaprilat, BQ123, and methotrexate across MDCK II/Oatp4 and MDCK II/Mrp2 monolayers, along with that across the double-transfected monolayer, was compared with that across the control monolayer. As shown in Fig. 3, symmetrical flux of E217G, pravastatin, and LTC4 was observed across the control, Oatp4, and Mrp2-expressing MDCK II monolayer, whereas the transfection of Oatp4 cDNA to MDCK II cells resulted in the appearance of vectorial transport from the basal to apical compartment for BQ123, temocaprilat, and TLC-S. Additional expression of Mrp2 on the apical membrane resulted in the marked vectorial transport of all ligands examined in the present study; the basal-to-apical flux of E217G, pravastatin, LTC4, TLC-S, temocaprilat, and BQ123 was approximately 18, 8, 8, 28, 3, and 6 times higher than that in the opposite direction in the double-transfected cells, respectively (Fig. 3). In contrast, the significant vectorial transport was not observed for methotrexate (Fig. 3).

The extent of metabolism of ligands was also determined at the end of experiments. Although the metabolism was negligible for E217G, pravastatin, TLC-S, BQ123, and temocaprilat, for LTC4 and methotrexate, 54% and 46% of the isotope content in the apical solution was accounted for by their metabolites, respectively. For the following analysis, the extent of metabolism was also taken into consideration.

Then, the kinetics of the transcellular transport of E217G was examined. The basal-to-apical flux of E217G across the
double-transfectant was saturable (Fig. 4). Kinetic analysis revealed that the saturation can be best described by assuming the presence of one saturable and one nonsaturable component. The analysis gave $K_m$, $V_{max}$, and $P_{diff}$ values of $22.4 \pm 3.1 \mu M$, $353 \pm 51 \text{ pmol/min/mg of protein}$, and $4.02 \pm 0.41 \mu l/min/mg of protein$, respectively, for the double-transfectant (Fig. 4A). Furthermore, to quantitatively evaluate the transport activity across the apical membrane, the $PS_{apical}$ of E$_{217}\beta G$ was also determined. As shown in Fig. 4B, the $PS_{apical}$ in the double-transfectant was significantly higher than that in the control monolayer. However, the $PS_{apical}$ of E$_{217}\beta G$ in the double transfectant was not saturated even if the concentration of E$_{217}\beta G$ in the basal compartment was increased to $150 \mu M$ (Fig. 4B).

In the same manner, the transcellular transport of pravastatin was kinetically analyzed. The transcellular transport of pravastatin across the monolayer was saturable (Fig. 5), with $K_m$, $V_{max}$ and $P_{diff}$ values of $48.3 \pm 7.0 \mu M$, $338 \pm 39 \text{ pmol/min/mg of protein}$, and $0.51 \pm 0.05 \mu l/min/mg of protein$, respectively (Fig. 5A). This $P_{diff}$ value was comparable with that determined in the parental MDCK II monolayer ($0.68 \pm 0.07 \mu l/min/mg of protein$). As shown in Fig. 5B, the $PS_{apical}$ of pravastatin in the double-transfectant was significantly higher than that in the control monolayer, as observed for E$_{217}\beta G$ (Fig. 4A). Moreover, the $PS_{apical}$ of pravastatin in the double-transfected cells was saturated at approximately $300 \mu M$ in the basal compartment.

To demonstrate the function of Oatp4 and Mrp2, we determined the cellular content of E$_{217}\beta G$ and pravastatin. It was found that the cellular accumulation of E$_{217}\beta G$, determined at 2 min, in control, Oatp4-expressing, Mrp2-expressing, and Oatp4/Mrp2-expressing MDCK II cells was $13.7 \pm 2.9$, $45.9 \pm 8.9$, $11.9 \pm 1.3$, and $24.6 \pm 5.9 \text{ pmol/mg of protein}$, respectively (Fig. 4B). For these cells, the cellular accumulation of pravastatin at 2 min was also determined to be $6.1 \pm 1.8$, $22.2 \pm 2.5$, $6.8 \pm 1.0$, and $10.6 \pm 1.2 \text{ pmol/mg of protein}$, respectively. These results also support the function of Oatp4 and Mrp2 in taking up and extruding compounds from the cells, respectively.

**Comparison of Transcellular Transport between Rat and Human Double-Transfectants.** We have previously determined the transcellular transport of E$_{217}\beta G$, pravastatin, LTC$_4$ and TLC-S across MDCK II cells expressing human OATP2 and MRP2 (human double transfectant; Sasaki et al., 2002). In the present study, we have additionally examined the flux of methotrexate and BQ-123 across the human double-transfectant (Fig. 5). The $PS_{apical}$ of pravastatin in the double-transfectant was significantly higher than that in the control monolayer, as observed for E$_{217}\beta G$ (Fig. 4A). Moreover, the $PS_{apical}$ of pravastatin in the double-transfected cells was saturated at approximately $300 \mu M$ in the basal compartment.

**Fig. 3.** Time-profiles for the transcellular transport of organic anions across MDCK II monolayers. Transcellular transport of $[^{3}H]E_{217}\beta G$ (1 mM), $[^{3}H]$pravastatin (1 mM), $[^{3}H]$LTC$_4$ (5 nM), $[^{3}H]$BQ123 (1 mM), $[^{3}H]$TLC-S (1 mM), $[^{14}C]$temocaprilat (1 mM), and $[^{3}H]$methotrexate (1 mM) across the control MDCK II (A), MDCK II/Oatp4 (B), MDCK II/Mrp2 (C), and the double-transfectant (D) was studied as a function of time. $E$, transcellular transport in the apical-to-basal direction; $F$, transcellular transport in the basal-to-apical direction. Points and vertical bars represent the mean $\pm$ S.E. of three independent determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.
ble transfectant, for the purpose of comparing the flux between the human and rat double transfectants. As a positive control for the present experiment, we examined the transcellular transport of E217βG. As shown in Fig. 6, the basal-to-apical transport of E217βG, methotrexate, and BQ123 was approximately 8, 2, and 5 times higher than that in the opposite direction in the human double transfectant, respectively. The absolute values for the basal-to-apical and apical-to-basal flux of E217βG (12.6 and 1.64 pmol/min/mg of protein, respectively) were almost the same as those determined previously (12.1 and 1.51 pmol/min/mg of protein, respectively; Sasaki et al., 2002).

The relationship of transcellular transport of a series of organic anions between rat and human double transfectants is shown in Fig. 7. Transcellular transport of six kinds of ligands correlated well between rat and human double transfectants (r² = 0.92).

**Relationship between in Vivo Biliary Excretion and in Vitro Transcellular Transport Clearance.** To examine the relationship between in vivo biliary excretion and in vitro transcellular transport, we have examined the disposition of E217βG, pravastatin, methotrexate and TLC-S. Figure 8 shows the time profiles for the blood concentration and biliary excretion rate after intravenous infusion of these ligands. Table 1 shows the pharmacokinetic parameters determined in rats and also the clearance for the transcellular transport across the rat double-transfectant. Based on these parameter values, the relationship between in vivo and in vitro clearance values was examined. For determining this relationship, the extent of in vivo metabolism was also taken into considerations. For E217βG, pravastatin, TLC-S, BQ123, and temocaprilat, in vivo metabolism was also negligible, whereas the in vivo biliary clearance values of LTC₄ and methotrexate were corrected by considering that 23 and 81% of the biliary excreted isotope after the intravenous administration of [³H]LTC₄ and [³H]methotrexate are responsible for unmetabolized LTC₄ and methotrexate, respectively (Fahrig et al., 1989; Wettstein et al., 1989). According to eq. 7, the α value was calculated to be 17.9 ± 1.9. As shown in Fig. 9, it was shown that we can quantitatively obtain the in vivo clearance from the results of in vitro transcellular transport experiments by considering the α value for a series of Oatp4 substrates.

**Discussion**

We have previously established MDCK II cells that express both human OATP2 and MRP2 (Sasaki et al., 2002) as an in vitro model to determine the transcellular transport of many organic anions. In the present study, we established MDCK II cells that express both rat Oatp4 and Mrp2 and compared the transport activity with the in vivo biliary excretion clearance. Although the uptake of organic anions in rat hepatocytes is mediated by Oatp4, Oatp1, and Oatp2, we have focused on Oatp4. Because rat Oatp4, a homolog of human OATP2, has a broad substrate specificity and is expressed in human hepatic cells, we have used it as a model for in vitro transport studies.
the liver to a much higher extent than other Oatp family proteins, Oatp4 may be important in the hepatic uptake of organic anions (Cattori et al., 2000, 2001; Li et al., 2002). Although we have also established the double-transfected MDCKII monolayer expressing rat Oatp1 and Mrp2, both Oatp1 and Mrp2 were localized on the apical membrane of MDCK II cells and, therefore, we could not use this Oatp1/Mrp2 double-transfectant as an in vitro hepatic model.

Immunohistochemical analysis by confocal laser microscopy suggested the basal and apical expression of Oatp4 and Mrp2 in MDCKII cells, respectively (Fig. 1), which is consistent with the previously reported localization of these transporters (Evers et al., 1998; Cattori et al., 2001) and that of human OATP2 and MRP2, respectively. Western blot analysis revealed that the expression levels of Oatp4 and Mrp2 in the singly transfected MDCK II cells were approximately the same as those in double-transfected cells (Fig. 2). The expression level of Oatp4 and Mrp2 in double transfectants was approximately 2- and 5-fold higher than that in rat liver (Fig. 2), respectively, suggesting that the expression level of Mrp2

![Fig. 6. Time-profiles for the transcellular transport of organic anions across MDCK II monolayers expressing human OATP2 and MRP2. Transcellular transport of [3H]E217βG (1 μM), [3H]BQ123 (1 μM), and [3H]methotrexate (1 μM) was examined across the control MDCK II (A), MDCK II/OATP2 (B), MDCK II/MRP2 (C), and the double-transfectant (D). ○, transcellular transport in the apical-to-basal direction; ●, transcellular transport in the basal-to-apical direction. Points and vertical bars represent the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.]

![Fig. 7. Comparison of transcellular transport of several organic anions by rat (Oatp4/Mrp2) and human double-transfectants (OATP2/MRP2). The transcellular transport of [3H]E217βG (1 μM), [3H]pravastatin (1 μM), [3H]LTC4 (5 nM), [3H]methotrexate (1 μM), [3H]TLC-S (1 μM), and [3H]BQ123 (1 μM) across MDCK II monolayers expressing Oatp4/Mrp2 (rat double transfectant) and OATP2/MRP2 (human double transfectant) was compared. Data for pravastatin, LTC4, and TLC-S in the human double-transfectant were taken from Sasaki et al. (2002). Other data were from Figs. 3 and 6. Symbols and bars represent the mean ± S.E. of three determinations.]


was enough to extrude compounds from the double-transfected cells in the present in vitro experimental system.

The basal-to-apical flux of E_{217}G was stimulated to a greater extent by coexpressing Mrp2 with Oatp4 (Fig. 3), suggesting that E_{217}G molecules taken up into the monolayer by Oatp4 are efficiently extruded into the apical compartment via Mrp2. This suggestion is further supported by the fact that the cellular accumulation of E_{217}G was significantly lower in the double-transfected cells compared with Oatp4-expressing cells (see Results). The \( K_m \) value for the transcellular transport of E_{217}G across the double-transflectant was 22.8 \( \pm \) 6.1 \( \mu \)M (Fig. 4A), which is similar to that reported for Oatp4-mediated uptake of E_{217}G in cRNA-injected Xenopus laevis oocytes (32 \( \mu \)M; Cattori et al., 2001). In contrast, the \( PS_{\text{apical}} \) was not saturated even if the concentration of E_{217}G in the basal compartment was increased up to 150 \( \mu \)M (Fig. 4B). Considering all these observations, the saturation observed in the transcellular transport of E_{217}G may be ascribed to the saturation of Oatp4-mediated uptake, suggesting that the uptake may be the rate-determining step in the transcellular transport of E_{217}G. This hypothesis is also consistent with our in vivo experiments in rats. The rate-determining process for the biliary excretion of E_{217}G may be the uptake across the sinusoidal membrane, because the uptake clearance of E_{217}G (39.6 ml/min/kg of bw; J. Aoki, H. Suzuki, and Y. Sugiyama, unpublished observations) determined according to the method described in Yamazaki et al. (1996) is similar to the biliary excretion clearance determined in the present study (44.3 ml/min/kg of bw; Fig. 8).

In the same manner, vectorial transport across the double transfectant was observed for pravastatin, TLC-S, and temocaprilat, indicating that these ligands are substrates of rat Oatp4 and Mrp2 (Fig. 3). We performed a kinetic analysis of the transcellular transport of pravastatin. The \( K_m \) value for the transcellular transport of pravastatin (48.3 \( \pm \) 7.0 \( \mu \)M; Fig. 5A) also agreed with the uptake of this drug determined in isolated rat hepatocytes (40 \( \mu \)M; Ishigami et al., 1995). Moreover, the \( PS_{\text{apical}} \) was saturated at approximately 300 \( \mu \)M in the basal compartment (Fig. 5B), which is consistent with the fact that pravastatin is a low-affinity substrate of Mrp2 (220 \( \mu \)M; Yamazaki et al., 1997). Given these kinetic parameters, it was also suggested that the rate-determining step in the transcellular transport of this compound may also be the uptake-mediated by Oatp4. This hypothesis is also consistent with our previous in vivo observations that uptake is the rate-determining step for the biliary excretion of pravastatin in rats (Yamazaki et al., 1997). Our previous in vivo experiments also suggested that the hepatic uptake is the rate-determining process for the biliary excretion of BQ123,

![Fig. 8. Time profile for blood concentration and biliary excretion rate during constant intravenous infusion to rats. Blood concentration and biliary excretion rate of E_{217}G, pravastatin, TLC-S, and methotrexate were examined in rats which received the i.v. infusion of respective compounds at the infusion rates of 7.57 pmol/min/kg of bw, 500 nmol/min/kg of bw, 666 pmol/min/kg of bw, and 168 nmol/min/kg of bw, respectively. ○, blood concentration; ●, biliary excretion rate. Points and vertical bars represent the mean \pm S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.](image-url)

### TABLE 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>( CL_{\text{basal}} )</th>
<th>( CL_{\text{apical}} )</th>
<th>( CL_{\text{basal}} )</th>
<th>( f_B )</th>
<th>Transcellular Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/min/kg</td>
<td>ml/min/kg</td>
<td>ml/min/kg/g of liver</td>
<td></td>
<td>( \mu )l/min/mg of protein</td>
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<tr>
<td>E_{217}G</td>
<td>42.9 ( \pm ) 2.5</td>
<td>27.8 ( \pm ) 3.5</td>
<td>1.23 ( \pm ) 0.12</td>
<td>0.16</td>
<td>19.4 ( \pm ) 1.3</td>
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<tr>
<td>Pravastatin</td>
<td>35.2 ( \pm ) 3.2</td>
<td>27.5 ( \pm ) 5.1</td>
<td>1.32 ( \pm ) 0.28</td>
<td>1.00</td>
<td>9.3 ( \pm ) 0.4</td>
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<tr>
<td>LTCG</td>
<td>90.9 ( \pm ) 9.1</td>
<td>24.2 ( \pm ) 5.6</td>
<td>0.96 ( \pm ) 0.22</td>
<td>0.16</td>
<td>10.4 ( \pm ) 1.0</td>
</tr>
<tr>
<td>BQ123</td>
<td>31.4 ( \pm ) 3.2</td>
<td>28.2 ( \pm ) 4.2</td>
<td>1.37 ( \pm ) 0.21</td>
<td>1.00</td>
<td>7.4 ( \pm ) 1.2</td>
</tr>
<tr>
<td>TLC-S</td>
<td>37.0 ( \pm ) 1.5</td>
<td>33.7 ( \pm ) 1.7</td>
<td>1.56 ( \pm ) 0.05</td>
<td>0.45</td>
<td>28.5 ( \pm ) 3.7</td>
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<tr>
<td>Temocaprilat</td>
<td>12.5 ( \pm ) 1.6</td>
<td>9.7 ( \pm ) 1.2</td>
<td>0.42 ( \pm ) 0.05</td>
<td>0.10</td>
<td>4.0 ( \pm ) 1.5</td>
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<tr>
<td>Methotrexate</td>
<td>12.7 ( \pm ) 1.9</td>
<td>10.7 ( \pm ) 1.7</td>
<td>0.46 ( \pm ) 0.07</td>
<td>0.94</td>
<td>0.9 ( \pm ) 1.0</td>
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</table>

In vivo pharmacokinetic parameters and in vitro transcellular transport of several organic anions.

Data shown in Figs. 3 and 8 were analyzed to quantify the kinetic parameters. In vivo data for LTCG, BQ123, and temocaprilat were taken from Sathirakul et al. (1994), Kato et al. (1999), and Ishizuka et al. (1997), respectively.
LTC₄ and temocaprilat (Sathirakul et al., 1994; Ishizuka et al., 1997; Kato et al., 1999).

Then, the correlation was examined in the transport activity between rat and human double-transfected. As shown in Fig. 7, the transcellular transport of six kinds of organic anions exhibited a good correlation ($r^2 = 0.92$) between rat and human double transfecteds. The exception is methotrexate, which showed no significant transcellular transport across the rat double transfected (Fig. 3). This finding is consistent with the previous report that this compound was not significantly taken up by X. laevis oocytes injected with rat Oatp4 cRNA (Cattori et al., 2001). The rate-limiting process for the biliary excretion of these compounds is the uptake in both in vivo and in vitro experiments, which suggests that the presence of good correlation in the transcellular transport across the rat and human double transfecteds can be accounted for by the similarity in the transport properties between rat Oatp4 and human OATP2. Although it has been established that the substrate specificity of human OATP2 and rat Oatp4 resemble each other, the results of the present study suggest that the absolute values and/or the rank order for the cellular uptake activity of these substrates may also be similar. However, to quantitatively predict human biliary excretion, the comparison of the expression level of OATP2 and MRP2 between the transfecteds and human liver is required.

In addition, for the purpose of examining whether the extent of in vivo biliary excretion can be quantitatively predicted from the double transfected, we also compared the clearance values determined in vitro with those determined in vivo. Because uptake is the rate-determining process for the in vitro transport and in vivo biliary excretion, we analyzed the correlation between in vivo and in vitro situations according to eq. 7. By considering the $a$ value of 17.9, the in vivo biliary clearance could be quantitatively predicted from the in vitro results determined in the double transfected, irrespective of the fact that other transporters (such as oatp1-2) are expressed under in vivo conditions. This result may be accounted for by the hypothesis that the transport activity of the other uptake transporters is similar to that of Oatp4. It is also possible that Oatp4 is the major transporter for a series of ligands. One of the possible factors used to account for the need to consider the $a$ value of 17.9 may be the difference in morphology between in vivo rat liver and in vitro MDCK II cells. Without considering the $a$ value, the extrapolation from in vitro data resulted in the underestimation of in vivo disposition (Fig. 9).

In conclusion, we have established an MDCK II cell line that expresses both rat Oatp4 and Mrp2 on the basal and apical membranes, respectively. In addition, when normalized by the amount of protein of the uptake transporter and the appropriate scaling factor, rat double transfected seems to be a good tool for the quantitative prediction of in vivo rat biliary excretion of substrates of these transporters. This result suggests that, in the same way, in vivo human biliary excretion, which is difficult to study, might be predicted by using human double transfected (OATP2/MPR2).

Acknowledgments

We thank Dr. Piet Borst (The Netherlands Cancer Institutes) for providing the parent MDCKII cells and Sankyo Co. for providing labeled and unlabeled pravastatin.

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that is deficient in Eisai hyperbilirubinemic mutant rats (EHBR). J Pharmacol Exp Ther 280:1304–1311.

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