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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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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-estradiol-17β-D-glucuronide, pravastatin, leukotriene C_4, cyclo-[D-Asp-Pro-D-Val-Leu-D-Trp] (BQ123), temocaprilat, and taurolithocholate 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., 2000a,b; 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 ($E_2$17βG), estrone-3-sulfate, dehydroepiandrosterone sulfate, bilirubin glucuronides, and clinically important drugs such as pravastatin. In rats, Oatp1/Slc21a1, Oatp2/Slc21a5, and Oatp4/Slc21a10 play important roles in the hepatic uptake of previously

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ABBREVIATIONS: OATP, organic anion transporting polypeptide; $E_2$17βG, 17β-estradiol-17β-D-glucuronide; MRP, multidrug resistance associated protein; MDCK, Madin-Darby canine kidney; LTC_4, leukotriene C_4; BQ123, cyclo-[D-Asp-Pro-D-Val-Leu-D-Trp]; TLC-S, taurolithocholate 3-sulfate; BSA, bovine serum albumin; TBS-T, Tris-buffered saline-Tween 20; PBS, phosphate-buffered saline; bw, body weight.

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; Keppler and Kartenbeck, 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.

Materials and Methods

Materials. [^{3}H]$E_2$17βG (1.6 TBq/mmol) and [^{3}H]leukotriene C_4 (LTC_4; 4.81 TBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]BQ123 (1.15TBq/mmol) and [^{3}H]methotrexate (1.5 TBq/mmol) were purchased from Amersham Bioscience (Little Chalfont, Buckinghamshire, UK). [^{3}H]Taurolithocholate sulfate (TLC-S) was synthesized from lithocholate-3-sulfate using [2-^{3}H]taurine (1.12 TBq/mmol; PerkinElmer Life and Analytical Sciences) as described previously (Sasaki et al., 2002). [^{3}H]Pravastatin (1.6 TBq/mmol) and [^{14}C]temocaprilat (1.3 MBq/mmol) were kindly donated by Sankyo Co. (Tokyo, Japan). pcDNA3.1/Zeo, Zeocin, and Geneticin were purchased from Invitrogen (Carlsbad, CA). All other chemicals were commercially available and of reagent grade.

For in vivo studies, male Sprague-Dawley rats weighing approximately 240 to 260 g were purchased from Japan SLC (Tokyo, Japan). This study was carried out in accordance with 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/Zeo 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/Zeo, resulting in the production of pcDNA3.1-Oatp4. The structure of Mrp2 cDNA expression vector is described previously (Kinoshita 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's medium with 10% FBS at 37°C under 5% CO_2. 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 pepstain 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 (NGYYCVPYDEQSNETPL) 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 (CEASTKATVKSSDLQEL) (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 FluorR680) 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).

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, Läufelfingen, Switzerland) for 60 min at room temperature. Cells were then washed three times with PBS and incubated with Goat anti-rabbit 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 SYTO61 (diluted 1000-fold in TBS; Molecular Probes). Membranes were cut from the inserts and mounted onto 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 1.4×10^5 cells per well and cultured with 10 mM sodium butyrate for 24 h (Konig et al., 2000a). For uptake studies, cells were washed three times and preincubated 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 Na_2CO_3, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$ 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 complete medium contained [^{3}H]$E_2$17βG (1 μM), [^{3}H]pravastatin (1 μM), [^{3}H]LTC_4 (5 nM), [^{3}H]TLC-S (1 μM), [^{3}H]methotrexate (1 μM), [^{14}C]temocaprilat (1 μM), or [^{3}H]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. $\times$ 150 mm) (Shiseido, Tokyo, Japan) was used. The mobile phase consisted of 50 mM 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 40°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 $E_2$17βG, pravastatin, TLC-S, BQ123, LTC_4, 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 [^{3}H]$E_2$17βG and [^{3}H]pravastatin were estimated from the following equation:

$$v_0 = (V_{max} \times S)/(K_m + S) + P_{diff} \times S \quad (1)$$

where v_0 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_{max} is the maximum uptake rate (picomoles per minute per milligram of protein), and P_{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 (Yamaoka 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_{apical}) was calculated by dividing the rate for the transcellular transport of [^{3}H]$E_2$17βG or [^{3}H]pravastatin determined over 2 h by the cellular concentration of [^{3}H]$E_2$17βG or [^{3}H]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, [^{3}H]$E_2$17βG, [^{3}H]pravastatin, [^{3}H]methotrexate, and [^{3}H]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

$$CL_{total} = I/C_{pss} \quad (2)$$

$$CL_{bile,\,p} = V_{bile}/C_{pss} \quad (3)$$

where I, C_{pss}, V_{bile}, C_{bss}, CL_{total}, and $CL_{bile,\,p}$ 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_{pss} was determined as the mean values of the plasma concentrations between 60 and 120 min. V_{bile} was determined as the biliary excretion rate between 60 and 120 min.

Determination of Plasma Protein Binding. The plasma protein binding of $E_2$17βG, 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 present in the in vivo infusion study (0.3 nM, 18 μM, 15 μM, and 20 nM for $E_2$17βG, pravastatin, methotrexate and TLC-S, respectively). The mixture was incubated at 37°C for 30 min to ensure binding equilibrium. After incubation, 40-μl aliquots were taken to determine the total plasma concentration. Then, the plasma was placed in an ultrafiltration apparatus (Centrifee, Amicon, Inc., Beverly, MA) with a molecular mass cutoff of 13 kDa and centrifuged at 3000 rpm (TOMY RL-100, Tokyo, Japan) for 10 min. After centrifugation, the concentration in the filtrate was also determined by oxsida (Aloka, Tokyo, Japan) as the unbound concentration. The plasma unbound fraction (f_u) was calculated by dividing the unbound concentration by the total plasma concentration. The recoveries of $E_2$17βG, pravastatin, methotrexate, and TLC-S filtered through the system were 91.1, 97.0, 97.4, and 92.2%, respectively. All the binding was normalized with respect to the filter blank.

Determination of Red Blood Cell Distribution. [^{3}H]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 combusted 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_{bss} was calculated from C_{pss} according to the following equation:

$$C_{bss} = C_{pss} \times R_b \quad (4)$$

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

$$CL_{bile,\ b} = V_{bile}/C_{bss} \quad (5)$$

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

$$f_B = f_u \times C_{pss}/C_{bss} = f_u/R_B \quad (6)$$

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:

$$CL_{bile,\ b} = \{Q_h \times f_B \times CL_{int,\ in\ vitro}/(Q_h + f_B \times CL_{int,\ in\ vitro})\} \times \alpha \quad (7)$$

where Q_h, $CL_{int,\ in\ vitro}$, and α represent the hepatic blood flow rate (1.6 ml/min/g of liver), in vitro 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.

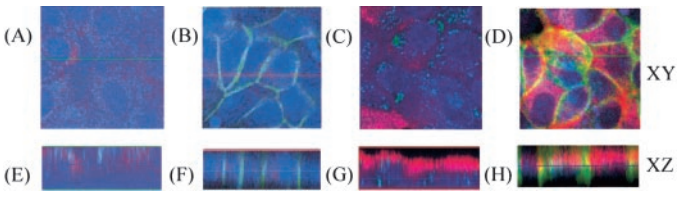

Fig. 1. Immunolocalization of recombinant rat Oatp4 and Mrp2 in stably transfected cells. Control MDCK II cells (A), MDCK II/Oatp4 (B), MDCK II/Mrp2 (C), and the double-transfectant (D) were stained with polyclonal antibody against rat Oatp4 (green fluorescence) and against rat Mrp2 (red fluorescence). Nuclei were stained with SYTO61 (blue fluorescence). A to D, 0.8-μm optical sections in the x-y plane. E to H, vertical sections in the x-z plane indicated by the green lines in A to D.

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 $E_2$17βG, pravastatin, LTC_4, 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 $E_2$17βG, pravastatin, and LTC_4 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 $E_2$17βG, pravastatin, LTC_4, 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 $E_2$17βG, pravastatin, TLC-S, BQ123, and temocaprilat, for LTC_4 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 $E_2$17βG was examined. The basal-to-apical flux of $E_2$17βG across the

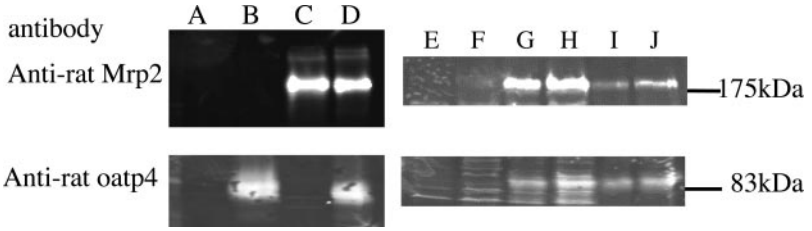

Fig. 2. Western blot analysis of Oatp4 and Mrp2. 20 μg crude membrane fraction from control MDCK II cells (A), MDCK II/Oatp4 (B), MDCK II/Mrp2 (C), and double-transfectant (D) were loaded and separated by SDS-PAGE (8.5% separating gel). Homogenate (50 and 100 μg) from control MDCK II cells (E and F), double-transfectant (G and H), and rat liver homogenate (I and J) were separated by SDS-PAGE (8.5% separating gel). Oatp4 and Mrp2 were detected by their respective polyclonal antibodies.

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 ± 3.1 μM, 353 ± 51 pmol/min/mg of protein, and 4.02 ± 0.41 μl/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_2$17βG 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_2$17βG in the double transfectant was not saturated even if the concentration of $E_2$17βG in the basal compartment was increased to 150 μM (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 ± 7.0 μM, 338 ± 39 pmol/min/mg of protein, and 0.51 ± 0.05 μl/min/mg of protein, respectively (Fig. 5A). This P_{diff} value was comparable with that determined in the parental MDCK II monolayer (0.68 ± 0.07 μl/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_2$17βG (Fig. 4A). Moreover, the PS_{apical} of pravastatin in the double-transfected cells was saturated at approximately 300 μM in the basal compartment.

To demonstrate the function of Oatp4 and Mrp2, we determined the cellular content of $E_2$17βG and pravastatin. It was found that the cellular accumulation of $E_2$17βG, determined at 2 min, in control, Oatp4-expressing, Mrp2-expressing, and Oatp4/Mrp2-expressing MDCK II cells was 13.7 ± 2.9, 45.9 ± 8.9, 11.9 ± 1.3, and 24.6 ± 5.9 pmol/mg of protein (n = 3), respectively. For these cells, the cellular accumulation of pravastatin at 2 min was also determined to be 6.1 ± 1.8, 22.2 ± 2.5, 6.8 ± 1.0, and 10.6 ± 1.2 pmol/mg of protein (n = 3), 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_2$17βG, 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 dou-

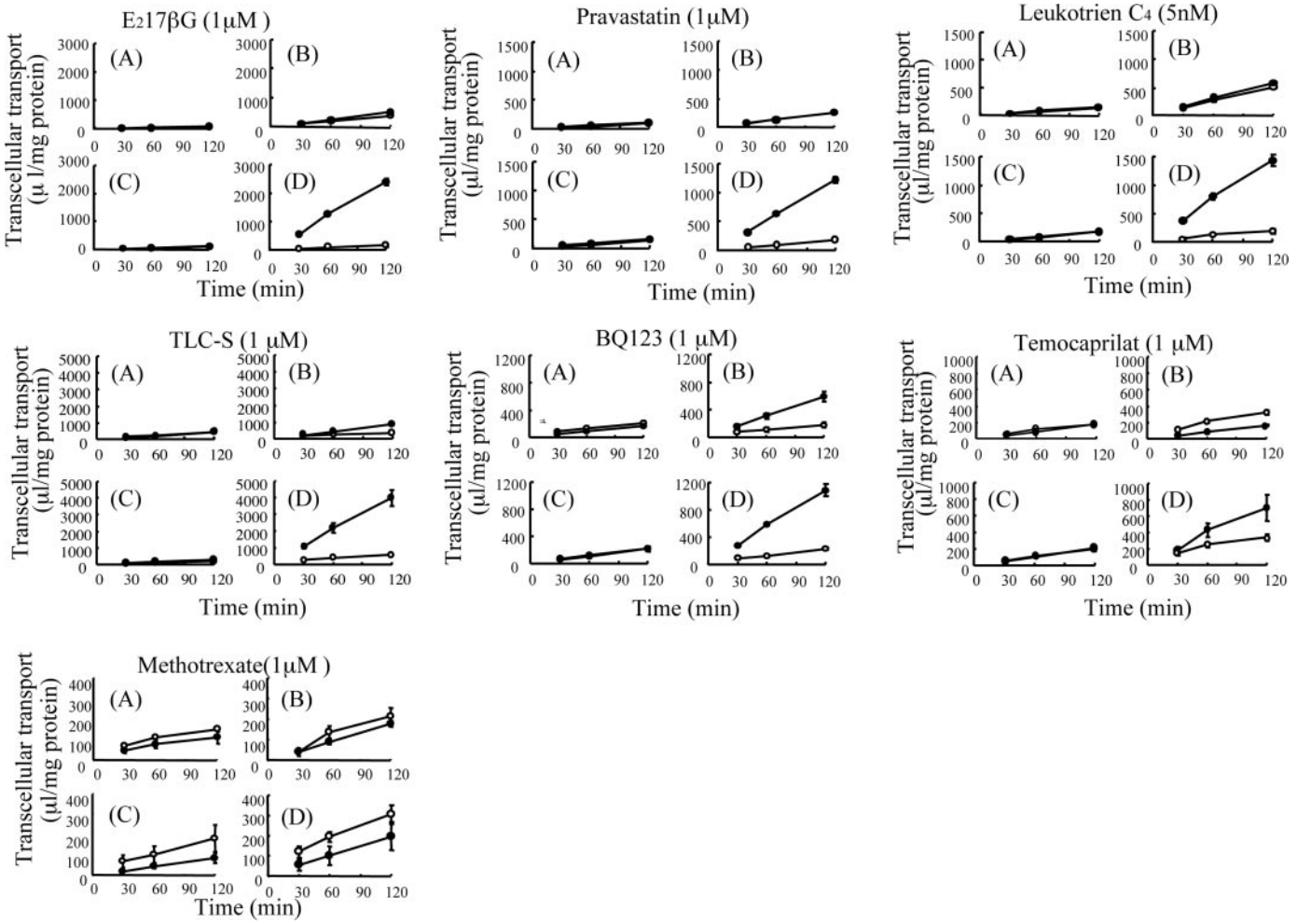

Fig. 3. Time-profiles for the transcellular transport of organic anions across MDCK II monolayers. Transcellular transport of [^{3}H]$E_2$17βG (1 μM), [^{3}H]pravastatin (1 μM), [^{3}H]LTC_4 (5 nM), [^{3}H]BQ123 (1 μM), [^{3}H]TLC-S (1 μM), [^{14}C]temocaprilat (1 μM), and [^{3}H]methotrexate (1 μM) 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. ○, 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 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 $E_2$17βG. As shown in Fig. 6, the basal-to-apical transport of $E_2$17βG, 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 $E_2$17βG (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 ($r^2 = 0.92$).

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 $E_2$17βG, 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 $E_2$17βG, pravastatin, TLC-S, BQ123, and temocaprilat, in vivo metabolism was also negligible, whereas the in vivo biliary clearance values of LTC_4 and methotrexate were corrected by considering that 23 and 81% of the biliary excreted isotope after the intravenous administration of [^{3}H]LTC_4 and [^{3}H]methotrexate are responsible for unmetabolized LTC_4 and methotrexate, respectively (Fahrig et al., 1989; Wettestein et al., 1989). According to eq. 7, the α value was calculated to be 17.9 $\pm$ 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

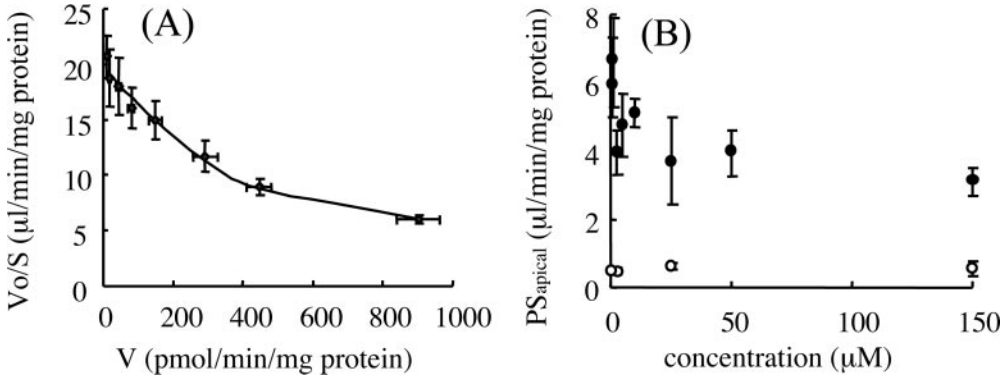

Fig. 4. Concentration-dependence of the transcellular transport and PS_{apical} of [^{3}H]$E_2$17βG across the double-transfectant. A, concentration-dependence of the transcellular transport of [^{3}H]$E_2$17βG (1 μM) across the double-transfectant, studied for 2 h in the presence and absence of unlabeled $E_2$17βG at 37°C. 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. B, concentration-dependence of PS_{apical} of $E_2$17βG in the control MDCK II monolayer (○) and that in the double-transfectant (●). The horizontal axis represents the medium concentration (basal compartment) of $E_2$17βG.

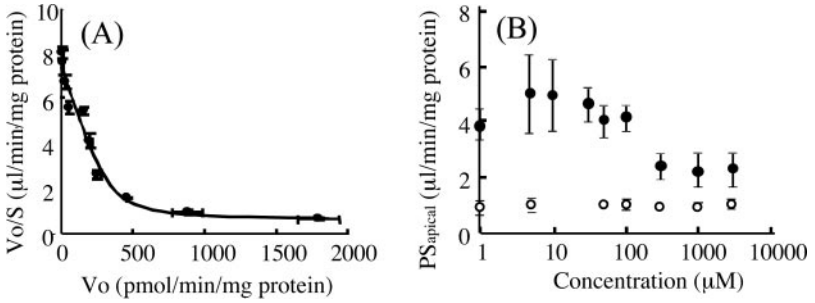

Fig. 5. Concentration dependence of transcellular transport and PS_{apical} of [^{3}H]pravastatin across the double-transfectant. A, concentration-dependence of the transcellular transport of [^{3}H]pravastatin (1 μM) across the double transfectant, studied for 2 h in the presence and absence of unlabeled pravastatin at 37°C. Each point and vertical bar represenst 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. B, concentration dependence of PS_{apical} of pravastatin in the control MDCK II monolayer (○) and in the double-transfectant (●). The horizontal axis represents the medium concentration (basal compartment) of pravastatin.

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

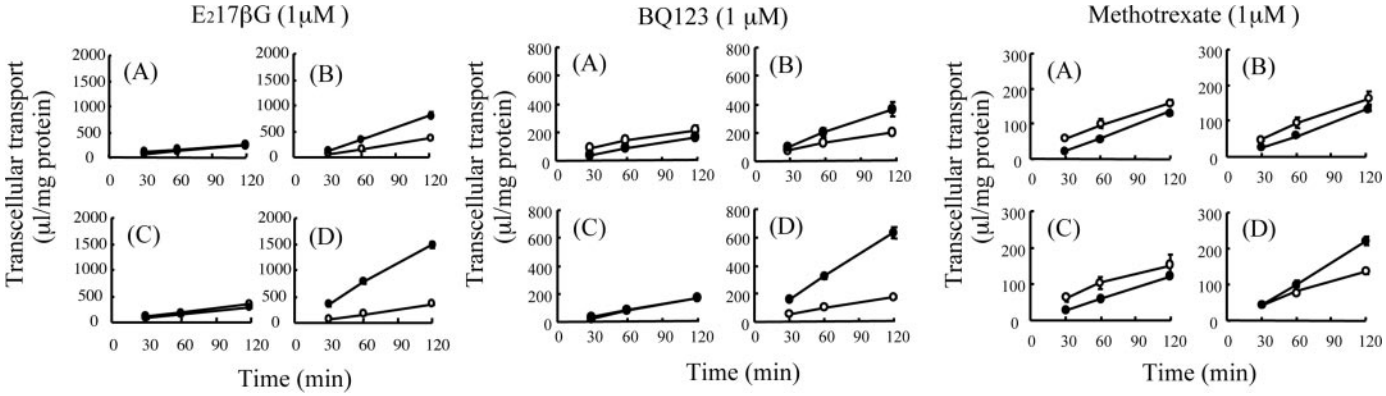

Fig. 6. Time-profiles for the transcellular transport of organic anions across MDCK II monolayers expressing human OATP2 and MRP2. Transcellular transport of [^{3}H]$E_2$17βG (1 μM), [^{3}H]BQ123 (1 μM), and [^{3}H]methotrexate (1 μM) was examined across the control MDCK II (A), MDCK II/OATP2 (B), MDCK 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.

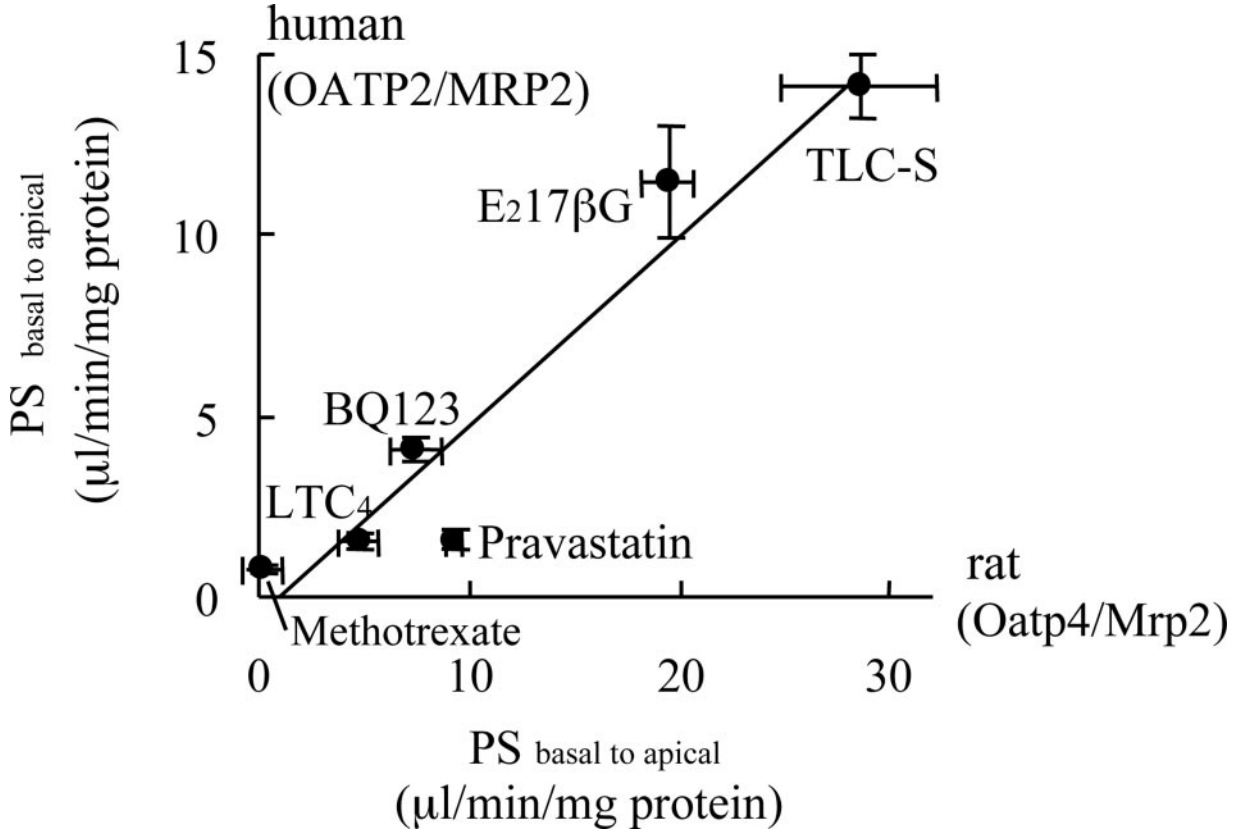

Fig. 7. Comparison of transcellular transport of several organic anions by rat (Oatp4/Mrp2) and human double-transfectants (OATP2/MRP2). The transcellular transport of [^{3}H]$E_2$17βG (1 μM), [^{3}H]pravastatin (1 μM), [^{3}H]LTC_4 (5 nM), [^{3}H]methotrexate (1 μM), [^{3}H]TLC-S (1 μM), and [^{3}H]BQ123 (1 μM) across MDCK II monolayers expressing Oatp4/Mrp2 (rat double transfectant) and OATP2/MRP2 (human double transfectant) was compared. Data for pravastatin, LTC_4, 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_2$17βG was stimulated to a greater extent by coexpressing Mrp2 with Oatp4 (Fig. 3), suggesting that $E_2$17βG 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_2$17βG 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_2$17βG across the double-transfectant was 22.8 ± 6.1 μM (Fig. 4A), which is similar to that reported for Oatp4-mediated uptake of $E_2$17βG in cRNA-injected *Xenopus laevis* oocytes (32 μM; Cattori et al., 2001). In contrast, the PS_{apical} was not saturated even if the concentration of $E_2$17βG in the basal compartment was increased up to 150 μM (Fig. 4B). Considering all these observations, the saturation observed in the transcellular transport of $E_2$17βG 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_2$17βG. This hypothesis is also consistent with our in vivo experiments in rats. The rate-determining process for the biliary excretion of $E_2$17βG may be the uptake across the sinusoidal membrane, because the uptake clearance of $E_2$17βG (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 ± 7.0 μM; Fig. 5A) also agreed with the uptake of this drug determined in isolated rat hepatocytes (40 μM; Ishigami et al., 1995). Moreover, the PS_{apical} was saturated at approximately 300 μM in the basal compartment (Fig. 5B), which is consistent with the fact that pravastatin is a low-affinity substrate of Mrp2 (220 μM; 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,

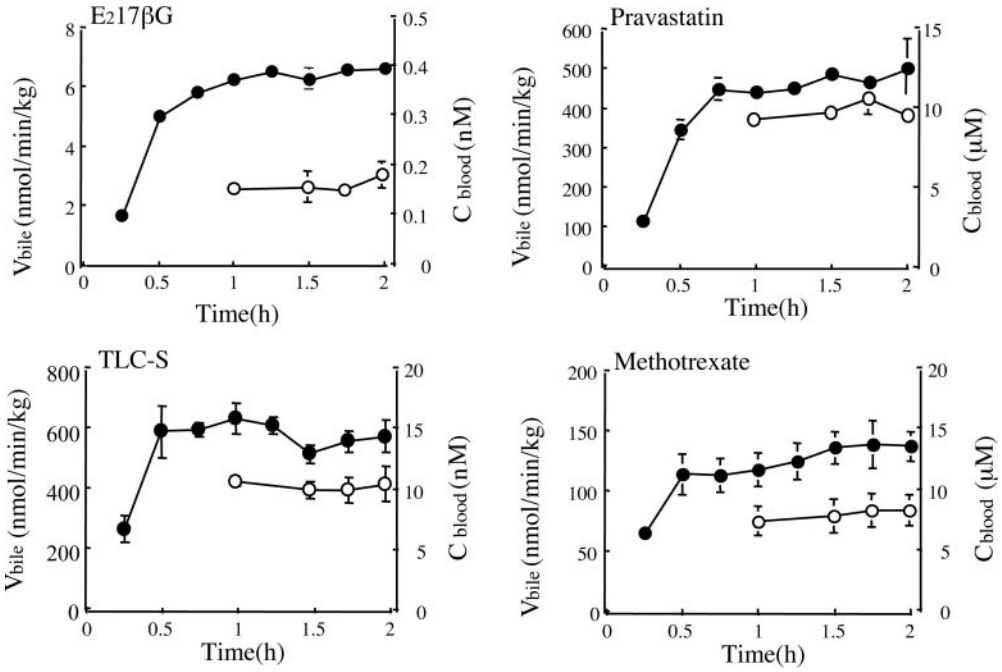

Fig. 8. Time profile for blood concentration and biliary excretion rate during constant intravenous infusion to rats. Blood concentration and biliary excretion rate of $E_2$17βG, pravastatin, TLC-S, and methotrexate were examined in rats which received the i.v. infusion of respective compounds at the 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 ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

TABLE 1

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 LTC_4, BQ123, and temocaprilat were taken from Sathirakul et al. (1994), Kato et al. (1999), and Ishizuka et al. (1997), respectively.

Compounds	CL_{total}	$CL_{bile,p}$	$CL_{bile,b}$	f_B	Transcellular Transport
	ml/min/kg	*ml/min/kg*	*ml/min/g of liver*		*μl/min/mg of protein*
$E_2$17βG	42.9 ± 2.5	27.8 ± 3.5	1.23 ± 0.12	0.16	19.4 ± 1.3
Pravastatin	35.2 ± 3.2	27.5 ± 5.1	1.32 ± 0.28	1.00	9.3 ± 0.4
LTC_4	90.9 ± 9.1	24.2 ± 5.6	0.96 ± 0.22	0.16	10.4 ± 1.0
BQ123	31.4 ± 3.2	28.2 ± 4.2	1.37 ± 0.21	1.00	7.4 ± 1.2
TLC-S	37.0 ± 1.5	33.7 ± 1.7	1.56 ± 0.05	0.45	28.5 ± 3.7
Temocaprilat	12.5 ± 1.6	9.7 ± 1.2	0.42 ± 0.05	0.10	4.0 ± 1.3
Methotrexate	12.7 ± 1.9	10.7 ± 1.7	0.46 ± 0.07	0.94	0.9 ± 1.0

LTC_4 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-transfectant. 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 transfectants. The exception is methotrexate, which showed no significant transcellular transport across the rat double transfectant (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 transfectants 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 transfectants 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 transfectant, 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 α value of 17.9, the in vivo biliary clearance could be quantitatively predicted from the in vitro results determined in the double transfectant, irrespective of the fact that other transporters (such as oatp1 and -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 α value of 17.9 may be the difference in morphology between in vivo rat liver and in vitro MDCK II cells. Without considering the α value, the extrapolation from in vitro data resulted in the underestimation of in vivo disposition (Fig. 9).

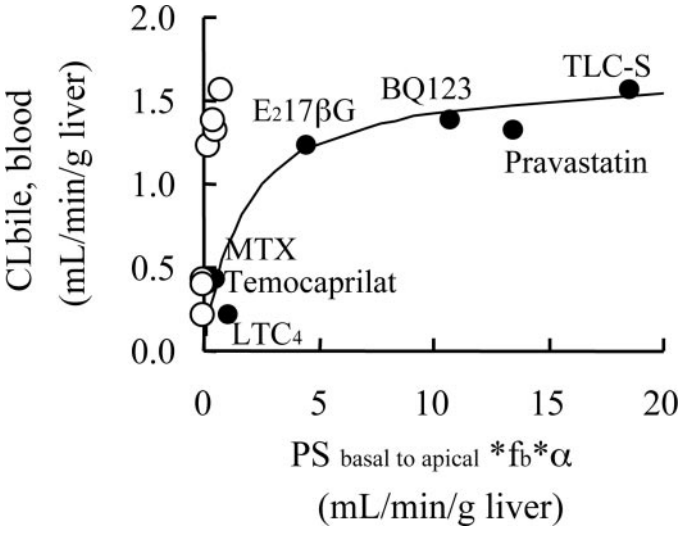

Fig. 9. Comparison of in vivo biliary excretion clearance and in vitro transcellular transport clearance across the double-transfectant. The in vivo biliary excretion clearance of $E_2 17\beta G$, pravastatin, LTC_4, TLC-S, temocaprilat, methotrexate, and BQ-123 was compared with the transcellular transport clearance of respective ligands across the double-transfectant. Data were taken from Figs. 3 and 8 and Table 1. The x-axis represents CL_{int} determined in vitro multiplied by f_B and the scaling factor, and the y-axis represents the in vivo biliary clearance defined for the blood ligand concentrations. ●, data whose x-axis values were corrected for the scaling factor (α = 17.9). The solid line represents the theoretical curve. ○, observed data.

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 transfectant 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 transfectant (OATP2/MRP2).

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