References

Abdul-Ghaffar NU and El-Sonbaty MR (1995) Pancreatitis and rhabdomyolysis associated with lovastatin-gemfibrfozil therapy. *J Clin Gastroenterol* **21**:340–341.

Backman JT, Kyrklund C, Kivistö KT, Wang J-S, and Neuvonen PJ (2000) Plasma concentrations of active simvastatin acid are increased by gemfibrozil. *Clin Pharmacol Ther* **68**:122–129.

Backman JT, Kyrklund C, Neuvonen M, and Neuvonen PJ (2002) Gemfibrozil greatly increases plasma concentrations of cerivastatin. *Clin Pharmacol Ther* **72**:685–691.

Bruno-Joyce J, Dugas JM, and MacCausland OE (2001) Cerivastatin and gemfibrozil-associated rhabdomyolysis. *Ann Pharmacother* **35**:1016–1019.

Cui Y, König J, Leier I, Buchholz U, and Keppler D (2001) Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. *J Biol Chem* **276**:9626–9630.

Fujino H, Yamada I, Shimada S, Yoneda M, and Kojima J (2003) Metabolic fate of pitavastatin, a new inhibitor of HMG-CoA reductase: human UDP-glucuronosyltransferase enzymes involved in lactonization. *Xenobiotica* **33**:27–41.

Galetin A, Clarke SE, and Houston JB (2002) Quinidine and haloperidol as modifiers of CYP3A4 activity: multisite kinetic model approach. *Drug Metab Dispos* **30**: 1512–1522.

Galetin A, Clarke SE, and Houston JB (2003) Multisite kinetic analysis of interactions between prototypical CYP3A4 subgroup substrates: midazolam, testosterone and nifedipine. *Drug Metab Dispos* **31**:1108–1116.

Hasunuma T, Nakamura M, Yachi T, Arisawa N, Fukushima K, and Iijima H (2003) The drug-drug interactions of pitavastatin (NK-104), a novel HMG-CoA reductase inhibitor and cyclosporine. *J Clin Ther Med* **19**:381–389.

Hengy H and Kölle EU (1985) Determination of gemfibrozil in plasma by high performance liquid chromatography. *Arzneimittelforschung* **35**:1637–1639.

Hirano M, Maeda K, Shitara Y, and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *J Pharmacol Exp Ther*, in press.

Hsiang B, Zhu Y, Wang Z, Wu Y, Sasseville V, Yang WP, and Kirchgessner TG (1999) A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *J Biol Chem* **274**:37161–37168.

König J, Cui Y, Nies AT, and Keppler D (2000a) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol* **278**:G156–G164.

König J, Cui Y, Nies AT, and Keppler D (2000b) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem* **275**:23161–23168.

Kyrklund C, Backman JT, Kivistö KT, Neuvonen M, Latila J, and Neuvonen PJ (2001) Plasma concentrations of active lovastatin acid are markedly increased by gemfibrozil but not by bezafibrate. *Clin Pharmacol Ther* **69**:340–345.

Kyrklund C, Backman JT, Neuvonen M, and Neuvonen PJ (2003) Gemfibrozil increases plasma pravastatin concentrations and reduces pravastatin renal clearance. *Clin Pharmacol Ther* **73**:538–544.

Mathew P, Cuddy T, Tracewell WG, and Salazar D (2004) An open-label study on the pharmacokinetics (PK) of pitavastatin (NK-104) when administered concomitantly with fenofibrate or gemfibrozil in healthy volunteers (Abstract PI-115). *Clin Pharmacol Ther* **75**:P33.

Matzno S, Tazuya-Murayama K, Tanaka H, Yasuda S, Mishima M, Uchida T, Nakabayashi T, and Matsuyama K (2003) Evaluation of the synergistic adverse effects of concomitant therapy with statins and fibrates on rhabdomyolysis. *J Pharm Pharmacol* **55**:795–802.

Moghadasian MH, Mancini GB, and Frohlich JJ (2000) Pharmacotherapy of hypercholesterolaemia: statins in clinical practice. *Expert Opin Pharmacother* **1**:683–695.

Mück W (2000) Clinical pharmacokinetics of cerivastatin. *Clin Pharmacokinet* **39**: 99–116.

Nakagawa A, Shigeta A, Iwabuchi H, Horiguchi M, Nakamura K, and Takahagi H (1991) Simultaneous determination of gemfibrozil and its metabolites in plasma and urine by a fully automated high performance liquid chromatographic system. *Biomed Chromatogr* **5**:68–73.

Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, and Nishimura K (2001) Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. *J Pharmacol Exp Ther* **297**:861–867.

Okerholm RA, Keeley FJ, Peterson FE, and Glazko AJ (1976) The metabolism of gemfibrozil. *Proc R Soc Med* **69 (Suppl 2)**:11–14.

Prueksaritanont T, Subramanian R, Fang X, Ma B, Qiu Y, Lin JH, Pearson PG, and Baillie TA (2002a) Glucuronidation of statins in animals and humans: a novel mechanism of statin lactonization. *Drug Metab Dispos* **30**:505–512.

Prueksaritanont T, Tang C, Qiu Y, Mu L, Subramanian R, and Lin JH (2002b) Effects of fibrates on metabolism of statins in human hepatocytes. *Drug Metab Dispos* **30**:1280–1287.

Prueksaritanont T, Zhao JJ, Ma B, Roadcap BA, Tang C, Qiu Y, Liu L, Lin JH, Pearson G, and Baillie TA (2002c) Mechanistic studies on metabolic interactions between gemfibrozil and statins. *J Pharmacol Exp Ther* **301**:1042–1051.

Regazzi MB, Campana IC, Raddato V, Lesi C, Perani G, Gavazzi A, and Vigano M (1993) Altered disposition of pravastatin following concomitant drug therapy with cyclosporin A in transplant recipients. *Transplant Proc* **25**:2732–2734.

Roca B, Calvo B, and Monferrer R (2002) Severe rhabdomyolysis and cerivastatin-gemfibrozil combination therapy. *Ann Pharmacother* **36**:730–731.

Sabordo L, Sallustio BC, Evans AM, and Nation RL (1999) Hepatic disposition of the acyl glucuronide1-O-gemfibrozil β-D-glucuronide: effects of dibromosulfophthalein on membrane transport and aglycone formation. *J Pharmacol Exp Ther* **288**:414–420.

Sabordo L, Sallustio BC, Evans AM, and Nation RL (2000) Hepatic disposition of the acyl glucuronide 1-O-gemfibrozil-β-D-glucuronide: effects of clofibric acid, acetaminophen and acetaminophen glucuronide. *J Pharmacol Exp Ther* **295**:44–50.

Sallustio BC, Fairchild BA, Shanahan K, Evans AM, and Nation RL (1996) Disposition of gemfibrozil and gemfibrozil acyl glucuronide in the rat isolated perfused liver. *Drug Metab Dispos* **24**:984–989.

Sasaki M, Suzuki H, Ito K, Abe T, and Sugiyama Y (2002) Transcellular transport of organic anions across double-transfected MDCKII cell monolayer expressing both human organic anion transporting polypeptide (OATP2/SLC21A6) and multidrug resistance associated protein 2 (MRP2/ABCC2). *J Biol Chem* **277**:6497–6503.

Shitara Y, Itoh T, Sato H, Li AP, and Sugiyama Y (2003) Inhibition of transporter-mediated hepatic uptake as a mechanism for drug-drug interaction between cerivastatin and cyclosporin A. *J Pharmacol Exp Ther* **304**:610–616.

Spence JD, Munoz CE, Hendricks L, Latchinian L, and Khouri HE (1995) Pharmacokinetics of the combination of fluvastatin and gemfibrozil. *Am J Cardiol* **76**:80A–83A.

Staffa JA, Chang J, and Green G (2002) Cerivastatin and reports of fatal rhabdomyolysis. *N Engl J Med* **346**:539–540.

Todd PA and Ward A (1988) Gemfibrozil: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in dyslipidaemia. *Drugs* **36**:314–339.

Ueda K, Kato Y, Komatsu K, and Sugiyama Y (2001) Inhibition of biliary excretion of methotrexate by probenecid in rats: quantitative prediction of interaction from in vitro data. *J Pharmacol Exp Ther* **297**:1036–1043.

Wang J-S, Neuvonen M, Wen X, Backman JT, and Neuvonen PJ (2002) Gemfibrozil inhibits CYP2C8-mediated cerivastatin metabolism in human liver microsomes. *Drug Metab Dispos* **30**:1352–1356.

Wen X, Wang J-S, Backman JT, Kivistö KT, and Neuvonen PJ (2001) Gemfibrozil is a potent inhibitor of human cytochrome P450 2C9. *Drug Metab Dispos* **29**:1359–1361.

Wierzbicki AS, Mikhalidis DP, Wray R, Schacter M, Cramb R, Simpson WG, and Byrne CB (2003) Statin-fibrate combination: therapy for hyperlipidemia: a review. *Curr Med Res Opin* **19**:155–168.

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IN VITRO AND IN VIVO CORRELATION OF THE INHIBITORY EFFECT OF CYCLOSPORIN A ON THE TRANSPORTER-MEDIATED HEPATIC UPTAKE OF CERIVASTATIN IN RATS

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ABSTRACT:

Previously, we have shown that the inhibition of the transporter-mediated hepatic uptake of cerivastatin (CER) by cyclosporin A (CsA) could, at least partly, explain a pharmacokinetic interaction between these drugs in humans. In the present study, we have examined the effect of CsA on the in vivo disposition of CER in rats and the in vitro uptake of [^{14}C]CER in isolated rat hepatocytes in an attempt to evaluate the effect of inhibition of transporter-mediated hepatic uptake on the in vivo CER disposition. The steady-state plasma concentration of CER increased 1.4-fold when coadministered with CsA up to a steady-state blood concentration of 4 μM. Studies of [^{14}C]CER uptake into isolated rat hepatocytes showed saturable transport, with the saturable portion accounting for more than 80% of the total uptake. CsA competitively inhibited the uptake of [^{14}C]CER with a K_i of 0.3 μM. The IC_{50} for the uptake of [^{14}C]CER in the absence and presence of rat plasma was 0.2 and 2.3 μM, respectively. The in vivo hepatic uptake of [^{14}C]CER evaluated by the liver uptake index method was also inhibited by CsA in a dose-dependent manner. On the other hand, CsA did not inhibit the metabolism of [^{14}C]CER in rat microsomes. The in vitro and in vivo correlation analysis revealed that this pharmacokinetic interaction between these drugs in rats could be quantitatively explained by the inhibition of transporter-mediated hepatic uptake. Thus, this drug-drug interaction in rats is predominantly caused by the transporter-mediated uptake process.

Cerivastatin (CER) is a potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitor (statin), which was used as an effective therapeutic agent for the treatment of hypercholesterolemia (Moghadasian, 1999). However, this drug was voluntarily withdrawn from the market in 2001 due to a severe side effect, myotoxicity, which sometimes caused deaths (Charatan, 2001). Among the 31 patients who died from this side effect in the United States, 12 were concomitantly taking gemfibrozil, a fibrate (Charatan, 2001), suggesting that this side effect may be, at least partly, caused by a drug-drug interaction (DDI). Gemfibrozil has been reported to increase the area under the plasma concentration-time curve (AUC) of CER by 4- to 6-fold (Mueck et al., 2001; Backman et al., 2002). Some reports have been published claiming that this interaction is caused by inhibition of the cytochrome P450 2C8 (CYP2C8)- and uridine diphosphate glucuronosyltransferase-mediated metabolism by gemfibrozil and its metabolite (Prueksaritanont et al., 2002a,b; Wang et al., 2002; Shitara et al., 2004).

The AUC of CER was also increased when coadministered with cyclosporin A (CsA) (Mück et al., 1999). We have previously shown that this clinically relevant DDI is, at least partly, caused by the inhibition by CsA of the transporter-mediated hepatic uptake but not its metabolism (Shitara et al., 2003). CsA inhibited the transporter-mediated uptake of CER in cryopreserved human hepatocytes and in organic anion-transporting polypeptide 1B1 (OATP1B1, formerly referred to as OATP2/OATP-C)-expressing cells (Shitara et al., 2003) with the inhibition constant (K_i) of 0.2 to 0.7 μM. Although this K_i value was higher than the unbound concentration of CsA in the circulating blood in clinical situations (at most 0.1 μM), it was lower than, or similar to, the estimated maximum unbound concentration of CsA at the inlet to the liver (0.66 μM) (Ito et al., 1998a,b; Kanamitsu et al., 2000; Hirota et al., 2001). Based on this analysis, we believe that inhibition of the transporter-mediated uptake also takes place in

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ABBREVIATIONS: CER, cerivastatin; AUC, area under the plasma concentration-time curve; CL_H, hepatic clearance; CL_{int}, intrinsic hepatic clearance; $CL_{int,all}$, overall intrinsic hepatic clearance; CL_{tot}, total body clearance; CL_{uptake}, uptake clearance; CsA, cyclosporin A; CYP2C8, cytochrome P450 2C8; DDI, drug-drug interaction; f_b, blood unbound fraction; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; IC_{50}, inhibitor concentration to produce a 50% reduction in the transport; KHB, Krebs-Henseleit buffer; K_i, inhibition constant; K_m, Michaelis constant; LUI, liver uptake index; OATP/Oatp, organic anion transporting polypeptide; P_{dif}, nonsaturable uptake clearance; $PS_{u,efflux}$, membrane permeability clearance of the unbound drug for the efflux process; $PS_{u,influx}$, membrane permeability clearance of the unbound drug for the influx process; SD rat, Sprague-Dawley rat; V_{max}, maximum uptake rate.

clinical situations and causes a DDI. However, it is impossible to measure the unbound concentration of CsA at the inlet to the liver and extrapolate from an in vitro inhibition study to the situation of in vivo DDI.

In the present study, to extrapolate the inhibition by CsA of the transporter-mediated uptake of CER from the in vitro to the in vivo situation, we analyzed the effect of CsA on the in vitro uptake of CER and in vivo disposition of CER in rats. Hirayama et al. (2000) examined the saturable uptake of CER in a primary culture of rat hepatocytes, and it was also taken up into human hepatocytes in a saturable manner (Shitara et al., 2003). In addition, the Oatp family transporters are conserved in rats, whereas OATP1B1 is, at least partly, responsible for the hepatic uptake of CER in humans (Shitara et al., 2003). Among the Oatps, Oatp1a1 (Oatp1), 1a4 (Oatp2), and 1b2 (Oatp4) are localized in the liver (Jacquemin et al., 1994; Noé et al., 1997; Cattori et al., 2000), although it is unknown which of them is the counterpart of OATP1B1. OATP/Oatp family transporters have similar substrate and inhibitor specificities, with some exceptions (Meier et al., 1997; Hagenbuch and Meier, 2003). In fact, CsA, an inhibitor of human OATP1B1, also inhibits rat Oatp1a1 and 1a4, although its effect on Oatp1b2 is unknown (Shitara et al., 2002). These facts suggest that the rat appears to be a good animal model for measuring the hepatic uptake of CER, although the molecular mechanisms governing the hepatic uptake in rats and humans are different. However, the metabolic profiles of CER in humans and rats are different. Both in rats and in humans, CER is mainly excreted in the form of metabolites. In humans, CER is mainly metabolized to M1 and M23, with a lesser amount of M24 by CYP2C8 and 3A4, whereas it is metabolized into many different products in rats (Mück et al., 1999; Mück, 2000; Boberg et al., 1998). Also, in rats, the isoform of cytochrome P450 responsible for its metabolism is unknown. Therefore, there is an interspecies difference between rats and humans in the molecular mechanism of the disposition and elimination of CER. However, as far as the transporter-mediated hepatic uptake is concerened, rats can be used as test models for extrapolating from in vitro to in vivo situations.

In the present study, we examined the inhibitory effect of CsA on the in vivo plasma CER concentration at steady state after intravenous administration, as well as the hepatic uptake of CER and the in vitro uptake of CER in isolated rat hepatocytes and rat Oatp1a1-expressing cells. The data obtained in the in vivo study were compared with those obtained in the in vitro study.

Materials and Methods

Reagents and Animals. [^{14}C]CER (2.03 GBq/mmol) and unlabeled CER were kindly provided by Bayer Healthcare AG (Wuppertal, Germany). CsA was purchased from Sigma-Aldrich (St. Louis, MO), and all other reagents were of analytical grade. Male Sprague-Dawley (SD) rats were purchased from Nihon SLC (Hamamatsu, Japan).

Determination of CER Plasma Concentrations in Rats. The studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. CER (1.32 and 4.95 μM for administration by bolus and infusion, respectively) was dissolved in saline. CsA was dissolved in a mixture of Cremophor EL (Sigma-Aldrich) and 94% ethanol (Wako, Osaka, Japan) (containing 0.65 g of Cremophor EL/ml), and subsequently diluted with 10 volumes of saline. Under light ether anesthesia, the right and left femoral veins of male SD rats weighing from 220 to 260 g were cannulated with polyethylene tubing (PE-50; BD Biosciences, Franklin Lakes, NJ). To avoid enterohepatic recirculation, which increases the inhibitor concentration in the portal vein and at the inlet to the liver, the bile duct was also cannulated with polyethylene tubing (PE-10: BD Biosciences). CER (1.32 nmol/kg) and CsA (0, 1.16, and 3.99 μmol/kg) were administered intravenously as a bolus via the right and left femoral veins, respectively, followed by infusion of CER (4.95 nmol/h/kg) and CsA (0, 0.175, and 0.599 μmol/h/kg). At 5 h after infusion, 400 μl of blood was collected from the tail vein, and EDTA (1 mg/ml) was added. At this time point, the plasma and blood concentrations of CER and CsA had both reached steady state (data not shown). The samples were stored at -20°C until analysis. Plasma concentrations of CER were determined by a validated method using atmospheric pressure ionization liquid chromatography-tandem mass spectrometry. To a 50-μl sample, 500 μl of 1 M phosphate buffer (pH 5.5) and 5 ml of diethyl ether/dichloromethane (2:1) were added; the sample was shaken for 10 min and centrifuged. Then, the organic layer was evaporated to dryness under N_2 gas at 40°C and the residue was dissolved in a 200-μl mobile phase. A 20-μl portion of each sample was then subjected to atmospheric pressure ionization liquid chromatography-tandem mass spectrometry. A Shimadzu 10A high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) combined with a model API 365 MS/MS (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a Turbo IonSpray probe was used. The analytes were separated on an Inertsil ODS-3 column (5 μm, 2.1 mm i.d.$\times$150 mm; GL Sciences Inc., Tokyo, Japan) using a mobile phase (acetonitrile/0.2% formic acid, 60:40, v/v) at a flow rate of 0.2 ml/min. Selected reaction monitoring was used to detect the analytes and internal standard (positive mode, *m/z* 460.6/356.1). The quality control sample showed an analytical variance of less than 8.8%. Blood concentrations of CsA were determined by radioimmunoassay using CYCLO-Trac (DiaSorin, Stillwater, MN), with quality control samples that were included in the kit and confirmed the precision of assay.

Uptake of [^{14}C]CER into hepatocytes. Isolated rat hepatocytes were prepared from SD rats weighing from 220 to 250 g by the collagenase perfusion method described previously (Yamazaki et al., 1993). Isolated hepatocytes (viability >90%) were suspended in Krebs-Henseleit buffer (KHB), adjusted to 4.0×10^6 viable cells/ml, and stored on ice. Before the uptake study, hepatocytes were incubated at 37°C for 3 min and the uptake reaction was started by adding an equal volume of KHB prewarmed at 37°C containing 0.6 μM [^{14}C]CER (final concentration, 0.3 μM) with unlabeled CER or CsA. At 0.5 and 2 min, the reaction was terminated by separating the cells from the substrate solution. For this purpose, an aliquot of 100 μl of incubation mixture was collected and placed in a centrifuge tube (250 μl) containing 50 μl of 2 N NaOH under a layer of 100 μl of oil (density, 1.015; a mixture of silicone oil and mineral oil, Sigma-Aldrich), and, subsequently, the sample tube was centrifuged for 10 s using a tabletop centrifuge (10,000*g*; Beckman Microfuge E; Beckman Coulter, Fullerton, CA). During this process, the hepatocytes pass through the oil layer into the alkaline solution. After an overnight incubation at room temperature to dissolve the cells in alkali, the centrifuge tube was cut and each compartment was transferred to a scintillation vial. The compartment containing dissolved cells was neutralized with 50 μl of 2 N HCl, mixed with scintillation cocktail (Clearsol II; Nakalai Tesque, Kyoto, Japan), and the radioactivity was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter). For the estimation of the inhibitory effects of CsA, it was added at the same time as [^{14}C]CER. In the present investigation, the uptake study was also conducted in incubation buffer containing 90% rat plasma to evaluate the effect of plasma protein. For this purpose, isolated hepatocytes were suspended in KHB, adjusted to 2.0×10^7 cells/ml, and prewarmed at 37°C before the uptake study. The reaction was started by addition of 9 volumes of rat plasma containing 0.25 μM [^{14}C]CER.

Uptake of CER into Rat Oatp1a1-Expressing Cells. The rat Oatp1a1-expression vector was constructed as described previously (Kouzuki et al., 1999). Rat Oatp1a1-expressing HEK293 cells and control cells were constructed by the transfection of expression vector and control pcXN2 vector, respectively, into cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instruction, and selection by 800 μg/ml antibiotic G418 sulfate (Promega, Madison, WI) for 3 weeks. Oatp1a1-expressing cells and control cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1000 U/ml penicillin G sodium, 1 mg/ml streptomycin sulfate, 2.5 μg/ml amphotericin B and 200 mg/liter G418 sulfate. For the uptake study, cells were seeded on 12-well plates coated with poly-L-lysine/poly-L-ornithine at 1.2×10^5 cells/well and cultured. After 2 days, culture medium was replaced with the same culture medium containing 10 mM sodium butyrate

(Wako) for the induction of Oatp1a1 expression (Schroeder et al., 1998), followed by culturing for one more day. Before the uptake study, cells were washed twice with ice-cold KHB. Then the ice-cold KHB was replaced with KHB at 37°C followed by prewarming at 37°C for 10 min. The uptake reaction was started by the replacement of KHB with a solution containing 0.3 μM [^{14}C]CER, with unlabeled CER or CsA. The reaction was terminated by removing the substrate solution by suction and, subsequently, cells were washed twice with ice-cold KHB after 5 min, since we confirmed the linearity of the uptake for at least 5 min. Cells were dissolved in 500 μl of 0.1 N NaOH overnight, followed by neutralization with 500 μl of 0.1 N HCl. Then, 800-μl aliquots were transferred to scintillation vials, and the radioactivity associated with cells and the medium was determined (LS6000SE). In addition, 50 μl of cell lysate was used for the protein assay by the Lowry method with bovine serum albumin as a standard (Lowry et al., 1951).

Protein Binding of CER in Rat Plasma. To estimate the fraction not bound to plasma protein, 3 μM [^{14}C]CER was added to rat plasma supplemented with 10% KHB and incubated for 30 min at 37°C. After that, the sample underwent ultrafiltration (Amicon Centrifree; Millipore Corporation, Bedford, MA). The radioactivity in the 90% plasma and filtrate was determined (LS6000SE) and the plasma protein unbound fraction was calculated. In a pilot study, no significant difference of protein binding was confirmed up to its concentration of 1000 μM.

Liver Uptake Index (LUI) Method. Under light ether anesthesia, the femoral vein of male SD rats (weighing from 220 to 280 g) was cannulated with polyethylene tubing (PE-50). Before the LUI study, a 2 ml/kg bolus of CsA (0, 2.4, 4.8, and 9.6 mg/kg) was administered via the femoral vein. At 5 min after intravenous administration of CsA, approximately 100 μl of blood was collected from the jugular vein for determination of the concentration of CsA. [^{14}C]CER and [^{3}H]inulin dissolved in rat plasma (approximately 18.5 and 100 kBq/ml/kg for [^{14}C]CER and [^{3}H]inulin, respectively) containing a 1:1500 dilution of CsA solution, which was used for the bolus intravenous administration, was rapidly injected into the portal vein immediately after ligation of the hepatic artery. After 18 s of bolus administration of radiolabeled compounds, which is long enough for the bolus to pass completely through the liver but short enough to prevent recirculation of the isotope (Partridge et al., 1985), the portal vein was cut and the liver was excised. The excised liver was minced, and approximately 100 mg of sample was transferred to a scintillation vial and dissolved in a solubilizing agent (Soluene-350; PerkinElmer Life and Analytical Sciences, Boston, MA) at 55°C, followed by the addition of liquid scintillation cocktail (Hionic-Fluor; PerkinElmer Life and Analytical Sciences). Also, 100 μl of injectate was transferred to a scintillation vial and liquid scintillation cocktail (Hionic-Fluor) was added. After that, the radioactivity taken up by the liver and in the injectate was determined in a liquid scintillation counter (LS6000SE).

Metabolism of CER and Testosterone in Rat Microsomes. Before the metabolism study, rat liver microsomes (final concentration 0.5 mg of protein/ml; BD Gentest, Woborn, MA) were incubated at 37°C for 10 min in 100 mM potassium phosphate buffer (pH 7.4) containing 3.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate deydrogenase, 1.3 mM NADPH, and 0.8 mM NADH. A 500- μl volume of incubation mixture was transferred to a polethyelene tube, and [^{14}C]CER (final 0.25 μM) or testosterone (final concentration 30 μM; Wako) was added to initiate the reaction with or without inhibitors. After incubation at 37°C for a designated time, the reaction was terminated by the addition of 500 μl of ice-cold acetonitrile and 20 μl of ice-cold methanol for the metabolism of [^{14}C]CER and testosterone, respectively, followed by centrifugation. To measure the metabolic rate of [^{14}C]CER, the supernatant was collected and concentrated to approximately 20 μl in a centrifugal concentrator, followed by thin-layer chromatography. The separation was carried out on a silica gel $60F_{254}$ plate (Merck, Darmstadt, Germany) using a suitable mobile phase (toluene/acetone/acetic acid, 70:30:5, v/v/v). The intensity of the bands for intact [^{14}C]CER separated by TLC was determined using the BAS 2000 system (Fuji Film, Tokyo, Japan). To measure the metabolic rate of testosterone, 6β- and 16α-hydroxytestosterone in the incubation mixture were determined by an HPLC-UV method. To a 100-μl volume of supernatant, 100 μl of internal standard (10 μg/ml phenacetin) was added followed by HPLC (VP-5 system; Shimadzu). The analyte was separated on a C18 column (Cosmosil 5C18-AR, 5 mm, 4.6 mm i.d. × 250 mm; Nakalai Tesque) at 45°C. The mobile phase consisted of a mixture of solvent A (20% tetrahydrofuran, 80% water) and solvent B (methanol). A 20-min linear gradient from 20% B to 30% B was applied at a flow rate of 1.0 ml/min. The products were detected by their absorbance at 254 nm and quantified by comparison with the absorbance of a standard curve for 6β- and 16α-hydroxytestosterone.

Data Analysis. The time courses of the uptake of [^{14}C]CER into hepatocytes were expressed as the uptake volume [μl/10^6 viable cells] for the radioactivity taken up into cells [dpm/10^6 cells] divided by the concentration of radioactivity in the incubation buffer [dpm/μl]. The initial uptake velocity of [^{14}C]CER was calculated from a slope of the uptake volume versus time plot obtained at 0.5 and 2 min and expressed as the uptake clearance (CL_{uptake}; μl/min/10^6 cells). The time courses of the uptake of [^{14}C]CER into rat Oatp1-expressing cells and vector-transfected cells were also expressed as the uptake volume (μl/mg protein) for the radioactivity in the cell lysate (dpm/mg protein) divided by the concentration of radioactivity in the incubation buffer (dpm/μl). Rat Oatp1-mediated uptake was calculated by using the uptake volume at 5 min in rat Oatp1-expressing cells and vector-transfected cells and expressed as the uptake clearance (CL_{uptake}; μl/min/mg protein), i.e., the CL_{uptake} in rat Oatp1-expressing cells minus that in vector-transfected cells.

The kinetic parameters for the uptake of [^{14}C]CER were calculated using the following equation:

$$v_0 = \frac{V_{max} \times S}{K_{m+S}} + P_{dif} \times S \quad (1)$$

where v_0 is the initial uptake rate (pmol/min/mg protein), S is the substrate concentration (μM), K_m is the Michaelis constant (μM), V_{max} is the maximum uptake rate (pmol/min/mg protein), and P_{dif} is the nonsaturable uptake clearance (μl/min/mg protein).

The uptake clearance in the isolated hepatocytes obtained in the presence of CsA was fitted to the following equation to calculate the inhibitor concentration to produce a 50% reduction (IC_{50}) in the uptake of [^{14}C]CER.

$$CL_{uptake}(+CsA) = \frac{CL_{uptake}(control) - CL_{uptake}(resistant)}{1 + I/IC_{50}} + CL_{uptake}(resistant) \quad (2)$$

where CL_{uptake}(+CsA) is the CL_{uptake} estimated in the presence of CsA, CL_{uptake}(control) is the CL_{uptake} estimated in the absence of CsA, CL_{uptake}(resistant) is the CL_{uptake} which is not affected by CsA, and I is the CsA concentration. In the presence of 90% rat plasma, these parameters were calculated based on the total concentration, not the estimated free concentration, of substrate (CER) and inhibitor (CsA). Parameters calculated based on the total concentrations of CER or CsA are referred to as $CL_{uptake,app}$, $K_{m,app}$, $P_{dif,app}$ and $IC_{50,app}$.

To determine the inhibition constant (K_i), the initial uptake rate of [^{14}C]CER into rat hepatocytes determined in the presence and absence of CsA was fitted to the following equations in which competitive inhibition was assumed:

$$v_0 = \frac{V_{max} \times S}{K_m \times \left(1 + \frac{I}{K_i}\right) + S} + P_{dif} \times S \quad (3)$$

These equations were fitted to the data obtained in the present study using a computerized version of the nonlinear least-squares method, WinNonlin (Pharsight, Mountain View, CA), to obtain the kinetic parameters or inhibition constant with a computer-calculated standard error of each estimate (computer-calculated S.E.), which means the precision of the estimated parameter, but not an estimate of inter-rat variability. The input data were weighted as the reciprocal of the observed values, and the Gauss Newton (Levenberg and Hartley) method was used as the fitting algorithm.

The data obtained in the LUI study were expressed as %LUI [%], which represents the ratio of the hepatic extraction of [^{14}C]CER to that of [^{3}H]inulin. The %LUI was obtained by the following equation:

$$\%LUI = \frac{([^{14}C]CER \text{ taken by liver}/[^{3}H]inulin \text{ taken by liver})}{([^{14}C]CER \text{ in injectate}/[^{3}H]inulin \text{ in injectate})} \times 100[\%] \quad (4)$$

where, [^{14}C]CER and [^{3}H]inulin taken up by the liver was measured based on the radioactivity associated with the liver [dpm/mg tissue] and [^{14}C]CER and

[^{3}H]inulin in the injectate were measured based on their concentrations [dpm/μl].

Statistical comparisons among multiple groups were carried out using Dunnett's test.

Results

In Vivo Study. The steady-state plasma concentrations of CER and the blood concentrations of CsA in rats 5 h after their intravenous infusion are shown in Table 1. The plasma concentration of CER significantly increased as the blood concentration of CsA increased (1.40- and 1.44-fold for 1.2 and 3.0 μM CsA; Table 1). The total body clearance (CL_{tot}) of CER was significantly reduced from 1.51 to 1.09 and 1.05 [l/h/kg] at steady-state blood concentrations of 1.2 and 3.0 μM CsA, respectively.

Uptake into Isolated Rat Hepatocytes. The uptake of CER into isolated rat hepatocytes incubated with KHB is shown in Fig. 1a. Kinetic analyses revealed that the K_m, V_{max}, and P_{dif} for the uptake of CER into isolated rat hepatocytes were 9.20 ± 2.24 μM, 1510 ± 330 pmol/min/10^6 viable cells, 35.2 ± 4.2 μl/min/10^6 viable cells (mean ± computer-calculated S.E.), respectively. The saturable component of the hepatic uptake, estimated by V_{max}/K_m, accounted for 82.4%. Uptake studies were also conducted in the presence of 90% rat plasma to investigate the effect of plasma protein binding. The apparent CL_{uptake} of CER was reduced in the presence of plasma (Fig. 1b). The kinetic parameters were 16.1 ± 2.4 μM, 481 ± 53 pmol/min/10^6 viable cells, and 2.37 ± 0.22 μl/min/10^6 viable cells (mean ± computer-calculated S.E.) for $K_{m,app}$, $V_{max,app}$, and $P_{dif,app}$, respectively (Fig. 1b). The saturable component accounted for 92.6% (Fig. 1b). Correcting for the CER-free fraction (4.32 ± 0.24%) gave unbound K_m and P_{dif} values of 0.696 μM and 0.102 μl/min/10^6 viable cells, respectively.

TABLE 1

Plasma concentration of CER and blood concentration of CsA after intravenous administration in rats

Plasma and blood concentrations of CER and CsA, respectively, were measured at 5 h after intravenous bolus injection and infusion of both compounds. All data are mean ± S.E. (n = 3–4).

Dose of CsA	Concentration of CsA	Concentration of CER
	μM	nM
0	0	3.27 ± 0.12
1.16 μmol/kg bolus injection → 0.175 mmol/h/kg infusion	1.24 ± 0.02	4.56 ± 0.52
3.99 mmol/kg bolus injection → 0.599 μmol/h/kg infusion	3.02 ± 0.22	4.71 ± 0.29**

** $p < 0.01$; a statistically significant difference from control by Dunnett's test.

Inhibition of the Uptake of [^{14}C]CER into Isolated Rat Hepatocytes by CsA. The uptake of [^{14}C]CER was examined in the presence of CsA (Fig. 2). CsA inhibited the uptake of CER into isolated rat hepatocytes in a concentration-dependent manner (Fig. 2a). The IC_{50} value was 0.198 ± 0.028 μM (mean ± computer-calculated S.E.). In the presence of 90% rat plasma, CsA inhibited the uptake of CER; however, the apparent IC_{50} values based on the total, but not free, concentration of CsA ($IC_{50,app}$) was increased. The $IC_{50,app}$ estimated in the presence of 90% rat plasma was 2.32 ± 0.33 μM (mean ± computer-calculated S.E.) (Fig. 2b). To determine the K_i value, kinetic analysis was performed in the presence of 0.1 and 0.3 μM CsA (Fig. 3). As shown in Fig. 3a, CsA affected the K_m value of CER rather than the V_{max} value, suggesting that CsA competitively inhibits the uptake of CER. This suggestion was also supported by a Lineweaver-Burk plot (Fig. 2b). Based on the competitive inhibition model (eq. 3), the K_i value of CsA was estimated to be 0.180 ± 0.023 μM (mean ± computer-calculated S.E.).

Uptake of [^{14}C]CER into Rat Oatp1a1-Expressing cells. The rat Oatp1a1-mediated uptake of [^{14}C]CER, estimated by its uptake in Oatp1a1-expressing cells minus that in control cells, is shown in Fig. 4. The K_m and V_{max} for the rat Oatp1-mediated uptake of CER were 6.42 ± 1.16 μM and 33.4 ± 4.2 pmol/min/mg protein (mean ± computer-calculated S.E.), respectively. Rat Oatp1a1-mediated uptake was also inhibited by CsA (Table 2).

LUI Study. The hepatic uptake of CER in vivo was also examined by an LUI study in rats (Fig. 5). The %LUI (eq. 4) was reduced following coadministration of CsA in a concentration-dependent manner up to 4 μM (Fig. 5). The reduction in %LUI was 66.9, 54.0, and

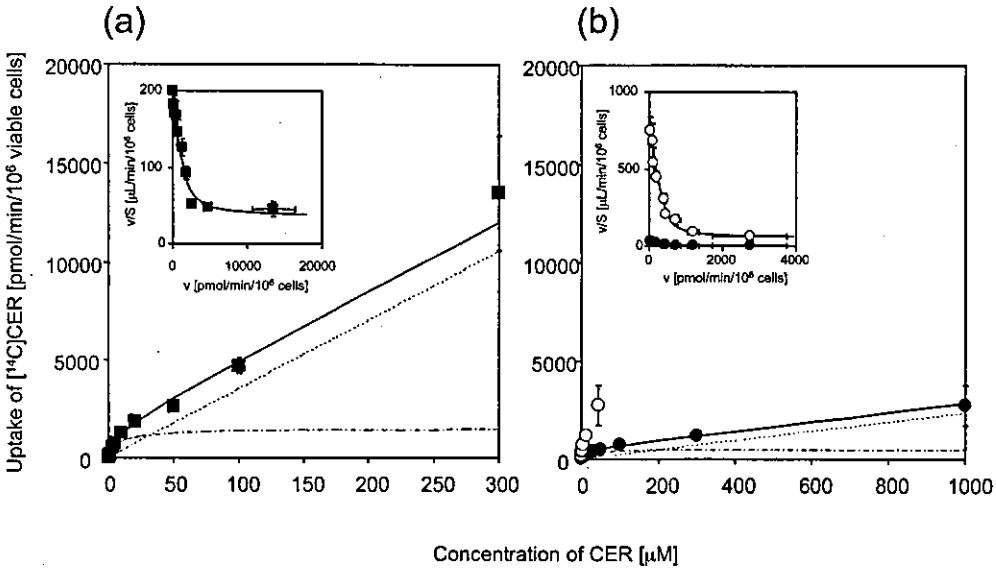

FIG. 1. The uptake of CER in isolated rat hepatocytes in the absence or presence of 90% rat plasma. Uptake of CER in isolated rat hepatocytes was examined in KHB (a) or 90% rat plasma containing KHB (b). The uptake rate versus CER concentration plot is shown. In the presence of 90% rat plasma, the data are shown with (○) and without (●) correction for CER plasma protein binding. Each point represents the mean ± S.E. (n = 3, three cell preparations). Solid lines, dotted lines, and dashed lines represent the fitted lines for total, nonsaturable, and saturable transport of CER. Eadie-Hofstee plot for the uptake of CER is also shown.

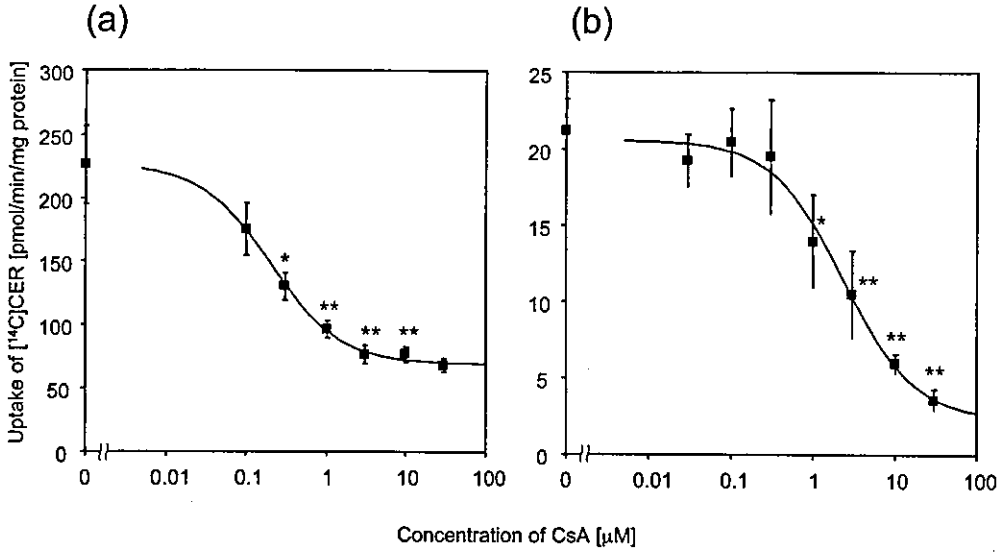

FIG. 2. Inhibitory effect of CsA on the uptake of [^{14}C]CER in isolated rat hepatocytes. The inhibitory effect of CsA on the uptake of CER (0.3 μM) in isolated rat hepatocytes was examined in the absence (a) or presence (b) of 90% rat plasma. Each point represents the mean ± S.E. (n = 3, three cell preparations). Solid lines represent the fitted lines. The asterisk represents a statistically significant difference from control shown by Dunnett's test (*, $p < 0.05$; **, $p < 0.01$).

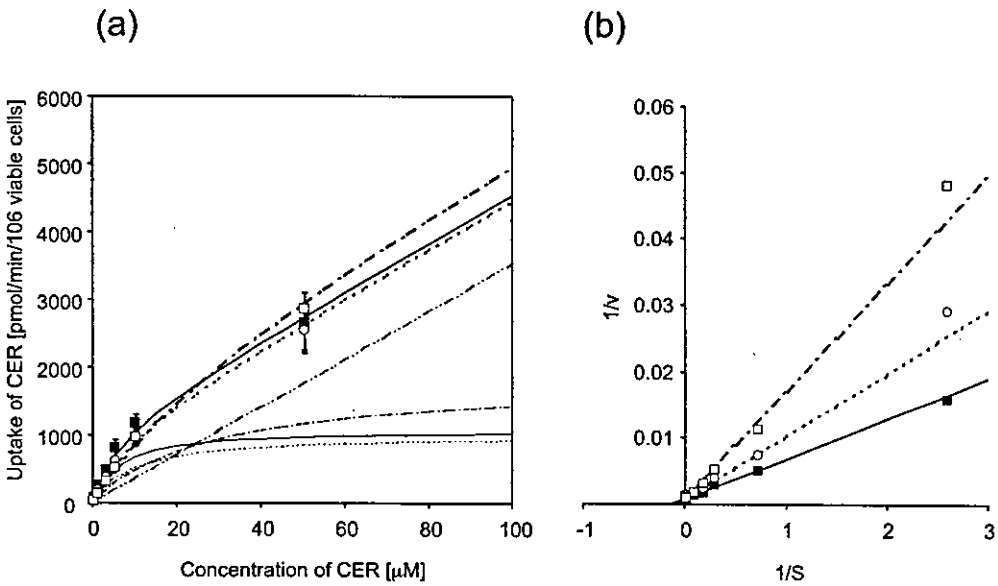

FIG. 3. The concentration-dependent uptake of CER in the presence or absence of CsA. The uptake of CER in isolated rat hepatocytes was examined in the presence of 0 (■), 0.1 (○), and 0.3 (□) μM CsA. The uptake rate versus the CER concentration plot (a) and a Lineweaver-Burk plot (b) are shown. Each symbol represents mean ± S.E. of three independent experiments. In a, thick solid, dotted, and dashed lines represent fitted curves for the total transport of CER in the presence of 0, 0.1, and 0.3 μM CsA, respectively. Thin solid, dotted, and dashed lines represent fitted curves for the saturable transport of CER in the presence of 0, 0.1, and 0.3 μM CsA, respectively. A chain double-dashed line represents the fitted curve for the nonsaturable transport of CER. In b, solid, dotted, and dashed lines represent fitted lines in the presence of 0, 0.1, and 0.3 μM CsA, respectively.

49.7% of the control for CsA blood concentrations of 2.0, 2.7, and 4.0 μM, respectively (Fig. 5).

Metabolism of [^{14}C]CER. The metabolism of [^{14}C]CER in rat microsomes was examined. After a 2 h-incubation, approximately 74% of [^{14}C]CER remained unchanged in the absence of inhibitors. The metabolism of [^{14}C]CER was not inhibited by CsA up to a concentration of 30 μM, whereas it was significantly inhibited by 0.2 μM ketoconazole (to 24.8% of the control) (Fig. 6a). The metabolism of testosterone in rat liver microsomes was also examined, and the formation rates of 6β- and 16α-hydroxylation were, respectively, 996 ± 22 and 1520 ± 10 pmol/min/mg protein (mean ± S.E.) in the absence of inhibitors (Fig. 6b). Both 6β- and 16α-hydroxylations were significantly inhibited by CsA (Fig. 6b). Ketoconazole (0.2 μM) significantly inhibited 6β-hydroxylation of testosterone, whereas it did not inhibit 16α-hydroxylation (Fig. 6b).

Discussion

The present study shows that CsA increases the plasma concentration of CER in rats in a dose-dependent manner (Table 1). It also shows that CsA inhibits the uptake of CER in rat hepatocytes, whereas it does not affect the in vitro metabolism of CER in microsomes (Figs. 2 and 6). Also in humans, CsA increases the plasma concentration of CER (Mück et al., 1999) and it potently inhibits the uptake of CER in hepatocytes with a minimal effect on the microsomal metabolism (Shitara et al., 2003). Therefore, the effect of CsA in rats appears to be similar to that in humans, although the molecular mechanisms of

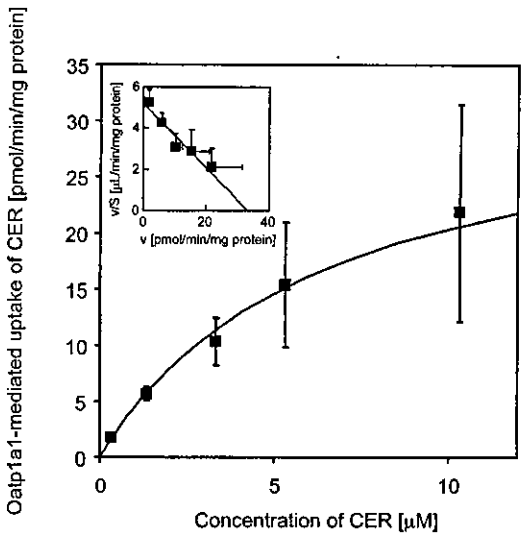

FIG. 4. The rat Oatp1a1-mediated uptake of CER. The rat Oatp1a1-mediated uptake rate of CER, which represented the uptake in Oatp1-expressing cells minus that in control cells, was plotted versus the CER concentration. Each symbol represents mean ± S.E. of three independent experiments. Solid line represents the fitted curve. The Eadie-Hofstee plot for the Oatp1a1-mediated uptake is also shown.

TABLE 2

Inhibitory effects of CsA on the Oatp1a1-mediated uptake of [^{14}C]CER

Uptake of [^{14}C]CER (0.3 μM) was observed in rat Oatp1a1-transfected and vector-transfected HEK293 cells.

Concentration of CsA	Oatp1a1-mediated uptake of [^{14}C]CER
μM	*μl/min/mg protein*
0	5.93 ± 1.01
0.1	3.66 ± 0.28**
1	3.65 ± 0.91
10	1.78 ± 0.63**

** $p < 0.01$; a statistically significant difference from control by Dunnett's test.

drug elimination in rats and humans are basically different. CsA inhibits the uptake of CER in rat hepatocytes with an unbound IC_{50} value similar to that in human hepatocytes (0.2–0.7 μM: Shitara et al., 2003). However, the in vivo effect of CsA on the disposition of CER was different between rats and humans, and the increase in the steady-state plasma concentration of CER in rats was minimal compared with that in humans. In fact, 3 μM blood CsA increased the steady-state plasma concentration of CER in rats only 1.4-fold (Table 1), whereas, in humans, CsA increased the AUC and the maximum plasma concentration of CER 3.8- and 5.0-fold, respectively, when the maximum blood concentration of CsA was approximately 1 μM (Mück et al., 1999). This difference in the severity of the interaction by these drugs between rats and humans may be explained by the different dosage regimen and experimental system. In rats, CsA, the inhibitor, was given intravenously and the biliary-excreted CsA was eliminated via cannulation without returning to the body via enterohepatic circulation, whereas, in humans, it was given orally and biliary excreted, with the CsA being transported to the portal vein and the inlet to the liver via enterohepatic circulation. In this case, the concentration of CsA at the inlet to the liver in humans may be higher than that in the circulating blood (Ito et al., 1998a,b). Therefore, in humans, a higher concentration of CsA at the inlet to the liver compared with that in the circulating blood may potently inhibit the hepatic uptake of CER, although its concentration in the circulating blood is lower in humans than in rats. However, there may be other mechanisms governing the DDI, and/or renal failure or kidney transplantation in patients may also have affected the plasma concentration of CER in the clinical case reported by Mück et al. (1999).

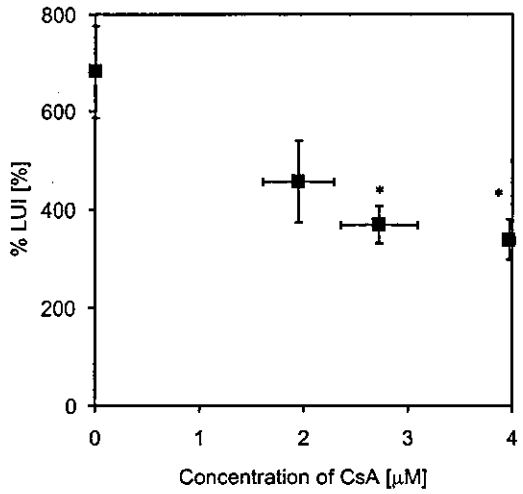

FIG. 5. Inhibitory effect of CsA on the hepatic uptake of [^{14}C]CER in rats in vivo. Inhibitory effect of CsA on the hepatic extraction of [^{14}C]CER normalized by that of [^{3}H]inulin (%LUI) during a single pass after intraportal bolus injection in rats was examined. %LUI was calculated from eq. 4. Concentration of CsA was measured just before the LUI study. Each point represents the mean ± S.E. (n = 4 and 8, with and without coadministration of CsA, respectively). The asterisk represents a statistically significant difference from control shown by Dunnett's test (*, $p < 0.05$).

In vitro uptake studies in isolated hepatocytes revealed saturable transport of CER in rat hepatocytes both in the absence and presence of rat plasma (Fig. 1). Saturable transport of CER in primary cultured rat hepatocytes has already been reported by Hirayama et al. (2000), although their CL_{uptake} (44.4 μl/min/mg protein calculated by V_{max}/K_m) was lower than that in the present study (165 μl/min/10^6 viable cells calculated by V_{max}/K_m), assuming that 10^6 cells from our studies correspond to 1 mg of protein. This may be due to differences in the experimental system (i.e., isolated and primary cultured hepatocytes), since the transporter function can be affected by the primary culture (Ishigami et al., 1995). In the present study, we have found that the transporter-mediated uptake accounted for more than 80% and 90% of the total hepatic uptake in the absence and presence of plasma, respectively, at concentrations of CER lower than the K_m (Fig. 1). The large saturable portion of the uptake of [^{14}C]CER in hepatocytes was similar to that in humans (70–80%; Shitara et al., 2003), suggesting that transporters play an important role in the hepatic uptake and disposition of CER both in rats and humans. Kinetic parameters, i.e., K_m, V_{max}, and P_{dif}, based on the free, or estimated free, concentrations of CER were different in the presence and absence of rat plasma. This difference was due to the different range of CER free concentrations under these two sets of experimental conditions (Fig. 1).

In the present study, the Oatp1a1-mediated uptake of [^{14}C]CER was examined (Fig. 4). In our pilot studies, it was shown that other Oatp family transporters in rats, i.e., Oatp1a4 and 1b2, also accepted CER as a substrate (data not shown). However, we performed the kinetic analysis and inhibition study using CsA only in Oatp1a1-expressing cells because the highest saturable transport was observed in these cells among all the Oatp family transporter-expressing cells we possess. Kinetic analyses revealed that CER was taken up into rat Oatp1a1-expressing cells with a K_m value (6.4 μM; Fig. 3) similar to that in isolated rat hepatocytes (9.2 μM; Fig. 1). Thus, Oatp family transporter(s) appears to be responsible for the hepatic uptake of CER in rats, whereas OATP1B1, at least partly, mediates its hepatic uptake in humans (Shitara et al., 2003). The inhibition by CsA of the Oatp1a1-mediated uptake of CER was also examined (Table 2). It was

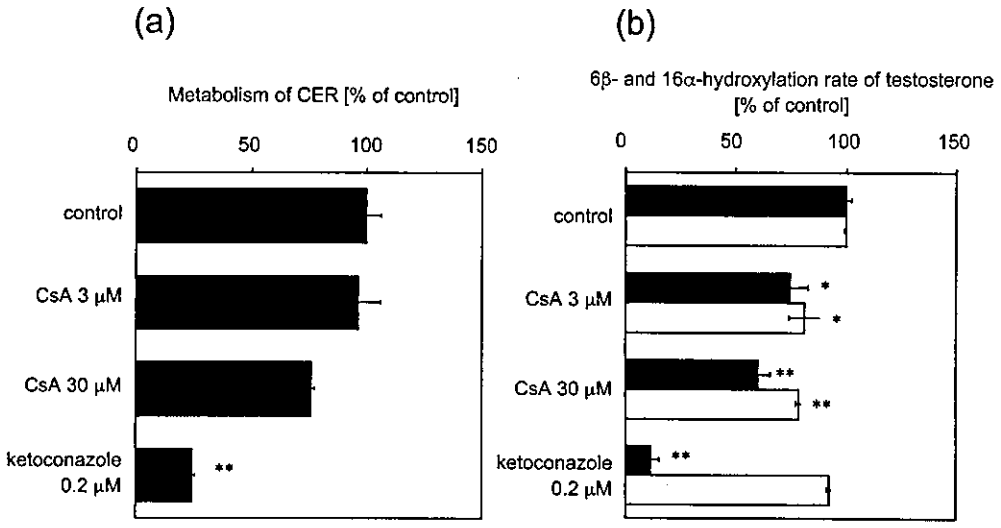

FIG. 6. Metabolic stability of CER and testosterone 6β- and 16α-hydroxylation in rat liver microsomes in the presence or absence of CsA or ketoconazole. In a, the effects of CsA and ketoconazole on the metabolism of CER in rat liver microsomes were examined. In b, those for testosterone 6β- (■) and 16α-hydroxylation (□) were examined. The metabolic rate in the presence or absence of inhibitors is shown in each figure. Each bar represents the mean ± S.E. (n = 3). The asterisk represents a statistically significant difference from control shown by Dunnett's test (*, $p < 0.05$; **, $p < 0.01$).

found that the Oatp1a1-mediated uptake of CER was inhibited by CsA in a concentration-dependent manner (Table 2), also supporting the involvement of Oatp transporter(s) in the hepatic uptake of CER.

We investigated the inhibitory effect of CsA on the uptake of CER in rat hepatocytes in the presence of 90% rat plasma, which was similar to in vivo conditions. In the presence of 90% rat plasma, the $IC_{50,app}$ value was approximately 12 times higher than the IC_{50} value in its absence (Fig. 2). Lemaire and Tillement (1982) reported that approximately 90% of CsA is bound to plasma proteins, mainly lipoprotein in rats. Taking this into consideration, the IC_{50} value, based on the estimated free concentration of CsA in the presence of 90% rat plasma, was calculated to be 0.232 μM, which is close to the IC_{50} value obtained in the study without rat plasma. The LUI study confirmed that the hepatic CER uptake measured in vivo was also affected by CsA (Fig. 5). When the blood concentration of CsA was 4 μM, the hepatic uptake of CER in the LUI study was reduced to 50% of the control value (Fig. 5), suggesting that the IC_{50} value for the in vivo hepatic uptake of CER was approximately 4 μM, which was similar to the $IC_{50,app}$ value estimated in the presence of plasma (Fig. 2b).

We have also examined the effect of CsA on the metabolism of CER in rat liver microsomes (Fig. 5). Only 26% of [^{14}C]CER was metabolized following a 2-h incubation in rat liver microsomes, whereas more than 50% was metabolized in human liver microsomes within 45 min (Shitara et al., 2003), suggesting slower metabolism in rat liver microsomes compared with their human counterparts. Since this metabolism of [^{14}C]CER was not significantly inhibited by CsA up to its concentration of 30 μM (Fig. 5), microsomal metabolism was not the mechanism for the pharmacokinetic interaction between CER and CsA in rats examined in the present study.

The results obtained in the in vitro studies should be quantitatively discussed in relation to those in vivo. Without administration of CsA, the CL_{tot} was estimated to be 1.51 l/h/kg (Table 1). In the case of CER, the urinary excretion is negligible (Boberg et al., 1998) and, therefore, the CL_{tot} is close to the hepatic clearance (CL_H). Assuming a well stirred model, the CL_H can be described by the following equation (Miyauchi et al., 1993; Yamazaki et al., 1996):

$$CL_H = \frac{Q_H \times f_b \times CL_{int,all}}{Q_H + f_b \times CL_{int,all}} \quad (5)$$

where Q_H is the hepatic blood flow and f_b is the blood unbound fraction. The $CL_{int,all}$ represents the overall intrinsic hepatic clearance, which includes membrane permeability, metabolism, and biliary excretion, as described by the following equation:

$$CL_{int,all} = PS_{u,influx} \times \frac{CL_{int}}{PS_{u,efflux} + CL_{int}} \quad (6)$$

where $PS_{u,influx}$ and $PS_{u,efflux}$ represent the membrane permeability clearance of the unbound drug for the influx and efflux process from outside and inside the cells, respectively, and CL_{int} represents the "exact" intrinsic clearance which includes metabolism and/or biliary excretion of the unbound drug. When the CL_{tot} is 1.51 l/h/kg, the $f_b \cdot CL_{int,all}$ is calculated to be 3.04 l/h/kg from eq. 6, assuming the hepatic blood flow rate is 3 l/h/kg. As shown in eq. 6, the $CL_{int,all}$ will be reduced in proportion to the decrease in $PS_{u,influx}$. In the presence of 1.2 and 3.0 μM CsA, the $PS_{u,influx}$ of CER fell to 66 and 44% of the control, respectively, when the hepatocytes were incubated in the presence of rat plasma (Fig. 2b), and, therefore, the in vivo $f_b \cdot CL_{int,all}$ is reduced to 2.01 and 1.34 l/h/kg (i.e., 66 and 44% of the control), which gives a predicted CL_H value of 1.20 and 0.93 l/h/kg, respectively, from eq. 5. This predicted CL_H is comparable with the CL_H observed in the present in vivo study (1.09 and 1.05 l/h/kg, respectively) (Table 1), suggesting that the pharmacokinetic interaction between CER and CsA in rats can be quantitatively explained by inhibition of the transporter-mediated uptake of CER.

In conclusion, the increased plasma concentration of CER in rats when coadministered with CsA can be quantitatively explained by inhibition of transporter-mediated uptake. The present study strongly suggests that the inhibition of the transporter-mediated uptake in the liver affects the drug disposition. Also, in humans, it is possible that inhibition of the transporter-mediated uptake of drugs may lead to a clinically relevant DDI.

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References

Backman JT, Kyrklund C, Neuvonen M, and Neuvonen PJ (2002) Gemfibrozil greatly increases plasma concentrations of cerivastatin. *Clin Pharmacol Ther* **72**:685–691.

Boberg M, Angerbauer R, Kanhai WK, Karl W, Kern A, Radtke M, and Steinke W (1998) Biotransformation of cerivastatin in mice, rats and dogs *in vivo*. *Drug Metab Dispos* **26**:640–652.

Cattori V, Hagenbuch B, Hagenbuch N, Stieger B, Ha R, Winterhalter KE, and Meier PJ (2000) Identification of organic anion transporting polypeptide 4 (Oatp4) as a major full-length isoform of the liver-specific transporter-1 (rlst-1) in rat liver. *FEBS Lett* **474**:242–245.

Charatan F (2001) Bayer decides to withdraw cholesterol lowering drug. *Br Med J* **323**:359.

Hagenbuch B and Meier PJ (2003) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609**:1–18.

Hirayama M, Yoshimura Y, and Moriyasu M (2000) Carrier-mediated uptake of cerivastatin in primary cultured rat hepatocytes. *Xenobio Metab Dispos* **15**:219–225.

Hirota N, Ito K, Iwatsubo T, Green CE, Tyson CA, Shimada N, Suzuki H, and Sugiyama Y (2001) *In vitro/in vivo* scaling of alprazolam metabolism by CYP3A4 and CYP3A5 in humans. *Biopharm Drug Dispos* **22**:53–71.

Ishigami M, Tokui T, Komai T, Tsukahara K, Yamazaki M, and Sugiyama Y (1995) Evaluation of the uptake of pravastatin by perfused rat liver and primary cultured rat hepatocytes. *Pharm Res (NY)* **12**:1741–1745.

Ito K, Iwatsubo T, Kanamitsu S, Nakajima Y, and Sugiyama Y (1998a) Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annu Rev Pharmacol Toxicol* **38**:461–499.

Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, and Sugiyama Y (1998b) Prediction of pharmacokinetics alterations caused by drug-drug interactions: metabolic interaction in the liver. *Pharmacol Rev* **50**:387–411.

Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW, and Meier PJ (1994) Expression cloning of a rat liver Na^+-independent organic anion transporter. *Proc Natl Acad Sci USA* **91**:133–137.

Kanamitsu S, Ito K, and Sugiyama Y (2000) Quantitative prediction of *in vivo* drug-drug interactions from *in vitro* data based on physiological pharmacokinetics: use of maximum unbound concentration of inhibitor at the inlet to the liver. *Pharm Res (NY)* **17**:336–343.

Kouzuki H, Suzuki H, Ito K, Ohashi R, and Sugiyama Y (1999) Contribution of organic anion transporting polypeptide to uptake of its possible substrates into rat hepatocytes. *J Pharmacol Exp Ther* **288**:627–634.

Lemaire M and Tillement JP (1982) Role of lipoproteins and erythrocytes in the *in vitro* binding and distribution of cyclosporin A in the blood. *J Pharm Pharmacol* **34**:715–718.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275.

Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B, and Stieger B (1997) Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **26**:1667–1677.

Miyauchi S, Sawada Y, Iga T, Hanano M, and Sugiyama Y (1993) Comparison of the hepatic uptake clearances of fifteen drugs with a wide range of membrane permeabilities in isolated rat hepatocytes and perfused rat livers. *Pharm Res (NY)* **10**:434–440.

Moghadasian MH (1999) Clinical pharmacology of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Life Sci* **65**:1329–1337.

Mück W, Mai I, Fritsche L, Ochmann K, Rohde G, Unger S, Johne A, Bauer S, Budde K, Roots I, et al. (1999) Increase in cerivastatin systemic exposure after single and multiple dosing in cyclosporin-treated kidney transplant recipients. *Clin Pharmacol Ther* **65**:251–261.

Mück W (2000) Clinical pharmacokinetics of cerivastatin. *Clin Pharmacokinet* **39**:99–116.

Mueck W, Frey R, Boix O, and Voith B (2001) Gemfibrozil/cerivastatin interaction. *AAPS Pharm Sci* **3 (Suppl.)**:abstract No. 3566.

Noé B, Hagenbuch B, Stieger B, and Meier PJ (1997) Isolation of multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc Natl Acad Sci USA* **94**:10346–10350.

Partridge WM, Premachandra BN, and Fierer G (1985) Transport of thyroxine bound to human prealbumin into rat liver. *Am J Physiol* **248**:G545–G550.

Prueksaritanont T, Tang C, Qiu Y, Mu L, Subramanian R, and Lin JH (2002a) Effects of fibrates on metabolism of statins in human hepatocytes. *Drug Metab Dispos* **30**:1280–1287.

Prueksaritanont T, Zhao JJ, Ma B, Roadcap BA, Tang C, Qiu Y, Liu L, Lin JH, Pearson G, and Baillie TA (2002b) Mechanistic studies on metabolic interactions between gemfibrozil and statins. *J Pharmacol Exp Ther* **301**:1042–1051.

Schroeder A, Eckhardt U, Stieger B, Tynes R, Schteingart CD, Hofmann AF, Meier PJ, and Hagenbuch B (1998) Substrate specificity of the rat liver Na^+-bile salt cotransporter in *Xenopus laevis* oocytes and in CHO cells. *Am J Physiol* **274**:G370–G375.

Shitara Y, Hirano M, Sato H, and Sugiyama Y (2004) Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (OATP2/OATP1B1: *SLC21A6*)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin: analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. *J Pharmacol Exp Ther* **311**:228–236.

Shitara Y, Itoh T, Sato H, Li AP, and Sugiyama Y (2003) Inhibition of transporter-mediated hepatic uptake as a mechanism for drug-drug interaction between cerivastatin and cyclosporin A. *J Pharmacol Exp Ther* **304**:610–616.

Shitara Y, Sugiyama D, Kusuhara H, Kato Y, Abe T, Meier PJ, Itoh T, and Sugiyama Y (2002) Comparative inhibitory effects of different compounds on rat Oatp1 (*Slc21a1*)- and Oatp2 (*Slc21a5*)-mediated transport. *Pharm Res (NY)* **19**:159–163.

Yamazaki M, Suzuki H, Hanano M, Tokui T, Komai T, and Sugiyama Y (1993) Na^+-independent multispecific anion transporter mediates active transport of pravastatin into rat liver. *Am J Physiol* **264**:G36–G44.

Yamazaki M, Suzuki H, and Sugiyama Y (1996) Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. *Pharm Res (NY)* **13**:497–513.

Wang J-S, Neuvonen M, Wen X, Backman JT, and Neuvonen PJ (2002) Gemfibrozil inhibits CYP2C8-mediated cerivastatin metabolism in human liver microsomes. *Drug Metab Dispos* **30**:1352–1356.

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Transporter Database, TP-Search: A Web-Accessible Comprehensive Database for Research in Pharmacokinetics of Drugs

This Letter to the Editor informs the readers of TP-Search, a unique comprehensive database for membrane transporter proteins that we have constructed to facilitate the study of drug transporters on a broad scale in the world and to provide a research tool for optimization of pharmacokinetic properties in terms of transporters during the early stage of drug development in pharmaceutical companies.

During the past decade, there has been a significant increase in the molecular characterization of transporter proteins in animals and humans (1). With newer information on the genetic/genomic studies, this has led to a better understanding of the importance of such transporter proteins as one of the main determinant factors to play a key role in drug disposition; that is, absorption, distribution, and excretion (ADE) of drugs (2–4). Because the amount of available data is rapidly increasing, a need for a publicly accessible database with comprehensive information about all of the known membrane transporters becomes increasingly important.

We have constructed TP-Search, a Web-accessible relational database on ADE-associated transporter proteins (http://www.tp-search.jp/), enabling users to search dynamically transporter-related information by chemical structures/names of substrate/inhibitor/inducers, gene expression, functions, drug-drug interaction involving transporters, and so on.

The other databases on transporters, which are currently available, are http://nutrigen.4t.com/humanabc.htm (database on ABC transporters by M. Müller), http://www.med.rug.nl/mdl/ (database of University Hospital Groningen), http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html (HGNC gene family nomenclature ABC transporters), http://lab.digibench.net/transporter/ (human membrane transporter database), http://xin.cz3.nus.edu.sg/group/adment/adment.asp (ADME-associated proteins database), and http://www.mhc.com/PGP/index.html (P-glycoprotein interaction). These have appeared to provide only certain aspects of specific class or group of membrane transporter proteins, whereas TP-Search aims at providing a comprehensive database on drug-transporters. Among those databases, the human membrane transporter database has been intended to support pharmacogenomic studies and so provides much information on sequence variants, altered functions caused by polymorphisms/mutations, and (patho)physiological role and associated diseases (5). The ADME-associated protein database provides comprehensive information on ADME-associated proteins, which include not only membrane transporter proteins involved in drug disposition, but also other proteins, such as plasma proteins, intracellular binding proteins, and drug metabolizing enzymes (6). Other transporter databases listed above are mainly focused on the information for ABC-transporters.

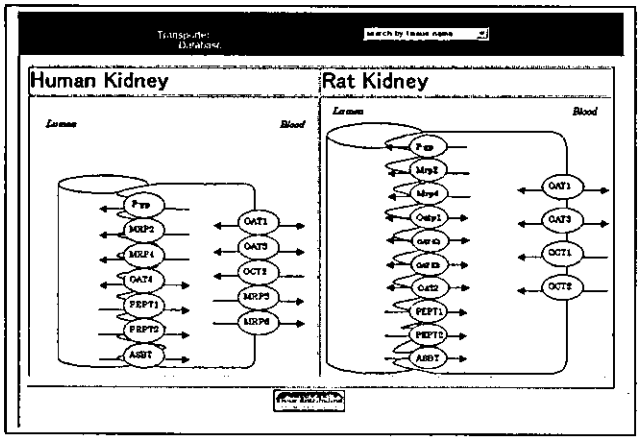

Fig. 1. Example of search results on transporters expressed in human and rat kidney.

Our methodology was as follows. Membrane transporter proteins were selected from a comprehensive search of available literature consisting of research papers, review articles, pharmacology textbooks, and other relevant publications (via PubMed; http://www.ncbi.nlm.gov/PubMed/), resulting in approximately 1,940 articles published from 1968 to 2002. The system is a typical Web application built on Application server, Web server, and Relational Database Management System (RDMS) to provide the services via the Internet. The user connects with the URL at http://www.tp-search.jp/ by a Web browser such as Netscape Navigator or Microsoft Internet Explorer.

The database, TP-Search, contains information on more than 75 membrane transporters (Table I), including cDNA and amino acid sequences, gene family, putative membrane topology, driving force, transport direction, substrate/inhibitor/inducer (chemical structures and kinetic data, i.e., Km/Ki), and tissue distribution in humans as well as in mice and rats, and drug-drug interactions involving transporters. All information available in this database is linked to the original references in PubMed, which ensures the users can confirm the validity of data and to obtain more detailed information available in the original references.

Terminology regarding genes often causes confusion. In our database, we use primarily the "Nomenclature of Mammalian Transporter Genes," (http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl/), such as the solute carrier superfamily (SLC) and ATP-binding cassette transporters (ABC). These standardized gene names, accompanied by conventional names, are both given in this database. The sequential information for transporters posted in the database was available through "Locus-Link" (http://www.ncbi.nlm.nih.gov/LocusLink/), and names and structures of the compounds, such as substrates/inhibitors/inducers, were searched through "Chem-Link" and "Japanese Accepted Names for Pharmaceuticals" (http://moldb.nihs.go.jp/jan/).

TP-Search is searchable by transporter name, tissue name (liver, kidney, intestine, brain, and expression in cell line; Fig. 1), substrate/inhibitor/inducer name, and drug-drug interaction. Implemented as a relational database, searches involving any combination of these options or selection field are also supported.

Because drug transporters have demonstrated a broad substrate specificity, drug-drug interaction involving these transporters is considered very likely. Approximately 1,200

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Table I. Membrane Transporters Archived in TP-Search

Transporters	Human	Mouse	Rat
P-glycoprotein (MDR)	MDR1	Mdr1a, Mdr1b	Mdr1a, Mdr1b
Multidrug resistance–associated protein	MRP1, MRP2, MRP3, MRP4, MRP5, MRP6	Mrp1, Mrp2, Mrp5	Mrp1, Mrp2, Mrp3, Mrp6
Breast cancer resistant protein	BCRP	Bcrp	Bcrp
Bile salt export pump	BSEP	Bsep	Bsep
Organic anion transporting polypeptide	OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-8, PGT	Oatp1, Oatp2, Oatp4, Oatp5, Pgt	Oatp1, Oatp2, Oatp3, Oatp4, OAT-K1, OAT-K2, Pgt
Bile acid transporter	NTCP, ASBT	Ntcp, Asbt	Ntcp, Asbt
Organic anion transporter	OAT1, OAT2, OAT3, OAT4	OAT1, OAT2, OAT3	OAT1, OAT2, OAT3
Organic cation transporter	OCT1, OCT2, OCT3	OCT1, OCT2, OCT3	OCT1, OCT2, OCT3
Organic cation/carnitine transporter	OCTN1, OCTN2	OCTN1, OCTN2, OCTN3	OCTN1, OCTN2
Peptide transporter	PEPT1, PEPT2	PEPT1, PEPT2	PEPT1, PEPT2

compounds known as substrates/inhibitors/inducers, including their names and structures, are currently archived in this database. With respect to the information currently available in this database, the exact role of transporter proteins in the observed *in vivo* drug-drug interaction has not always been clearly defined. It might be just extrapolated from *in vitro* information, such as substrate affinity, inhibitory effect, and so forth. In addition, because the substrate specificity of CYP3A and P-glycoprotein overlaps, many drugs may be a substrate of both (7,8). In such cases, it is difficult to distinguish the contribution to the increased oral bioavailability between CYP3A and P-glycoprotein. When the role of transporter proteins in the *in vivo* drug-drug interaction was suggested or indicated in peer-reviewed references, such information was incorporated into this database, even though there might be the limitation of scientific validity of such data.

This database will be updated periodically (bimonthly or quarterly), so that information regarding newly identified ADE-associated membrane transport proteins and additional knowledge about function or related proteins will be added. Toward that end, a data submission interface will be available in the database.

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REFERENCES

1. R. B. Kim. Transporters in drug disposition. *Curr. Opin. Drug Discov. Devel.* **3**:94–101 (2000).
2. V. H. Lee, J. L. Sporty, and T. E. Fandy. Pharmacogenomics of drug transporters: the next drug delivery challenge. *Adv. Drug Deliv. Rev.* **50**:S33–S40 (2001).
3. A. Ayrton and P. Morgan. Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica* **31**:469–497 (2001).
4. H. Kusuhara and Y. Sugiyama. Role of transporters in the tissue-selective distribution and elimination of drugs: transporters in the liver, small intestine, brain and kidney. *J. Controlled Release* **78**: 43–54 (2002).
5. Q. Yan and W. Sadée. Human membrane transporter database: A web-accessible relational database for drug transport studies and pharmacogenomics. *AAPS Pharmsci.* **2**: 1–7 (2000).
6. L. Z. Ji Sun, X. Chen, J. F. Wang, and Y. Z. Chen. ADME-AP: a database of ADME associated proteins. *Bioinformatics* **18**: 1699–1700 (2002).
7. H. Suzuki and Y. Sugiyama. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* **12**:3-12 (2000).
8. V. J. Wacher, L. Salphati, and L. Z. Benet. Active secretion and enterocytic drug metabolism barriers to drug absorption. *Adv. Drug Deliv. Rev.* **46**:89–102 (2001).

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Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the Hepatic Uptake of Pitavastatin in Humans

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ABSTRACT

Pitavastatin, a novel potent 3-hydroxymethylglutaryl-CoA reductase inhibitor, is selectively distributed to the liver in rats. However, the hepatic uptake mechanism of pitavastatin has not been clarified yet. In the present study, we investigated the contribution of organic anion transporting polypeptide 2 (OATP2/OATP1B1) and OATP8 (OATP1B3) to pitavastatin uptake using transporter-expressing HEK293 cells and human cryopreserved hepatocytes. Uptake studies using OATP2- and OATP8-expressing cells revealed a saturable and Na^+-independent uptake, with K_m values of 3.0 and 3.3 μM for OATP2 and OATP8, respectively. To determine which transporter is more important for its hepatic uptake, we proposed a methodology for estimating their quantitative contribution to the overall hepatic uptake by comparing the uptake clearance of pitavastatin with that of reference compounds (a selective substrate for OATP2 (estrone-3-sulfate) and OATP8 (cholecystokinin octapeptide) in expression systems and human hepatocytes. The concept of this method is similar to the so-called relative activity factor method often used in estimating the contribution of each cytochrome P450 isoform to the overall metabolism. Applying this method to pitavastatin, the observed uptake clearance in human hepatocytes could be almost completely accounted for by OATP2 and OATP8, and about 90% of the total hepatic clearance could be accounted for by OATP2. This result was also supported by estimating the relative expression level of each transporter in expression systems and hepatocytes by Western blot analysis. These results suggest that OATP2 is the most important transporter for the hepatic uptake of pitavastatin in humans.

Pitavastatin is a highly potent inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Aoki et al., 1997; Kajinami et al., 2003). Previously, Kimata et al. (1998) have demonstrated that [^{14}C]pitavastatin is selectively distributed to the liver in rats with the liver/plasma concentration ratio of more than 53. Shimada et al. (2003) have recently reported that the uptake of pitavastatin by rat hepatocytes is saturable and temperature dependent, suggesting that pitavastatin might be transported by carrier-mediated systems. Pitavastatin is scarcely metabolized in human liver microsomes (Fujino et al., 2003) and is excreted into the bile in unchanged form (Kojima et al., 2001). In humans, the fraction excreted in urine was less than 2% (Kajinami et al., 2003). Therefore, when considering the pharmacokinetics of pitavastatin, we should focus on the hepatic clearance of unchanged pitavastatin.

Statins reduce the plasma level of low-density lipoprotein cholesterol and triglycerides in a dose-dependent manner, whereas one of the severe adverse effects, rhabdomyolysis, also appears to be dose-dependent (Davidson et al., 1997). Since liver is a major clearance organ as well as a pharmacological target organ of pitavastatin, it is essential to clarify the uptake mechanism of pitavastatin by hepatocytes to predict the pharmacological and toxicological effects.

At the present time, several transporters are thought to be involved in the hepatic uptake of a variety of drugs in human liver. Na^+-taurocholate cotransporting polypeptide, organic anion transporting polypeptide (OATP) 2 (OATP1B1, OATP-C/LST-1), OATP8 (OATP1B3, LST-2), OATP-B (OATP2B1), organic anion transporter (OAT) 2, and organic cation transporter 1 are localized on the basolateral membrane of human hepatocytes (Hagenbuch and Meier, 1996, 2003; Muller and Jansen, 1997). In particular, OATP2 and OATP8 are selectively expressed in the human liver and exhibit broad sub-

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ABBREVIATIONS: HMG, 3-hydroxy-3-methylglutaryl; OATP, organic anion transporting polypeptide; $E_2 17\beta G$, estradiol 17β-D-glucuronide; E-sul, estrone-3-sulfate; CCK-8, cholecystokinin octapeptide; PCR, polymerase chain reaction; TBS-T, Tris-buffered saline containing 0.05% Tween 20; RAF, relative activity factor; CL, clearance.

strate specificities, which suggest that they play an important role in the hepatic uptake of several anionic endogenous compounds and drugs (Hagenbuch and Meier, 2003). OATP2 accepts statins including pravastatin, cerivastatin, and rosuvastatin as substrates (Hsiang et al., 1999; Brown et al., 2001; Nakai et al., 2001; Sasaki et al., 2002; Shitara et al., 2003a). The substrate specificity of OATP2 commonly overlaps that of OATP8, and several compounds can be bisubstrates of both OATP2 and OATP8. However, there are some differences as far as substrate recognition and transcriptional regulation are concerned (Hagenbuch and Meier, 2003; Kullak-Ublick et al., 2004). Therefore, it is essential to evaluate their quantitative contribution to the total hepatic uptake to estimate the overall hepatic clearance for individuals when there are changes in expression level or function caused by pathological conditions, single-nucleotide polymorphisms, and transporter-mediated drug-drug interactions. This kind of information will help us predict the distribution of pitavastatin in the target organ, as well as the plasma concentrations, subsequent pharmacological effects, and adverse events under these conditions. However, so far, no studies have been published describing how to estimate the contribution of each uptake transporter quantitatively in human liver.

In the present study, we analyzed the involvement of the transporters, OATP2 and OATP8, in the hepatic uptake of pitavastatin along with estradiol 17β-D-glucuronide ($E_2$17βG), a typical substrate of both OATP2 and OATP8 (Cui et al., 2001; Ismair et al., 2001). In addition, we developed a methodology for determining the contribution of each transporter to the hepatic uptake of test compounds by comparing their uptake clearance with that of reference compounds (a selective substrate for OATP2 (estrone-3-sulfate) and OATP8 (cholecystokinin octapeptide) in expression systems and human hepatocytes. We also used another approach by directly comparing expression levels of OATP2 and OATP8 in expression systems and hepatocytes using Western blot analysis and compared the results obtained by these two methods.

Materials and Methods

Materials. Pitavastatin (monocalcium bis[(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]3,5-dihydroxy-6-hepteonate]) was synthesized by Nissan Chemical Industries (Chiba, Japan). [Fluorobenzene-*U*-^{14}C]pitavastatin (11.7 mCi/mmol) was synthesized by Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). $E_2$17βG and estrone-3-sulfate (E-sul) (45 and 46 Ci/mmol, respectively) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), whereas cholecystokinin octapeptide (CCK-8) (77 Ci/mmol) was purchased from Amersham Biosciences UK, Ltd.. Unlabeled $E_2$17βG, E-sul, and CCK-8 were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and commercially available.

Construction of Stably Transfected HEK293 Cells Expressing Human OATP2 and OATP8. The human OATP2 gene was isolated by PCR using the human liver cDNA purchased from Takara Bio Inc. (Shiga, Japan). The first half (1–1125) of the gene was amplified using a forward primer containing a KpnI site (5′-GGGGTACCATGGACCAAAATCAACATTTGAAT-3′) and a reverse primer (5′-GTTAGCCTTAGATGAAGGCTGACC-3′). The second half (1041–2076) of the gene was amplified using a forward primer (5′-ACAAGTAAGCAGCTATATTGGTGC-3′) and a reverse primer containing a NotI site (5′-GGGCGGCCGCTTAACAATGTGTTTCACTATCTG-3′). Each PCR product was TA cloned into the pGEM vector (Promega, Madison, WI), respectively. Then, the pGEM vector containing the first half was digested with KpnI and Eco105 I, and the pGEM vector with the second half was digested with Eco105 I and NotI. Subsequently, the first and second halves of the OATP2 cDNA were ligated into KpnI and NotI sites of the pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) to obtain the full-length human OATP2 gene.

The human OATP8 gene was isolated by PCR using human liver cDNA purchased from BD Biosciences Clontech (Palo Alto, CA) as a template. The C-terminal fragment of the OATP8 gene was amplified using a forward primer (5′-AGAGTCAGCATCTTCAGAG-3′) and a reverse primer (5′-TTTTCCTTTTGCGGCCGCATTGTCAGTGAAAGACCAGG-3′) and TA cloned into pGEM vector. The N-terminal fragment was amplified using a forward primer containing a HindIII site and Kozak sequence just before the start codon (5′-CCCAAGCTTCCGCCATGGACCAACATCAAC-3′) and a reverse primer (5′-CTTCGGTCTGTGTAGTTTAG-3′). To subclone the full length of the OATP8 cDNA, fragment 1 was prepared by digesting the pGEM vector containing the C-terminal fragment with HindIII and NotI, and fragment 2 was created by digesting the pGEM vector containing the N-terminal fragment with HindIII. Then, fragments 1 and 2 were ligated into linearized pcDNA3.1 (+) digested with HindIII and NotI.

OATP2- and OATP8-expressing HEK293 cells and control cells were constructed by the transfection of expression vector and control pcDNA3.1 vector, respectively, into cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instruction and the selection by 800 μg/ml Antibiotic G418 sulfate (Promega, Madison, WI) for 3 weeks.

Cell Culture. Transporter-expressing or vector-transfected HEK293 cells were grown in Dulbecco's modified Eagle's medium low glucose (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% CO_2 and 95% humidity. Cells were then seeded in 12-well plates at a density of 1.5×10^5 cells/well. For the transport study, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium-butyrate for 24 h before transport assay to induce the expression of OATP2 and OATP8.

Transport Study Using Transporter Expression Systems. The transport study was carried out as described previously (Sugiyama et al., 2001). Uptake was initiated by adding Krebs-Henseleit buffer containing radiolabeled and unlabeled substrates after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer consisted of 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$ adjusted to pH 7.4. The uptake was terminated at a designated time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then, cells were washed twice with 1 ml of ice-cold Krebs-Henseleit buffer, solubilized in 500 μl of 0.2 N NaOH, and kept overnight at 4°C. Aliquots (500 μl) were transferred to scintillation vials after adding 250 μl of 0.4 N HCl. The radioactivity associated with the cells and incubation buffer was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter, Fullerton, CA) after adding 2 ml of scintillation fluid (Clear-sol I; Nacalai Tesque, Kyoto, Japan) to the scintillation vials. The remaining 50 μl of cell lysate was used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

Transport Study Using Human Cryopreserved Hepatocytes. This experiment was performed as described previously (Shitara et al., 2003a). Cryopreserved human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD). In this experiment, we selected three batches of human hepatocytes (Lot. OCF, 094, and ETR) that show relatively high uptake amount of $E_2$17βG and E-sul among eight independent batches of hepatocytes. Immediately before the study, the hepatocytes (1-ml suspension) were thawed at 37°C, then quickly suspended in 10 ml of ice-cold Krebs-Henseleit buffer and centrifuged (50*g*) for 2 min at 4°C, followed by

removal of the supernatant. This procedure was repeated once more to remove cryopreservation buffer, and then the cells were resuspended in the same buffer to give a cell density of 1.0×10^6 viable cells/ml for the uptake study. The number of viable cells was determined by trypan blue staining. To measure the uptake in the absence of Na^+, sodium chloride and sodium bicarbonate in Krebs-Henseleit buffer were replaced with choline chloride and choline bicarbonate. Prior to the uptake studies, the cell suspensions were prewarmed in an incubator at 37°C for 3 min. The uptake studies were initiated by adding an equal volume of buffer containing labeled and unlabeled substrates to the cell suspension. After incubation at 37°C for 0.5, 2, or 5 min, the reaction was terminated by separating the cells from the substrate solution. For this purpose, an aliquot of 80 μl of incubation mixture was collected and placed in a centrifuge tube (450 μl) containing 50 μl of 2 N NaOH under a layer of 100 μl of oil (density, 1.015, a mixture of silicone oil and mineral oil; Sigma-Aldrich), and subsequently the sample tube was centrifuged for 10 s using a tabletop centrifuge (10,000*g*, Beckman Microfuge E; Beckman Coulter). During this process, hepatocytes passed through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes, the centrifuge tube was cut and each compartment was transferred to a scintillation vial. The compartment containing the dissolved cells was neutralized with 50 μl of 2 N HCl, mixed with scintillation cocktail, and the radioactivity was measured in a liquid scintillation counter.

Antiserum and Western Blot Analysis. As shown in previous reports, anti-OATP2 and anti-OATP8 sera were raised in rabbits against a synthetic peptide consisting of the 21 and 15 carboxyl-terminal amino acids, respectively, of OATP2 and OATP8 coupled to keyhole limpet hemocyanine at its N terminus via an additional cysteine (Konig et al., 2000a,b). Crude membrane fractions were prepared from human hepatocytes and transporter-expressing HEK293 cells as described previously (Sasaki et al., 2002). The crude membrane fractions were diluted with 3× Red loading buffer (Bio-Labs, Hertfordshire, UK) and loaded onto a 7% SDS-polyacrylamide gel with a 4.4% stacking gel. Proteins were electroblotted onto a polyvinylidene diflouride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad, Hercules, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with anti-OATP2 serum (dilution 1:500) or anti-OATP8 serum (dilution 1:1000). The membrane was incubated with a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Biosciences UK, Ltd.) diluted 1:2000 in TBS-T for 1 h at room temperature followed by washing with TBS-T. The band was detected, and its intensity was quantified using an image analyzer (LAS-1000 plus; Fuji Film, Tokyo, Japan).

Kinetic Analyses. Ligand uptake was expressed as the uptake volume (microliters per milligram of protein), given as the amount of radioactivity associated with the cells (disintegrations per minute per milligram of protein) divided by its concentration in the incubation medium (disintegrations per minute per microliter). Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into cDNA-transfected cells. Kinetic parameters were obtained using the following equation:

$$v = \frac{V_{max} \times S}{K_m + S} + P_{dif} \times S \quad (1)$$

where v is the uptake velocity of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the 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_{dif} is the nonsaturable uptake clearance (microliters per minute per milligram of protein). Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981), and the Damping Gauss Newton Method algorithm was used for fitting. The input data were weighted as the reciprocal of the observed values.

To determine saturable hepatic uptake clearance in human hepatocytes, we first determined the hepatic uptake clearance [$CL_{(2min-0.5min)}$] (microliters per minute per 10^6 cells) by calculating the slope of the uptake volume (V_d) (microliters per 10^6 cells) between 0.5 and 2 min (eq. 2). The saturable component of the hepatic uptake clearance (CL_{hep}) was determined by subtracting $CL_{(2\ min-0.5\ min)}$ in the presence of 100 μM substrate (excess) from that in the presence of 1 μM substrate (tracer) (eq. 3).

$$CL_{(2\ min-0.5\ min)} = \frac{V_{d,2\ min} - V_{d,0.5\ min}}{2 - 0.5} \quad (2)$$

$$CL_{hep} = CL_{(2\ min-0.5\ min),tracer} - CL_{(2\ min-0.5\ min),excess} \quad (3)$$

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

Estimation of Uptake Clearance in Human Hepatocytes from cDNA Transfectants. The use of this method for estimating the contribution of each molecule to the overall reaction [relative activity factor (RAF) method] has been described for cytochrome P450 (Crespi and Penman, 1997), and it has also been applied to renal uptake transporters (Hasegawa et al., 2003). Based on this strategy, we estimated the contribution of OATP2 and OATP8 to the overall uptake by human hepatocytes. Because E-sul and CCK-8 could be selective substrates for OATP2 and OATP8, respectively, they were used as reference compounds for OATP2- and OATP8-mediated uptake (Cui et al., 2001; Ismair et al., 2001). The ratio of the uptake clearance of reference compounds in human hepatocytes to that in the expression system was calculated and defined as $R_{act,\ OATP2}$ and $R_{act,\ OATP8}$. The uptake clearance by OATP2 and OATP8 was separately calculated by multiplying the uptake clearance of the test compounds (pitavastatin and $E_2$17βG) in transporter-expressing cells ($CL_{OATP2,test}$ and $CL_{OATP8,test}$) by $R_{act,\ OATP2}$ and $R_{act,\ OATP8}$, respectively, as described in the following equations:

$$R_{act,OATP2} = \frac{CL_{Hep,E-sul}}{CL_{OATP2,E-sul}} \quad (4)$$

$$R_{act,OATP8} = \frac{CL_{Hep,CCK-8}}{CL_{OATP8,CCK-8}} \quad (5)$$

$$CL_{hep,test,OATP2} = CL_{OATP2,test} \cdot R_{act,OATP2} \quad (6)$$

$$CL_{hep,test,OATP8} = CL_{OATP8,test} \cdot R_{act,OATP8} \quad (7)$$

$$CL_{hep,test} = CL_{hep,test,OATP2} + CL_{hep,test,OATP8} \quad (8)$$

In addition, the ratio of the expression levels of OATP2 and OATP8 in human hepatocytes (per 10^6 cells) to transporter-expressing cells (per milligram of protein) ($R_{exp,\ OATP2}$ and $R_{exp,\ OATP8}$) could be calculated from the intensity of specific bands in Western blot analysis and the amount of crude membrane prepared from each cell type as follows. The relative expression level per 10^6 hepatocytes or milligram of protein in HEK293 whole cells was given by multiplying the band density per unit protein amount in crude membrane of each batch of hepatocytes or transporter-expressing HEK293 cells by protein amount in crude membrane obtained by 10^6 hepatocytes or 1 mg of whole-cell protein in HEK293 transfectants. The R_{exp} value was calculated as the relative expression level per 10^6 hepatocytes divided by that per milligram of protein in HEK293 cells. The OATP2- and OATP8-mediated hepatic uptake of test compounds (pitavastatin and $E_2$17βG) was calculated from the following equation:

$$CL_{hep,test} = CL_{OATP2,test} \cdot R_{exp,OATP2} + CL_{OATP8,test} \cdot R_{exp,OATP8} \quad (9)$$

Results

Uptake of $E_2$17βG, E-sul, CCK-8, and Pitavastatin by Transporter-Expressing Cells. The saturation kinetics of $E_2$17βG, E-sul, CCK-8, and pitavastatin by OATP2- and OATP8-expressing cells and vector-transfected HEK293 are shown as Eadie-Hofstee plots in Fig. 1. Pitavastatin as well as $E_2$17βG were significantly taken up into both OATP2- and OATP8-expressing HEK293 cells compared with vector-transfected cells (Fig. 1, A and D). The transfection of OATP2 resulted in an increase in the uptake of E-sul but did not affect the uptake of CCK-8, whereas transfection of OATP8 resulted in an increase in the uptake of CCK-8 but not E-sul (Fig. 1, B and C). The concentration dependence of the uptake of pitavastatin, $E_2$17βG, and E-sul could be explained by a one-saturable component (Fig. 1, A, B, and D). However, a saturable and a nonsaturable component could be accounted for even in the specific uptake of CCK-8 determined by subtraction of the uptake by vector-transfected cells from that by OATP8-expressing cells (Fig. 1C). Their kinetic parameters are summarized in Table 1. The rank order in the uptake clearance by OATP2 was E-sul > pitavastatin > $E_2$17βG, whereas that by OATP8 was pitavastatin > CCK-8 > $E_2$17βG.

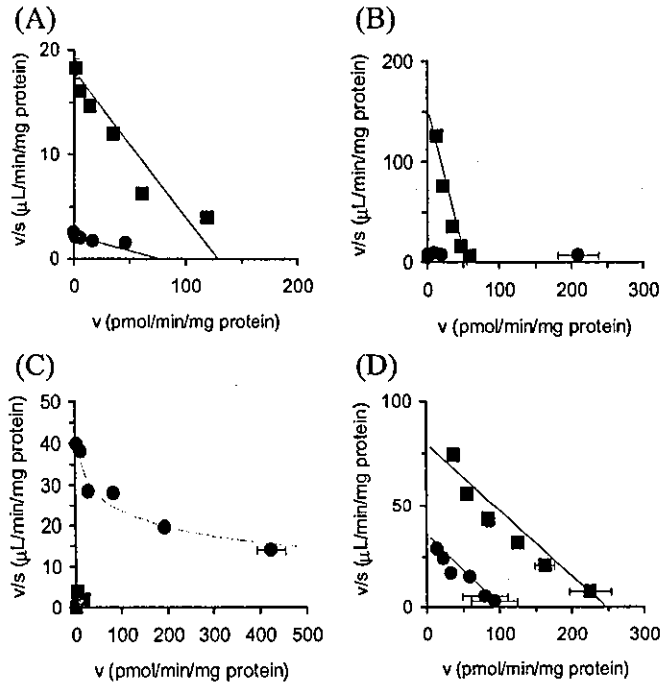

Fig. 1. Eadie-Hofstee plots of the uptake of [^{3}H]$E_2$17βG, [^{3}H]E-sul, [^{3}H]CCK-8, and [^{14}C]pitavastatin by OATP2- and OATP8-expressed HEK293 cells. Squares and circles represent the uptake in OATP2- and OATP8-expressed cells, respectively. The concentration dependence of OATP2- and OATP8-mediated uptake of [^{3}H]$E_2$17βG (A), [^{3}H]E-sul (B), [^{3}H]CCK-8 (C), and [^{14}C]pitavastatin (D) is shown as Eadie-Hofstee plots. The uptake of [^{3}H]$E_2$17βG, [^{3}H]E-sul, [^{3}H]CCK-8, and [^{14}C]pitavastatin for 5, 0.5, 2, and 2 min, respectively, was determined at various concentrations (0.1–30 μM). The OATP2- and OATP8-mediated transport was obtained by subtracting the uptake in vector-transfected cells from that in OATP2- or OATP8-expressing cells. Lines represent the fitted curves obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

Uptake of $E_2$17βG, E-sul, CCK-8, and Pitavastatin by Human Cryopreserved Hepatocytes. Typical time profiles of the uptake of $E_2$17βG, E-sul, CCK-8, and pitavastatin in one batch of human hepatocytes (Lot. OCF) are shown in Fig. 2. Time-dependent uptake of all ligands was observed at 1 μM, and it was decreased in the presence of 100 μM unlabeled ligands in all batches of hepatocytes examined in the present study (data not shown). The uptake clearance of these substrates in each donor is listed in Table 2. The uptake clearance by human hepatocytes was in the order E-sul > pitavastatin > $E_2$17βG > CCK-8.

The Effect of Sodium Ion on the Uptake of Pitavastatin by Human Cryopreserved Hepatocytes. To determine whether the uptake of pitavastatin into hepatocytes is Na^+ dependent or not, we investigated the pitavastatin uptake in the presence and absence of Na^+ in human hepatocytes. As shown in Fig. 3, replacement of Na^+ with choline in the transport buffer had no effect on the uptake of pitavastatin in human hepatocytes.

Western Blot Analysis. The relative expression levels of OATP2 and OATP8 in crude membrane from transfected cells and hepatocytes were investigated by Western blot analyses. An antiserum against OATP2 recognized approximately 80-kilodalton proteins in the crude membrane fractions prepared from human hepatocytes and OATP2-expressing cells, respectively (Fig. 4A). Anti-OATP8 antiserum could detect the specific band of approximately 120-kilodalton proteins in the crude membrane fractions prepared from human hepatocytes and OATP8-expressing cells, respectively (Fig. 4B). The molecular weights of OATP2 and OATP8 in the human hepatocytes were almost the same as those in OATP2- and OATP8-expressing cells, respectively. No expression of OATP2 or OATP8 was observed in vector-transfected HEK293 cells (Fig. 4, A and B). Figure 4, C and D showed the linear relationship between the applied amount of crude membrane obtained from transporter-expressing cells and human hepatocytes and the intensity of the specific band measured by digital densitometer. The slope of the

TABLE 1

Kinetic parameters of the uptake of compounds by transporter-expressing HEK293 cells

Data shown in Fig. 1 were used to determine these parameters calculated by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

Transporter	Substrate	K_m	V_{max}	V_{max}/K_m	P_{dif}
		μM	*pmol/min/mg*	*μl/min/mg*	*μl/min/mg*
OATP2	E-sul	0.458 ± 0.154	60.3 ± 8.8	132 ± 19	
	CCK-8	Not significantly transported			
	Pitavastatin	3.00 ± 0.39	230 ± 17	76.6 ± 5.6	
	$E_2$17βG	8.29 ± 0.42	131 ± 5	15.8 ± 0.6	
OATP8	E-sul	Not significantly transported			
	CCK-8	3.82 ± 2.20	102 ± 60	26.7 ± 15.7	11.4 ± 2.6
	Pitavastatin	3.25 ± 0.44	100 ± 8	30.6 ± 2.3	
	$E_2$17βG	24.6 ± 7.6	56.8 ± 16.1	2.31 ± 0.65	

P_{dif}, nonsaturable uptake clearance (microliters per minute per milligram of protein).

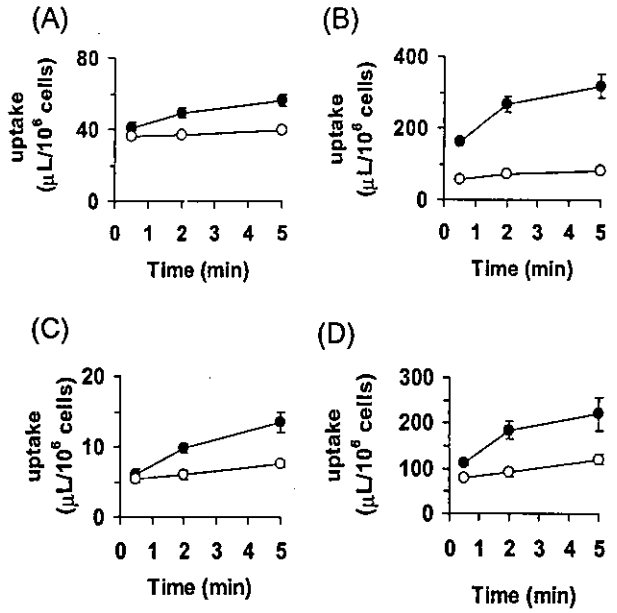

Fig. 2. Time profiles of the uptake of [^{3}H]E_2 17βG, [^{3}H]E-sul, [^{3}H]CCK-8, and [^{14}C]pitavastatin by human hepatocytes (Lot. ETR). The uptake of these substrates for 0.5, 2, and 5 min was determined at two concentrations (closed circle, 1 μM; open circle, 100 μM) at 37°C. Each point represents the mean ± S.E. (n = 3).

TABLE 2

Uptake clearance of reference compounds (E-sul and CCK-8) and test compounds (pitavastatin and $E_2$17βG) in human hepatocytes

Substrate	Uptake Clearance (CL_{hep})[a]		
	Lot. OCF	Lot. 094	Lot. ETR
	μl/min/10^6 cells		
E-sul	110	134	57.7
CCK-8	7.89	3.50	2.02
pitavastatin	61.3	113	39.2
$E_2$17βG	13.5	17.0	5.5

[a] Uptake clearance was determined using three independent batches of human cryopreserved hepatocytes.

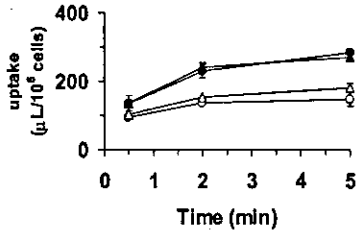

Fig. 3. Time profiles of the uptake of [^{14}C]pitavastatin by human hepatocytes (Lot. OCF) in the presence and absence of Na^+. The uptake of pitavastatin for 0.5, 2, and 5 min was determined at two concentrations (closed symbol, 1 μM; open symbol, 100 μM) at 37°C. Circles and triangles indicate the pitavastatin uptake in the presence and absence of Na^+, respectively. Each point represents the mean ± S.E. (n = 3).

regression line in Fig. 4, C and D reflected the relative expression level of each transporter in the transfectants and hepatocytes.

Estimation of Uptake Clearance and Contribution of OATP2 and OATP8 in Human Hepatocytes. We calculated the estimated uptake clearance of OATP2 and OATP8 in human hepatocytes by two approaches (Tables 3 and 4). In the first approach, by comparing the uptake clearance of reference compounds (E-sul for OATP2 and CCK-8 for OATP8) in transfectants and hepatocytes, we were able to

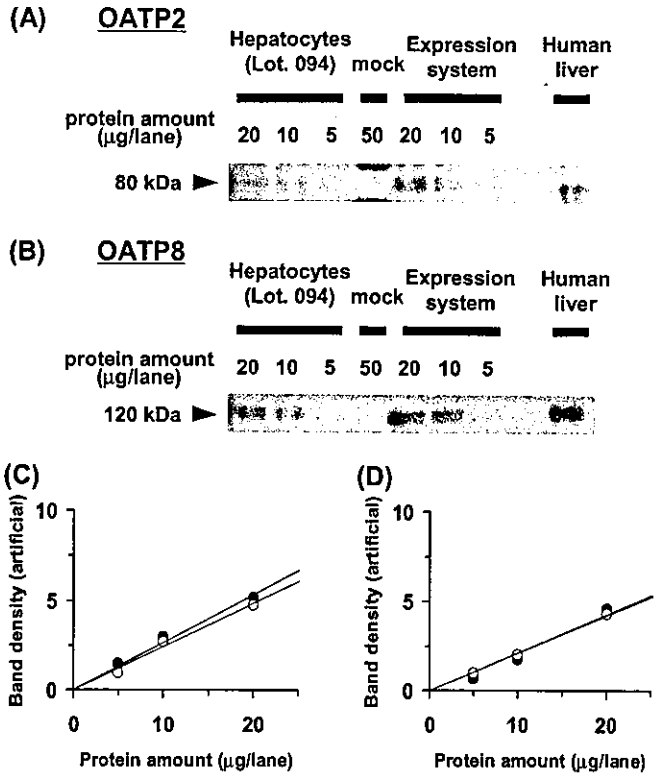

Fig. 4. Western blot analysis of OATP2 and OATP8. Crude membrane fractions (5, 10, and 20 μg) prepared from OATP2- and OATP8-expressed HEK293 cells and human hepatocytes (Lot. 094) were loaded and separated by SDS-PAGE (7% separating gel). The sample indicated "Human liver" means the crude membrane vesicles prepared from a human frozen liver block as a positive control. OATP2 and OATP8 were detected by preimmune antisera raised against the carboxyl terminus of human OATP2 (A) and OATP8 (B), respectively. Comparison of the relative expression levels of OATP2 (C) and OATP8 (D) between transfectants and hepatocytes is shown. The x-axis and y-axis represent the amount of crude membrane obtained from transfectants and human hepatocytes and the intensity of the specific band in Western blot analysis, respectively. Closed circles and open circles indicate the band density of human hepatocytes (Lot. 094) and OATP2- (C) or OATP8- (D) expressing HEK293 cells, respectively.

calculate the ratio of the clearance (R_{act}) and estimated clearance of pitavastatin and $E_2$17βG mediated by OATP2 and OATP8 based on the RAF method (Table 3). In the second approach, we compared the intensity of specific bands of transfectants and hepatocytes in Western blot analysis. We could obtain 62.1 and 96.7 μg of protein in crude membrane from 1 mg of whole cell protein in OATP2- and OATP8-expressing HEK293 cells, respectively, whereas 178, 89, and 82 μg of protein in crude membrane were obtained from 10^6 hepatocytes of Lot. OCF, 094, and ETR, respectively. When the band density per unit protein amount in crude membrane of OATP2- or OATP8-expressing HEK293 cells was defined as 1, the relative expression level of OATP2 or OATP8 per unit protein amount in crude membrane of hepatocytes of Lot. OCF, 094, and ETR was 1.01, 1.10, and 0.673 (per microgram) for OATP2 and 0.659, 1.01, and 0.872 (per microgram) for OATP8, respectively. Using these values, the relative ratio of the expression level (R_{exp}) and estimated clearance could be calculated (Table 4). The estimation by the two approaches suggested that both pitavastatin and $E_2$17βG are taken up into human hepatocytes predominantly by OATP2 (Tables 3 and 4).

TABLE 3
Contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin and $E_2$17βG determined using transporter-selective substrates

Hepatocyte Lot	Ratio of Uptake Clearance $CL_{hep}/CL_{transporter}$		Estimated Clearance[a]			
			Pitavastatin		$E_2$17βG	
	$R_{act,OATP2}$	$R_{act,OATP8}$	OATP2	OATP8	OATP2	OATP8
			μl/min/10⁶ cells			
OCF	0.833	0.291	63.8	8.92	13.2	0.218
			87.7%	12.3%	98.4%	1.63%
094	1.02	0.131	77.8	4.01	16.0	0.0979
			95.1%	4.91%	99.4%	0.607%
ETR	0.437	0.0757	33.5	2.32	6.91	0.0565
			93.5%	6.47%	99.2%	0.812%

[a] The second line entry under "Estimated Clearance" shows the percentage of OATP2- or OATP8-mediated uptake clearance relative to the sum of the estimated clearance mediated by OATP2 and OATP8. The details of this estimation are described under *Materials and Methods*.

TABLE 4
Contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin and $E_2$17βG determined by the relative expression level

Hepatocyte Lot	Ratio of Expression Level[a] Hepatocyte Transporter		Estimated Clearance[b] E-sul	CCK-8	Pitavastatin		$E_2$17βG	
	$R_{exp,OATP2}$	$R_{exp,OATP8}$	OATP2	OATP8	OATP2	OATP8	OATP2	OATP8
					μl/min/10⁶ cells			
OCF	2.90	1.21	383	32.3	222	37.0	45.8	2.80
					85.7%	14.3%	94.2%	5.76%
094	1.58	0.930	208	24.8	121	28.4	24.9	2.15
					80.9%	19.1%	92.1%	7.95%
ETR	0.89	0.737	117	19.7	68.1	22.6	14.0	1.71
					75.1%	24.9%	89.2%	10.8%

[a] Ratio of the expression level was determined by the intensity of the specific band in the crude membrane prepared from human hepatocytes (per 10^6 cells) divided by that in the crude membrane from transporter-expressing cells (per milligram) in Western blot analysis.
[b] The second line entry under "Estimated Clearance" shows the percentage of the OATP2- or OATP8-mediated uptake clearance relative to the sum of the estimated clearance mediated by OATP2 and OATP8. The details of this estimation are described under *Materials and Methods*.

Discussion

In the present study, we have clarified that pitavastatin is taken up into human hepatocytes via OATP2 and OATP8, and we also investigated the transport properties of pitavastatin using transporter-expressing HEK293 cells and human cryopreserved hepatocytes to estimate the contribution of these transporters to the total uptake in human hepatocytes.

Significant uptake of pitavastatin via OATP2 and OATP8 was observed compared with control cells, and this became saturated on increasing its concentration in the medium (Fig. 1). It has been shown that, in a series of statins, pravastatin, cerivastatin, and rosuvastatin are substrates of OATP2 (Brown et al., 2001; Nakai et al., 2001; Sasaki et al., 2002; Shitara et al., 2003a). The K_m value of pitavastatin for OATP2 is comparable with that of cerivastatin (K_m = 4.3 μM) (in-house data) and rosuvastatin (K_m = 7.3 μM) (Brown et al., 2001) and smaller than that of pravastatin (K_m = 24.3 μM) (Sasaki et al., 2002).

We demonstrated here for the first time that, as far as statins are concerned, pitavastatin is a substrate for OATP8 as well as OATP2 (Fig. 1D). K_m values of pitavastatin for OATP2 and 8 were comparable (Fig. 1; Table 1); therefore, we were not able to determine which transporter is mainly responsible for its uptake in human hepatocytes only by saturation kinetics in human hepatocytes.

It has been reported that E-sul and CCK-8 are selective substrates of OATP2 and OATP8, respectively (Cui et al., 2001; Ismair et al., 2001). We confirmed these findings in our experimental systems (Fig. 1). We also confirmed that $E_2$17βG was a good substrate for both OATP2 and OATP8 by using transporter-expressing cells (Fig. 1) (König et al., 2000a, b). As shown in Table 1, the K_m values of $E_2$17βG, E-sul, and CCK-8 were comparable with the reported values (Ismair et al., 2001; Tamai et al., 2001).

Next, we performed the uptake study in human cryopreserved hepatocytes. Shitara et al. (2003b) reported that large interbatch differences in uptake activity were observed in human cryopreserved hepatocytes probably due to differences in the conditions of isolation of the hepatocytes and cryopreservation as well as the interindividual variability in the expression and function of transporters. So, we carried out a study using three batches of hepatocytes prepared from three independent donors. In all three batches examined in the present study, pitavastatin, $E_2$17βG, E-sul, and CCK-8 were taken up in a time-dependent and saturable manner (Fig. 2).

We examined the uptake of pitavastatin in the presence and absence of Na^+ to determine whether Na^+-dependent uptake of pitavastatin was observed in human hepatocytes. In this experiment, we confirmed that the batch (Lot. OCF) used in the present study exhibited Na^+-dependent uptake of taurocholate (data not shown) to the same extent as shown in the previous report (Shitara et al., 2003b). However, the uptake of pitavastatin by hepatocytes did not change regardless of the presence of Na^+, suggesting that pitavastatin is mainly taken up in a Na^+-independent way, suggesting that Na^+-taurocholate-cotransporting polypeptide is not mainly involved in hepatic uptake of pitavastatin. Previous reports demonstrated that both OATP2 and OATP8 can transport substrates in an Na^+-independent manner (König et al.,

2000a,b), and these Na^+-independent transporters are involved in hepatic uptake of pitavastatin.

To estimate the contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin, we developed a method using reference compounds that were selective substrates of OATP2 or OATP8. Crespi and Penman (1997) proposed the RAF method using the ratio of the metabolic activity in human liver microsomes divided by the activity in each isoform-specific cytochrome P450 reaction. Applying the RAF concept to transporter research, Hasegawa et al. (2003) calculated the contribution of Oat1 and Oat3 to the renal uptake in rats by comparing the uptake clearance of reference compounds (*p*-aminohippurate for Oat1 and benzylpenicillin for Oat3) in kidney slices and Oat1- and Oat3-transfected cells. According to this method, assuming that both for OATP2 and OATP8, the intrinsic clearance per unit molecule in human hepatocytes is the same as that in the expression system, the ratio of the transport activity of reference compounds in human hepatocytes to that in transporter-expressing cells (R_{act}) should correspond to the relative expression level of each transporter. Multiplying R_{act} by the uptake clearance of pitavastatin and $E_2 17\beta G$ in each transfectant, we can estimate the OATP2- and OATP8-mediated portion of the uptake of pitavastatin and $E_2 17\beta G$ in hepatocytes. From our estimation described above, the sum of the calculated OATP2- and OATP8-mediated uptake was almost identical to the experimentally observed uptake clearances in the cases of both pitavastatin and $E_2 17\beta G$ (Tables 2 and 3). Therefore, the uptake of pitavastatin and $E_2 17\beta G$ by human hepatocytes could be fully explained by OATP2 and OATP8, and more than 87% of their total hepatic uptake could be accounted for by OATP2 in all three independent batches of human cryopreserved hepatocytes (Tables 2 and 3).

To validate our estimation, we directly compared the expression level of OATP2 and OATP8 between transfectants and hepatocytes by Western blot analysis and calculated the ratio of the expression level in these cells (R_{exp}) as described above. As a result of our estimation using R_{exp} values, the sum of the predicted OATP2- and OATP8-mediated uptake of pitavastatin and $E_2 17\beta G$ was 5 to 10 times higher than their observed clearance in hepatocytes (Tables 2 and 4), a finding that is not consistent with our earlier estimation based on the relative uptake clearance of the reference compounds. There are two possibilities to account for this discrepancy. Firstly, the recovery of each transporter protein in samples for Western blot analysis in transfectants and hepatocytes may be different. Secondly, the total amount of protein in the whole-cell crude membrane, which we measured in the present study, may not indicate the expression level of a functional transporter on the cell surface. On the other hand, our results indicated that the ratio of the R_{exp} values for OATP2 and OATP8 was comparable with that of the R_{act} values and therefore, the estimated contribution of each transporter was almost the same when using the two calculation approaches. The contribution of OATP2 to the hepatic uptake of pitavastatin and $E_2 17\beta G$ estimated from the R_{exp} value also indicated more than 75 and 85% of the overall uptake in all three batches of hepatocytes, respectively, suggesting that the results obtained by the two approaches are consistent, and OATP2 appears to be mainly responsible for the hepatic uptake of pitavastatin and $E_2 17\beta G$ although both compounds are partly taken up by OATP8.

Alcorn et al. (2002) have reported that the mRNA level of OATP-B is almost the same as that of OATP2 in human liver. Strictly speaking, E-sul is not a selective substrate of OATP2 because some reports have indicated that it can be taken up by OATP-B and OATP8 (Kullak-Ublick et al., 2001; Kobayashi et al., 2003; Ismair et al., 2003), although we could not observe significant uptake of E-sul via OATP8 (Fig. 1), and Cui et al. (2001) reported that OATP8-expressing HEK293 cells did not significantly take up E-sul. However, we believe that E-sul can be used as a specific substrate of OATP2 in hepatocytes for the following reason. Previous reports have demonstrated that CCK-8 can be transported by OATP8, but not OATP-B and OATP2 (Ismair et al., 2001), and that $E_2 17\beta G$ can be a substrate of OATP2 and OATP8, but not OATP-B (Kullak-Ublick et al., 2001; Tamai et al., 2001). Taking these facts into consideration, we believe that the hepatic uptake of $E_2 17\beta G$ can be almost completely accounted for by OATP2 because the contribution of OATP8 to the overall hepatic uptake of $E_2 17\beta G$ was negligible in our estimation based on the uptake clearance of CCK-8 (Table 3). On the other hand, the uptake clearance of $E_2 17\beta G$ in human hepatocytes was almost comparable with the OATP2-mediated uptake clearance of $E_2 17\beta G$ estimated from the uptake clearance of E-sul in human hepatocytes and the OATP2 expression system. Therefore, we believe that E-sul is taken up into human hepatocytes mainly by OATP2.

In clinical situations, Shitara et al. (2003a) suggested that the drug-drug interaction between cerivastatin and cyclosporin A is mediated by OATP2. Nishizato et al. (2003) reported that OATP-C*15 polymorphism affects plasma concentration of pravastatin. Therefore, OATP2 may play a major role in the hepatic uptake of statins, and we should pay attention to functional change of OATP2 in pharmacokinetics of statins.

In conclusion, we have evaluated the contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin and $E_2 17\beta G$ by transporter-expressing HEK293 cells and human hepatocytes. When we estimated their contribution by two approaches using the uptake of transporter-selective substrates and the relative expression level measured by Western blot analysis, most of the total hepatic clearance of pitavastatin and $E_2 17\beta G$ could be accounted for by OATP2. These methods will be useful for rapidly identifying the separate contributions of OATP2 and OATP8 to the hepatic uptake.

References

Alcorn J, Lu X, Moscow JA, and McNamara PJ (2002) Transporter gene expression in lactating and nonlactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. *J Pharmacol Exp Ther* **303**:487–496.

Aoki T, Nishimura H, Nakagawa S, Kojima J, Suzuki H, Tamaki T, Wada Y, Yokoo N, Sato F, Kimata H, et al. (1997) Pharmacological profile of a novel synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Arzneimittelforschung* **47**:904–909.

Brown CDA, Windass A, Bleasby K, and Lauffart B (2001) Rosuvastatin is a high affinity substrate of hepatic organic anion transporter OATP-C. *Atheroscler Suppl* **2**:90.

Crespi CL and Penman BW (1997) Use of cDNA-expressed human cytochrome P450 enzymes to study potential drug-drug interactions. *Adv Pharmacol* **43**:171–188.

Cui Y, Konig J, Leier I, Buchholz U, and Keppler D (2001) Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. *J Biol Chem* **276**:9626–9630.

Davidson MH, Stein EA, Dujovne CA, Hunninghake DB, Weiss SR, Knopp RH, Illingworth DR, Mitchel YB, Melino MR, Zupkis RV, et al. (1997) The efficacy and six-week tolerability of simvastatin 80 and 160 mg/day. *Am J Cardiol* **79**:38–42.

Fujino H, Saito T, Tsunenari Y, and Kojima J (2003) Interaction between several medicines and statins. *Arzneimittelforschung* **53**:145–153.

Hagenbuch B and Meier PJ (1996) Sinusoidal (basolateral) bile salt uptake systems of hepatocytes. *Semin Liver Dis* **16**:129–136.
Hagenbuch B and Meier PJ (2003) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609**:1–18.
Hasegawa M, Kusuhara H, Endou H, and Sugiyama Y (2003) Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat. *J Pharmacol Exp Ther* **305**:1087–1097.
Hsiang B, Zhu Y, Wang Z, Wu Y, Sasseville V, Yang WP, and Kirchgessner TG (1999) A novel human hepatic organic anion transporting polypeptide (OATP2): identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *J Biol Chem* **274**:37161–37168.
Ismair MG, Stanca C, Ha HR, Renner EL, Meier PJ, and Kullak-Ublick GA (2003) Interactions of glycyrrhizin with organic anion transporting polypeptides of rat and human liver. *Hepatol Res* **26**:343–347.
Ismair MG, Stieger B, Cattori V, Hagenbuch B, Fried M, Meier PJ, and Kullak-Ublick GA (2001) Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver. *Gastroenterology* **121**:1185–1190.
Kajinami K, Takekoshi N, and Saito Y (2003) Pitavastatin: efficacy and safety profiles of a novel synthetic HMG-CoA reductase inhibitor. *Cardiovasc Drug Rev* **21**:199–215.
Kimata H, Fujino H, Koide T, Yamada Y, Tsunenari Y, Yonemitsu M, and Yanagawa Y (1998) Studies on the metabolic fate of NK-104, a new inhibitor of HMG-CoA reductase: I. Absorption, distribution, metabolism and excretion in rats. *Xenobio Metabol Dispos* **13**:484–498.
Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* **306**:703–708.
Kojima J, Ohshima T, Yoneda M, and Sawada H (2001) Effect of biliary excretion on the pharmacokinetics of pitavastatin (NK-104) in dogs. *Xenobio Metab Disp* **16**: 497–502.
König J, Cui Y, Nies AT, and Keppler D (2000a) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem* **275**:23161–23168.
König J, Cui Y, Nies AT, and Keppler D (2000b) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol Gastrointest Liver Physiol* **278**:G156–164.
Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**:525–533.
Kullak-Ublick GA, Stieger B, and Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322–342.
Muller M and Jansen PL (1997) Molecular aspects of hepatobiliary transport. *Am J Physiol* **272**:G1285–1303.
Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, and Nishimura K (2001) Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. *J Pharmacol Exp Ther* **297**:861–867.
Nishizato Y, Ieiri I, Suzuki H, Kimura M, Kawabata K, Hirota T, Takane H, Irie S, Kusuhara H, Urasaki Y, et al. (2003) Polymorphisms of OATP-C (SLC21A6) and OAT3 (SLC22A8) genes: consequences for pravastatin pharmacokinetics. *Clin Pharmacol Ther* **73**:554–565.
Sasaki M, Suzuki H, Ito K, Abe T, and Sugiyama Y (2002) Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and multidrug resistance-associated protein 2 (MRP2/ABCC2). *J Biol Chem* **277**:6497–6503.
Shimada S, Fujino H, Morikawa T, Moriyasu M, and Kojima J (2003) Uptake mechanism of pitavastatin, a new inhibitor of HMG-CoA reductase, in rat hepatocytes. *Drug Metab Pharmakokinet* **18**:245–251.
Shitara Y, Itoh T, Sato H, Li AP, and Sugiyama Y (2003a) Inhibition of transporter-mediated hepatic uptake as a mechanism for drug-drug interaction between cerivastatin and cyclosporin A. *J Pharmacol Exp Ther* **304**:610–616.
Shitara Y, Li AP, Kato Y, Chuang LU, Ito K, Itoh T, and Sugiyama Y (2003b) Function of uptake transporters for taurocholate and estradiol 17β-D-glucuronide in cryopreserved human hepatocytes. *Drug Metab Pharmakokinet* **18**:33–41.
Sugiyama D, Kusuhara H, Shitara Y, Abe T, Meier PJ, Sekine T, Endou H, Suzuki H, and Sugiyama Y (2001) Characterization of the efflux transport of 17beta-estradiol-D-glucuronide from the brain across the blood-brain barrier. *J Pharmacol Exp Ther* **298**:316–322.
Tamai I, Nozawa T, Koshida M, Nezu J, Sai Y, and Tsuji A (2001) Functional characterization of human organic anion transporting polypeptide B (OATP-B) in comparison with liver-specific OATP-C. *Pharm Res (NY)* **18**:1262–1269.
Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**:879–885.

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Involvement of Multiple Transporters in the Efflux of 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitors across the Blood-Brain Barrier

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ABSTRACT

Statins, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, are frequently used for the treatment of hypercholesterolemia. The present study aimed to examine the involvement of organic anion transporters in the efflux transport of pravastatin and pitavastatin across the blood-brain barrier (BBB). Transport studies using cDNA-transfected cells revealed that these statins are substrates of multispecific organic anion transporters expressed at the BBB (rOat3:*Slc22a8* and rOatp2:*Slco1a4*). The efflux of these statins across the BBB was characterized using the brain efflux index method. The efflux clearance of pitavastatin across the BBB, obtained from the elimination rate constant and the distribution volume in the brain, was greater than that of pravastatin (364 versus 59 μl/min/g brain). The efflux of pravastatin and pitavastatin was saturable (apparent K_m values: 18 and 5 μM, respectively) and inhibited by probenecid but unaffected by tetraethylammonium. Furthermore, an inhibitor of the efflux pathway for hydrophilic organic anions across the BBB (*p*-aminohippurate), and inhibitors of the efflux pathway for amphipathic organic anions (taurocholate and digoxin) inhibited the efflux of both statins. The degree of inhibition by *p*-aminohippurate was similar and partial for the efflux of pravastatin and pitavastatin. Taurocholate and digoxin completely inhibited the efflux of pitavastatin, whereas their effect was partial for the efflux of pravastatin. The results of the present study suggest the involvement of multiple transporters, including rOat3 and rOatp2, in the efflux transport of pravastatin and pitavastatin across the BBB, each making a different contribution.

The brain capillary endothelial cells are characterized by highly developed tight junctions and the expression of xenobiotic transporters (Kusuhara and Sugiyama, 2001a,b; Lee et al., 2001; Golden and Pollack, 2003; Sun et al., 2003). These transporters include the member(s) of the Oat family (Oat3) (Ohtsuki et al., 2002; Kikuchi et al., 2003), the Oatp family (Oatp2) (Asaba et al., 2000; Sugiyama et al., 2001), and the ATP-binding cassette transporters (Mdr1 and Mrp1) (Kusuhara et al., 1997; Sugiyama et al., 2003). Cumulative evidence suggests that these transporters facilitate the elimination of xenobiotics and endogenous compounds from the central nervous system (CNS) across the BBB, providing the barrier function between the blood and the brain.

Statins, HMG-CoA reductase inhibitors, have been used for the treatment of hypercholesterolemia. HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, is present in the liver and nonhepatic tissues, catalyzing the early conversion of HMG-CoA to mevalonic acid, and the enzyme inhibition in the liver by statins results in the lower serum level of total cholesterol (Reinoso et al., 2002). The adverse effects of statins include CNS side effects, such as sleep disturbance, as well as myopathy (Schaefer, 1988; Barth et al., 1990). Therefore, the liver selectivity of statins must be given priority in clinical situations to reduce the undesired toxicological effects on the body. On the other hand, several reports suggest that statins have a potentially neuroprotective effect (Cucchiara and Kasner, 2001). Thus, it is possible that statins could be used for the treatment of CNS diseases. Previously, Saheki et al. (1994) showed that the brain uptake clearance of pravastatin was quite low, almost comparable with that of sucrose using the in situ brain perfusion technique. However, taking into consideration the chronic administration of statins, it is necessary to

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ABBREVIATIONS: Oat, organic anion transporter; Oatp, organic anion-transporting polypeptide; CNS, central nervous system; BBB, blood-brain barrier; HMG, 3-hydroxy-3-methylglutaryl; BEI, brain efflux index; PAH, *p*-aminohippurate; TCA, taurocholate.