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

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Abbreviations:

HEK, human embryonic kidney

OATP, organic anion transporting polypeptide

NTCP, Na\(^+\)-taurocholate cotransporting polypeptide

AUC, area under the plasma concentration curve

HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A

E\(_2\)17\(\beta\)G, estradiol 17\(\beta\)-D-glucuronide

E-sul, estrone-3-sulfate

CCK-8, cholecystokinin octapeptide

DDI, drug-drug interaction

RAF, relative activity factor

SNPs, single nucleotide polymorphisms

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Abstract

Pitavastatin, a novel potent HMG-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 Michaelis-Menten constants (Km) 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 (cholcystokinin octapeptide)) in expression systems and human hepatocytes. The concept of this method is similar to the so-called relative activity factor (RAF) 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.
Introduction

Pitavastatin is a highly potent inhibitor of 3-hydroxymethylglutaryl coenzyme A (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 [14C]-pitavastatin is selectively distributed to the liver in rats with the liver-to-plasma concentration ratio of more than 54. 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, while 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 pharmacological
target organ of pitavastatin, it is essential to clarify the uptake mechanism of pitavastatin by hepatocytes in order 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 co-transporting polypeptide (NTCP), 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 (OCT) 1 are localized on the basolateral membrane of human hepatocytes (Hagenbuch and Meier, 1996; Muller and Jansen, 1997; Hagenbuch and Meier, 2003). In particular, OATP2 and OATP8 are selectively expressed in the human liver and exhibit broad substrate specificities, which suggests 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 in order to estimate the overall hepatic clearance for individuals when there are changes in expression level or function caused by pathological conditions, single nucleotide polymorphisms (SNPs) 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 (E217βG), a typical substrate of both OATP2 and OATP8 (Cui et al., 2001; Ismaier 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 2 methods.
Materials and Methods

Materials. Pitavastatin, monocalcium bis [(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate] was synthesized by Nissan Chemical Industries (Chiba, Japan). [Fluorobenzene-U-"C] pitavastatin (11.7 mCi/mmol) was synthesized by Amersham Co. (Little Chalfort, UK). [3H] Estradiol 17β-α-glucronide, (E217βG) and [3H] estrone-3-sulfate (E-sul), (45 Ci/mmol and 46 Ci/mmol, respectively) were purchased from New England Nuclear (Boston, MA), while [3H] cholecystokinin octapeptide (CCK-8) (77 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Unlabeled E217βG, E-sul, and CCK-8 were purchased from Sigma (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 Kpn I site
(5'-GGGGTACCAGACCCAAATCAACATTGAAT-3') and a reverse primer (5'-GTTAGCCTTAGATGAAGGCTGACC-3'). The second half (1041-2076) of the gene was amplified using a forward primer (5'-ACAAGTAAGCAGCTATATTGGTG-3') and a reverse primer containing a Not I site (5'-GGGCGGCCGCTTAACAATGTGTTTCACTATCTG-3'). Each PCR product was TA-cloned into the pGEM vector (Promega, CA, USA), respectively. Then, the pGEM vector containing the first half was digested with Kpn I and Eco105 I and the pGEM vector with the second half was digested with Eco105 I and Not I. Subsequently, the first and second halves of the OATP2 cDNA were ligated into Kpn I and Not I 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'-TTTTCCCTTTTGGCAGGCTTTTCGTGAAGACCAGG-3') and TA-cloned into pGEM vector. The N-terminal fragment was amplified using a forward primer containing a Hind III 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 Hind III and Not I and fragment #2 was created by digesting the pGEM vector containing the N-terminal fragment with Hind III. Then, fragments #1 and #2 were ligated into linearized pcDNA3.1 (+) digested with Hind III and Not I.

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 Corp., Indianapolis, IN), according to the manufacturer’s instruction and the selection by 800 µg/mL Antibiotic G418 sulfate (Promega Corp., Madison, WI) for 3 weeks.

**Cell Culture.** Transporter-expressing or vector-transfected HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) low glucose (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (Sigma, St. Louis, MO), 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37°C with 5 % CO₂ and 95 % humidity. Cells were then seeded in 12-well plates at a density of 1.5 × 10⁵ cells/well. For the transport
study, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium-butyrate 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 NaHCO₃, 4.8 mM KCl, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl₂ 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 ice-cold Krebs-Henseleit buffer, solubilized in 500 µL 0.2 N NaOH, and kept overnight at 4°C. Aliquots (500 µL) were transferred to scintillation vials after adding 250 µL 0.4 N HCl. The radioactivity associated with the cells and incubation buffer was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter, Inc.) after adding 2 mL scintillation fluid.
(Clear-sol I, NACALAI TESQUE, Kyoto, Japan) to the scintillation vials. The remaining 50 µL 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, USA). In this experiment, we selected three batches of human hepatocytes (Lot. OCF, 094 and ETR) which show relatively high uptake amount of E₂17βG 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 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 × 10⁶ 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 biocarbonate. 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 incubation mixture was collected and placed in a centrifuge tube (450 µL) containing 50 µL 2 N NaOH under a layer of 100 µL 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 (10,000g; Beckman Microfuge E; Beckman Coulter, Inc., Fullerton, CA). 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 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-terminal 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 (BioLabs, Hertfordshire, UK) and loaded onto a 7% SDS-polyacrylamide gel with a 4.4% stacking gel. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad, Richmond, 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 Pharmacia Biotech, Buckinghamshire, UK) 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 [µL/mg protein], given as the amount of radioactivity associated with the cells [dpm/mg protein] divided by its concentration in the incubation medium [dpm/µL]. 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:

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

where $v$ is the uptake velocity of the substrate (pmol/min/mg protein), $S$ is the substrate concentration in the medium (µM), $K_m$ is the Michaelis constant (µM), $V_{\text{max}}$ is the maximum uptake rate (pmol/min/mg protein) and $P_{\text{dif}}$ is the non-saturable uptake clearance (µL/min/mg 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 \( (\text{CL}_{(2\text{min}-0.5\text{min})}) \) (µL/min/10^6 cells) by calculating the slope of the uptake volume \( (V_d) \) (µL/10^6 cells) between 0.5 and 2 min (eq. 2). The saturable component of the hepatic uptake clearance \( (\text{CL}_{\text{hep}}) \) was determined by subtracting \( \text{CL}_{(2\text{min}-0.5\text{min})} \) in the presence of 100 µM substrate (excess) from that in the presence of 1 µM substrate (tracer) (eq. 3).

\[
\text{CL}_{(2\text{min}-0.5\text{min})} = \frac{V_{d,2\text{min}} - V_{d,0.5\text{min}}}{2 - 0.5}
\]

\[
\text{CL}_{\text{hep}} = \text{CL}_{(2\text{min}-0.5\text{min}),\text{tracer}} - \text{CL}_{(2\text{min}-0.5\text{min}),\text{excess}}
\]

where \( \text{CL}_{(2\text{min}-0.5\text{min}),\text{tracer}} \) and \( \text{CL}_{(2\text{min}-0.5\text{min}),\text{excess}} \) represent \( \text{CL}_{(2\text{min}-0.5\text{min})} \) 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 estrone-3-sulfate (E-sul) and
cholecystokinin octapeptide (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 $E_217βG$) 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:

\[
R_{act, OATP2} = \frac{CL_{Hep,E-sul}}{CL_{OATP2,E-sul}} \quad \text{(eq. 4)}
\]

\[
R_{act, OATP8} = \frac{CL_{Hep,CCK-8}}{CL_{OATP8,CCK-8}} \quad \text{(eq. 5)}
\]

\[
CL_{hep, test, OATP2} = CL_{OATP2, test} \cdot R_{act, OATP2} \quad \text{(eq. 6)}
\]

\[
CL_{hep, test, OATP8} = CL_{OATP8, test} \cdot R_{act, OATP8} \quad \text{(eq. 7)}
\]

\[
CL_{hep, test} = CL_{hep, test, OATP2} + CL_{hep, test, OATP8} \quad \text{(eq. 8)}
\]

In addition, the ratio of the expression levels of OATP2 and OATP8 in human hepatocytes (per $10^6$ cells) to transporter-expressing cells (per mg protein) ($R_{exp, OATP2}$ and $R_{exp, OATP8}$, respectively, as described in the following equations:
OATP2 and $R_{\text{exp, OATP8}}$ 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 mg 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 whole cell protein in HEK293 transfectants. The $R_{\text{exp}}$ value was calculated as the relative expression level per $10^6$ hepatocytes divided by that per mg protein in HEK293 cells. The OATP2- and OATP8-mediated hepatic uptake of test compounds (pitavastatin and $E_217\beta G$) was calculated from the following equation:

$$\begin{align*}
CL_{\text{hep, test}} &= CL_{\text{OATP2, test}} \cdot R_{\text{exp, OATP2}} + CL_{\text{OATP8, test}} \cdot R_{\text{exp, OATP8}} \\
&= \text{(eq. 9)}
\end{align*}$$
RESULTS

Uptake of $E_2\beta G$, E-sul, CCK-8 and Pitavastatin by transporter-expressing cells.

The saturation kinetics of $E_2\beta G$, 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 $E_2\beta G$ were significantly taken up into both OATP2- and OATP8-expressing HEK293 cells compared with vector-transfected cells (Fig. 1A and 1D). The transfection of OATP2 resulted in an increase in the uptake of E-sul, but did not affect the uptake of CCK-8, while transfection of OATP8 resulted in an increase in the uptake of CCK-8, but not E-sul (Fig. 1B and 1C). The concentration-dependence of the uptake of pitavastatin, $E_2\beta G$ and E-sul could be explained by a one-saturable component (Fig. 1A, B and D). However, a saturable and a non-saturable 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 > $E_2\beta G$, while that by OATP8 was pitavastatin > CCK-8 > $E_2\beta G$. 

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Uptake of $E_217\beta G$, E-sul, CCK-8 and Pitavastatin by Human Cryopreserved Hepatocytes.

Typical time-profiles of the uptake of $E_217\beta G$, 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 $\mu$M and it was decreased in the presence of 100 $\mu$M 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 > $E_217\beta G$ > 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 kDa 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 kDa 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. 4A, B). Fig. 4C and 4D 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 regression line in Fig. 4C and 4D 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 calculate the ratio of the clearance ($R_{act}$) and estimated clearance of pitavastatin and $E_2 17\beta G$ 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 protein in crude membrane from 1 mg whole cell protein in OATP2- and OATP8-expressing HEK293 cells, respectively, while 178, 89 and 82 µg 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 (µg) for OATP2 and 0.659, 1.01 and 0.872 (µg) 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 \( \text{E}_2 \text{17βG} \) are taken
up into human hepatocytes predominantly by OATP2 (Tables 3 and 4).
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 Km value of pitavastatin for OATP2 is comparable with that of cerivastatin (Km=4.3 µM) (in-house data) and rosuvastatin (Km=7.3 µM) (Brown et al., 2001), and smaller than that of pravastatin (Km=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). Km values of pitavastatin for OATP2 and 8 were comparable (Fig. 1, Table 1) and 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 $E_217\beta G$ was a good substrate for both OATP2 and OATP8 by using transporter-expressing cells (Fig. 1) (Konig et al., 2000a, b). As shown in Table 1, the $K_m$ values of $E_217\beta G$, 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 inter-batch 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 inter-individual 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, $E_217\beta G$, 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 NTCP is not mainly involved in hepatic uptake of pitavastatin. Previous reports demonstrated that both OATP2 and OATP8 can transport substrates in a Na⁺-independent manner (Konig 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 which were selective substrates of OATP2 or OATP8. Crespi and Penman (1997) proposed the relative activity factor (RAF) method using the ratio of the metabolic activity in human liver microsomes divided by the activity in each isoform-specific cytochrome P-450 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 (Ract) should correspond to the relative expression level of each transporter. Multiplying Ract by the uptake clearance of pitavastatin and E2\textsubscript{17}\beta G in each transfectant, we can estimate the OATP2- and OATP8-mediated portion of the uptake of pitavastatin and E2\textsubscript{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 E2\textsubscript{17}\beta G (Tables 2 and 3). Therefore, the uptake of pitavastatin and E2\textsubscript{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 ($R_{\text{exp}}$) as described above. As a result of our estimation using $R_{\text{exp}}$ values, the sum of the predicted OATP2- and OATP8-mediated uptake of pitavastatin and E$_2$17βG was 5-10 times higher than their observed clearance in hepatocytes (Tables 2 and 4), a finding which 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 $R_{\text{exp}}$ values for OATP2 and OATP8 was comparable with that of the $R_{\text{act}}$ 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<sub>217</sub>βG 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<sub>217</sub>βG 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<sub>217</sub>βG 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 E₂₁₇βG can be almost completely accounted for by OATP2 because the contribution of OATP8 to the overall hepatic uptake of E₂₁₇βG was negligible in our estimation based on the uptake clearance of CCK-8 (Table 3). On the other hand, the uptake clearance of E₂₁₇βG in human hepatocytes was almost comparable with the OATP2-mediated uptake clearance of E₂₁₇βG 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 DDI 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 E₂₁₇βG 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 E₂₁⁷βG 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


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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.


Footnotes

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Legends for figures

Fig. 1  Eadie-Hofstee plots of the uptake of $[\text{3H}] \text{E}_2\text{17}\beta\text{G}, [\text{3H}] \text{E-sul}, [\text{3H}] \text{CCK-8}$ and $[\text{14C}] \text{pitavastatin}$ 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 $[\text{3H}] \text{E}_2\text{17}\beta\text{G}$ (A), $[\text{3H}] \text{E-sul}$ (B) $[\text{3H}] \text{CCK-8}$ (C) and $[\text{14C}] \text{pitavastatin}$ (D) is shown as Eadie-Hofstee plots. The uptake of $[\text{3H}] \text{E}_2\text{17}\beta\text{G}, [\text{3H}] \text{E-sul}, [\text{3H}] \text{CCK-8}$ and $[\text{14C}] \text{pitavastatin}$ 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 non-linear regression analysis.

Each point represents the mean ± S.E. (n = 3).

Fig. 2  Time-profiles of the uptake of $[\text{3H}] \text{E}_2\text{17}\beta\text{G}, [\text{3H}] \text{E-sul}, [\text{3H}] \text{CCK-8}$ and $[\text{14C}] \text{pitavastatin}$ 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).

Fig. 3 Time-profiles of the uptake of [14C] 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).

Fig. 4 Western blot analysis of OATP2 and OATP8. 5, 10 and 20 µg crude membrane fractions 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 and Y axis represents 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.
Table. 1  Kinetic parameters of the uptake of compounds by transporter-expressing HEK293 cells.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Km  µM</th>
<th>Vmax pmol/min/mg</th>
<th>Vmax/Km µL/min/mg</th>
<th>Pdif µL/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP2</td>
<td>E-sul</td>
<td>0.458 ± 0.154</td>
<td>60.3 ± 8.8</td>
<td>132 ± 19</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CCK-8</td>
<td>Not significantly transported</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pitavastatin</td>
<td>3.00 ± 0.39</td>
<td>230 ± 17</td>
<td>76.6 ± 5.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E217βG</td>
<td>8.29 ± 0.42</td>
<td>131 ± 5</td>
<td>15.8 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>OATP8</td>
<td>E-sul</td>
<td>3.82 ± 2.20</td>
<td>102 ± 60</td>
<td>26.7 ± 15.7</td>
<td>11.4 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Not significantly transported</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCK-8</td>
<td>3.25 ± 0.44</td>
<td>100 ± 8</td>
<td>30.6 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pitavastatin</td>
<td>24.6 ± 7.6</td>
<td>56.8 ± 16.1</td>
<td>2.31 ± 0.65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E217βG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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).

Pdif means the non-saturable uptake clearance (µL/min/mg protein).
Table 2  Uptake clearance of reference compounds (E-sul and CCK-8) and test compounds (pitavastatin and E$_2$17βG) in human hepatocytes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Uptake clearance (CL$_{\text{hep}}$)$^a$ (µL/min/10$^6$ cells)</th>
<th>Lot.OCF</th>
<th>Lot.094</th>
<th>Lot.ETR</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-sul</td>
<td>110</td>
<td>134</td>
<td>57.7</td>
<td></td>
</tr>
<tr>
<td>CCK-8</td>
<td>7.89</td>
<td>3.50</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>pitavastatin</td>
<td>61.3</td>
<td>113</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>E$_2$17βG</td>
<td>13.5</td>
<td>17.0</td>
<td>5.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Uptake clearance was determined using three independent batches of human cryopreserved hepatocytes.
Table 3 Contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin and E217βG determined using transporter-selective substrates.

<table>
<thead>
<tr>
<th>Hepatocyte Lot</th>
<th>Ratio of uptake clearance</th>
<th>Estimated clearance $^a$ (µL/min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{CL}<em>{\text{hep}}/\text{CL}</em>{\text{transporter}}$</td>
<td>$\text{R}_{\text{act, OATP2}}$</td>
</tr>
<tr>
<td>OCF</td>
<td>0.833</td>
<td>0.291</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>094</td>
<td>1.02</td>
<td>0.131</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETR</td>
<td>0.437</td>
<td>0.0757</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The lower column in the “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 in the Materials and Methods.
Table 4  Contribution of OATP2 and OATP8 to the hepatic uptake of pitavastatin and E217βG determined by the relative expression level

<table>
<thead>
<tr>
<th>Hepatocyte Lot</th>
<th>Ratio of expression level ( \frac{R_{\text{exp, OATP2}}}{R_{\text{exp, OATP8}}} )</th>
<th>Estimated clearance ( (\mu L/min/10^6 \text{ cells}) )</th>
<th>E-sul CCK-8</th>
<th>pitavastatin OATP2</th>
<th>pitavastatin OATP8</th>
<th>E217βG OATP2</th>
<th>E217βG OATP8</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCF</td>
<td>2.90</td>
<td>383</td>
<td>32.3</td>
<td>222</td>
<td>37.0</td>
<td>45.8</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85.7 %</td>
<td>14.3 %</td>
<td>94.2 %</td>
<td>5.76 %</td>
</tr>
<tr>
<td>094</td>
<td>1.58</td>
<td>208</td>
<td>24.8</td>
<td>121</td>
<td>28.4</td>
<td>24.9</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80.9 %</td>
<td>19.1 %</td>
<td>92.1 %</td>
<td>7.95 %</td>
</tr>
<tr>
<td>ETR</td>
<td>0.89</td>
<td>117</td>
<td>19.7</td>
<td>68.1</td>
<td>22.6</td>
<td>14.0</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75.1 %</td>
<td>24.9 %</td>
<td>89.2 %</td>
<td>10.8 %</td>
</tr>
</tbody>
</table>

\( a \) 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 mg) in Western blot analysis.

\( b \) The lower column in the “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 in the Materials and Methods.
Figure 1

(A) 

(B) 

(C) 

(D) 

v/s (µL/min/mg protein)

v (pmol/min/mg protein)

v (pmol/min/mg protein)

v (pmol/min/mg protein)
Figure 3

Uptake (µL/10^6 cells) vs. Time (min)

- Solid circles: Group A
- Open triangles: Group B
- Open circles: Group C

The graph shows an increase in uptake over time for all groups. Group A has the highest uptake, followed by Group B and then Group C.
Figure 4

(A) OATP2

Hepatocytes (Lot. 094)  mock  Expression system  Human liver

protein amount  (µg/lane)  20  10  5  50  20  10  5

80 kDa  ▶

(B) OATP8

Hepatocytes (Lot. 094)  mock  Expression system  Human liver

protein amount  (µg/lane)  20  10  5  50  20  10  5

120 kDa  ▶

(C)

Band density (artificial)

0  0  10  10  20  20

Protein amount (µg/lane)

(D)

Band density (artificial)

0  0  10  10  20  20

Protein amount (µg/lane)