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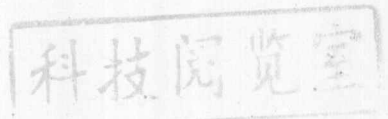
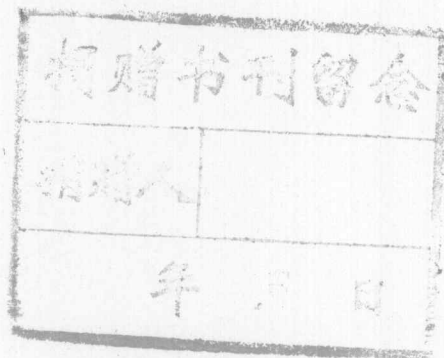
(English Article)

1999

Department of Biopharmaceutics
Graduate School of Pharmaceutical Sciences
The University of Tokyo

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Contribution of Organic Anion Transporting Polypeptide to Uptake of Its Possible Substrates into Rat Hepatocytes¹

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ABSTRACT

Organic anion transporting polypeptide (oatp1) has been cloned from rat liver as one of the transporters responsible for the hepatic uptake of ligands, and its substrate specificity has been determined. However, the contribution of oatp1 to the Na⁺-independent uptake of ligands into rat hepatocytes remains to be investigated. In the present study, we determined the contribution of oatp1 and examined the uptake of ligands into primary cultured hepatocytes (cultured for 4 h) and into COS-7 cells transiently expressing oatp1 and normalized using estradiol-17 β -D-glucuronide as a reference compound. Western blot analysis indicated that oatp1 was less extensively glycosylated in transfected COS-7 cells, and the expression level in transfectant was one-seventh that in rat liver. The K_m values for the uptake of estradiol-17 β -D-glucuronide were similar for cultured hepatocytes and oatp1-transfected COS-7 cells (K_m = 12.3 versus 20.4 μ M), although the V_{max} value for oatp1-transfected COS-7 cells was one-seventh that for cultured

hepatocytes (V_{max} = 1.30 versus 0.175 nmol/min/mg protein). The contribution of oatp1 to the Na⁺-independent uptake of taurocholic acid and cholic acid into rat hepatocytes was more than 50 to 60%, whereas the corresponding values for the sulfate-conjugates of estrone and 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole were 20 to 30%. In addition, the analysis indicated that the contribution of oatp1 to the Na⁺-independent uptake of several ligands [glucuronide-conjugate of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole, ibuprofen, pravastatin, ouabain, and 2,4-dinitrophenyl-S-glutathione] was minimal. Collectively, the transfected COS-7 cells may be used to quantitatively predict oatp1 activity in hepatocytes after correction of its expressed amount. It is also suggested that multiple transport mechanisms are responsible for the Na⁺-independent uptake of organic anions into hepatocytes.

Along with renal excretion, hepatic elimination is one of the major pathways involved in the detoxification of xenobiotics. Hepatic uptake is the initial process for the elimination of xenobiotics mediated by metabolism and/or biliary excretion. Previously, the mechanism for the hepatic uptake of ligands has been studied by kinetic analysis of the experimental data obtained *in vivo*, *in situ* using perfused liver, and *in vitro* in isolated and/or cultured hepatocytes and isolated sinusoidal membrane vesicles (Elferink et al., 1995). Cumulative evidence indicates that organic anions such as bilirubin (Paumgartner and Reichen, 1976), bromosulphophthalein (BSP; Wolkoff et al., 1987), dibromosulphophthalein (DBSP; Blom et al., 1981), 1-anilino-8-naphthalene sulfonate (Sug-

iyama et al., 1983), and benzylpenicillin (Tsuji et al., 1986) are taken up by hepatocytes via a Na⁺-independent transport system. In addition, analysis of the hepatic uptake of bile acids revealed that the uptake of taurocholic acid (TC) and cholic acid (CA) is mediated by both Na⁺-dependent and -independent transport system, and approximately 20% and 60% of TC and CA uptake is mediated by a Na⁺-independent mechanism, respectively (Yamazaki et al., 1993b). In addition, recently, Yamazaki et al. (1993a) and Nakamura et al. (1996) found that a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (pravastatin) and a cyclic peptide (BQ-123; an endothelin antagonist) are taken up by isolated hepatocyte via a Na⁺-independent transport system and reported the mutual inhibition between these compounds and DBSP or bile acids (such as TC and CA). These results are consistent with the hypothesis that Na⁺-independent bile acid transport and organic anion transport are mediated by a common transport carrier. Based on its wide range of sub-

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ABBREVIATIONS: Ntcp, sodium taurocholate cotransporting polypeptide; oatp, organic anion transporting polypeptide; E₂-17 β G, estradiol-17 β -D-glucuronide; TC, taurocholate, taurocholic acid; CA, cholate, cholic acid; DNP-SG, 2,4-dinitrophenyl-S-glutathione; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole; BSP, bromosulphophthalein; DBSP, dibromosulphophthalein; DMEM, Dulbecco's modified Eagle's medium; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TBS-T, Tris-buffered saline containing 0.05% Tween 20; SD, Sprague-Dawley; K_m , Michaelis constant; V_{max} , maximum transport velocity; CL_{uptake} , uptake clearance.

strate specificity, this putative transporter has been referred to as the "Na⁺-independent multispecific organic anion transporter" (Meier, 1988).

To obtain more detailed information on the mechanism of hepatic uptake, the cDNA species for Na⁺-TC cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (oatp1) were isolated from rat liver based on expression cloning with *Xenopus laevis* oocytes (Hagenbuch et al., 1991; Jacquemin et al., 1994). Moreover, the human homologs of these transporters (NTCP and OATP) have been cloned (Hagenbuch and Meier, 1994; Kullak-Ublick et al., 1995). Using antibodies, in a rat liver study, it has been shown that oatp1 is selectively confined to the basolateral plasma membrane of hepatocytes (Bergwerk et al., 1996). The transport properties of oatp1 have been characterized by using oocytes injected with cRNA and mammalian cells transfected with cDNA (Meier, 1995); it has been shown that oatp1 mediates the Na⁺-independent uptake of TC and CA and can undergo *cis*-inhibition by neutral and unconjugated bile salts as well as a variety of nonbile salt amphipathic organic anions (Jacquemin et al., 1994; Kullak-Ublick et al., 1994). Moreover, it has been demonstrated that the affinity of oatp1 for estradiol 17 β -D-glucuronide (E₂17 β G; K_m = 3 μ M) was significantly higher than that for TC (K_m = 27 μ M) (Kanai et al., 1996). In addition, oatp1 has recently been demonstrated to transport anionic steroid conjugates (estrone-3-sulfate), neutral steroids (ouabain, aldosterone, cortisol), and even some amphipathic organic cations such as *N*-(4,4-azo-*n*-pentyl)-21-deoxyajmalinium, a permanently charged photolabile derivative of the antiarrhythmic drug *N*-propylajmaline (Bossuyt et al., 1996). Hence, oatp1 represents a polyspecific and multivalent transport system able to accept a large variety of structurally unrelated and differently charged amphipathic organic substrates.

The purpose of the present study is to examine whether several organic anions, which are taken up by hepatocytes via a Na⁺-independent mechanism, can be oatp1 substrates. In addition, to clarify the contribution of oatp1 to the Na⁺-independent uptake of ligands, we examined the uptake of ligands into primary cultured hepatocytes and into COS-7 cells transiently expressing oatp1 and normalized the uptake using E₂17 β G as a reference compound.

Experimental Procedures

Materials. COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). [³H]E₂17 β G (1813 GBq/mmol), [³H]TC (128.4 GBq/mmol), [³H]CA (906.5 GBq/mmol), [³H]estrone-3-sulfate (1883 GBq/mmol), and [³H]ouabain (758.5 GBq/mmol) were purchased from New England Nuclear (Boston, MA). [³H]Ibuprofen (18.5 GBq/mmol) was purchased from Amersham International (Buckinghamshire, UK). The glucuronide- and sulfate-conjugates of [¹⁴C]6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)-benzothiazole (E3040; 1.85 GBq/mmol), prepared according to the method described previously (Hibi et al., 1994), were kindly donated by Eisai Co., Ltd (Tokyo, Japan). [³H]Pravastatin was kindly donated by Sankyo Co., Ltd (Tokyo, Japan). [³H]2,4-Dinitrophenyl-S-glutathione (DNP-SG) was synthesized according to the method described by Saxena and Henderson (1995) using [³H]glutathione (1739 GBq/mmol; New England Nuclear). All other chemicals were commercially available and of reagent grade.

Transient Expression of oatp1 cDNA in COS-7 Cells. Full-length cDNA for oatp1, kindly donated by Dr. Peter J. Meier, was initially cloned in the plasmid pSPORT1 (BRL, Gaithersburg, MD)

(Jacquemin et al., 1994). oatp1 cDNA was excised with *Mlu*I (Takara, Tokyo, Japan) to perform the subcloning into the *Xho*I site in the pCAGGS vector, which has an SV40 origin of replication to allow duplication of the plasmid in COS-7 cells (Niwa et al., 1991) after converting to blunt ends.

For transfection, COS-7 cells were cultured in 150-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. At 30% confluence, cells were exposed to serum-free DMEM containing plasmid (1 μ g/ml) and Lipofectamine (1 μ g/ml; BRL, Gaithersburg, MD). At 8 h after transfection, the plasmid-Lipofectamine solution was removed, and the medium, consisting of DMEM supplemented with 5% fetal bovine serum, was cultured overnight. Then, the transfected cells were treated with trypsin and approximately 1.6×10^5 cells were seeded onto 22-mm dishes and cultured overnight. An uptake study was performed at 48 h after transfection.

Primary Cultured Rat Hepatocytes. Rat hepatocytes were isolated from male Sprague-Dawley (SD) rats (200–250 g; Nihon Ika-gaku Dobutsu Shizai Kenkyusyo, Tokyo, Japan) after perfusion of the liver with collagenase. Cell viability was routinely checked by the trypan blue [0.4% (w/v)] exclusion test. After preparation, freshly isolated cells were suspended in Williams' medium E. Approximately 5×10^5 cells were placed onto collagen-coated 22-mm dishes and cultured for 4 h.

Northern Blot Analysis. Northern blot analysis was performed as described previously (Ito et al., 1997). Either 0.5 or 2 μ g of poly(A)⁺ RNA, prepared from COS-7 cells 48 h after transfection, and SD rat liver were separated on a 0.8% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane, before fixation by baking for 2 h at 80°C. Blots were prehybridized in medium containing 4 \times saline sodium citrate (SSC), 5 \times Denhardt's solution, 0.2% sodium dodecyl sulfate (SDS), 0.1 mg/ml sonicated salmon sperm DNA, and 50% formamide at 42°C for 2 h. We used 2 kbp of oatp1 cDNA (nucleic acid, 69–2102 bp) as a hybridization probe, and hybridization was performed overnight in the same buffer containing 10^6 cpm/ml ³²P-labeled cDNA prepared by the random primed labeling method. As a control, ³²P-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech Laboratories, Inc., Palo Alto, CA) was used. The hybridized membrane was washed in 2 \times SSC and 0.1% SDS at 55°C for 20 min and then in 0.1 \times SSC and 0.1% SDS at 55°C for 20 min. Then, the membrane was exposed to imaging plates (Fuji Film, Tokyo, Japan) for 1 h at room temperature. The intensity of the specific band was quantified using a Bio-Image Analyzer (Bas 2000, Fuji Film).

Western Blot Analysis. For the Western blot analysis, crude membrane was prepared from COS-7 and SD rat liver according to the method of Gant et al. (1991). Cells and liver were homogenized in five volumes of 0.1 M Tris-HCl buffer, pH 7.4, containing 1 μ g/ml leupeptin and pepstatin A and 50 μ g/ml phenylmethylsulfonyl fluoride with 20 strokes of a Dounce homogenizer. After centrifugation (1500g for 10 min) of homogenate, the supernatant was recentrifuged (100,000g for 30 min). The precipitate was suspended in Tris-HCl buffer and recentrifuged (100,000g for 30 min). The crude membrane fraction was resuspended in the 0.1 M Tris-HCl buffer containing the proteinase inhibitors with five strokes of a Dounce homogenizer and stored at –80°C before being used for Western blot analysis. All procedures were performed at 0 to 4°C. The membrane protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as a standard. Then, either 25 or 50 μ g of crude membrane was dissolved in 10 ml of 2 \times 0.25 M Tris-HCl buffer containing 2% SDS, 30% glycerol, and 0.01% bromophenol blue, pH 6.8, and loaded onto a 7.5% SDS-polyacrylamide gel electrophoresis plate with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (NEB, Beverly, MA). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA) using a blotter (BioRad Laboratories, 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% BSA for 1 to 2 h at room temperature. After washing with TBS-T (3×5 min), the membrane was incubated overnight with anti-oatp1 rabbit serum (dilution 1:5000), which was kindly donated by Dr. Peter J. Meier (Bergwerk et al., 1996), in TBS-T containing 5% BSA at 4°C and then washed with TBS-T (3×5 min). The membrane was allowed to bind to 125 I-labeled sheep anti-rabbit Ig in TBS-T containing 5% BSA for 1 h at room temperature and then washed with TBS-T (3×5 min). Then, the membrane was exposed to imaging plates (Fuji Film, Tokyo, Japan) overnight at room temperature. The intensity of the specific band was quantified using a Bio-Image Analyzer (Bas 2000, Fuji Film).

Uptake Study. Uptake was initiated by adding the radiolabeled ligands to the medium after washing the culture dishes three times and preincubation with Krebs-Henseleit buffer or choline buffer at 37°C for 5 min. The Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM Na_2CO_3 , 4.83 mM KCl, 0.96 mM KH_2PO_4 , 1.20 mM MgSO_4 , 12.5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 5 mM glucose, and 1.53 mM CaCl_2 adjusted to pH 7.3. The composition of the choline buffer was the same as the Krebs-Henseleit buffer except that NaCl and NaHCO_3 were replaced with isotonic choline chloride and choline bicarbonate, respectively. The final concentration of [^3H]E $_2$ 17 β G, [^3H]TC, [^3H]CA, [^3H]estrone-3-sulfate, [^3H]ouabain, [^3H]ibuprofen, [^3H]pravastatin, and [^3H]DNP-SG was 1 μM , whereas that of [^{14}C]E3040 sulfate and [^{14}C]E3040 glucuronide was 2 and 10 μM , respectively. At designated times, the reaction was terminated by adding ice-cold Krebs-Henseleit buffer. Just before the designated times, 50 μl of medium was transferred to scintillation vials. Then, cells were washed three times with 2 ml of ice-cold Krebs-Henseleit buffer and solubilized in 500 μl of 1 N NaOH. After adding 500 μl of distilled water, 800- μl aliquots were transferred to scintillation vials. The radioactivity associated with the cells and medium was determined in a liquid scintillation counter (LS 6000SE; Beckman Instruments, Inc., Fullerton, CA) after adding 8 ml of scintillation fluid (Hionic flow; Packard Instrument Co., Downers Grove, IL) to the scintillation vials. The remaining 100- μl aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with BSA as a standard. Ligand uptake is given as the cell-to-medium concentration ratio, determined as the amount of ligands associated with the cells divided by the medium concentration.

Determination of Kinetic Parameters. The uptake of E $_2$ 17 β G for 1 min was studied to determine the kinetic parameters because the initial velocity of the uptake of E $_2$ 17 β G is linear during this period. The kinetic parameters were estimated from the following equation;

$$V_0 = (V_{\max} \times S) / (K_m + S)$$

where V_0 is the initial uptake velocity of E $_2$ 17 β G (nmol/min/mg protein), S is the concentration of E $_2$ 17 β G in the medium (μM), K_m is the Michaelis constant (μM), and V_{\max} is the maximum uptake rate (nmol/min/mg protein). The uptake data were fitted to this equation by a nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

Estimation of Contribution of oatp1 to Na $^+$ -Independent Uptake of Ligands into Rat Hepatocytes. To determine the Na $^+$ -independent uptake by hepatocytes, uptake was measured in choline buffer. oatp1-mediated uptake was determined by subtracting the uptake into COS-7 cells transfected with pCAGGS vector from that into COS-7 cells transfected with pCAGGS containing oatp1 (measured in Krebs-Henseleit buffer). The uptake clearance of ligands (CL_{uptake}) was calculated using linear regression applied to the initial two or three data points. R_{hep} and R_{COS} were defined by $R_{\text{hep}} = CL_{\text{uptake}}$ of ligands into hepatocytes/ CL_{uptake} of E $_2$ 17 β G into hepatocytes and $R_{\text{COS}} = CL_{\text{uptake}}$ of ligands into COS-7 cells/ CL_{uptake} of E $_2$ 17 β G into COS-7 cells.

The contribution of oatp1 to the Na $^+$ -independent uptake of ligands into rat hepatocytes was estimated from the following equation:

$$\text{Contribution (\%)} = R_{\text{COS}} / R_{\text{hep}} \times 100$$

Results

Expression of oatp1 in COS-7 Cells. The expression of transfected oatp1 in COS-7 cells was examined by Northern and Western blot analyses. As shown in Fig. 1, the oatp1 transcript was found at approximately 4.3 and 2.5 kb in transfected COS-7 cells (lanes c and d), whereas it was found at approximately 4.3 kb in rat liver (lane a). However, Western blot analysis (Fig. 1) indicated that the molecular mass of the oatp1 product in COS-7 cells (lanes j and k) was approximately 72 kDa, which was significantly lower than that in liver (83 kDa) (lanes h and i). Although the amount of oatp1 transcript was approximately 10-fold higher in oatp1-transfected COS-7 cells than in liver, the amount of oatp1 expressed on the membrane prepared from oatp1-transfected

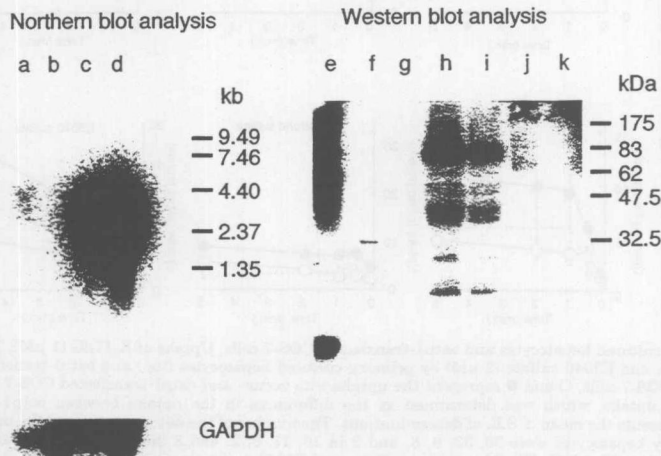


Fig. 1. Expression of oatp1. Expression of oatp1 in transfected COS-7 cells was examined by Northern (lanes a-d) and Western (lanes e-k) blot analyses. Poly(A) $^+$ RNA from SD rat liver and oatp1- and vector-transfected COS-7 cells were used in Northern blot analysis. The membrane hybridized with ^{32}P -labeled oatp1 cDNA fragment (nucleic acid, 69-2102 bp) and rehybridized with ^{32}P -labeled GAPDH cDNA was exposed for 1 h at room temperature with an intensifying screen. Lanes a, b, and d were loaded with 2 μg of poly(A) $^+$ RNA from rat liver and vector- and oatp1-transfected COS-7 cells, respectively. Lane c was loaded with 0.5 μg of poly(A) $^+$ RNA from oatp1-transfected COS-7 cells. The crude membrane from SD rat liver and oatp1- and vector-transfected COS-7 cells were used in Western blot analysis. The membrane incubated with anti-rabbit oatp1 serum was exposed overnight at room temperature with an intensifying screen. Lanes e, f, g, h, i, j, and k were loaded with a marker, 50 and 25 μg of crude membrane from vector-transfected COS-7 cells, 50 and 25 μg of crude membrane from liver, and 50 and 25 μg of crude membrane from oatp1-transfected COS-7 cells, respectively.

COS-7 cells was approximately one-seventh that of liver (Fig. 1). No expression of oatp1 was observed in COS-7 cells transfected with pCAGGS vector (lanes f and g).

Quantification of Ligand Transport. Na^+ -independent uptake of $\text{E}_217\beta\text{G}$, TC, CA, estrone-3-sulfate, and E3040 sulfate was observed in rat hepatocytes (Fig. 2). In addition, the uptake of these ligands into COS-7 cells was stimulated by transfection of oatp1 (Fig. 2). Kinetic analysis of the Na^+ -independent uptake of $\text{E}_217\beta\text{G}$ by cultured hepatocytes gave a K_m of $12.9 \pm 1.3 \mu\text{M}$ and a V_{\max} of $1.30 \pm 0.10 \text{ nmol/min/mg protein}$ (Fig. 3). In the same manner, the K_m and V_{\max} of oatp1-mediated $\text{E}_217\beta\text{G}$ uptake was $20.4 \pm 9.0 \mu\text{M}$ and $0.175 \pm 0.051 \text{ nmol/min/mg protein}$, respectively (Fig. 3).

The CL_{uptake} for the Na^+ -independent uptake of $\text{E}_217\beta\text{G}$, TC, CA, estrone-3-sulfate, and E3040 sulfate by cultured hepatocytes was 63, 13, 40, 78, and $29 \mu\text{l/min/mg protein}$, respectively (Table 1). In the same manner, the oatp1-mediated CL_{uptake} of $\text{E}_217\beta\text{G}$, TC, CA, estrone-3-sulfate, and E3040 sulfate was calculated to be 19, 2.3, 6.2, 6.3, and $1.8 \mu\text{l/min/mg protein}$, respectively (Table 1). These results gave R_{hep} and R_{COS} values of 0.2 and 0.12 for TC, 0.63 and 0.33 for CA, 1.2 and 0.33 for estrone-3-sulfate, and 0.46 and 0.095 for E3040 sulfate, respectively (Table 1). The contribution of oatp1 to the Na^+ -independent uptake of TC, CA, estrone-3-sulfate, and E3040 sulfate into cultured hepatocytes was 60%, 52%, 27%, and 21%, respectively (Table 1).

Transfection of oatp1 did not affect the uptake of E3040 glucuronide, ibuprofen, pravastatin, DNP-SG, and ouabain by COS-7 cells (Fig. 4), although the Na^+ -independent uptake of these ligands into rat hepatocytes was observed. Accordingly, the contribution of oatp1 to the uptake of these ligands into hepatocytes was minimal (Table 1).

Discussion

In the present study, we compared the ligand transport between primary cultured rat hepatocytes and oatp1-transfected COS-7 cells. Because it has been reported that the expression of transporters and their function is reduced in hepatocytes cultured for more than 6 h (Ishigami et al., 1995; Liang et al., 1993), the culture period was restricted to 4 h or less in the present study (Torchia et al., 1996).

The expression of oatp1 cDNA was studied in transfected COS-7 cells (Fig. 1). Northern blot analysis indicated that the length of the transcript (approximately 4.3 and 2.5 kb; Fig. 1) was similar to that observed in liver (approximately 4.3 kb; Fig. 1), which is in agreement with previous reports. Jacquemin et al. (1994) reported that oatp1 cDNA hybridized with several mRNAs from rat liver (4.3, 3.3, 2.5, and 1.4 kb), and the strongest hybridization signal was observed with mRNA of 4.3 and 3.3 kb. In addition, Bergwerk et al. (1996) indicated that oatp1 cDNA hybridized with RNA of 4.3 and 3.3 kb isolated from rat liver by Northern blot analysis under high-stringency conditions. Western blot analysis indicated that the molecular mass of oatp1 expressed in COS-7 cells (approximately 72 kDa; Fig. 1) was smaller than that in rat liver (approximately 83 kDa; Fig. 1). This lower molecular mass of oatp1 in the transfected COS-7 cells may be accounted for by a much lower degree of glycosylation of this transporter; previous Western blot analysis indicated that the molecular mass of oatp1 in rat liver was 80 kDa and that in oatp1-transfected HeLa cells with vaccinia virus was 70 kDa. In addition, preincubation of rat sinusoidal membrane subfractions with *N*-glycanase resulted in a shift of oatp1 migration from 80 to 65 kDa (Bergwerk et al., 1996). Collectively, the

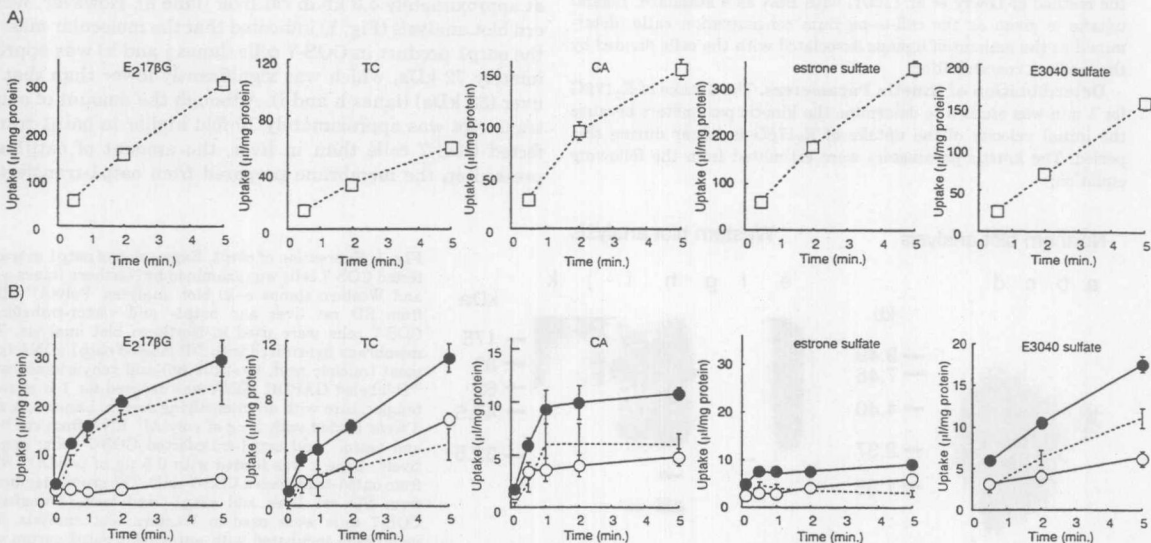


Fig. 2. Time profiles for the uptake of ligands by primary cultured hepatocytes and oatp1-transfected COS-7 cells. Uptake of $\text{E}_217\beta\text{G}$ ($1 \mu\text{M}$), TC ($1 \mu\text{M}$), CA ($1 \mu\text{M}$), estrone-3-sulfate (estrone sulfate; $1 \mu\text{M}$), and E3040 sulfate ($2 \mu\text{M}$) by primary cultured hepatocytes (top) and oatp1-transfected COS-7 cells (bottom) was examined. For experiments in COS-7 cells, \circ and \bullet represent the uptake into vector- and oatp1-transfected COS-7 cells, respectively. Dotted lines represent the oatp1-mediated uptake, which was determined as the differences in the uptake between oatp1- and vector-transfected cells. Each symbol and vertical bar represents the mean \pm S.E. of determinations. The number of the determinations of the uptake of $\text{E}_217\beta\text{G}$, TC, CA, estrone sulfate, and E3040 sulfate by hepatocytes were 30, 33, 9, 6, and 9 in 10, 11, 3, 2, and 3 independent preparations, respectively. The number of the determinations of the uptake of $\text{E}_217\beta\text{G}$, TC, CA, estrone sulfate, and E3040 sulfate by COS-7 cells were 9, 9, 6, 6, and 6 in 3, 3, 2, 2, and 2 independent preparations, respectively.

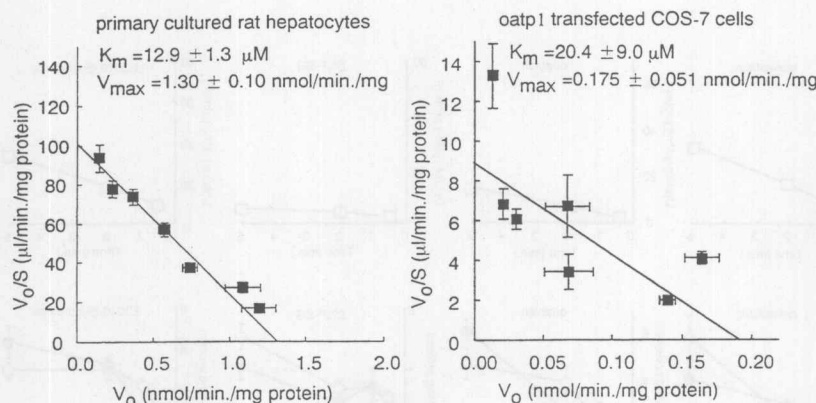


Fig. 3. Saturation of the Na^+ -independent and oatp1-mediated uptake of $\text{E}_217\beta\text{G}$. Saturation of the Na^+ -independent uptake of $\text{E}_217\beta\text{G}$ by primary cultured hepatocytes was determined in the absence of Na^+ (left). oatp1-mediated uptake of $\text{E}_217\beta\text{G}$ by transfected COS-7 cells was determined as the differences in the uptake between oatp1- and vector-transfected COS-7 cells (right). Each symbol and vertical bar represents the mean \pm S.E. of three independent determinations.

TABLE 1

Contribution of oatp1 to Na^+ -independent uptake of ligands into rat hepatocytes

Data shown in Figs. 2 and 4 were used to determine the clearance for the Na^+ -independent uptake by hepatocytes and that for oatp1-mediated uptake by transfected COS-7 cells. Based on these clearance values, R_{hep} and R_{COS} values were determined to calculate the contribution of oatp1.

Compound	CL_{uptake} (Hepatocyte) $\mu\text{l/min/mg protein}$	R_{hep}	CL_{uptake} (COS-7) $\mu\text{l/min/mg protein}$	R_{COS}	Contribution of oatp1 %
$\text{E}_217\beta\text{G}$	63.1	1.00	19.0	1.00	100
Taurocholate	12.8	0.203	2.33	0.123	60
Cholate	39.8	0.631	6.24	0.329	52
Estrone sulfate	77.7	1.23	6.30	0.332	27
E3040 sulfate	29.1	0.461	1.80	0.0947	21
E3040 glucuronide	5.61	0.0889	ND	ND	ND
Ibuprofen	24.5	0.388	ND	ND	ND
Pravastatin	6.20	0.0983	ND	ND	ND
Ouabain	2.80	0.0444	ND	ND	ND
DNP-SG	1.30	0.0206	ND	ND	ND

results of the present study may be accounted for if the glycosylation of oatp1 in transfected COS-7 cells is minimal. In our previous study, Western blot analysis indicated that the molecular mass of Ntcp expressed in COS-7 cells (approximately 33 kDa) was also much smaller than that in cultured hepatocytes (approximately 51 kDa), which may be due to the lower degree of glycosylation (Kouzuki et al., 1998). As shown in Fig. 1, we found that although the mRNA levels in COS-7 cells are approximately 10-fold higher than that in liver, the expression of oatp1 in oatp1-transfected COS-7 cells is approximately one-seventh that in liver. Such a difference in the expression level between mRNA and protein has been observed frequently. The results can be accounted for by low efficiency of transcription in COS-7 cells and/or by less stability of oatp1 protein in COS-7 cells. The latter explanation is also plausible because 1) we found that oatp1 product in COS-7 cells was less glycosylated and 2) it has been demonstrated that less glycosylation results in the instability of proteins (Schinkel et al., 1993). These results are also consistent with the expression of Ntcp in the transfected COS-7 cells (Kouzuki et al., 1998).

Our kinetic analysis indicated that the K_m value for $\text{E}_217\beta\text{G}$ was similar in cultured hepatocytes and oatp1-transfected COS-7 cells (12.9 versus 20.4 μM), although the deviation was associated with the data (Fig. 3). These values are in agreement with previous reports in which the K_m of oatp1 for $\text{E}_217\beta\text{G}$ was examined in cRNA-injected oocytes (3 μM) (Bossuyt et al., 1996), in cDNA-transfected HeLa cells with vaccinia virus (3.2 μM) (Kanai et al., 1996), and in sinusoidal

membrane vesicles isolated from rat liver (4.5–13 μM) (Brouwer et al., 1987; Vore and Hoffman, 1994). We found that the V_{max} for $\text{E}_217\beta\text{G}$ in oatp1-transfected COS-7 cells was approximately one-seventh that in hepatocytes (1.30 versus 0.175 nmol/min/mg protein) (Fig. 3). Because the Western blot analysis revealed that the expression of oatp1 in the transfected COS-7 cells is one-seventh that in the liver after correction of the background level (Fig. 1), this suggested that the glycosylation of oatp1 may have no effect on either the affinity or the velocity of transport. In addition, the transfected COS-7 cells may be used to quantitatively predict oatp1-activity in hepatocytes after correction of its expressed amount by Western blot analysis.

Using oatp1-transfected cells, we showed that TC, CA, estrone-3-sulfate, and E3040 sulfate are substrates for oatp1 (Fig. 2). These results are consistent with previous works; Jacquemin et al. (1994) and Bossuyt et al. (1996) found that injection of oatp1 cRNA into oocytes stimulated the uptake of TC, CA and estrone-3-sulfate. Kanai et al. (1996) demonstrated that the CL_{uptake} of TC into oatp1-transfected HeLa cells with vaccinia virus was approximately 6-fold lower than that of $\text{E}_217\beta\text{G}$ (0.53 versus 3.1 $\mu\text{l/min/mg protein}$), which is comparable with the present observation; in oatp1-transfected COS-7 cells, CL_{uptake} of TC was approximately 8-fold lower than that of $\text{E}_217\beta\text{G}$ (2.3 versus 19 $\mu\text{l/min/mg protein}$; Table 1). By using $\text{E}_217\beta\text{G}$ as a reference compound, we could determine the contribution of oatp1 to the hepatic uptake of ligands. Our kinetic analysis indicated that 50 to 60% of the Na^+ -independent hepatic uptake of TC and CA is accounted

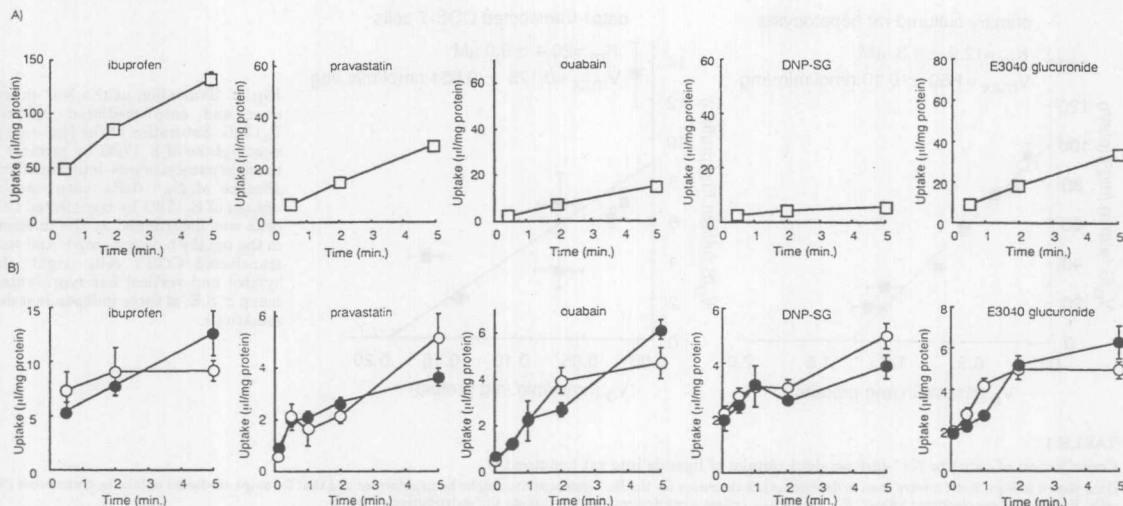


Fig. 4. Time profiles for the uptake of ligands by primary cultured hepatocytes and oatp1-transfected COS-7 cells. Uptake of ibuprofen (1 μ M), pravastatin (1 μ M), ouabain (1 μ M), DNP-SG (1 μ M), and E3040 glucuronide (10 μ M) by primary cultured hepatocytes (top) and oatp1-transfected COS-7 cells (bottom) was examined. For experiments in COS-7 cells, \circ and \bullet represent the uptake into vector- and oatp1-transfected COS-7 cells, respectively. Each symbol and vertical bar represents the mean \pm S.E. of determinations. The number of the determinations of the uptake of ibuprofen, pravastatin, ouabain, DNP-SG, and E3040 glucuronide by hepatocytes were 9, 9, 9, 3, and 3 in 3, 3, 3, 1, and 1 independent preparations, respectively. The number of the determinations of the uptake of ibuprofen, pravastatin, ouabain, DNP-SG, and E3040 glucuronide by COS-7 cells were 6, 9, 6, 3, and 3 in 2, 3, 2, 1, and 1 independent preparations, respectively.

for by oatp1 (Table 1). Because it has been shown that approximately 65% and 70% of hepatocellular uptake of TC and CA in the absence of Na^+ are accounted for by a saturable processes (Yamazaki et al., 1993b), the contribution of oatp1 to the Na^+ -independent specific uptake of these bile acids may be higher than 50 to 60%. In contrast, oatp1 contributed approximately 27% and 21% to the Na^+ -independent uptake of estrone-3-sulfate and E3040 sulfate, respectively (Table 1), suggesting that another transporter(s) may be involved in the Na^+ -independent uptake of these ligands. For E3040 sulfate, more than 80% of Na^+ -independent hepatocellular uptake was accounted for by a carrier-mediated mechanism (Takenaka et al., 1997).

We also examined the uptake of E3040 glucuronide, ibuprofen, pravastatin, DNP-SG, and ouabain in oatp1-transfected COS-7 cells. Although these ligands were taken up by the cultured hepatocytes, and indeed, the contribution of carrier-mediated uptake has been determined as more than 80% for E3040 glucuronide, pravastatin, and ouabain (Eaton and Klaassen, 1978; Takenaka et al., 1997; Yamazaki et al., 1993a), transfection of oatp1 did not stimulate the uptake into COS-7 cells (Fig. 4). Some of the present results are inconsistent with previously accepted ideas; we demonstrated that the hepatic uptake of pravastatin is mediated by a Na^+ -independent mechanism and that the uptake is competitively inhibited by DBSP and bile acids (such as TC and CA; Yamazaki et al., 1993a). These results are consistent with the hypothesis that the hepatic uptake of pravastatin is mediated by a "multispecific organic anion transporter" (Meier, 1988). The results of the present study, however, indicate that oatp1 does not predominantly mediate the transport of pravastatin (Table 1). It is therefore necessary to assume the presence of multiple transporters to provide a molecular basis for the concept that the hepatic uptake of

organic anions is mediated by a multispecific organic anion transporter, which has been established from a kinetic analysis of the experimented data obtained in isolated/cultured hepatocytes and isolated sinusoidal membrane vesicles (Meier, 1988). It is plausible that oatp2, a homolog of oatp1, may also responsible for the hepatic uptake of organic anions (Noe et al., 1997). Such inconsistency in the observations between the hepatocytes and gene product has been reported previously; although mutual competitive inhibition is observed between TC and bumetanide in hepatocytes (Blitzer et al., 1982; Petzinger et al., 1989), Ntcp does not mediate the Na^+ -dependent uptake of bumetanide (Petzinger et al., 1996).

Although Bossuyt et al. (1996) demonstrated that injection of oatp1 cRNA into oocytes stimulated the uptake of ouabain, we found that transfection of oatp1 cDNA did not affect the uptake of this compound into COS-7 cells. This discrepancy may be accounted for by considering the background level of uptake in COS-7 cells. The CL_{uptake} of $E_{217\beta}G$ and ouabain into oocytes injected with oatp1 cRNA was approximately 9.7 and 0.21 nl/min/oocyte, respectively (Bossuyt et al., 1996). If the uptake of ouabain is mediated by oatp1 in the transfected COS-7 cells, the CL_{uptake} of ouabain is calculated to be 0.41 μ l/min/mg protein from that of $E_{217\beta}G$ (19 μ l/min/mg protein; Table 1), whereas that of ouabain into vector-transfected COS-7 cells was 1.8 μ l/min/mg protein (Fig. 4). It is possible that the background level of uptake in COS-7 cells prevented detection of the oatp1-mediated transport of ouabain. Because it was recently demonstrated that oatp2 mediates the uptake of ouabain, the contribution of oatp2 to the hepatic uptake of the cardiac glycoside may be predominant (Noe et al., 1997).

The limitation of the present method to determine quantitatively the contribution of oatp1 to the hepatic uptake of

substrates is related to the assumption that $E_217\beta G$ is predominantly taken up by hepatocytes via oatp1. However, Brouwer et al. (1987) indicated that at least two transport systems are involved in the hepatocellular uptake of $E_217\beta G$ (kinetic parameters: $K_{m1} = 4.54 \mu M$; $V_{max1} = 0.149 \text{ nmol/min/mg protein}$; $K_{m2} = 149 \mu M$; $V_{max2} = 0.641 \text{ nmol/min/mg protein}$). More recently, oatp2 has been cloned from rat brain as a homolog of oatp1. Indeed, oatp2 is also expressed in hepatocytes and accepts $E_217\beta G$ as the substrate (Noe et al., 1997). Collectively, we must be cautious in the interpretation of the data because the magnitude of the contribution of oatp1 may be overestimated if other identified or unidentified membrane protein or proteins are responsible for the hepatic uptake of $E_217\beta G$. Although it was suggested that the uptake of $E_217\beta G$ by hepatocytes is predominantly mediated by oatp1 based on the agreement in the V_{max} value for the uptake of $E_217\beta G$ and the expression level of oatp1 protein between hepatocytes and transfected COS-7 cells, a full answer to this question may be obtained by investigation of the effect of antisense against oatp1 on the uptake of $E_217\beta G$ by oocytes given an injection of total rat liver mRNA. No such experiment has been performed for $E_217\beta G$, whereas it was shown that the simultaneous injection of antisense against oatp1 with total liver mRNA into the oocytes resulted in a reduction in the Na^+ -independent uptake of TC and BSP by 80% and 50%, respectively (Hagenbuch et al., 1996). Regardless of the limitation, the use of the present method should be still meaningful because we could demonstrate the role of additional transporters for the Na^+ -independent hepatic uptake of estrone-3-sulfate, E3040 sulfate, E3040 glucuronide, ibuprofen, pravastatin, DNP-SG, and ouabain.

In conclusion, a comparison of the uptake by rat hepatocytes and oatp1-transfected COS-7 cells suggested that more than 50 to 60% of the Na^+ -independent uptake of TC and CA is mediated by oatp1, whereas oatp1 accounted for only 20 to 30% of the Na^+ -independent uptake of estrone-3-sulfate and E3040 sulfate. In addition, the contribution of oatp1 to the uptake of E3040 glucuronide, ibuprofen, pravastatin, DNP-SG, and ouabain was minimal, suggesting the presence of multispecificity for the Na^+ -independent transport mechanism across the sinusoidal membrane.

Acknowledgments

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Carrier-Mediated Hepatic Uptake of Peptidic Endothelin Antagonists in Rats¹

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ABSTRACT

The endothelin antagonist BQ-123, an anionic cyclopentapeptide, is taken up by rat hepatocytes through active transport systems. Here, we have examined the hepatocellular uptake mechanism for several BQ-123 derivatives with anionic charges using isolated rat hepatocytes. BQ-485, a linear peptide, BQ-518, a cyclic peptide, and compound A, a cyclic peptide with a cationic moiety, were taken up by hepatocytes in a concentration-dependent manner. The uptake of BQ-485 was most efficient, whereas compound A showed comparable uptake with BQ-123. The uptake of these peptides was Na⁺- and energy-dependent, suggesting that active transport mechanisms are involved in their uptake into hepatocytes. BQ-485, BQ-518, and compound A can almost completely inhibit both the Na⁺-de-

pendent and -independent uptake of [³H]BQ-123, with inhibition constants (*K_i*) that are comparable to the Michaelis-Menten constants (*K_m*) for their Na⁺-dependent and -independent uptake, respectively. Inhibition by BQ-485 was competitive, and the uptake of BQ-485 can be inhibited by BQ-123, with *K_i* values that are comparable with the *K_m* values for BQ-123 uptake. The uptake of BQ-123 by COS-7 cells transfected with either Na⁺-dependent taurocholate-cotransporting polypeptide (Ntcp) or Na⁺-independent basolateral organic anion-transporting polypeptide (oatp1) was minimal. Thus, these three peptides share the transporters that also recognize BQ-123 but appear to differ from Ntcp and oatp1.

BQ-123, initially developed as a selective receptor antagonist for endothelin (ET) receptor subtype ET_A is a cyclic pentapeptide with a single carboxyl group (Mino et al., 1992; Itoh et al., 1993). BQ-123 exhibits a variety of biological and therapeutic activities, including an antihypertensive effect as well as a protective effect against proximal tubular necrosis in acute renal failure due to ischemia in rats (Mino et al., 1992; Itoh et al., 1993). It is also stable to enzymatic degradation in the gastrointestinal tract and the liver. In spite of its therapeutic potential and stability, the compound is quickly eliminated from the systemic circulation with a short half-life because of rapid transport from the circulating plasma into the bile (Shin et al., 1997). Almost 90% of an i.v. dose is recovered in the bile unchanged in rats (Nakamura et al., 1996). This short plasma half-life limits its usefulness as a therapeutic agent.

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Studies have recently reported, as mechanisms for the hepatic uptake of peptides, specific carrier-mediated transport systems on the sinusoidal membrane of hepatocytes for some peptides, e.g., octreotide (Terasaki et al., 1995; Yamada et al., 1996), cholecystokinin (Gores et al., 1986; Gores et al., 1989), renin inhibitors (Bertrams and Ziegler, 1991a,b), and cyclosomatostatin (Ziegler et al., 1988, 1991). A hepatic uptake mechanism of BQ-123 has also been studied recently (Nakamura et al., 1996). The compound is taken up extensively by isolated rat hepatocytes in both a Na⁺-dependent and Na⁺-independent manner. The Na⁺-dependent system transports BQ-123 with high affinity and low capacity, whereas the Na⁺-independent system has low affinity and high capacity (Nakamura et al., 1996). Both of these uptake systems were found to be active transport systems and could be inhibited by bile acid (taurocholate) and an organic anion (dibromosulphophthalein) (Nakamura et al., 1996). The existence of these transport systems may explain the large hepatic distribution and short half-life of this compound in plasma (Nakamura et al., 1996; Shin et al., 1996).

To find a long-acting ET receptor antagonist, we synthe-

ABBREVIATIONS: ET, endothelin; Ntcp, Na⁺-dependent taurocholate-cotransporting polypeptide; oatp, Na⁺-independent basolateral organic anion-transporting polypeptide; DBSP, dibromosulphophthalein; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; TCA, taurocholic acid; E2-17G, estradiol 17β-D-glucuronide; a008, azidobenzamido-008.