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# Uptake of Ursodeoxycholate and Its Conjugates by Human Hepatocytes: Role of $Na^+$-Taurocholate Cotransporting Polypeptide (NTCP), Organic Anion Transporting Polypeptide (OATP) 1B1 (OATP-C), and OATP1B3 (OATP8)

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**Abstract:** Ursodeoxycholate (UDCA) is widely used for the treatment of cholestatic liver disease. After oral administration, UDCA is absorbed, taken up efficiently by hepatocytes, and conjugated mainly with glycine to form glycoursodeoxycholate (GUDC) or partly with taurine to form tauroursodeoxycholate (TUDC), which undergo enterohepatic circulation. In this study, to check whether three basolateral transporters—$Na^+$-taurocholate cotransporting polypeptide (NTCP, SLC10A1), organic anion transporting polypeptide (OATP) 1B1 (OATP-C), and OATP1B3 (OATP8)—mediate uptake of UDCA, GUDC, and TUDC by human hepatocytes, we investigated their transport properties using transporter-expressing HEK293 cells and human cryopreserved hepatocytes. TUDC and GUDC could be taken up via human NTCP, OATP1B1, and OATP1B3, whereas UDCA could be transported significantly by NTCP, but not OATP1B1 and OATP1B3 in our expression systems. We observed a time-dependent and saturable uptake of UDCA and its conjugates by human cryopreserved hepatocytes, and more than half of the overall uptake involved a saturable component. Kinetic analyses revealed that the contribution of $Na^+$-dependent and -independent pathways to the uptake of UDCA or TUDC was very similar, while the $Na^+$-independent uptake of GUDC was predominant. These results suggest that UDCA and its conjugates are taken up by both multiple saturable transport systems and nonsaturable transport in human liver with different contributions. These results provide an explanation for the efficient hepatic clearance of UDCA and its conjugates in patients receiving UDCA therapy.

**Keywords:** Ursodeoxycholate; organic anion transporting polypeptide; OATP; $Na^+$-taurocholate cotransporting polypeptide (NTCP); hepatic uptake; human hepatocytes

## Introduction

Ursodeoxycholate (UDCA) is a natural, noncytotoxic bile acid that is used as a drug for the treatment of various cholestatic disorders,[1,2] though the exact molecular mechanisms of its pharmacological actions have not been clarified.[2] Under normal conditions, UDCA accounts for only about 3% of the total bile acids in human bile. However, the fraction of UDCA conjugates in total bile acids increases up to 30−50% in patients receiving UDCA.[3] Lindor et al. reported that, after repetitive administration of UDCA for 2

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(1) Hofmann, A. F. The continuing importance of bile acids in liver and intestinal disease. *Arch. Intern. Med.* **1999**, *159*, 2647−2658.

(2) Paumgartner, G.; Beuers, U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. *Hepatology* **2002**, *36*, 525−531.



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years to primary biliary cirrhosis patients, the percentage of UDCA in serum bile acids is well correlated with that in biliary bile acids, but in some patients plasma bile acids were enriched with UDCA, but not biliary bile acids.[4] They also reported that changes in biliary UDCA concentration, rather than in serum concentration, correlated well with the decrease in the concentration of alkaline phosphatase, aspartate aminotransferase (AST), and bilirubin in the serum, which are clinical markers of hepatic function.[4] Therefore, in order to predict the effectiveness of the treatment for individual patients, it is important to understand the molecular mechanism of UDCA accumulation in the bile.

After oral administration of UDCA, it is absorbed from the small intestine, efficiently extracted by hepatocytes, and conjugated mainly with glycine to give glycoursodeoxycholate (GUDC) and partly with taurine, to give tauroursodeoxycholate (TUDC). GUDC and TUDC are secreted into bile and join endogenous bile acids circulating enterohepatically. So the hepatic uptake process of UDCA and its conjugates partly contributes to their long-time retention in bile.

Traditionally, the hepatic uptake of bile acids consists of $Na^+$-independent and $Na^+$-dependent pathways.[5] For example, cholate is taken up into isolated rat hepatocytes mainly in a $Na^+$-independent manner, while about 80% of the taurocholate uptake is mediated by the $Na^+$-dependent route.[6] At the present time, it is known that $Na^+$-taurocholate cotransporting polypeptide (NTCP) is involved in $Na^+$-dependent uptake,[7] whereas the organic anion transporting polypeptide (OATP) family, also present on the basolateral membrane, is responsible for $Na^+$-independent uptake.[8] OATP1B1 (OATP-C) and OATP1B3 (OATP8) are expressed exclusively in the liver and transport a wide variety of endogenous compounds as well as drugs.[8] They can also transport not only unconjugated bile acids (e.g., cholate) but also conjugated bile acids (e.g., glycocholate and taurocholate).[8] Regarding the uptake mechanism of UDCA and its conjugates in humans, it has been reported that TUDC can be a substrate of NTCP and OATP1A2 (OATP-A),[9,10] although the role of OATP1A2 in its hepatic uptake is thought to be minor because the expression level of OATP1A2 in human liver is very low.[11] On the other hand, some previous reports have shown that TUDC can be transported by rat Ntcp, Oatp1a1 (Oatp1),[12] Oatp1a4 (Oatp2),[13] and Oatp1a5 (Oatp3),[14] and GUDC can be transported by Oatp1a5 in rats.[14] Which transporters, if any, mediate the transport of UDCA has not been clarified. Poupon et al. used perfused rat liver to demonstrate that UDCA can be taken up in a saturable manner, which indicates the involvement of transporters in the hepatic uptake of UDCA.[15] Because there is no strict one-to-one relationship between rodent Oatps and human OATPs and the composition of conjugated bile acids in bile differs markedly between rats and humans, it seemed to be important for us to perform transport analyses of UDCA and its conjugates using human gene products and human hepatocytes. In this study, we have characterized the transport of UDCA, GUDC, and TUDC by NTCP, OATP1B1, and OATP1B3 using transporter expression systems and human cryopreserved hepatocytes.

## Experimental Section.

**Materials.** [$^3$H]Ursodeoxycholate (20 Ci/mmol) was obtained from SibTech Inc. (Newington, CT) by customized synthesis. 22,23-[$^3$H]Tauroursodeoxycholate (10 Ci/mmol) and 22,23-[$^3$H]glycoursodeoxycholate (11 Ci/mmol) were synthesized by reductive tritiation of the unsaturated precursor (see ref 16). [$^3$H]Taurocholate (2 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled sodium ursodeoxycholate, sodium tauroursode-


(3) Invernizzi, P.; Setchell, K. D.; Crosignani, A.; Battezzati, P. M.; Larghi, A.; O'Connell, N. C.; Podda, M. Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. *Hepatology* **1999**, *29*, 320−327.

(4) Lindor, K. D.; Lacerda, M. A.; Jorgensen, R. A.; DeSotel, C. K.; Batta, A. K.; Salen, G.; Dickson, E. R.; Rossi, S. S.; Hofmann, A. F. Relationship between biliary and serum bile acids and response to ursodeoxycholic acid in patients with primary biliary cirrhosis. *Am. J. Gastroenterol.* **1998**, *93*, 1498−1504.

(5) Van Dyke, R. W.; Stephens, J. E.; Scharschmidt, B. F. Bile acid transport in cultured rat hepatocytes. *Am. J. Physiol.* **1982**, *243*, G484−492.

(6) Anwer, M. S.; Hegner, D. Effect of Na on bile acid uptake by isolated rat hepatocytes. Evidence for a heterogeneous system. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 181−192.

(7) Hagenbuch, B.; Meier, P. J. Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *J. Clin. Invest.* **1994**, *93*, 1326−1331.

(8) Hagenbuch, B.; Meier, P. J. The superfamily of organic anion transporting polypeptides. *Biochim. Biophys. Acta* **2003**, *1609*, 1−18.

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

(10) Kullak-Ublick, G. A.; Hagenbuch, B.; Stieger, B.; Schteingart, C. D.; Hofmann, A. F.; Wolkoff, A. W.; Meier, P. J. Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology* **1995**, *109*, 1274−1282.

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

(12) Eckhardt, U.; Schroeder, A.; Stieger, B.; Hochli, M.; Landmann, L.; Tynes, R.; Meier, P. J.; Hagenbuch, B. Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. *Am. J. Physiol.* **1999**, *276*, G1037−1042.

(13) Reichel, C.; Gao, B.; Van Montfoort, J.; Cattori, V.; Rahner, C.; Hagenbuch, B.; Stieger, B.; Kamisako, T.; Meier, P. J. Localization and function of the organic anion-transporting polypeptide Oatp2 in rat liver. *Gastroenterology* **1999**, *117*, 688−695.

(14) Walters, H. C.; Craddock, A. L.; Fusegawa, H.; Willingham, M. C.; Dawson, P. A. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol.* **2000**, *279*, G1188−1200.

(15) Poupon, R.; Chretien, Y.; Parquet, M.; Ballet, F.; Rey, C.; Infante, R. Hepatic transport of bile acids in the isolated perfused rat liver. Structure-kinetic relationship. *Biochem. Pharmacol.* **1988**, *37*, 209−212.

oxycholate, and sodium glycoursodeoxycholate were kindly provided by Mitsubishi Pharma Co. (Osaka, Japan). Unlabeled taurocholate was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were commercially available and of reagent grade.

**Construction of Human NTCP-Expressing Cells.** The human NTCP cDNA was amplified by PCR using human liver cDNA purchased from BD Biosciences Clontech (Palo Alto, CA) as a template. The sequences of the forward and reverse primer to obtain the full-length NTCP cDNA were 5′-GCTAGCATCGATGCCGCCATGGAGGCCCACAACGCGTC-3′ and 5′-AAGCTTGCGGCCGCCTAGGCTGTGCAAGGGGAGC-3′. The amplified cDNA fragment was TA-cloned into the pGEM vector (Promega, CA). Then, human NTCP-cloned vector was digested with *Nhe*I and *Hin*dIII and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). HEK293 cells were transfected with expression vector using FuGENE6 (Roche Diagnostics Corp., Indianapolis, IN), according to the manufacturer's instruction. After G418 selection (800 $\mu$g/mL) for 3 weeks, single colonies were screened using the transport activity of taurocholate, and the clone with the highest activity was maintained and used for further analyses.

**Cell Culture.** Transporter-expressing HEK293 cells and vector-control cells were grown in Dulbecco's modified Eagle's medium (low glucose version) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 $\mu$g/mL streptomycin at 37 °C with 5% $CO_2$ and 95% humidity.

**Transport Assay in Transporter Expression Systems.** The transport study was carried out as described previously.[17] Cells were seeded in 12-well plates at a density of $1.5 \times 10^5$ cells/well. Cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate 24 h before transport assay to induce the expression of OATP1B1, OATP1B3, and OAT2. 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. To measure the $Na^+$-independent uptake, sodium chloride and sodium bicarbonate in Krebs−Henseleit buffer were replaced with choline chloride and choline bicarbonate. The Krebs−Henseleit buffer consists of 118 mM NaCl, 23 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM $KH_2PO_4$, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4. 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 $\mu$L of 0.2 N NaOH, and kept overnight at 4 °C. Aliquots (500 $\mu$L) were transferred to scintillation vials after addition of 250 $\mu$L of 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 addition of 2 mL of scintillation fluid (Clear-sol I, NACALAI TESQUE, Kyoto, Japan) to the scintillation vials. The remaining 50 $\mu$L of the aliquots of cell lysate was used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

**Transport by Human Cryopreserved Hepatocytes.** This experiment was performed as described previously.[18] Cryopreserved human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD). Just before the study, the hepatocytes (1 mL suspension) were thawed at 37 °C, then immediately 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 cells were resuspended in the same buffer to give a cell density of $1.0 \times 10^6$ viable cells/mL for the uptake study. The number of viable cells was determined by trypan blue staining. The average fraction of viable cells in human cryopreserved hepatocytes was 65−80%. 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 addition of 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 $\mu$L of incubation mixture was collected and placed in a centrifuge tube (450 $\mu$L) containing 50 $\mu$L of 2 N NaOH under a layer of 100 $\mu$L of oil (density, 1.015, a mixture of silicone oil and mineral oil; Sigma-Aldrich, St. Louis, MO), and subsequently, the sample tube was centrifuged for 10 s using a tabletop centrifuge (10000*g*; 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 $\mu$L of 2 N HCl, mixed with scintillation cocktail, and the radioactivity was measured in a liquid scintillation counter.

**Data Analyses.** The uptake was expressed as the uptake volume ($\mu$L/mg of protein), given as the amount of radioactivity associated with the cells (dpm/mg of protein) divided

(16) Sorscher, S.; Lillienau, J.; Meinkoth, J. L.; Steinbach, J. H.; Schteingart, C. D.; Feramisco, J.; Hofmann, A. F. Conjugated bile acid uptake by Xenopus laevis oocytes induced by microinjection with ileal Poly A+ mRNA. *Biochem. Biophys. Res. Commun.* **1992**, *186*, 1455−1462.

(17) Hirano, M.; Maeda, K.; Shitara, Y.; Sugiyama, Y. Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the Hepatic Uptake of Pitavastatin in Humans. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 139−146.

(18) Shitara, Y.; Li, A. P.; Kato, Y.; Lu, C.; Ito, K.; Itoh, T.; Sugiyama, Y. Function of uptake transporters for taurocholate and estradiol-17beta-D-glucuronide in cryopreserved human hepatocytes. *Drug Metab. Pharmacokinet.* **2003**, *18*, 33−41.

by its concentration in the incubation medium (dpm/$\mu$L). To estimate the hepatic uptake clearance of the substrate in human hepatocytes, uptake clearance [$CL_{2-0.5min}$ ($\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$)] was calculated by subtracting the uptake volume [$V_d$ ($\mu$L ($10^6$ cells)$^{-1}$)] at 0.5 min from that at 2 min as shown in eq 1, because the uptake at the *Y*-intercept (time 0) of the graph is thought to represent nonspecific adsorption to the culture dish or the cell surface of the hepatocytes and 0.5 min is the shortest measurable sampling point from a practical point of view. Also, the saturable hepatic uptake clearance [$CL_{hep}$ ($\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$)] was determined by subtracting the uptake clearance in the presence of excess unlabeled substrate (300 $\mu$M) ($CL_{2-0.5min,excess}$) from that in the presence of 1 $\mu$M substrate ($CL_{2-0.5min,tracer}$) as shown in eq 2.

$$CL_{2-0.5min} = \frac{V_{d,2min} - V_{d,0.5min}}{2 - 0.5} \quad (1)$$

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

Kinetic parameters were obtained using the following equation:

$$v = \frac{V_{max}S}{K_m + S} + P_{dif}S \quad (3)$$

where $v$ is the initial uptake velocity of substrate [pmol min$^{-1}$ (mg of protein (or $10^6$ cells))$^{-1}$], $S$ is the substrate concentration in the incubation buffer ($\mu$M), $K_m$ is the Michaelis−Menten constant ($\mu$M), $V_{max}$ is the maximum uptake velocity [pmol min$^{-1}$ (mg of protein (or $10^6$ cells))$^{-1}$], and $P_{dif}$ is the nonspecific uptake clearance [$\mu$L min$^{-1}$ (mg of protein (or $10^6$ cells))$^{-1}$]. The uptake data were fitted to this equation by a nonlinear least-squares method using a MULTI program.[19] The input data were weighted as the reciprocal of the observed values, and the Damping Gauss Newton method was used as the fitting algorithm.

## Results

**Uptake of Taurocholate, UDCA, and UDCA Conjugates TUDC and GUDC by Human OATP1B1-, OATP1B3-, and OAT2-Expressing HEK293 Cells.** The time course of the uptake of UDCA and its conjugates, TUDC and GUDC, by human OATP1B1- and OATP1B3-expressing HEK293 cells is shown in Figure 1A, B, and C, respectively. TUDC and GUDC showed significantly greater uptake in HEK293 cells expressing OATP1B1 or OATP1B3 compared with vector-transfected cells. The time-dependent uptake of UDCA was observed even in vector-transfected cells, and transfection of OATP1B1 or OATP1B3 to HEK293 cells did not enhance the intracellular accumulation of UDCA. As previously described,[8] significant uptake of taurocholate by our OATP1B1 and OATP1B3 expression system was also observed (Figure 1D). On the other hand, the significant uptake of UDCA, TUDC, GUDC, and TCA was not observed in human OAT2-transfected cells compared with vector-transfected cells (Figure 5).

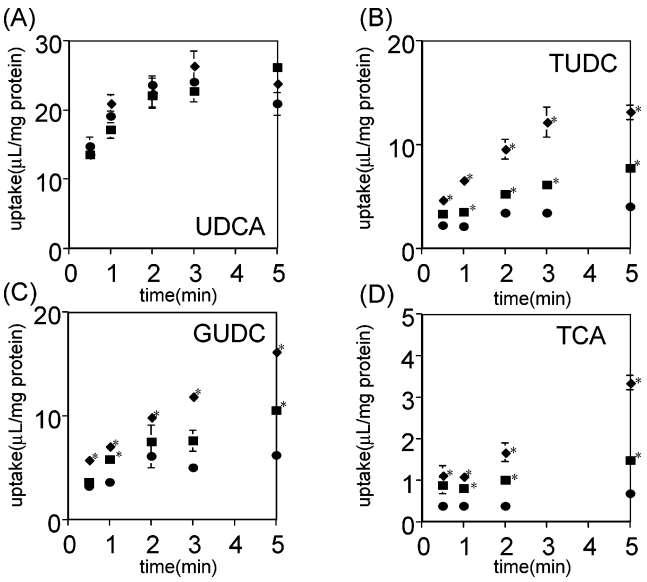


***Figure 1.*** Time course of the uptake of UDCA (A), TUDC (B), GUDC (C), and taurocholate (TCA) (D) by OATP1B1-expressing, OATP1B3-expressing, and vector-transfected HEK293 cells. The concentration of bile acids is 1 $\mu$M. Uptake in OATP1B1-expressing cells is indicated by squares, in OATP1B3-expressing cells by diamonds, and in vector-transfected cells by circles. Each data point and bar represents the mean ± SE ($n = 3$). *$p < 0.05$, significantly different from vector-transfected cells by Student's *t* test.

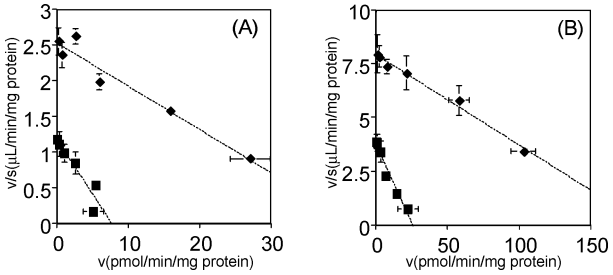


***Figure 2.*** Eadie−Hofstee plots of the OATP1B1- and OATP1B3-mediated uptake of TUDC and GUDC. The concentration dependence (1−100 $\mu$M) of OATP1B1- (squares) and OATP1B3-mediated uptake (diamonds) of TUDC (A) and GUDC (B) was determined. Transporter-mediated uptake was calculated by subtracting the uptake into vector-transfected cells from that into transporter-expressing cells. Each data point and bar represents the mean ± SE ($n = 3$).

Because the uptake of TUDC and GUDC was linear up to 2 min (Figure 1B,C), the initial uptake was investigated as the uptake for 2 min at various substrate concentrations (Figure 2). The uptake of TUDC and GUDC by HEK293 cells expressing OATP1B1 and OATP1B3 was saturated at higher concentration. The Eadie−Hofstee plots shown in Figure 2 indicate that the uptake of TUDC and GUDC by OATP1B1- and OATP1B3-expressed HEK293 cells

(19) Yamaoka, K.; Tanigawara, Y.; Nakagawa, T.; Uno, T. A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobio-Dyn.* **1981**, *4*, 879−885.

***Table 1.*** Kinetic Parameters of OATP1B1-, OATP1B3-, and NTCP-Mediated Uptake of TUDC and GUDC[a]

| | OATP1B1 | | | OATP1B3 | | | NTCP | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ [$\mu$M] | $V_{max}$ [pmol min$^{-1}$ (mg of protein)$^{-1}$] | $V_{max}/K_m$ [$\mu$L min$^{-1}$ (mg of protein)$^{-1}$] | $K_m$ [$\mu$M] | $V_{max}$ [pmol min$^{-1}$ (mg of protein)$^{-1}$] | $V_{max}/K_m$ [$\mu$L min$^{-1}$ (mg of protein)$^{-1}$] | $K_m$ [$\mu$M] | $V_{max}$ [pmol min$^{-1}$ (mg of protein)$^{-1}$] | $V_{max}/K_m$ [$\mu$L min$^{-1}$ (mg of protein)$^{-1}$] |
| TUDC | 7.47 ± 0.99 | 8.71 ± 1.02 | 1.17 ± 0.21 | 15.9 ± 3.3 | 40.2 ± 7.6 | 2.54 ± 0.71 | 3.49 ± 0.34 | 10.1 ± 0.6 | 2.90 ± 0.31 |
| GUDC | 5.17 ± 0.71 | 20.6 ± 2.5 | 3.99 ± 0.73 | 24.7 ± 1.8 | 195 ± 13 | 7.90 ± 0.79 | 0.376 ± 0.174 | 0.358 ± 0.145 | 0.953 ± 0.587 |
| | | | | | | | 25.3 ± 1.8 | 36.7 ± 1.2 | 1.45 ± 0.11 |

[a] The uptake of TUDC and GUDC into OATP1B1, OATP1B3, and NTCP-expressing HEK293 cells was measured at different substrate concentrations. Kinetic parameters were obtained by fitting the Michaelis−Menten equation as described in the Experimental Section. Data represent the mean ± computer calculated SD.

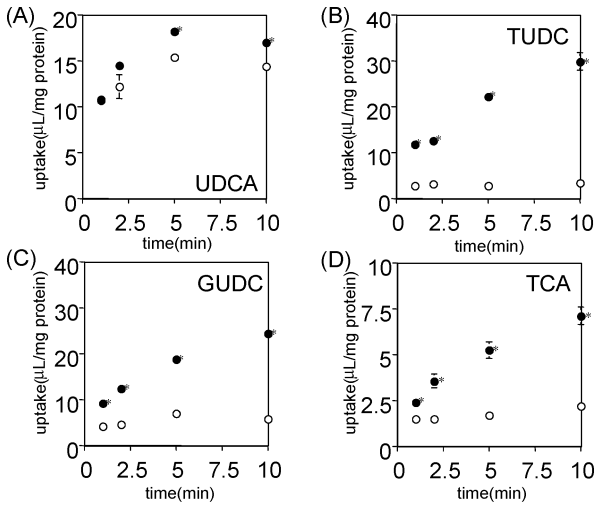


***Figure 3.*** Time course of the uptake of UDCA (A), TUDC (B), GUDC (C), and taurocholate (TCA) (D) by NTCP-expressing and vector-transfected HEK293 cells. The concentration of bile acids is 1 $\mu$M. Uptake in NTCP-expressing cells is indicated by closed circles and in vector-transfected cells by open circles. Each data point and bar represents the mean ± SE ($n = 3$). *$p < 0.05$, significantly different from vector-transfected cells by Student's $t$ test.

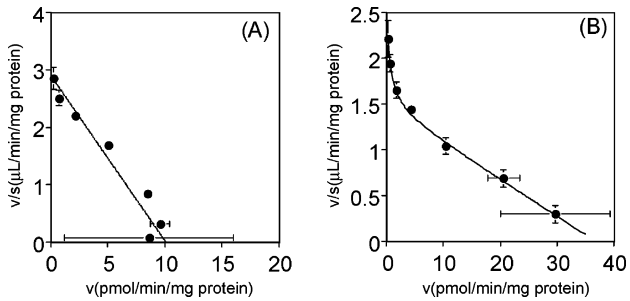


***Figure 4.*** Eadie−Hofstee plots of the NTCP-mediated uptake of TUDC and GUDC. The concentration dependence (1−100 $\mu$M) of NTCP-mediated uptake of TUDC (A) and GUDC (B) was determined. NTCP-mediated uptake was calculated by subtracting the uptake into vector-transfected cells from that into NTCP-expressing cells. Each data point and bar represents the mean ± SE ($n = 3$).

consists of one saturable component. The $K_m$ and $V_{max}$ values are summarized in Table 1. The uptake clearance ($V_{max}/K_m$) of GUDC by OATP1B1- and OATP1B3-expressed cells was about 3 times higher than that of TUDC.

**Uptake of Taurocholate, UDCA, and UDCA Conjugates TUDC and GUDC by Human NTCP-Expressing HEK293 Cells.** The time course of the uptake of UDCA and its conjugates, TUDC and GUDC, by human NTCP-expressing HEK293 cells is shown in Figure 3, plots A, B, and C, respectively. TUDC and GUDC were taken up by HEK293 cells expressing human NTCP more rapidly than vector-transfected cells. NTCP-mediated significant uptake of UDCA was observed, although it was very low. Eadie−Hofstee plots of the NTCP-mediated uptake of TUDC and GUDC are shown in Figure 4. It appears that GUDC exhibits biphasic saturation kinetics, while TUDC has only one saturable component. The $K_m$ and $V_{max}$ values are listed in Table 1.

**Uptake of Taurocholate, UDCA, and UDCA Conjugates TUDC and GUDC by Human Cryopreserved Hepatocytes.** The uptake of UDCA, TUDC, GUDC, and taurocholate by human cryopreserved hepatocytes prepared from three independent donors was investigated. Typical uptake time courses by human cryopreserved hepatocytes from one donor (Lot OCF) are shown in Figure 6. Time-dependent uptake of all compounds was observed, and this decreased in the presence of an excess of unlabeled compounds (Figure 6). The proportion of the saturable uptake clearance of UDCA, TUDC, GUDC, and taurocholate calculated as explained in the Experimental Section under tracer conditions was 64%, 74%, 47%, and 43% of the total uptake clearance, respectively. Replacement of $Na^+$ with choline in the incubation buffer resulted in a partial reduction of their uptake (Figure 6). The results of the uptake experiments using the other two lots of hepatocytes showed the same pattern as described above. The saturable uptake clearance of each compound in the three lots of human hepatocytes in the presence or absence of $Na^+$ is given in Table 2. The average $Na^+$-dependent fraction of the total uptake clearance of UDCA, TUDC, GUDC, and taurocholate obtained from the three lots of hepatocytes was 49%, 55%, 21%, and 63%, respectively.

**Concentration Dependence of $Na^+$-Dependent and -Independent Uptake Clearance of UDCA and Its Conjugates by Human Cryopreserved Hepatocytes.** Figure 7 shows the Eadie−Hofstee plots of the uptake of UDCA, TUDC, and GUDC in the presence or absence of $Na^+$ using

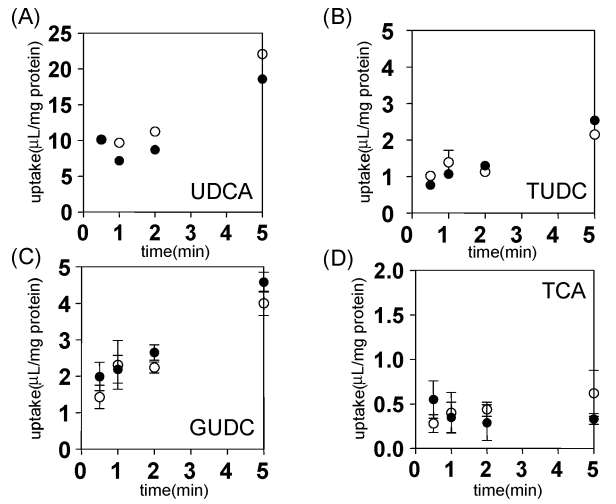


***Figure 5.*** Time course of the uptake of UDCA (A), TUDC (B), GUDC (C), and taurocholate (TCA) (D) by human OAT2-expressing and vector-transfected HEK293 cells. The concentration of bile acids is 1 $\mu$M. Uptake in human OAT2-expressing cells is indicated by closed circles and in vector-transfected cells by open circles. Each data point and bar represents the mean ± SE ($n$ = 3).

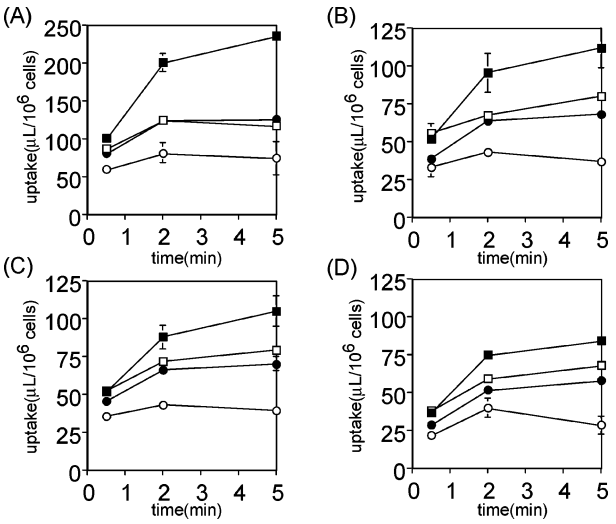


***Figure 6.*** Time profiles for the uptake of UDCA, TUDC, GUDC, and TCA in human cryopreserved hepatocytes (Lot OCF). Uptake of UDCA (A), TUDC (B), GUDC (C), and taurocholate (TCA) (D) into human cryopreserved hepatocytes was measured by incubating cells with 1 $\mu$M (closed symbols) or 300 $\mu$M (open symbols) of each ligand at 37 °C in the presence (squares) or absence of $Na^+$ (circles). The viability of the human hepatocytes used in this experiment was 70%. Each time point and bar represents the mean ± SE of 3 separate determinations.

human cryopreserved hepatocytes of OCF, the lot with the highest uptake activity. One saturable and one nonsaturable model can explain the concentration dependence of each experiment so well. Kinetic parameters for UDCA, TUDC, and GUDC are summarized in Table 3. The $K_m$ and $V_{max}$ values for the $Na^+$-independent uptake of all compounds were smaller than those for uptake in the presence of $Na^+$.

## Discussion

In this study, we focused on the hepatic uptake of UDCA and its conjugates, which is one of the important processes in their enterohepatic circulation. To clarify the involvement of transporters and their contributions to the hepatic uptake, we performed kinetic analyses using transporter-expressing HEK293 cells and human cryopreserved hepatocytes.

First we checked the involvement of transporters in the hepatic uptake of UDCA and its conjugates in human NTCP-, OATP1B1-, OATP1B3-, and OAT2-expressing HEK293 cells. It is generally believed that NTCP is responsible for the $Na^+$-dependent uptake, while OATP1B1 and OATP1B3 are mainly involved in the $Na^+$-independent uptake. Our results indicate that NTCP, OATP1B1, and OATP1B3 transport TUDC and GUDC in a saturable manner. The $K_m$ values for OATP1B1 were lower than those for OATP1B3. The $K_m$ values of TUDC and GUDC obtained in the present study were very similar to reported $K_m$ values for rat Oatps.[12−14] In the case of NTCP, the uptake of GUDC could be explained by a two-saturable model, while TUDC exhibited monophasic saturation. The $K_m$ value of TUDC for human NTCP was lower than that for rat Ntcp (14 $\mu$M).[20] Comparing the reported $K_m$ values of taurocholate for NTCP (6.2, 7.9 $\mu$M)[7,21] and OATP1B1 (10, 33.8 $\mu$M),[22,23] TUDC and GUDC showed higher affinity than taurocholate in human NTCP and OATP1B1 and the relative uptake clearance of TUDC and GUDC was higher than that of taurocholate (Figures 1 and 3).

On the other hand, NTCP showed a very small but significant uptake of UDCA, while no significant uptake via OATP1B1 and OATP1B3 was observed. This result was consistent with the recent report showing that rat Oatp1a1 can transport TUDC and GUDC, but not UDCA.[24] We observed time-dependent association of UDCA in the control cells. Because the uptake of 1 $\mu$M UDCA was not signifi-

(20) Schroeder, A.; Eckhardt, U.; Stieger, B.; Tynes, R.; Schteingart, C. D.; Hofmann, A. F.; Meier, P. J.; Hagenbuch, B. Substrate specificity of the rat liver Na(+)-bile salt cotransporter in Xenopus laevis oocytes and in CHO cells. *Am. J. Physiol.* **1998**, *274*, G370−375.

(21) Kim, R. B.; Leake, B.; Cvetkovic, M.; Roden, M. M.; Nadeau, J.; Walubo, A.; Wilkinson, G. R. Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity. *J. Pharmacol. Exp. Ther.* **1999**, *291*, 1204−1209.

(22) Hsiang, B.; Zhu, Y.; Wang, Z.; Wu, Y.; Sasseville, V.; Yang, W. P.; Kirchgessner, T. G. 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.* **1999**, *274*, 37161−37168.

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

(24) Hata, S.; Wang, P.; Eftychiou, N.; Ananthanarayanan, M.; Batta, A.; Salen, G.; Pang, K. S.; Wolkoff, A. W. Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake. *Am. J. Physiol.* **2003**, *285*, G829−839.

***Table 2.*** $Na^+$-Dependent and -Independent Uptake of TCA, UDCA, TUDC, and GUDC in Human Cryopreserved Hepatocytes[a]

| substrate | | Lot OCF | Lot ETR | Lot 094 | average |
|---|---|---|---|---|---|
| TCA | $Na^+$ (+) | 10.7 ± 2.3 | 4.01 ± 0.93 | 7.49 ± 0.28 | 7.40 ± 0.83 |
| | $Na^+$ (−) | 3.64 ± 4.30 | 1.05 ± 1.08 | 3.62 ± 1.52 | 2.77 ± 1.56 |
| | $Na^+$ dependent | 7.07 ± 4.90 | 2.97 ± 1.43 | 3.87 ± 1.55 | 4.64 ± 1.78 |
| UDCA | $Na^+$ (+) | 42.4 ± 8.2 | 21.5 ± 4.7 | 9.17 ± 3.06 | 24.4 ± 3.3 |
| | $Na^+$ (−) | 15.7 ± 9.8 | 16.1 ± 2.3 | 5.57 ± 2.74 | 12.5 ± 3.5 |
| | $Na^+$ dependent | 26.7 ± 12.8 | 5.43 ± 5.25 | 3.59 ± 4.11 | 11.9 ± 4.8 |
| TUDC | $Na^+$ (+) | 21.5 ± 7.7 | 7.80 ± 2.01 | 6.43 ± 2.62 | 11.9 ± 2.8 |
| | $Na^+$ (−) | 9.86 ± 3.66 | 4.51 ± 0.60 | 1.79 ± 1.25 | 5.39 ± 1.30 |
| | $Na^+$ dependent | 11.7 ± 8.5 | 3.29 ± 2.09 | 4.65 ± 2.91 | 6.55 ± 3.07 |
| GUDC | $Na^+$ (+) | 11.2 ± 3.3 | 3.79 ± 2.57 | 3.24 ± 1.65 | 6.08 ± 1.50 |
| | $Na^+$ (−) | 8.70 ± 2.46 | 1.69 ± 0.95 | 3.94 ± 3.83 | 4.78 ± 1.55 |
| | $Na^+$ dependent | 2.52 ± 4.14 | 2.10 ± 2.73 | (<0) | 1.30 ± 2.16 |

[a] Unit: $\mu$L $min^{-1}$ $(10^6$ cells$)^{-1}$. The uptake of TCA, UDCA, TUDC, and GUDC in three independent lots of human cryopreserved hepatocytes was measured in the presence and absence of $Na^+$. $Na^+$-dependent uptake clearance was calculated by subtracting the clearance in the absence of $Na^+$ from that in the presence of $Na^+$. Data represent the mean ± SE of three separate determinations.

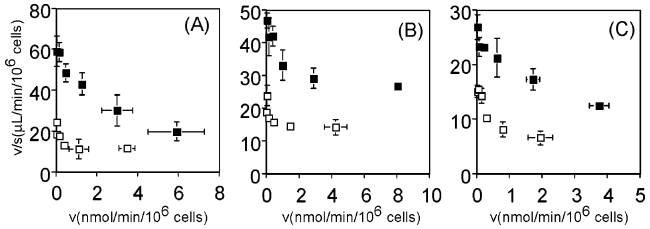


***Figure 7.*** Concentration dependence of the uptake of UDCA, TUDC, and GUDC in human cryopreserved hepatocytes (Lot OCF) in the presence or absence of $Na^+$. The uptake of UDCA (A), TUDC (B), and GUDC (C) into human cryopreserved hepatocytes was measured in the presence (closed squares) or absence of $Na^+$ (open squares). The clearance at each concentration (1−300 $\mu$M) was determined by eq 1 shown in the Experimental Section. The viability of the human hepatocytes used in this experiment was 70%. Each time point and bar represents the mean ± SE of 3 separate determinations.

cantly changed even in the presence of 300 $\mu$M UDCA or 300 $\mu$M probenecid, which was used as a typical inhibitor of organic anion transport systems, and the uptake at 37 °C was not very different from that at 4 °C (data not shown), we confirmed that UDCA is taken up not by an energy-driven active transport mechanism for organic anion in HEK293 cells.

Next, we characterized the uptake of UDCA and its conjugates by human cryopreserved hepatocytes. Although some interbatch differences were observed, more than half of the uptake clearance was saturable, which suggests the importance of transporters in the hepatic uptake of UDCA and its conjugates. The $Na^+$-dependent fraction in the overall uptake was 63% for taurocholate, and about 50% for TUDC and UDCA. On the other hand, it is interesting that the $Na^+$-dependent fraction in overall hepatic uptake of GUDC was only about 20%, which was relatively low compared with UDCA and TUDC. GUDC is the most important molecule as far as the enterohepatic circulation of UDCA is concerned because 70−80% of orally administered UDCA is conjugated with glycine.[3] In rats, UDCA is extensively conjugated with taurine and TUDC undergoes enterohepatic circulation.[25] The uptake of TUDC in isolated rat hepatocytes in the absence of $Na^+$ has been reported to be about $^1/_{13}$ of that in the presence of $Na^+$,[26] indicating that the contribution of the $Na^+$-dependent pathway to the uptake of TUDC in rat hepatocytes is greater than that in human hepatocytes. Taking these findings into consideration, it is possible that $Na^+$-dependent uptake of TUDC is responsible for the efficient enterohepatic circulation of UDCA in rats, while the $Na^+$-independent pathway is also important for the enterohepatic circulation of UDCA (as its glycine conjugate) in humans.

Then, we performed self-inhibition experiments in the presence and absence of $Na^+$ using human cryopreserved hepatocytes (Table 3). The $K_m$ values of TUDC and GUDC uptake in the presence of $Na^+$ were higher than that of taurocholate (2−8 $\mu$M),[18] while those in the absence of $Na^+$ were close to those of OATPs. We have already developed a method for determining the contribution of OATP1B1 and OATP1B3 to the hepatic uptake of a variety of compounds.[17] Applying this method to the estimation, the contribution of OATP1B1 was almost the same as that of OATP1B3 to the hepatic uptake of UDCA conjugates. On the other hand, the $K_m$ values of UDCA conjugates in the presence of $Na^+$ were greater than those of NTCP. It has been shown that the $K_m$ value of taurocholate uptake in cryopreserved human hepatocytes (2−8 $\mu$M)[18] was comparable with that in human NTCP-expression systems (6.2, 7.9 $\mu$M),[7,21] implying that the saturation kinetics of taurocholate uptake in human hepatocytes could be explained by the result obtained from the NTCP-expression system. Therefore, this discrepancy between the $K_m$ values of UDCA conjugates in human hepatocytes and the NTCP expression system suggests that

(25) Crosignani, A.; Setchell, K. D.; Invernizzi, P.; Larghi, A.; Rodrigues, C. M.; Podda, M. Clinical pharmacokinetics of therapeutic bile acids. *Clin. Pharmacokinet.* **1996**, *30*, 333−358.
(26) Takikawa, H.; Takemura, T.; Yamanaka, M. The mechanism of tauroursodeoxycholate uptake by isolated rat hepatocytes. *Int. Hepatol. Commun.* **1995**, *3*, 20−25.

***Table 3.*** Kinetic Parameters of the Uptake of UDCA, TUDC, and GUDC in Human Cryopreserved Hepatocytes in the Presence or Absence of $Na^+$ [a]

| | $Na^+$ (+) | | | | $Na^+$ (−) | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ [$\mu$M] | $V_{max}$ [pmol min$^{-1}$ ($10^6$ cells)$^{-1}$] | $V_{max}/K_m$ [$\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$] | $P_{dif}$ [$\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$] | $K_m$ [$\mu$M] | $V_{max}$ [pmol min$^{-1}$ ($10^6$ cells)$^{-1}$] | $V_{max}/K_m$ [$\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$] | $P_{dif}$ [$\mu$L min$^{-1}$ ($10^6$ cells)$^{-1}$] |
| UDCA | 42.1 ± 13.1 | 1860 ± 670 | 44.3 ± 21.1 | 15.6 ± 3.1 | 4.30 ± 2.98 | 66.9 ± 38.3 | 15.5 ± 14.0 | 11.2 ± 1.2 |
| TUDC | 21.2 ± 13.0 | 453 ± 286 | 21.4 ± 18.9 | 25.3 ± 2.3 | 15.2 ± 27.3 | 122 ± 221 | 8.05 ± 20.54 | 13.5 ± 2.7 |
| GUDC | 84.1 ± 33.5 | 1350 ± 721 | 16.0 ± 10.7 | 9.31 ± 2.72 | 32.8 ± 14.1 | 366 ± 164 | 11.1 ± 6.9 | 5.40 ± 1.05 |

[a] The uptake of UDCA, TUDC, and GUDC into human cryopreserved hepatocytes (Lot OCF) was measured in the presence or absence of $Na^+$. Kinetic parameters were obtained by fitting the one-saturable one-nonsaturable model as described in Experimental Section. Data represent the mean ± computer calculated SD.

unknown transporters may be responsible for the $Na^+$-dependent uptake of UDCA conjugates. Microsomal epoxide hydrolase (mEH) might be one of the candidates for the low affinity $Na^+$-dependent transport system because the $K_m$ value of taurocholate uptake via mEH has been reported to be 26.3 $\mu$M,[27] which is greater than that via NTCP (6 $\mu$M), although the functional significance of mEH is controversial.

Regarding the hepatic uptake of UDCA, it has been reported that the contributions of the $Na^+$-dependent and -independent pathways to its hepatic uptake were almost identical in isolated hamster hepatocytes, which is consistent with our results.[28] On the other hand, we did not see any significant uptake of UDCA via OATP1B1 and OATP1B3, which we expected to work as high-affinity $Na^+$-independent transport systems. Britz et al. have reported that Bamet-UD2 (cisplatin conjugated with two molecules of UDCA) can be taken up by OATP1A2 (OATP-A), OATP1B1 (OATP-C), organic cation transporter (OCT) 1, OCT2, and NTCP.[29] Therefore, other transporters may play a role in the $Na^+$-independent uptake of UDCA. Moreover, the rank order of the $Na^+$-dependent uptake of UDCA and its conjugates in human hepatocytes was different from that in the NTCP expression system. Further characterization of the UDCA transport mechanisms will be needed.

In humans, most of UDCA is conjugated with glycine and the serum concentration of conjugated UDCA in portal venous blood was larger than that of unconjugated UDCA in gallstone patients treated with UDCA.[30] Therefore, the clearance of GUDC may be one of the determinant factors for the pharmacokinetics of UDCA. We showed that the hepatic uptake of GUDC is mediated mainly by $Na^+$-independent carriers, such as OATP1B1 and OATP1B3, so the function of OATP1B1 and OATP1B3 may partly determine the pharmacokinetics of UDCA. Some single nucleotide polymorphisms (SNPs) in OATP1B1 and OATP1B3 changed the transport activity compared with wild type, and especially SNPs in OATP1B1 markedly affected the pharmacokinetics of substrates of OATP1B1 such as pravastatin in clinical situations.[31] Therefore, the SNPs in these transporters may also alter the clinical pharmacokinetics of UDCA.

In conclusion, we confirmed that UDCA and its conjugates can be taken up in both a $Na^+$-dependent and a $Na^+$-independent manner and that their uptake is saturable, indicating the involvement of transporters in the hepatic uptake of UDCA and its conjugates in human cryopreserved hepatocytes. In particular, GUDC, which is the major component of UDCA administered clinically, can be transported mainly in a $Na^+$-independent manner in human hepatocytes. From the results of the transport assay using expression systems, UDCA can be recognized by NTCP, but not OATP1B1 and OATP1B3, while conjugated UDCA can be transported by at least NTCP, OATP1B1, and OATP1B3. We suggest that these transporters may play an important role in the enterohepatic circulation of UDCA and its subsequent therapeutic effects.

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(27) von Dippe, P.; Amoui, M.; Stellwagen, R. H.; Levy, D. The functional expression of sodium-dependent bile acid transport in Madin-Darby canine kidney cells transfected with the cDNA for microsomal epoxide hydrolase. *J. Biol. Chem.* **1996**, *271*, 18176−18180.

(28) Bouscarel, B.; Nussbaum, R.; Dubner, H.; Fromm, H. The role of sodium in the uptake of ursodeoxycholic acid in isolated hamster hepatocytes. *Hepatology* **1995**, *21*, 145−154.

(29) Briz, O.; Serrano, M. A.; Rebollo, N.; Hagenbuch, B.; Meier, P. J.; Koepsell, H.; Marin, J. J. Carriers involved in targeting the cytostatic bile acid-cisplatin derivatives cis-diammine-chlorocholylglycinate-platinum(II) and cis-diammine-bisursodeoxycholate-platinum(II) toward liver cells. *Mol. Pharmacol.* **2002**, *61*, 853−860.

(30) Ewerth, S.; Angelin, B.; Einarsson, K.; Nilsell, K.; Bjorkhem, I. Serum concentrations of ursodeoxycholic acid in portal venous and systemic venous blood of fasting humans as determined by isotope dilution-mass spectrometry. *Gastroenterology* **1985**, *88*, 126−133.

(31) Marzolini, C.; Tirona, R. G.; Kim, R. B. Pharmacogenomics of OATP and OAT families. *Pharmacogenomics* **2004**, *5*, 273−282.