Modulation by Drugs of Human Hepatic Sodium-Dependent Bile Acid Transporter (Sodium Taurocholate Cotransporting Polypeptide) Activity

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ABSTRACT

Adequate bile flow, maintained in part by the efficient enterohepatic recirculation of bile acids, is critical for normal liver function. One important component of this process is the uptake of bile acids from the portal circulation into hepatocytes by the bile acid uptake transporter sodium taurocholate cotransporting polypeptide (NTCP). Thus, the expression and functional activity of this transporter may affect the rate of bile acid removal from the portal circulation. Accordingly, we assessed NTCP mRNA expression from human livers using a sensitive RNase protection assay. In addition, the ability of various bile acids and drugs to inhibit NTCP activity was determined using a recombinant vaccinia expression system. A 40-fold interindividual variability was found in NTCP mRNA levels determined in eight liver samples of disease-free donors. Expressed NTCP exhibited high-affinity, sodium-dependent uptake of taurocholate, and as expected, this was markedly inhibited by bile acids and organic anions. A number of drugs, including peptidomimetic renin inhibitors, propranolol, ciclosporin, and progesterone, were found to be potent inhibitors, whereas antiarrhythmic agents, including bupivacaine, lidocaine, and quinidine, were found to enhance NTCP activity. Accordingly, these results indicate that large interindividual variability exists in NTCP mRNA level and that a number of drugs currently in clinical use have the potential to interact with and alter NTCP activity, thereby affecting hepatic bile acid uptake.

The molecular cloning and functional characterization of hepatic transport systems have greatly enhanced our knowledge of hepatic uptake and efflux of endobiotics and xenobiotics. In particular, the critical role of such transport systems in the vectorial movement of bile acids from the portal circulation and excretion into bile (Trauner et al., 1998). Bile acids are critical components in the maintenance of adequate bile flow, cholesterol metabolism, digestion, and removal of endobiotics and xenobiotics (Trauner et al., 1998). Moreover, mutations in the phospholipid flippase (MDR3) and in bile acid efflux transporter (sisp-gp) have been implicated in cholestatic liver diseases (de Vree et al., 1998; Strautnieks et al., 1998). Nascent bile acid synthesis is minimized by the efficient enterohepatic recycling of bile acids (Ostrow, 1993). One important component of this process is the rapid uptake of bile acids from the portal circulation into hepatocytes by bile acid transport systems located on the basolateral membrane of hepatocytes (Trauner et al., 1998). Molecular cloning approaches have identified a number of hepatic transporters capable of bile acid and xenobiotic uptake. Available data support the role of Na+/taurocholate cotransporting polypeptide, cloned initially from the rat (Ntcp) (Hagenbuch et al., 1991) and, more recently, from human liver (NTCP; Hagenbuch and Meier, 1994) as the key transporter for hepatic uptake of bile acids (Stieger and Meier, 1998). This notion is augmented by experimental data showing that coinjection of specific antisense oligonucleotides against Ntcp, along with the total rat liver mRNA, into Xenopus laevis oocytes, resulted in a 95% reduction in the expressed sodium-dependent taurocholate uptake activity (Hagenbuch et al., 1996). Thus, it is possible that alterations in NTCP transport activity may contribute to cholestasis.

Despite its potential importance in the maintenance of normal liver function, many aspects of NTCP activity, including the extent of its interaction with drugs in clinical use, have not been systematically evaluated. Identification and the avoidance of drug inhibitors of NTCP may be important with respect to drug-induced cholestasis, especially among those with preexisting cholestatic liver disease. Moreover, bile acids are frequently used as therapeutic agents in a

ABBREVIATION: NTCP, human sodium taurocholate cotransporting polypeptide.
number of liver diseases. In the present study, we show that a large interindividual variability in NTCP mRNA levels exists and that NTCP transport activity can be modulated by a variety of bile acids and nonbile acid drugs.

**Experimental Procedures**

**Materials.** $[^{3}H]$Taurocholate (specific activity, 2 Ci/mmole) was obtained from DuPont New England Nuclear (Boston, MA). $[^{3}H]$UT1038 (specific activity, 35 mCi/mg), unlabeled UT1038 and $[^{3}H]$EDMS1921 (specific activity, 79 mCi/mg), and unlabeled EMDS1921 were obtained from The Upjohn Company (Kalamazoo, MI) and E. Merck (Darmstadt, Germany), respectively. $[^{3}H]$Propranolol (specific activity, 26.4 Ci/mmol), unlabeled S- and R-enantiomers of propranolol, and its racemate had been previously obtained from ICN Pharmaceuticals (Aldelore, UK). $[^{3}P]$dCTP (specific activity, 3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Angiopeptin was provided by the Henri Beaufour Institute (Washington, DC). Recombinant vaccinia virus containing the T7-RNA polymerase gene (vtf-7) and the pTM1 expression vector was gifts provided by Dr. Bernard Moss (National Institutes of Health, Bethesda, MD). Human liver samples were provided by Dr. F. P. Guengerich at our institution. All other chemicals and reagents, unless stated otherwise, were obtained from Sigma-Aldrich Research (St. Louis, MO) and were of the highest grade available.

**Interindividual Variability in Hepatic NTCP mRNA Expression.** Human liver samples (Nashville Regional Organ Procurement Agency, Nashville, TN) from eight Caucasian donors (age range, 10–49 years; mean age, 29 years; five females and three males) without any history of underlying liver pathology or prior medications (cause of death: motor vehicle accident, $n = 5$; subarachnoid hemorrhage, $n = 2$; and self-inflicted gunshot wound, $n = 1$) were used in the total RNA extraction using Trizol reagent (Life Technologies, Rockville, MD). A ribonuclease protection assay was carried out on the derived total RNA samples to quantify NTCP mRNA levels. A 203-bp NTCP cDNA for use in the antisense riboprobe synthesis was obtained by PCR of a full-length NTCP cDNA containing the T7-RNA polymerase gene (vtf-7) and the pTM1 expression vector. The probe synthesis was obtained by PCR of a full-length NTCP cDNA insert in the sense orientation downstream of the T7-promoter sequence in the primer bypassed the need of the T7-promoter sequence in the primer. Inclusion of the T7-promoter sequence in the primer bypassed the need for subcloning or linearization. The resulting PCR product (100 ng) was used in the synthesis of T7-RNA polymerase-mediated antisense RNA using the RPA II kit (Ambion, Austin, TX) according to the recommended protocol supplied by the manufacturer. As a reference RNA, cyclophilin mRNA levels were assessed using pTRE-cyclophilin-human cDNA template (Ambion) designed to detect a 105-bp fragment of human cyclophilin, according to the same protocol. Subsequent to RNase digestion, samples were loaded onto a denaturing 4% acrylamide gel for electrophoresis. On the completion of electrophoresis, the gels were dried and overlaid with radiograph film for electrophoretic analysis.

**Cell Culture and Virus Preparation.** HeLa cells grown in 12-well plates ($\sim 0.8 \times 10^{5}$ cells/well) were infected with vaccinia (VTF-7) at a multiplicity of 10 PFU/cell in serum-free OptiMEM I medium (Life Technologies) and allowed to adsorb for 30 min at 37°C. Cells in each well were then transfected using the cationic lipid agent Lipofectin (Life Technologies) at a concentration of 1 µg/ml/well. After 30 min, the transfection medium was replaced with 800 µl of 1% FBS with antibiotic/antimycotic mixture. The cells were incubated for 48 h at 37°C, the infected cells were pelleted, homogenized, and recovered through centrifugation, followed by titration of viral stock as described by Blakely et al. (1991).

**NTCP Plasmid Construction.** The full open reading frame of human NTCP cDNA was obtained by polymerase chain reaction (PCR) using a combination of Taq and Deep Vent DNA polymerases from a cDNA library synthesized from human liver mRNA using oligonucleotide primers 5'-ATGGGAGGCCACACAGCCGTCT-3' as the forward and 5'-CTAGGGCTGTCAGGGGGA CCA-3' as the reverse primer. A single PCR product of expected size was visualized on an ethidium bromide-stained, 2% agarose gel. An aliquot of the PCR product was ligated into the PCR II vector (InVitrogen, Carlsbad, CA). Subsequent to Escherichia coli transformation and growth, the NTCP cDNA was released from the PCR II vector by digestion with the restriction endonuclease EcoRI. The NTCP insert cleaved from the PCR II vector was then recovered through agarose gel electrophoresis and extraction from the gel using the DNA binding resin Qiaex (Qiagen, Chatsworth, CA). The recovered cDNA was then ligated into the pTM1 vector, previously linearized with EcoRI, and dephosphorylated using calf intestinal alkaline phosphatase. After transformation and growth in E. coli, individual colonies containing the pTM1/NTCP construct were identified. A pTM1-NTCP with the NTCP cDNA insert in the sense orientation downstream from the T7-promoter region was fully sequenced using an ABI automated sequencer (Applied Biosystems Inc., Foster City, CA), and found to fully match the published sequence (Hagenbuch and Meier, 1994). This clone was used in the expression studies.

**Transport Studies Using Recombinant Vaccinia Virus.** HeLa cells grown in 12-well plates ($\sim 0.8 \times 10^{5}$ cells/well) were infected with vaccinia (VTF-7) at a multiplicity of 10 PFU/cell in serum-free OptiMEM I medium (Life Technologies) and allowed to adsorb for 30 min at 37°C. Cells in each well were then transfected using the cationic lipid agent Lipofectin (Life Technologies) with either 1 µg of pTM1 plasmid vector containing the transporter cDNA insert or pTM1 vector lacking any insert. A lipofectin/plasmid DNA ratio of approximately 3:1 yielded best results. After the application of this mixture, the cells were allowed to incubate at 37°C for 16 h. Incubation periods of less than 12 h or more than 20 h resulted in reduced transport activity. On the day of the transport study, the cells were washed with 2 ml of a pH 7.4 buffer (transport buffer A) containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl, 1 mM MgCl, and 10 mM HEPES-tris(hydroxymethyl) aminomethane. Cells were then preincubated for 15 min with 0.3 µl/well of this buffer. Then, 0.1 µl/well of the same buffer containing a 4× cocktail of desired concentrations of labeled taurocholate with or without unlabeled inhibitor/drug was added. Uptake was allowed to occur for a predetermined time at 37°C and then the transport activity was stopped by washing each well with 1 ml of ice-cold buffer (transport buffer A), repeated three times. Subsequently, the cells were lysed by the addition of 0.5 µl of 1% SDS to each well, followed by the measurement of lysate radioactivity with a Rackbeta liquid scintillation counter (Pharmacia-LKB Nuclear, Gaithersburg, MD). Protein concentrations were determined using a Coomassie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) with BSA as the standard.

**Characterization of Transport Kinetics.** To determine the transport kinetics of NTCP-mediated taurocholate uptake, the cellular accumulation of labeled taurocholate over time was assessed by stopping the transport activity at 1, 2, 3, 4, 5, 10, 15, 30, and 60 min after the addition of the substrate. The 1-min time point chosen as the best single time at which the rate of uptake appeared linear. In the subsequent experiments, varying concentrations of unlabeled substrate were also added to the wells. Uptake was allowed to continue for 1 min under the conditions outlined previously. A Michaelis-Menten type nonlinear curve-fitting was carried out using Prism (GraphPAD, San Diego, CA) to obtain the estimate of maximal uptake rate, $V_{\text{max}}$, and the concentration at which half-maximal uptake occurred, $K_{\text{m}}$. Inhibition of NTCP-mediated taurocholate transport by drugs was assessed by coinoculating a candidate drug inhibitor (100 µM), along with the labeled taurocholate (5 µM) for 30 min. Taurocholate uptake in the presence and the absence of the candidate inhibitor was compared in cells transfected with pTM1-
NTCP or only the parental pTM1 plasmid. In addition, selected drugs with inhibitory properties were assessed further at multiple inhibitor concentrations to define the inhibition constant IC₅₀ by using the Hill equation ( Prism). All experiments were carried out in triplicate, on at least three differing experiment days. Data are shown as mean ± S.E.

**Statistical Analysis.** Mean ± S.E. values were compared using a Student’s t test or Mann-Whitney U test, and a value of P < .05 was taken as the minimum level of significance.

**Results**

Quantification of hepatic NTCP mRNA expression, using an RNase protection assay on total RNA samples derived from eight disease-free livers obtained from donors of European-American descent (Fig. 1), showed that after normalization to their corresponding cyclophilin levels, the estimated levels of NTCP mRNA varied by 40-fold between the liver samples.

Recombinant vaccinia-mediated expression of NTCP indicated that uptake of [³H]taurocholate (0.2 μM) was rapid and very efficient relative to cells transfected with plasmids lacking NTCP, which failed to show any significant [³H]taurocholate uptake (Fig. 2). Replacement of sodium in the incubation buffer with potassium resulted in a marked (8-fold) reduction in NTCP-mediated taurocholate uptake (Fig. 2); however, a modest residual level of transport activity remained even in the absence of sodium at this tracer concentration. Interestingly, the sodium-independent component was no longer discernible at a higher substrate concentration (5 μM taurocholate, data not shown).

The uptake of [³H]taurocholate, in the presence of buffer sodium, at differing concentrations of unlabeled taurocholate showed that NTCP-mediated uptake of taurocholate was a saturable process that could be described using the Michaelis-Menten equation. The apparent Kₘ value was 7.9 ± 2.0 μM, whereas Vₘₐₓ was 49 ± 3 pmol/mg protein/min (Fig. 2).

A large number of bile acids and drugs were assessed for their effect on expressed NTCP activity. As expected, bile acids (100 μM) such as cholic acid, glycocholic acid, chenodeoxycholic acid, deoxycholic acid, and ursodeoxycholic acid were inhibitors of [³H]taurocholate (5 μM) uptake (Fig. 3). Bromosulfophthalein (100 μM), an organic anion, was also an inhibitor of taurocholate uptake (Fig. 3). Further concentration-dependent inhibition studies with cholic acid, ursodeoxycholic acid, and bromosulfophthalein revealed that all were potent inhibitors (Fig. 4) with IC₅₀ values of 3.6 to 7.3 μM (Table 1).

Although a variety of drugs, such as sodium valproate, nicotine, and salicylate, were without effect at the studied inhibitor concentration (100 μM), a number of drugs were found to significantly reduce NTCP-mediated taurocholate uptake; these agents included furosemide, bumetanide, ketonazole (400 μM), progesterone, cyclosporin, and the β-adrenoceptor blocker propranolol. Ketoconazole, furosemide, cyclosporin, and racemic-propranolol were further studied over a range of inhibitor concentrations (Fig. 4), and their IC₅₀ values were calculated (Table 1). Additionally, oligopeptides, including the linear oligopeptide renin inhibitor agents U71038 and EMD 51921 (Fig. 4), the somatostatin analog angiopeptin, and the mushroom poison α-amanitin, were also capable of inhibiting taurocholate transport (Fig. 3). By contrast, dipeptides/tripeptides were without effect (Fig. 3).

Interestingly, a number of drugs were found to significantly enhance NTCP-mediated taurocholate uptake (Figs. 3 and 5), such as bupivacaine, lidocaine, and quinidine, with the greatest enhancement being observed with quinidine. This effect of quinidine was concentration dependent and resulted in over 2-fold greater uptake compared with that in the absence of quinidine (Fig. 5). BSA-induced enhancement of taurocholate uptake was most notable at a low (1 μg/ml) concentration (Fig. 5); at concentrations above 20 μg/ml, BSA inhibited taurocholate uptake.

![Fig. 1.](image1.png)

![Fig. 2.](image2.png)
Large interindividual differences were found in the level of expressed NTCP mRNA obtained from livers of disease-free donors; the difference in expressed mRNA level between the sample exhibiting the lowest level (HL123, Fig. 1) and the highest (HL104, Fig. 1) was 40-fold. In studies of rats, a 10-fold increase in Ntcp mRNA level after prolactin treatment was associated with a 2-fold increase in the $V_{\text{max}}$ value for taurocholate uptake (Liu et al., 1994). Clearly, the measurement of NTCP protein will be an important next step in delineating whether this large variability in NTCP mRNA levels is similarly reflected at the protein level. However, this is currently difficult to determine because a high-affinity antibody against NTCP is not available.

Recombinant vaccinia-mediated expression of NTCP showed the uptake of taurocholate to be rapid and predominately sodium dependent (Fig. 2). The derived $K_m$ value of 7.9 ± 2.0 μM for taurocholate uptake is in close agreement to the $K_m$ value of 6.2 μM observed for NTCP expressed in X. laevis oocytes (Hagenbuch and Meier, 1994). As expected, bile acids and the organic anion bromosulfophthalein were potent inhibitors of NTCP-mediated taurocholate uptake. Interestingly, however, ursodeoxycholic acid appeared to be the most potent inhibitor (Figs. 3 and 4), with an estimated IC$_{50}$ value of 3.6 μM (Table 1). Ursodeoxycholic acid is currently used in a variety of liver diseases, particularly for the treatment of cholestatic liver diseases (Poupon and Poupon, 1995). It has been suggested that the mechanism of action of this dihydroxy bile acid involves competition for entry into the hepatocyte with other more hepatotoxic bile acids (Galle et al., 1990; Heuman, 1993). Also, it is generally considered that ursodeoxycholic acid uptake into hepatocyte is primarily passive, due to its lipophilicity (Scharschmidt and Lake, 1989), although more recent work carried out with isolated hamster hepatocytes suggests the involvement of a transporter for ursodeoxycholic acid at concentrations of less than 20 μM (Bouscarel et al., 1995). However, the data in this study suggest that ursodeoxycholic acid reduces the hepatic uptake of more polar and hepatotoxic bile acids through the inhibition of NTCP.

To define the extent of interaction between NTCP and nonbile acid drugs, a two-step approach was adopted. Initially, effects of drugs already known to alter the activity of sodium-dependent bile acid transport system were investigated to determine their relative potency. Subsequently, a number of structurally divergent drugs that are frequently used in the clinical setting were assessed for their effect on NTCP activity. Indeed, drugs previously identified as inhibitors of sodium-dependent bile acid uptake transport systems, including furosemide (Blitzer et al., 1982; Zimmerli et al., 1989), bumetanide (Zimmerli et al., 1989; Hagenbuch et al., 1991; Boyer et al., 1994; Hagenbuch and Meier, 1994),

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**TABLE 1**

Affinities for inhibition of NTCP-mediated taurocholate uptake by bile acids, organic anion (bromosulfophthalein), and drugs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin</td>
<td>1.0</td>
</tr>
<tr>
<td>Ursodeoxycholic acid</td>
<td>3.6</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>5.3</td>
</tr>
<tr>
<td>(R)-Propranolol</td>
<td>5.5</td>
</tr>
<tr>
<td>(S)-Propranolol</td>
<td>6.1</td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>7.3</td>
</tr>
<tr>
<td>Furosemide</td>
<td>15</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>264</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Effect of bile acids and xenobiotics on the modulation of NTCP-mediated taurocholate uptake transport expressed in HeLa cells. The uptake of [3H]taurocholate (5 μM) in the presence of various bile acids, bromosulfophthalein, drugs, oligopeptides, and dipeptide/tripeptide agents added to the transport buffer at 100 μM concentrations (unless noted otherwise), were compared with control samples containing only [3H]taurocholate (5 μM). *P < .05 and **P < .01, statistically significant difference from the control values.

**Fig. 4.** Dose-dependent inhibition of NTCP-mediated taurocholate uptake (5 μM) by bile acids and drugs added to transport buffer. A, bile acids ursodeoxycholic acid (○) and cholic acid (▲) and the organic anion bromosulfophthalein (▼). B, drugs that were assessed were (S)-propranolol (□), (R)-propranolol (△), ketoconazole (▼), furosemide (●), and cyclosporin (○). The degree of inhibition was derived by comparison to control studies carried out without any inhibitor agents. Data are mean ± S.E.
progesterone (Zimmerli et al., 1989), ketoconazole (Zimmerli et al., 1989), and cyclosporin (Stacey and Kotecka, 1988; Moseley et al., 1990; Azer and Stacey, 1993), were able to inhibit NTCP-mediated taurocholate uptake. However, the effects of ketoconazole (Fig. 3) were seen only at high concentrations (>100 μM). On the other hand, cyclosporin was a very potent NTCP inhibitor (Fig. 3), with an apparent IC₅₀ value of 1.0 μM (Table 1). Therapeutic trough plasma levels of cyclosporine in humans are approximately 150 to 400 ng/ml (0.125–0.33 μM), but it is likely that portal vein concentrations are much higher. Accordingly, inhibition of NTCP may be an additional mechanism for the observed cyclosporin-induced cholestasis (Arias, 1993). We also found that a number of other unrecognized drugs, including sodium valproate, nicotine, salicylate (Fig. 3), and the commonly prescribed β-adrenoceptor-blocking agent propranolol, were inhibitors of NTCP (Figs. 3 and 4). Both the (R)- and (S)-propranolol enantiomers appeared to possess similar inhibitory properties, with IC₅₀ values of 5.5 and 6.1 μM, respectively, despite the fact that propranolol is not a substrate of NTCP (data not shown). In addition, peptidomimetic agents, including the pseudotetrapeptide renin inhibitor U71038, a pseudotetrapeptide EMD51921, and the somatostatin analog angiopeptin, were also found to be capable of inhibiting NTCP-mediated taurocholate uptake (Fig. 4). However, like propranolol, neither U71038 nor EMD51921 was a transport substrate.

Albumin (Fig. 4), a known enhancer of sodium-dependent bile acid transport (Blitzer and Lyons, 1985), was able to enhance NTCP-mediated taurocholate uptake at low concentrations. However, this effect was significant only at a very low concentration (1 μg/μl), and the further addition of BSA resulted in reduced transport activity (Fig. 5). Human plasma albumin levels range from 30 to 40 μg/μl; therefore, it is unlikely the enhanced transport activity seen at the very low concentrations would have any in vivo clinical relevance. Surprisingly, a number of antiarrhythmic drugs, including bupivacaine, lidocaine, and quinidine, were found to enhance NTCP-mediated taurocholate uptake. In fact, quinidine exhibited a concentration-related enhancement in taurocholate uptake. This enhancement of bile acid uptake represents an interesting contrast to the well-recognized effect of quinidine as an inhibitor of ATP-dependent drug efflux transporter P-glycoprotein (Cardarelli et al., 1995; Leu and Huang, 1995; Fromm et al., 1999). The fact that only class I antiarrhythmic agents were found to possess this ability suggests that cell membrane electrical gradient or endogenous sodium/potassium channels present in HeLa cells may be altering NTCP transport activity. Also, it is possible that these drugs directly interact with NTCP at the level of the substrate binding domain and induce positive cooperativity. Regardless, delineation of mechanisms for the observed enhancing effects of such agents was beyond the scope of the current study.

In conclusion, data obtained in this study suggest that the key bile acid uptake transporter NTCP can interact with a variety of drugs in clinical use and not just with bile acids. Moreover, there may be a wide range in the expressed level of NTCP. Clearly, additional studies, particularly in humans, are needed to determine whether these findings reflect the in vivo situation. Nevertheless, the inclusion of NTCP as a potential etiological cause of cholestasis may prove to be therapeutically relevant.

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References


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