Inhibition of Bile Acid Transport across Na\textsuperscript{+}/Taurocholate Cotransporting Polypeptide (SLC10A1) and Bile Salt Export Pump (ABCB 11)-Coexpressing LLC-PK1 Cells by Cholestasis-Inducing Drugs

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ABSTRACT:

Vectorial transport of bile acids across hepatocytes is a major driving force for bile flow, and bile acid retention in the liver causes hepatotoxicity. The basolateral and apical transporters for bile acids are thought to be targets of drugs that induce cholestasis. Previously, we constructed polarized LLC-PK1 cells that express both a major bile acid uptake transporter human Na\textsuperscript{+}/taurocholate cotransporting polypeptide (SLC10A1) (NTCP) and the bile acid efflux transporter human bile salt export pump (ABCB 11) (BSEP) and showed that monolayers of such cells can be used to characterize vectorial transcellular transport of bile acids. In the present study, we investigated whether cholestasis-inducing drugs could inhibit bile acid transport in such cells. Because fluorescent substrates allow the development of a high-throughput screening method, we examined the transport by NTCP and BSEP of fluorescent bile acids as well as taurocholate. The aminofluorescein-tagged bile acids, chenodeoxycholylglycylamidofluorescein and chenodeoxycholylamidofluorescein, were substrates of both NTCP and BSEP, and their basal-to-apical transport rates across coexpressing cell monolayers were 4.3 to 4.5 times those of the vector control, although smaller than for taurocholate. The well known cholestatic drugs, rifampicin, rifamycin SV, glibenclamide, and cypensorin A, reduced the basal-to-apical transport and the apical efflux clearance of taurocholate across NTCP- and BSEP-coexpressing cell monolayers. Further analysis indicated that the drugs inhibited both NTCP and BSEP. Our study suggests that such coexpressing cells can provide a useful system for the identification of inhibitors of these two transport systems, including potential drug candidates.

Hepatotoxic adverse effects, often indicated by cholestasis, are a concern for every drug, and severe hepatotoxicity may cause a drug to be withdrawn from the market. Biliary excretion of bile acids is one of the principal driving forces for bile formation by generating an osmotic driving force favoring influx of water and electrolytes through the paracellular space (Wheeler et al., 1968; Wheeler, 1972). The transcellular transport is mediated by transporter proteins located on the sinusoidal (basolateral) and canalicular (apical) membranes of hepatocytes (Meier and Stieger, 2002; Trauner and Boyer, 2003). The basolateral Na\textsuperscript{+}/taurocholate cotransporting polypeptide (NTCP/SLC10A1) transports bile acids from the space of Disse into hepatocytes (Hagenbuch et al., 1991; Boyer et al., 1994). Human NTCP accepts most physiological bile acids and some organic anions, such as estrone-3-sulfate and bromosulphophthalein (Meier et al., 1997). Sodium-independent uptake of bile acids is carried out by members of the organic anion-transporting polypeptide family, such as rat Oatp1A1 and human OATP1B1. Although there are several carrier proteins capable of transporting bile acids, much evidence suggests, at least in the rodent, that NTCP-mediated transport accounts for a large part of the total bile acid uptake (Wolkoff and Cohen, 2003). At the canalicular membrane, the efflux of bile acids by the bile salt export pump (BSEP/ABCB11) mediates concentrative excretion (Boyer et al., 1994; Gerloff et al., 1998; Noe et al., 2002). Mutations of BSEP in humans causes progressive familial intrahepatic cholestasis type II, a fatal condition (Strautnieks et al., 1998).

One mechanism for cholestasis is thought to be inhibition of hepatocyte transport systems for bile acids and other organic anions by

ABBREVIATIONS: NTCP, Na\textsuperscript{+}/taurocholate cotransporting polypeptide (SLC10A1); BSEP, bile salt export pump (ABCB 11); CGamF, chenodeoxycholylglycylamidofluorescein; CamF, chenodeoxycholylamidofluorescein; CDCGamF, chenodeoxycholylglycylamidofluorescein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; UDC-1-NBD, ursodeoxycholyl-(N-ε-NBD)-lysine; 7β-NBD-NCT, 7β-NBD-cholyltaurine; PS, permeability-surface area product; LLC-NTCP/BSEP, NTCP- and BSEP-coexpressing LLC-PK1 cells; LLC-NTCP, NTCP-expressing LLC-PK1 cells.
drugs. The inhibitory effects of such drugs on the uptake and efflux of bile acids have been studied using isolated and primary cultured hepatocyte or canalicular membrane vesicles (Kukongviriyapan and Stacey, 1991), as well as the isolated perfused liver (Bolder et al., 1999). Recently, NTCP and BSEP, which generate bile salt-dependent bile flow, have been shown to be possible target molecules for cholestatic drugs (Kim et al., 1999; Stieger et al., 2000; Akita et al., 2001; Bohan and Boyer, 2002).

Previously, we constructed NTCP- and BSEP-coexpressing LLC-PK1 cells as an in vitro model of the vectorial transcellular transport of bile acids in hepatocytes (Mita et al., 2006). This approach is useful for the screening of choleretic bile acids, which are good substrates of these transporters. A second use of this system is to identify inhibitors of these transporters which might have cholestatic effects in vivo. The method should also be useful for defining structure-transport activity relationships of bile acids. In the present study, we assessed the inhibitory effects of cholestasis-inducing drugs on transport across coexpressing cells with the aim of developing a screening system for cholestatic compounds. We compared the transport of fluorescent bile acid derivatives with that of taurocholate in the hope that such fluorescent compounds would be efficiently transported and thereby permit the development of a high-throughput screening method for detecting the inhibitory effects of drug candidates.

Materials and Methods

Chemicals. [3H]Taurocholic acid (2 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled taurocholic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled cholic acid was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Unlabeled ursodeoxycholic acid, tauroursodeoxycholic acid, and glycoursodeoxycholic acid were kindly provided by Mitsubishi Pharma (Osaka, Japan). Unlabeled cholic acid was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Unlabeled taurocholic acid was obtained from Sigma-Aldrich (St. Louis, MO). 3H-Cholesterol, [3H]cholesteryl ether, and [3H]cholesterylamine were purchased from New England Nuclear (Boston, MA). Unlabeled cholic acid was purchased from Sigma Chemical. Other chemicals used were commercially available and of reagent grade.

Cell Culture and Transfection. Human NTCP- and human BSEP-expressing LLC-PK1 cells were established and maintained as described previously (Mita et al., 2006). Briefly, parental LLC-PK1 cells were grown in M199 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen; 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B) at 37°C under 5% CO2. Full-length NTCP cDNA was subcloned into pcDNA3.1 (Invitrogen) and transfection into human NTCP cells was identified with FuGENE 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer’s instructions. Transfectants expressing NTCP were selected with G418 (800 μg/ml) and the clone with the highest NTCP activity was screened by the uptake activity for taurocholate.

Transfected LLC-PK1 cells expressing NTCP were seeded on 12-well plates and cultured without viral infection. Cells were harvested 48 h after infection, and expression of NTCP was induced by 10 mM sodium butyrate for 24 h (Cui et al., 1999). To evaluate the integrity of the monolayer, transepithelial electrical resistance was measured using a Milllicell-ERS (Millipore Corp., Bedford, MA). The monolayers’ transepithelial electrical resistances before the experiments were 200 to 300 Ω cm2. Then, cells were washed with transport buffer (118 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2 adjusted to pH 7.4). Subsequently, 3H-labeled taurocholate or fluorescent bile acids were added to the transport buffer in the basal compartment (950 μl) for transcellular transport studies or 12-well plates for uptake studies. After the times indicated, the amount of substrates in the opposite apical compartment was measured by the radioactivity for taurocholate, or by the absorbance at 490 nm for fluorescent bile acids using a Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Potential inhibitors were added to both apical and basal compartments 30 min before the transport study. The accumulated radioactivity in the cell was determined at the end of the experiments by lysing the cells with 500 μl of 0.2 N NaOH and measuring the radioactivity in the cell lysates. Aliquots (50 μl) of cell lysates were used to determine protein concentrations by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The apparent intracellular concentration of taurocholate (Ccell) was determined by assuming that the cellular volume per milligram of cellular protein was 4 μl.

Data Analysis. The kinetic parameters were defined as follows: PSbasal, basal-to-apical (pmol/min/mg protein) is the permeability-surface area product (PS) for the basal-to-apical clearance defined for the ligand concentration in the medium. PSapical, (pmol/min/mg protein) is the PS product for the influx of ligand across the apical membrane, which is defined for Cmed; PSbasal (pmol/min/mg protein) is the PS product for the efflux of ligand across the apical membrane, which is defined for the ligand concentration in the basal membrane, Cbasal. These parameters were determined for each compound and each cell preparation. The PSbasal is a hybrid parameter consisting of PS1, PS2, and PS3.

In this study, PSbasal and PSapical were calculated as follows: PSbasal = Vbasal/Cmed and PS3 = Vapical/Ccell where Vbasal (pmol/min/mg protein) is the increasing velocity of taurocholate in the apical compartment. Vapical was determined by analyzing the transcellular transport for 1 h. Since the amount of taurocholate transported increased linearly as a function of time over the 2-h period and the intracellular concentration was constant during the incubation periods (Mita et al., 2006), we hypothesized that the initial transport velocity could be determined from the slope over the period 0 to 1 h.

Results

Transcellular Transport of Fluorescent Bile Acids. To identify a good substrate of NTCP and BSEP for the functional probe in the inhibition study, the basal-to-apical transport clearance (PSbasal) of taurocholate and fluorescent bile acids across NTCP- and BSEP-coexpressing LLC-PK1 cells (LLC-NTCP/BSEP) was compared...
FIG. 2. Transcellular transport of labeled and fluorescent bile acids across NTCP- and BSEP-coexpressing LLC-PK1 cells. $[^3]$H]Taurocholate (1 μM), CGamF, CamF, CDCGamF, UDC-L-NBD, and $7\beta$-NBD-NCT (10 μM) across the LLC-PK1 cell monolayers were determined. Open, hatched, and closed bars represent the basal-to-apical transcellular transport across the control (LLC), LLC-NTCP, and LLC-NTCP/BSEP monolayers, respectively. Vertical bars represent the S.E. of three determinations. At the bottom is a graph in which the transcellular transport data are expressed on the same scale.
The intracellular concentration \( C \) of \([3H]taurocholate\) was used. The absolute PS \( \alpha-a \) value of all the fluorescent bile acids was less than \( \frac{1}{6} \) that of \([3H]taurocholate\). The ratio of the intracellular content of the compounds, which is important for this study because it is needed to calculate the efflux clearance across the apical membrane (PS3).

### Inhibitory Effects of a Series of Cholestasis-Inducing Drugs

Next, the inhibitory effect of cholestasis-inducing drugs on the basal-to-apical transport clearance \( P_{S_{b-a}} \) of taurocholate was measured (Fig. 3). \( P_{S_{b-a}} \) was reduced by 100 \( \mu M \) rifampicin and rifamycin SV to 50% of the control level, and 10 \( \mu M \) glibenclamide reduced it to 70% (Fig. 3A). The intracellular concentration \( C_{cell} \) of taurocholate was determined for each compound at the end of the experiment (Fig. 3B). The \( C_{cell} \) of taurocholate was increased by 100 \( \mu M \) rifampicin to 160% of that of control cells (no inhibitor added). However, 100 \( \mu M \) rifamycin caused a 10% reduction in the apparent cellular concentration of taurocholate and 10 \( \mu M \) glibenclamide led to a 30% reduction in \( C_{cell} \) compared with control cells. Calculation of the efflux clearance across the apical membrane PS3 using the measured \( C_{cell} \) showed that 100 \( \mu M \) rifampicin, rifamycin SV, and glibenclamide produced a 70%, 44%, and 63% inhibition of PS3, respectively, indicating that the drugs inhibited the efflux of taurocholate by BSEP located in the apical membrane (Fig. 3C). When the efflux process is the only target of inhibition, \( C_{cell} \) should be increased by the drugs compared with untreated LLC-NTCP/BSEP cells. However, as mentioned above, \( C_{cell} \) was reduced by rifamycin SV and glibenclamide. This means that not only BSEP but also NTCP was inhibited in this experiment as far as rifamycin SV and glibenclamide were concerned. Of course, from these data, we cannot exclude the possibility that inhibition of NTCP is also involved in the case of rifampicin. A 100 \( \mu M \) concentration of captopril and cimetidine did not affect the transport and \( C_{cell} \) of taurocholate significantly (Fig. 3, A–C).

### Kinetics of the Inhibition by Cyclosporin A

To evaluate the inhibition kinetics involved in the transcellular transport when both the uptake and efflux processes are affected, cyclosporin A, an inhibitor of both NTCP and BSEP, was also examined (Fig. 4). The basal-to-apical transport clearance \( P_{S_{b-a}} \) of taurocholate was inhibited by cyclosporin A (and its metabolites) with a \( K_i \) value of 1.0 ± 0.2 \( \mu M \) (Fig. 4A). The intracellular concentration \( C_{cell} \) determined at the end of each experiment was also reduced by cyclosporin A, suggesting that uptake of taurocholate by NTCP was inhibited by cyclosporin A treatment. The inhibition of the uptake process was confirmed by evaluating the inhibitory effect of cyclosporin A on the uptake of taurocholate into only NTCP-expressing LLC-PK1 cells. The \( K_i \) value was determined as 0.27 ± 0.06 \( \mu M \) (Fig. 4C). At the same time, the calculated PS3 showed a reduction depending on the concentration of cyclosporin A, probably because of inhibition of BSEP by cyclosporin A (and/or its metabolites) (Fig. 4B). These results showed that both uptake and efflux processes are affected by 1 to 10 \( \mu M \) cyclosporin A.

### Discussion

In the present study, we assessed the inhibitory effects of cholestasis-inducing drugs on bile acid transport across LLC-NTCP/BSEP cells. Our hope was to develop a rapid screening system for drugs that inhibit these transporters.

Initially, we focused on the fluorescent bile acids as a probe of NTCP and BSEP function and investigated whether they were substrates of NTCP and BSEP using LLC-NTCP/BSEP. The fluorescent derivatives of bile acids used in this study were originally synthesized for the functional analysis of bile salt transport systems in isolated hepatocytes, immortalized cell lines derived from hepatocytes, or in vivo (Holzinger et al., 1998; Cantz et al., 2000). Direct demonstration of the transport of these bile acids via NTCP or BSEP has not yet been carried out, although sodium-dependent uptake for CGamF has been observed (Maglova et al., 1995).

Basal-to-apical transport across LLC-NTCP/BSEP was observed in a rank order of taurocholate > CGamF > CDCGamF, and no significant transport was observed for UDC-L-NBD, CamF, and 7β-NBD-NCT (Fig. 2). This order was similar to that of the maximum output rate of the bile acids in an isolated liver perfusion study: taurocholate 22.7 > CGamF 14.1 > CamF 7.7 > UDC-L-NBD 1.1 (mol/g liver/min) (Holzinger et al., 1998). This result supports the hypothesis that the transport of fluorescent derivatives of cholic acid in hepatocytes is mainly mediated by NTCP and BSEP, and showed that our in vitro system can reflect the physiological function of these transporters as far as transcellular transport is concerned. As for UDC-L-NBD, although uptake by LLC-NTCP inhibited by taurocholate was observed using fluorescent microscopy (data not shown), no significant transcellular transport across LLC-NTCP/BSEP was observed. This might be because of the nature of this bile salt, which is sequestered in the cells (Holzinger et al., 1998; Cantz et al., 2000). Nonetheless, fluorescent bile acids were transported in this system. Better fluorescent bile acids that will be transported as efficiently as taurocholate will make excellent tools for high-throughput screening.

Inhibition of BSEP by cholestasis-inducible drugs is one of the most frequently reported mechanisms of drug-induced cholestasis (Bohan and Boyer, 2002). Among such drugs, rifampicin, rifamycin SV, glibenclamide, and cyclosporin A (Stieger et al., 2000; Byrne et al., 2002) were used in this study. As shown in Fig. 3, PS3, the efflux clearance that reflects the function of BSEP, was reduced by all the drugs examined. The concentration needed for 50% inhibition of PS3 is between 10 and 100 \( \mu M \) for rifampicin and glibenclamide and approximately 100 \( \mu M \) for rifamycin SV. The reported \( K_i \) values for the inhibition of taurocholate uptake into human BSEP-expressing membrane vesicles are 31 \( \mu M \) for rifamycin SV and 31 \( \mu M \) for glibenclamide (Byrne et al., 2002). For rifampicin, only the \( K_i \) value of 12 \( \mu M \) for rat Bsep is available (Stieger et al., 2000). Compared
with these values, the inhibitory concentration was higher in our LLC-NTCP/BSEP cells than in other studies that used vesicles. One possible explanation for this is that the protein unbound concentrations of the drugs in cytoplasm are lower than in the medium because the drugs may not penetrate the plasma membrane efficiently and the drugs may also bind to intracellular proteins.

Inhibition of BSEP in the transcellular transport of taurocholate should be accompanied by an increase in the intracellular concentration of taurocholate. However, the increase was observed only in the case of rifampicin. This means that rifamycin SV and glibenclamide also inhibited NTCP-mediated uptake at the same time. Recently, it has been reported that 100 μM rifampicin or rifamycin SV can reduce
the uptake of taurocholate by rat Ntcp to 60% of the total uptake (Fattinger et al., 2000). However, in this study, after incubation with 100 μM rifampicin and rifamycin SV, the reduction in C\text{cell} was not as much as 60%. An increase by rifampicin and only a slight decrease by rifamycin SV were observed (Fig. 3). If we hypothesize there is no species difference in the inhibitory effect of these drugs between humans and rats, this result indicates that the inhibition of NTCP and BSEP balanced each other.

Captopril and cimetidine are reported to cause cholestasis (Mohi-ud-din and Lewis, 2004). However, their interactions with bile acid transporters have not been reported [cimetidine does not have a significant inhibitory effect on BSEP (Wang et al., 2003)], and other pathways are postulated as a possible mechanism. Corresponding to this, both captopril and cimetidine did not affect the transcellular transport and C\text{cell} of taurocholate at 100 μM (Fig. 3, A–C).

The inhibitory effect of cyclosporin A, an inhibitor of both NTCP and BSEP, was also examined as well as the inhibition kinetics of the transcellular transport when both the uptake and efflux processes are affected (Fig. 4). The baso-to-apical transport clearance PS\text{b-a} was inhibited with a K\text{i} value of 1.0 ± 0.2 (μM). The efflux clearance PS3 was inhibited depending on the medium concentration of cyclosporin A. Although estimation of the exact K\text{i} value is difficult, it appeared to be close to the reported K\text{i} value for the inhibition of the uptake of taurocholate into human BSEP-expressing membrane vesicles by cyclosporin A (9.5 μM) (Byrne et al., 2002).

The question that we must consider here is to what extent inhibition of the uptake and efflux process affects the net transcellular transport. It was estimated that the K\text{i} value for the inhibitory effect of cyclosporin A on the uptake of taurocholate into human NTCP-expressing LLC-PK1 cells was 0.27 ± 0.06 (μM) (Fig. 4). This value is similar to the K\text{i} value for PS\text{b-a}, which suggests that the inhibition of PS\text{b-a} reflects the inhibition of the uptake process mediated by NTCP. Although we do not know whether NTCP or BSEP is important for the cyclosporin A-induced cholestasis in physiological situations, the result of this study and the following aspects support the importance of NTCP. The transcellular transport clearance can be expressed as the hybrid of each transmembrane transport clearance as described under Data Analysis: PS\text{b-a} = PS1 · PS3/(PS2 + PS3). If the efflux clearance across the apical membrane, PS3, is far greater than that across the basal membrane, PS2, PS\text{b-a} is nearly equal to PS1. Thus, inhibition of the uptake process, PS1, can lead to inhibition of transcellular transport more easily than inhibition of the efflux process, PS3. The effect of inhibition of the uptake and/or efflux process on the net transcellular transport is simulated in Fig. 5. The ratio of PS2:PS3 is substituted by the measured value in the isolated rat liver perfusion studies: PS3, 69.2 ± 6.3 (μl/min/g liver); PS2, 8.4 ± 0.6 (μl/min/g liver) (Akita et al., 2002). If the efficacy of the inhibitory effect of the drug is similar for the uptake and efflux processes, inhibition of uptake is more effective than that of efflux as far as the net transcellular transport is concerned.

BSEP has been extensively studied as a target molecule of drug-induced cholestasis because it plays a role in the regulation of the concentration of bile acids in hepatocytes. Inhibition of BSEP leads to an intracellular accumulation of bile acids, resulting in cellular damage because of their cytotoxic effects. However, there should be some cases where the inhibition of NTCP plays a major role in drug-induced cholestasis, considering the importance of the uptake process in the overall transcellular transport of bile acids as described above. Cyclosporin A-induced cholestasis may be one of those. The plasma bile salt concentration was increased in rats after administration of 10 mg/kg cyclosporin A (Stone et al., 1987), indicating that cyclosporin A inhibits the uptake of bile acids from the portal blood into hepato-
cytes. Moreover, there was no change in liver histology in the cholestasis caused by cyclosporin A (Kukongviriyapan and Stacey, 1991), suggesting that the cytotoxicity brought about by intracellular bile acids here is not very severe. These facts indicate the importance of the inhibition of NTCP, at least in the case of cyclosporin A-induced cholestasis.

In conclusion, LLC-NTCP/BSEP cells were used for the detection of the inhibitory effect of drugs on NTCP and/or BSEP, although the quantitative evaluation of the inhibitory effect on BSEP appears to be difficult at the present time, compared with transport studies using membrane vesicles. Furthermore, to predict the effect of drugs under physiological conditions, we must consider the drug metabolites, which sometimes significantly inhibit BSEP (Funk et al., 2001). Because there is only a minor quantity of Hepatitis C virus in drug metabolism in LLC-PK1 cells (Gonzalez and Tarloff, 2004), the inhibitory effects observed in this study are speculated to be those produced by drugs in their unchanged forms. The additional expression of such enzymes and uptake transporters of drugs, such as OATP1B1 and OATP1B3, will provide a more useful tool for quantitative measurement of the inhibitory effect on BSEP.

References
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