Hepatobiliary Disposition of 3α,6α,7α,12α-Tetrahydroxy-Cholanoyl Taurine: A Substrate for Multiple Canalicular Transporters

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ABSTRACT:

Tetrahydroxy bile acids become major biliary bile acids in Bsep(−/−) mice and Fxr(−/−) mice fed cholic acid; we characterized disposition of these novel bile acids that also occur in patients with cholestasis. We investigated mouse Mrp2 (mMrp2) and P-glycoprotein ([P-gp] mMdr1a)-mediated transport of a tetrahydroxy bile acid, 6α,OH-taurocholic acid (6α-OH-TC), and its biliary excretion in wild-type and Mrp2(−/−) mice in the presence or absence of N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), a P-gp and breast cancer resistance protein inhibitor. 6α-OH-TC was rapidly excreted into bile of wild-type mice (78% recovery); confusion of GF120918 had no significant effect. In Mrp2(−/−) mice, biliary excretion was decreased (52% recovery) and confusion of GF120918 further decreased these values (34% recovery). In wild-type, but not Mrp2(−/−), mice, 6α-OH-TC increased bile flow 2.5-fold. Membrane vesicle transport studies of 6α-OH-TC (0.05–0.75 mM) yielded saturation kinetics with a higher apparent affinity for mMrp2 (Km = 0.13 mM) than for mMdr1a (Km = 0.33 mM); mBsep transported 6α-OH-TC with positive cooperativity (Hill slope = 2.1). Human multidrug resistance-associated protein (MRP) 2 and P-gp also transported 6α-OH-TC but with positive cooperativity (Hill slope = 3.6 and 1.6, respectively). After intrajejunal administration, the time course of 6α-OH-TC biliary recovery was similar to that of coinfused taurocholate, implying that 6α-OH-TC can undergo enterohepatic cycling. Thus, Mrp2 plays a key role in 6α-OH-TC biliary excretion, whereas P-glycoprotein plays a secondary role; Bsep likely mediates excretion of 6α-OH-TC in the absence of Mrp2 and P-gp. In Bsep(−/−) mice, efficient synthesis of tetrahydroxy bile acids that are Mrp2 and P-gp substrates can explain the noncholestatic phenotype.

Introduction

Biliary secretion is a complex process requiring transport of osmotically active solutes, primarily conjugated bile salts and glutathione, into the canalicular space (Blitzer and Boyer, 1982). Canalicular anion transport is mediated mostly by two unidirectional ATP-binding cassette (ABC) transport proteins: bile salt export pump (Bsep; Abcb11) and multidrug resistance-associated protein 2 (Mrp2; Abcc2). P-Glycoprotein ([P-gp] Mdr1a/b; Abcb1a/b) and Bcrp (Abcg2) are two additional ABC transporters that mediate canalicular efflux of drugs and their metabolites. Among the various canalicular transporters, Bsep plays a dominant role in biliary excretion of bile salts (Green et al., 2000; Wang et al., 2001), and it has a much greater transport capacity than any of the other canalicular transporters (Hofmann, 1994).

In humans, mutations in the BSEP gene that impair its function cause a fatal condition called type 2 progressive familial intrahepatic cholestasis (PFIC2) (Strautnieks et al., 1998; Jansen et al., 1999). PFIC2 is characterized by defective biliary salt secretion and extensive accumulation of bile salts in the liver, resulting in hepatic failure and death.

In contrast to the severe cholestasis occurring in man when BSEP is deficient, deletion of Bsep in the mouse causes only mild cholestasis. A major biliary bile acid in the Bsep(−/−) mouse was found to be the tauroine-conjugated 12α-hydroxy derivative of β-muricholic acid [3α,6β,7β,12α-(OH)4-5β-cholyl taurine; 12α-OH-βMC], indicating that this tetrahydroxy bile acid was formed in the mouse and could be excreted by one or more canalicular transporters other than Bsep (Wang et al., 2001). Another tetrahydroxy bile acid is formed in the Fxr(−/−) mouse when cholic acid

AABBREVIATIONS: ABC, ATP-binding cassette; BSEP/Bsep, bile salt export pump; MRP/Mrp, multidrug resistance-associated protein; P-gp, P-glycoprotein; Bcrp, breast cancer resistance protein; PFIC2, type 2 progressive familial intrahepatic cholestasis; 12α-OH-TβMC, tauroine-conjugated 12α-hydroxy β-muricholic acid; Fxr, farnesoid X receptor; mMrp2, mouse Mrp2; 6α-OH-TC, 6α-OH-taurocholic acid; MDR1, multidrug resistance 1; [3H]6β-TC, tauroine-conjugated 22, 23-3H-3α, 6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; HPLC, high-performance liquid chromatography; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; mP-gp, mouse P-gp; EV, empty vector; mBsep, mouse Bsep; hMRP, human MRP; hMDR1, human MDR1; TUDC, tauroursodeoxycholate; ANOVA, analysis of variance.
is added to the diet (Cho et al., 2009); this bile acid was shown to be 3α,6α-, or 6β,7α,12α-tetrahydroxy bile acid (as the taurine conjugate), and it is likely to be formed by 6-hydroxylation of cholic acid as such and its taurine conjugate. In patients with severe cholostasis, cholic acid is hydroxylated at C-6 to form 3α,6α,7α,12α-OH-tetrahydroxy-5β-cholanoic acid, which is excreted in part in urine (Bremmelgaard and Sjøvall, 1979, 1980).

We have shown that human MRP2 transports tauroursodeoxycholate (TUDC), a hydrophilic taurine-conjugated dihydroxy bile acid (Gerk et al., 2007). Lam et al. (2005) showed that the expression of Mrp2, Mdr2, Mrp2, and Mrp3 was markedly induced in Bsep−/− mice. Taken together, these observations suggested that Mrp2 might mediate the Bsep-independent biliary efflux of hydrophilic tetrahydroxy bile acids. In this study, we determined the role of both mouse Mrp2 (mMrp2) and P-glycoprotein (mMdr1a) in the hepatobiliary excretion of the tetrahydroxy bile salt, 6α-OH-taurocholic acid (6α-OH-TC). Single-pass liver perfusion studies (in wild-type and Mrp2−/− mice in the presence and absence of OH-TC). Single-pass liver perfusion studies (in wild)-type and

FIG. 1. Structure of cholic acid, 3α,6α,7α,12α-tetrahydroxy-5β-cholane-24-oic acid (6α-OH-CA), β-muricholic acid, and 12α-OH-β-muricholic acid as their corresponding taurine conjugates.
liver was severed and the inferior vena cava above the liver was cannulated with PE-60 tubing. After an 10-min period of equilibration of liver temperature and bile flow, \[^3H\]6α-OH-TC (21 nCi/nmol) was infused into the portal vein at a rate of 24 nmol/min over 4 min. For P-gp inhibition studies, GF120918 (10 μM, 0.3 ml of dimethyl sulfoxide) or vehicle was added to the perfusion medium 5 min before \[^3H\]6α-OH-TC infusion. Bile and perfusate outflow were collected in 5- (0–30 min) and 10-min intervals (30–90 min). The bile volume was determined gravimetrically, assuming a density of 1.0. At the end of the perfusion, livers were isolated and snap-frozen.

For the enterohepatic recirculation studies, intraduodenal administration of bile salts was performed as described previously, with slight modifications (Ballatori et al., 2008). In brief, the ileum was ligated at the ileocecal junction and 5 cm proximal to the ileocecal junction. Saline (150 μl) containing \[^3H\]6α-OH-TC (2μCi, 150 nmol) and a tracer dose of \[^14C\]taurocholate (1 μCi, 15 nmol, a recovery standard) were injected with a 26-gauge needle directly into the lumen of the ileum between the sutures. Bile was collected in 5- (0–30 min) and 10-min intervals (30–120 min). \[^3H\]6α-OH-TC and \[^14C\]taurocholate in the bile were quantitated by liquid scintillation spectroscopy. Reported transport values were corrected for that occurring in saline by subtracting the counts in bile from the counts in ileal perfusate.

Analytical Methods. Total radioactivity was quantitated in aliquots of bile and perfusate outflow after addition of 5 ml of Bio-Safe II cocktail (Research Products International Corp., Mt. Prospect, IL) by liquid scintillation spectroscopy. Liver radioactivity was counted as described previously (Chong-Rae Lim et al., 1996). Liver (0.2 g) was solubilized with 1 ml of Soluene-35 (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 50°C for 12 h, and then 0.2 ml of isopropyl alcohol and 0.4 ml of hydrogen peroxide (30%) were added to minimize the color quenching. After neutralizing the mixture with 5 N HCl, 10 ml of scintillation cocktail was added and incubated in the dark at 25°C for 24 h; the radioactivity was then measured.

Vesicular Transport Assay. ATP-dependent transport of \[^3H\]6α-OH-TC into the inside-out Sf9 membrane vesicles overexpressing the mouse and human canalicular transporters and control vesicles were determined as described previously (Gerke et al., 2004). ATP-dependent transport of \[^3H\]6α-OH-TC into membrane vesicles (10 μg/20 μl) was measured in incubations at 37°C for 2 min, transport stopped with 3.5 ml of ice-cold stop buffer, and the mixture was quickly filtered through Durapore 0.4-μm filters (Millipore, Bedford, MA). Radioactivity on the filters was detected by liquid scintillation spectroscopy. Reported transport values were corrected for that occurring in the presence of AMP as well as for any ATP-dependent transport in Sf9 vesicles expressing EV.

Statistical Analysis. Data are presented as the mean ± S.D. (n = 4–5 animals per group). Statistical significance was assessed by two-way analysis of variance (ANOVA) with Tukey’s post hoc test using GraphPad 4.0 (GraphPad Software Inc., San Diego, CA), as indicated in figure legends. For bile flow studies, one-way ANOVA with Bonferroni post hoc test was used. In all cases, P < 0.05 was considered to be statistically significant.

Kinetic Analysis. Curve fitting was done by nonlinear regression analysis with GraphPad Prism 4. Values are presented as mean ± S.E. To determine allostericity, data from concentration-dependent transport assays were analyzed according to the Hill equation:

\[
\nu = \frac{V_{\text{max}} \cdot [S]}{K_{\text{m}} + [S]} \tag{1}
\]

where \(\nu\) = velocity, \(V_{\text{max}}\) = maximal velocity, \([S]\) = initial substrate concentration, \(K_{\text{m}}\) = the substrate concentration at half-maximal velocity, and \(n\) = Hill coefficient (Hill slope). The data were then compared with a fit to a one-binding site version of eq. 1 (n = 1), the Michaelis-Menten equation. To determine which of the two models best fit the data, the extra sum-of-squares F test was used.

Results

Disposition and Choleretic Effect of 6α-OH-TC in the Single-Pass Perfused Mouse Liver. The involvement of Mrp2 and P-gp in the hepatobiliary transport of 6α-OH-TC was investigated by analyzing the uptake and biliary secretion of 6α-OH-TC in the perfused liver of wild-type and Mrp2(-/-) mice in the presence or absence of 10 μM GF120918 (Figs. 2 and 3). 6α-OH-TC was rapidly taken up from the perfusate and secreted in bile of wild-type mice. Sixty percent of the dose was secreted into the bile within 20 min of the start of infusion, and an additional 18% of the dose was secreted into bile over the next 40 min (Fig. 2, A and B; Table 1). Coinfusion of GF120918, a potent inhibitor of Mrd1 and Bcrp, did not significantly alter the biliary secretion of \[^3H\]6α-OH-TC. In Mrp2(-/-) mice, however, the rate of biliary secretion of 6α-OH-TC was significantly decreased (Fig. 2A) compared...
The concentration of 6α-OH-TC in perfusate outflow versus time is shown in Fig. 3. Approximately 8 to 10% of the dose was seen in the perfusate within the first 5 min after infusion of 6α-OH-TC in all treatment groups and indicates the portion of the dose that was not extracted by the liver. However, at later time points, the concentration of 6α-OH-TC in the perfusate outflow in Mrp2(-/-) mice was higher compared with wild-type mice (Fig. 3, A and B), suggesting that the accumulated 6α-OH-TC in the liver, and that which was not secreted into bile in Mrp2(-/-) mice, was in part effluxed across the basolateral membrane into the perfusate. 6α-OH-TC was not retained extensively in the livers of wild-type mice, either in the presence or absence of GF120918. As noted in Table 1, 6α-OH-TC accumulated >20-fold in the livers of Mrp2(-/-) compared with wild-type mice, and infusion of GF120918 further increased the retention of 6α-OH-TC in the liver in Mrp2(-/-) mice.

Kinetics of ATP-Dependent [3H]6α-OH-TC Transport by Mouse and Human Canaliculat Transporters in Vesicular Transport Assays. We characterized the transport of a broad concentration range (5–750 μM) of [3H]6α-OH-TC by membrane vesicles overexpressing mMrp2, mMDR1a, or mBsep to obtain the kinetic parameters. The transport of [3H]6α-OH-TC by mMrp2, mP-gp, and mBsep was linear with respect to incubation time (Fig. 4A); furthermore, linearity with respect to membrane protein concentration (5–20 μg) was established (data not shown). Membrane vesicles from 5FF cells transfected solely with EV did not transport [3H]6α-OH-TC, nor did membrane vesicles expressing mouse Bcrp (Fig. 4A).

Mrp2 mediated saturable transport of [3H]6α-OH-TC that was best fitted to classic Michaelis-Menten kinetics (Fig. 4B; Table 2). mP-gp also transported [3H]6α-OH-TC via classic Michaelis-Menten kinetics with a lower apparent affinity compared with mMrp2. mBsep transported [3H]6α-OH-TC with positive cooperativity, as shown in Fig. 4B and Table 2. These data indicate that [3H]6α-OH-TC is a substrate for multiple canalicular transporters, namely mMrp2, mP-gp, and mBsep.

In view of the formation of tetrahydroxy bile acids under cholestatic conditions in humans, we also investigated the [3H]6α-OH-TC transport by hMRP2 and hMDR1 overexpressing plasma membrane vesicles under conditions of linearity with respect to time and protein concentration. hMRP2 transported the bile acid with low apparent affinity but with strong evidence of positive cooperativity. hMDR1 showed lower apparent affinity for [3H]6α-OH-TC than mMDR1a and the transport was also positively cooperative, although to a lesser extent than that observed with hMRP2 (Fig. 4C; Table 2).

Biliary Secretion of [3H]6α-OH-TC and [3H]Taurocholate after Its Intralereal Administration in Wild-Type Mice. To determine the absorption of [3H]6α-OH-TC by the terminal ileum and therefore its potential for enterohepatic recirculation, [3H]6α-OH-TC was injected into the terminal ileum lumen of wild-type mice and its biliary recovery was measured. [3H]Taurocholate was coadministered as a recovery marker. The biliary secretion of [3H]6α-OH-TC and [3H]Taurocholate followed a similar time course, with the majority of the dose excreted in bile within the first 15 min of their intralaereal administration (Fig. 5A). However, the total recovery of [3H]6α-OH-TC radioactivity was lower (51%) than that of [3H]Taurocholate (91%) at the end of the 120-min collection period (Fig. 5B). Nonetheless, these data indicated that [3H]6α-OH-TC is absorbed from the terminal ileum and can undergo enterohepatic recirculation, as do other major conjugated bile acids.

Inhibition of 6α-OH-TC Transport by 12α-OH-TjMC. 12α-OH-TjMC is the predominant tetrahydroxyalted bile acid species in Bsep(-/-) mice (Perwaiz et al., 2003). To determine whether it might also be an Mrp2 substrate, we investigated its effect on 6α-
**TABLE 1**

Percent recovery of 6α-OH-TC in perfused mouse liver

<table>
<thead>
<tr>
<th></th>
<th>Bile</th>
<th>Perfusate</th>
<th>Liver</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>−GF120918</td>
<td>78 ± 5</td>
<td>14 ± 2</td>
<td>0.2 ± 0.03</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Mrp2(−/−) mice</td>
<td>52 ± 3*</td>
<td>26 ± 4*</td>
<td>5.4 ± 2*</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>+GF120918</td>
<td>75 ± 6</td>
<td>17 ± 3</td>
<td>0.23 ± 0.04</td>
<td>89 ± 8</td>
</tr>
<tr>
<td></td>
<td>34 ± 5‡</td>
<td>30 ± 2‡</td>
<td>10 ± 2.2‡</td>
<td>74 ± 5</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. wild-type mice.
‡p < 0.05 vs. Mrp2(−/−) mice.

**Discussion**

Tetrahydroxy bile acids occur very rarely as biliary bile acids in vertebrates (Hofmann et al., 2010). They have recently been identified in substantial amounts in mice under two circumstances. The first is the Bsep(−/−) mouse, in which β-muricholic acid (3α,6β,7β-trihydroxy) undergoes C-12 hydroxylation, presumably by the microsomal hydroxylase Cyp8b1. The second is the Fxr(−/−) mouse ingesting a diet containing cholic acid, in which cholic acid (3α,7α,12α-trihydroxy) undergoes hydroxylation at C-6, preferably by Cyp3A11 (Cho et al., 2009). In humans, genetic defects in the BSEP gene that result in loss of BSEP expression cause the potentially fatal condition PFIC2, a disorder associated with markedly elevated serum bile acid levels and a low content of bile acids in bile (Jansen et al., 1999). In contrast to the fatal condition caused by BSEP deficiency in man, targeted inactivation of Bsep in mice is associated with a much less-severe phenotype. Bsep(−/−) mice have mild intrahepatic cholestasis with biliary bile acid secretion ~30% of that in wild-type mice (Wang et al., 2001). The identification of tetrahydroxy biliary bile acids in the Bsep(−/−) mouse requires that such bile acids use canalicular transporters other than Bsep. Our data indicate that at least one tetrahydroxy bile acid is transported by at least three canalicular transporters in mice, Mrp2, Mdr1a, and Bsep. We also used vesicular transport studies to show that the human canalicular transporters, hMRP2 and hMDR1 also transport this tetrahydroxy bile acid.

6α-OH-TC was taken up efficiently and secreted rapidly into the bile of wild-type mice. A role of Mrp2 in its biliary secretion was clearly shown by the marked decrease in the cumulative biliary secretion of 6α-OH-TC in Mrp2(−/−) mice from 78 to 52% of the dose. However, the remaining 52% of the dose secreted in bile of Mrp2(−/−) mice implied the role of yet other canalicular transporters in 6α-OH-TC biliary secretion. Wang et al. (2009) recently generated the Bsep, Mdr1a, and Mdr1b triple knockout mice, which had a markedly increased severity of cholestasis, implying that P-gp can function as a compensatory mechanism in Bsep(−/−) mice. Therefore, we investigated the role of P-gp as a mechanism of biliary secretion of 6α-OH-TC using GF120918, a very potent inhibitor of P-gp and Bcrp (Hyafil et al., 1993; Allen et al., 2003). Infusion of 10 μM GF120918 has been shown to effectively inhibit P-gp in mouse liver (Tian et al., 2007). When GF120918 was coinfused with 6α-OH-TC in Mrp2(−/−) mice, cumulative biliary secretion of 6α-OH-TC was even further reduced to 34% of the dose, indicating that P-gp is likely another canalicular transporter for this bile salt. However, it should be noted that coinfusion of GF120918 in the wild-type mice did not alter biliary secretion of 6α-OH-TC. Based on these
observations and the fact that P-gp expression in Mrp2(−/−) mice is similar to that in wild-type mice (Chu et al., 2006), we conclude that P-gp plays a secondary role to Mrp2 in 6α-OH-TC biliary secretion. The canalicular transporter Bcrp is also inhibited by GF120918. Thus, decreased biliary secretion of [3H] 6α-OH-TC in the presence of GF120918 could suggest a role for Bcrp in canalicular transport of [3H]6α-OH-TC, although Bcrp expression is not changed in Mrp2(−/−) mice (Chu et al., 2006). Although human BCRP expressed in Lactococcus lactis was shown to transport cholate, deoxycholate, and taurocholate (Janvilisri et al., 2005), we did not detect ATP-dependent transport of 6α-OH-TC in plasma membrane vesicles overexpressing murine Bcrp.

6α-OH-TC was taken up efficiently into hepatocytes, as indicated by its extensive and rapid elimination into bile. The efficiency of uptake may be somewhat less than that observed for taurocholate in the perfused rat liver, which exceeds 90% (Holzinger et al., 1998). The sodium-dependent uptake (Ntcp) and sodium-independent uptake (Oatp) transporters in the basolateral membrane play an important role in the hepatic uptake of physiologic conjugated bile acids. However, identification of the specific carriers responsible for 6α-OH-TC uptake requires further investigation. The concentration of 6α-OH-TC in the perfusate outflow was also measured. The continuing regurgitation of 6α-OH-TC at later time points from the liver of Mrp2(−/−) mice was greater than in wild-type mice, consistent with its decreased biliary clearance, and is likely due to efflux of 6α-OH-TC by basolateral transporters.

The above in vivo findings obtained from perfusion experiments were confirmed by in vitro transport experiments. [3H]6α-OH-TC was transported by membrane vesicles from mMrp2 expressing Sf9 cells in an ATP-dependent manner that was saturable, confirming that Mrp2 was capable of transporting this hydrophilic bile salt. mMdr1a overexpressing Sf9 membrane vesicles also transported 6α-OH-TC but with a lower apparent affinity than mMrp2. Therefore, it is possible that P-gp can play a significant role as an alternative canalicular transporter for tetrahydroxy bile salts in Bsep(−/−) mice, where its expression level is significantly up-regulated (Wang et al., 2009). Characterization of transport of [3H]6α-OH-TC by mBsep-overexpressing membrane vesicles showed that Bsep transported 6α-OH-TC with lower apparent affinity but also with significant positive cooperativity. Thus, Bsep could account for 34% of the [3H]6α-OH-TC.
mouse, formation of tetrahydroxy bile acids and their elimination by multiple major canaliculi are life-saving adaptations to cholestasis, which do not occur in man.

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References


Bremmelgaard A and Svjoll J (1979) Bile acid profiles in urine of patients with liver diseases.


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