Drug- and Estrogen-Induced Cholestasis Through Inhibition of the Hepatocellular Bile Salt Export Pump (Bsep) of Rat Liver

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Background & Aims: **Drug-induced cholestasis is a frequent form of acquired liver disease. To elucidate the molecular pathogenesis of drug-induced cholestasis, we investigated the effects of prototypic cholestatic drugs on the canalicular bile salt export pump (Bsep) of rat liver.** ***Methods:*** **Vesicles were isolated from Bsep-, Mrp2-, and Bsep/Mrp2-expressing Sf9 cells. Canalicular plasma membrane (cLPM) vesicles from rat liver and Sf9 cell vesicles were used to study adenosine triphosphate (ATP)-dependent solute uptake by a rapid filtration technique.** ***Results:*** **Bsep-expressing Sf9 cell vesicles showed ATP-dependent transport of numerous monoanionic bile salts with similar Michaelis constant values as in cLPM vesicles, whereas several known substrates of the multispecific organic anion transporter Mrp2 were not transported by Bsep. Cyclosporin A, rifamycin SV, rifampicin, and glibenclamide *cis*-inhibited Bsep-mediated bile salt transport to similar extents as ATP-dependent taurocholate transport in cLPM vesicles. In contrast, the cholestatic estrogen metabolite estradiol-17β-glucuronide inhibited ATP-dependent taurocholate transport only in normal cLPM and in Bsep/Mrp2-coexpressing Sf9 cell vesicles, but not in Mrp2-deficient cLPM or in selectively Bsep-expressing Sf9 cell vesicles, indicating that it *trans*-inhibits Bsep only after its secretion into bile canaliculi by Mrp2.** ***Conclusions:*** **These results provide a molecular basis for previous in vivo observations and identify Bsep as an important target for induction of drug- and estrogen-induced cholestasis in mammalian liver.**

Ongoing bile formation is an important function of mammalian liver. It is an osmotic transport process that is driven by active secretion of bile salts, organic anions (e.g., glutathione), and lipids across the canalicular membrane of hepatocytes into the minute channels of bile canaliculi.[1] Water and electrolytes follow passively through aquaporin and tight junctions.[1] From the biliary canaliculi, bile is transported via the bile ducts into the duodenum where it is essential for normal fat solubilization and absorption. Defective bile secretion results in cholestasis with accumulation of bile salts and other toxic bile constituents within hepatocytes and blood plasma.[1] Clinically cholestatic liver disease is characterized by pruritus, jaundice caused by hyperbilirubinemia, altered lipid and cholesterol metabolism, and intestinal malabsorption of fat and fat soluble vitamins. Chronic cholestasis of long duration leads to biliary cirrhosis because of bile salt–induced toxic liver cell necrosis followed by fibrosis and cirrhosis.

Cholestasis may occur because of hereditary or acquired impairment of selective canalicular bile secretory processes or as a secondary consequence of structural hepatocyte or bile duct damage. Recently, specific molecular transport defects have been identified as the cause of certain forms of progressive familial intrahepatic cholestasis (PFIC).[1] For example, PFIC-2 is caused by absence of the liver bile salt export pump BSEP[2] and PFIC-3 is associated with the defective hepatocytic phosphatidylcholine transporter MDR3.[3,4] Furthermore, the conjugated hyperbilirubinemia associated with the Dubin–Johnson syndrome is caused by a hereditary mutation leading to defective expression of the canalicular multiorganic anion transporter or MRP2.[5] A similar Mrp2 defect is present in the transport mutant GY/TR$^-$ and Eisai hyperbilirubinemic rat strains.[6,7] Among these, ABC (adenosine triphosphate [ATP]-binding cassette) transporters, the rat liver bile salt export pump (Bsep) has been recently shown to functionally mediate canalicular ATP-dependent bile salt secretion.[8] Although PFIC and other hereditary forms of cholestasis are rare diseases, cholestasis induced by drugs and other xenobiotic substances is a frequent problem in clinical medicine.[9,10] Unfortunately, in most cases of drug-induced cholestasis the underlying pathogenetic mechanism(s) of liver damage remains unknown. However, identification of the molecular pathogenesis of drug-induced cholestasis is important because it provides the only possibility to detect potential

Abbreviations used in this paper: Bsep (BSEP), bile salt export pump of rat (human) liver; cLPM, canalicular liver plasma membrane; CyA, cyclosporin A; $E_2$17G, ethinylestradiol-17β-glucuronide; MDR, multidrug resistance gene product; MOI, multiplicity of infection; Mrp2 (MRP2), canalicular multidrug resistance protein 2 of rat (human) liver; PFIC, progressive familial intrahepatic cholestasis.

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cholestatic side effects already during drug development. In this study we have investigated whether inhibition of the canalicular bile salt export pump Bsep(rat)/BSEP(human) could account for some forms of drug-induced cholestasis. The results show that the prototypic drugs cyclosporin A (CyA), rifamycin SV, rifampicin, and glibenclamide all *cis*-inhibit ATP-dependent bile salt transport to similar extents in isolated canalicular rat liver plasma membranes (cLPMs) and in Bsep-expressing Sf9 cell vesicles (Bsep-vesicles). In contrast, the estrogen metabolite estradiol-17β-glucuronide ($E_2$17G) *trans*-inhibited Bsep-mediated bile salt transport activity and, therefore, exerts its cholestatic action only after its excretion by Mrp2 into the canalicular lumen. These data prove the principle and support the concept that inhibition of the canalicular Bsep represents an important mechanism of drug-induced cholestasis.

Materials and Methods

Cell Culture

Sf9 cells were obtained from Pharmingen (San Diego, CA) and kept at 27°C under standard conditions. Infections with baculoviruses encoding Bsep[8] were performed with a multiplicity of infection (MOI) of 10 and 3 days of incubation before subcellular fractionation. Mrp2 recombinant baculovirus[11] was generated with the Bac-to-Bac system (Life Technologies Inc., Gaithersburg, MD). Sf9 cells were infected with Mrp2-encoding baculovirus at MOI of 7.5 and incubated for 2 days before subcellular fractionation. Coexpression of both Mrp2 and Bsep (Mrp2/Bsep-vesicles) has been performed at MOIs of 8 and 10, respectively, and 3 days of incubation before subcellular fractionation.

Subcellular Fractionation

Male Sprague–Dawley rats were obtained from RCC Ltd. (Füllinsdorf, Switzerland). GY/TR$^-$ rats were a generous gift of Dr. F. Kuipers (Groningen, The Netherlands) and were bred in-house. The animals were kept under standard conditions and had free access to food and water. Rat liver cLPM vesicles were isolated as described.[12] A total membrane fraction from Sf9 cells infected with wild-type, Mrp2-, and/or Bsep-expressing baculovirus was isolated as described previously.[8] Canalicular and Sf9 cell–derived vesicles were resuspended in 50 mmol/L sucrose, 100 mmol/L KNO_3, and 10 mmol/L HEPES/Tris (pH 7.4) and stored in liquid nitrogen until used. The expression levels of Mrp2, Bsep, and Mrp2/Bsep in Sf9 cell–derived vesicles were routinely checked by Western blotting using rabbit polyclonal antibodies against the corresponding C-termini.[8,11] Protein was determined using a modification of the Lowry procedure.[13]

Transport Studies

Uptake experiments were performed by a rapid filtration technique.[14] The buffer system was optimized for ATP-dependent bile salt transport[15] and contained 50 mmol/L sucrose, 100 mmol/L KNO_3, and 10 mmol/L $Mg(NO_3)_2$ with or without 5 mmol/L ATP and various substrate inhibitors at the concentrations given in the figures. ATP-dependent uptake was calculated as the difference of uptake in the presence and absence of ATP. The inhibitors CyA and rifampicin were obtained from Sigma Chemical Co. (St. Louis, MO), rifamycin SV from Fluka AG (Buchs, Switzerland), and glibenclamide from ICN Biochemical Inc. (Aurora, Ohio). [^{14}C]Chlorodinitrobenzene, [^{3}H]leukotriene C_4, [^{3}H]glutathione, [^{3}H]taurocholic acid, [^{3}H]glycocholic acid, and [^{3}H]estradiol-17β-glucuronide were obtained from NEN Life Science Products (Boston, MA). All other radioactive bile acids were a generous gift of Dr. A. F. Hofmann (Department of Medicine, UCSD, San Diego, CA). The glutathione conjugate of chlorodinitrobenzene (DNP-SG) was synthesized as described previously.[16]

Results

Functional Expression of Bsep in Baculovirus-Infected Sf9 Cells

In mammalian (including human) liver, the canalicular secretion of anionic bile salts and nonbile salt organic anions is mediated by the 2 separate ABC transporters Bsep (BSEP) and Mrp2 (MRP2).[17] These complementary transport functions were confirmed by the separate expression of rat liver Bsep and Mrp2 in baculovirus-infected Sf9 cells (Table 1). Although membrane vesicles from Sf9 cells infected with the wild-type baculovirus did not exhibit any ATP-dependent transport activities, infection of Sf9 cells with Bsep complementary DNA–containing baculovirus resulted in the selective expression of ATP-dependent transport activity for the monoanionic primary and secondary bile salts taurocholate, glycocholate, taurochenodeoxycholate, glycochenodeoxycholate, taurodeoxycholate, and tauroursodeoxycholate (Table 1). In contrast, expression of Mrp2 conferred ATP-dependent transport activities for sulfated taurolithocholate, dinitrophenylglutathione (DNP-SG), leukotriene C_4, $E_2$17G, and oxidized glutathione (GSSG) (Table 1). Expression of Bsep and Mrp2 in the isolated Sf9 cell vesicles was independently verified in Western blot analysis using monospecific polyclonal antibodies (Figure 1). These data show that the substrate specificity of Bsep is confined to monoanionic bile salt derivatives and that Bsep exhibits the greatest transport activities for the primary dihydroxylated bile salts taurochenodeoxycholate and glycochenodeoxycholate.

Bsep Mediates Characteristic Canalicular Bile Salt Transport Activity

Because the cholestatic potential of Bsep inhibition is directly dependent on the overall significance of Bsep for canalicular bile salt secretion, we next compared

Table 1. Comparison of the Substrate Specificity of Bsep and mrp2

Substrate	Control vesicles	Bsep vesicles	Mrp2 vesicles
Taurocholate (2 μmol/L)	1.1 ± 0.8	69.2 ± 4.1[a]	1.1 ± 0.7
Glycocholate (20 μmol/L)	9.1 ± 6.9	59.7 ± 13.2[a]	10.3 ± 4.4
Taurochenodeoxycholate (2 μmol/L)	66.8 ± 14.1	150.1 ± 9.8[a]	ND
Glycochenodeoxycholate (2 μmol/L)	16.7 ± 10.8	118.5 ± 15.6[a]	ND
Taurodeoxycholate (2 μmol/L)	6.8 ± 3.5	27.2 ± 8.8[a]	ND
Tauroursodeoxycholate (2 μmol/L)	1.9 ± 1.1	22.1 ± 2.9[a]	ND
Taurolithosulfocholate (2 μmol/L)	3.2 ± 1.6	3.7 ± 1.4	41.1 ± 3.7[a]
DNP-SG (25 μmol/L)	6.5 ± 2.4	6.3 ± 3.4	81.4 ± 7.2[a]
Leukotriene C_4 (0.05 μmol/L)	0.7 ± 0.2	0.9 ± 0.3	3.6 ± 0.3[a]
$E_2$17G (10 μmol/L)	8.5 ± 1.2	7.9 ± 1.7	56.9 ± 8.1[a]
GSSG (100 μmol/L)	1.5 ± 1.5	1.0 ± 1.3	51.7 ± 2.7[a]

NOTE. Vesicles were isolated from Sf9 cells infected with either wild-type baculovirus (control) or baculovirus containing Bsep or mrp2, respectively. Transport studies were performed as described in Materials and Methods. ATP-dependent uptake rates are given as pmol substrate/mg protein × min. The data are given as means ± SD of triplicate determinations in 3 separate membrane preparations.
DNP-SG, dinitrophenylglutathione; GSSG, oxidized glutathione; ND, not determined.
[a] Values significantly different from control values ($P < 0.02$).

the kinetics of ATP-dependent bile salt transport activity in isolated cLPMs and in Bsep-expressing Sf9 cell vesicles. In both systems, the Michaelis constant (K_m) values of initial (linear) ATP-dependent uptake values were similar for the primary di- and trihydroxylated bile salts taurochenodeoxycholate and taurocholate, respectively (Table 2). The same was also true for tauroursodeoxycholate, which is frequently used as a therapeutic agent in cholestatic liver disease. Furthermore, the rank orders of the maximum velocity values were identical in cLPM vesicles and Bsep-expressing vesicles (taurochenodeoxycholate > taurocholate > tauroursodeoxycholate > glycocholate). Although glycocholate was transported by Bsep (Table 1), its ATP-dependent transport rate was considerably lower than that of taurine-conjugated bile salts (Tables 1 and 2), which may relate to the fact that the rat liver predominantly forms taurine-conjugated bile salts. The relatively low transport activity together with a low degree of expression (compared with cLPM) prevented the exact kinetic characterization of ATP-dependent glycocholate transport in Bsep vesicles (Table 2). Nevertheless, the close similarity of the ATP-dependent bile salt transport kinetics between cLPM vesicles and Bsep-expressing vesicles strongly indicates that Bsep represents the major, if not the only, canalicular Bsep in rat liver. This conclusion is further supported by similar *cis*-inhibitory effects of unlabeled bile salts (20 μmol/L) on ATP-dependent uptake of [^{3}H]taurocholate (2 μmol/L) in cLPM vesicles (taurocholate, 61% inhibition; taurodeoxycholate, 62% inhibition) and Bsep vesicles (taurocholate, 70% inhibition; taurodeoxycholate, 67% inhibition). The same was also true for CyA (3 μmol/L; cLPM, 91% inhibition; Bsep vesicles, 76% inhibition) and glibenclamide (40 μmol/L; cLPM, 83% inhibition; Bsep vesicles, 75% inhibition), providing first evidence that inhibition of Bsep might play a role in drug-induced cholestasis.

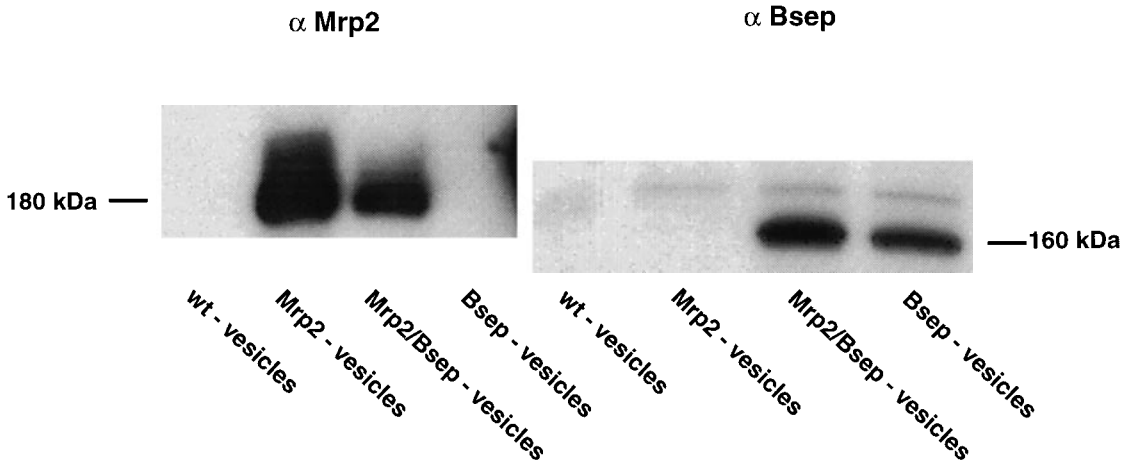

Figure 1. Western blot analysis of Mrp2, Bsep, and Mrp2/Bsep protein expression in baculovirus-infected Sf9 cell vesicles. Lanes were loaded with total membrane vesicles isolated from Sf9 cells infected with wild-type baculovirus (wt-vesicles), Mrp2-cDNA containing baculovirus (Mrp2-vesicles), Mrp2-cDNA and Bsep-cDNA containing baculoviruses (Mrp2/Bsep-vesicles), and Bsep-cDNA containing baculovirus (Bsep-vesicles). Western blotting was performed with antibodies against Mrp2 (αMrp2) and Bsep (αBsep).[8,11] The results show that both antibodies selectively recognize their specific antigens and that vesicles isolated from double-infected Sf9 cells (i.e., Mrp2/Bsep vesicles) contain both Mrp2 and Bsep.

Table 2. Comparison of Kinetic Parameters of ATP-Dependent Transport of Various Bile Salts in Rat Liver cLPM Vesicles or Vesicles Isolated From Sf9 Cells Expressing Bsep (Bsep Vesicles)

	cLPM		Bsep vesicles	
Bile salt	V_{max}	K_m	V_{max}	K_m
Taurochenodeoxycholate	927 ± 308	3.6 ± 2.8	241 ± 33	2.2 ± 0.7
Taurocholate	724 ± 120	2.1 ± 1.7	160 ± 8	5.3 ± 0.7
Tauroursodeoxycholate	431 ± 56	6.2 ± 2.0	62 ± 10	4.1 ± 1.6
Glycocholate	110 ± 26	3.8 ± 3.0	ND	ND

NOTE. Vesicles were isolated from rat liver (cLPM) or Sf9 cells infected with baculovirus containing Bsep. Initial (45 seconds) ATP-dependent uptakes of increasing bile salt concentrations were determined as described in Materials and Methods. Kinetic parameters were fitted to the uptake data using the Michaelis–Menten equation with nonlinear regression analysis (Systat; Systat Inc. Evanston, IL). V_{max} ± SE values are given as pmol bile salt/mg protein × min. K_m ± SE values are given as μmol/L. The values for taurocholate are taken from Stieger et al.[15] and Gerloff et al.[8] for cLPM and Bsep vesicles, respectively.

ND, not determined; V_{max}, maximum velocity.

cis-Inhibition of Bsep by Cholestatic Drugs

Numerous drugs can interfere with normal bile formation and induce cholestasis in mammalian liver,[9,10] including CyA,[18–20] rifamycin SV,[21,22] rifampicin,[23,24] and the sulfonylurea antidiabetics.[25–29] CyA has been previously shown to *cis*-inhibit ATP-dependent taurocholate transport in cLPM vesicles with a K_i value of 0.2 μmol/L.[30] We found a similar K_i value (0.3 μmol/L) for CyA-induced inhibition of Bsep-mediated taurocholate transport in Sf9-cell vesicles (Figure 2). In addition, the antibiotics rifamycin SV and rifampicin and the sulfonylurea antidiabetic glibenclamide competitively *cis*-inhibited ATP-dependent taurocholate uptake with similar K_i values in cLPM and Bsep vesicles (Figure 2). Only for rifamycin SV, a greater than 2-fold difference in the K_i values was observed, suggesting that this drug may exert additional inhibitory effects (e.g., *cis*- and t*rans*-inhibition) in cLPM vesicles. Nevertheless, the results support the concept that direct *cis*-inhibition of Bsep can induce intracellular accumulation of toxic bile salt concentrations and lead to cholestatic liver disease. Parenthetically, CyA (10 μmol/L) inhibited Mrp2-mediated dinitrophenylglutathione only moderately (~50% inhibition), whereas rifamycin SV and rifampicin (10–100 μmol/L) exerted no inhibitory effects on Mrp2-mediated transport (data not shown).

$E_2$17G *trans*-Inhibits Bsep From the Canalicular Lumen

$E_2$17G is a physiological estrogen metabolite that is predominantly excreted into bile by Mrp2 (Table 1).[31] However, at higher doses $E_2$17G can induce cholestasis in the rat.[31] Therefore, we investigated whether the cholestatic effect of $E_2$17G could also be explained by inhibition of Bsep. As shown in Figure 3, $E_2$17G exhibited a dose-dependent inhibition of ATP-dependent taurocholate uptake in cLPM vesicles of normal rat liver. However, no inhibitory effects of $E_2$17G on taurocholate transport were found in Bsep vesicles. A possible explanation for this discrepancy between cLPM vesicles of normal rat liver and Bsep-expressing vesicles could be that $E_2$17G must be first transported into the cLPM vesicles by Mrp2 and then *trans*-inhibits Bsep from the inside of the vesicle. If this assumption is correct, $E_2$17G should have no inhibitory effects on ATP-dependent taurocholate transport in cLPM vesicles isolated from Mrp2-deficient GY/TR$^-$ rats.[6] This was in fact the case, as further illustrated in Figure 3. Furthermore, coexpression of Mrp2 and Bsep in Sf9 cell vesicles (Figure 1) resulted in a concentration- and time-dependent inhibition of ATP-dependent taurocholate transport by $E_2$17G. As shown in Figure 4, although 10 μmol/L $E_2$17G exerted low inhibitory effects at early (45 seconds) and late (5 minutes) time periods of incubation, 100 μmol/L of $E_2$17G inhibited ATP-dependent taurocholate uptake by 32% and 42%, respectively. These data are consistent with the interpretation that Mrp2-mediated intravesicular accumulation of $E_2$17G is required to significantly inhibit Bsep-mediated transport function. They most probably reflect *trans*-inhibition of Bsep by intravesicular $E_2$17G, although an Mrp2-dependent intramembranous interaction between $E_2$17G and Bsep cannot be definitely ruled out. The longer incubation periods required for the accumulation of inhibitory $E_2$17G concentrations within Mrp2/Bsep-coexpressing Sf9 cell vesicles (Figure 4) compared with cLPM vesicles (Figure 3) can be explained by the differences in transport rates between the 2 vesicular preparations, which in the case of Sf9 cell vesicles consist of a total membrane fraction with a lower carrier number per milligram of protein compared with the highly purified cLPM preparation.[8] Hence, our studies are consistent with the interpretation that $E_2$17G-induced cholestasis results from the accumulation of $E_2$17G in the bile canaliculus as a result of its excretion by Mrp2 (Figure 5).

Discussion

Canalicular bile salt secretion is the major driving force of hepatic bile formation. In our previous study, the so-called sister of P-glycoprotein[32] was identified as a canalicular ATP-dependent Bsep in rat liver.[8] In parallel, the coding region of the human BSEP gene has been

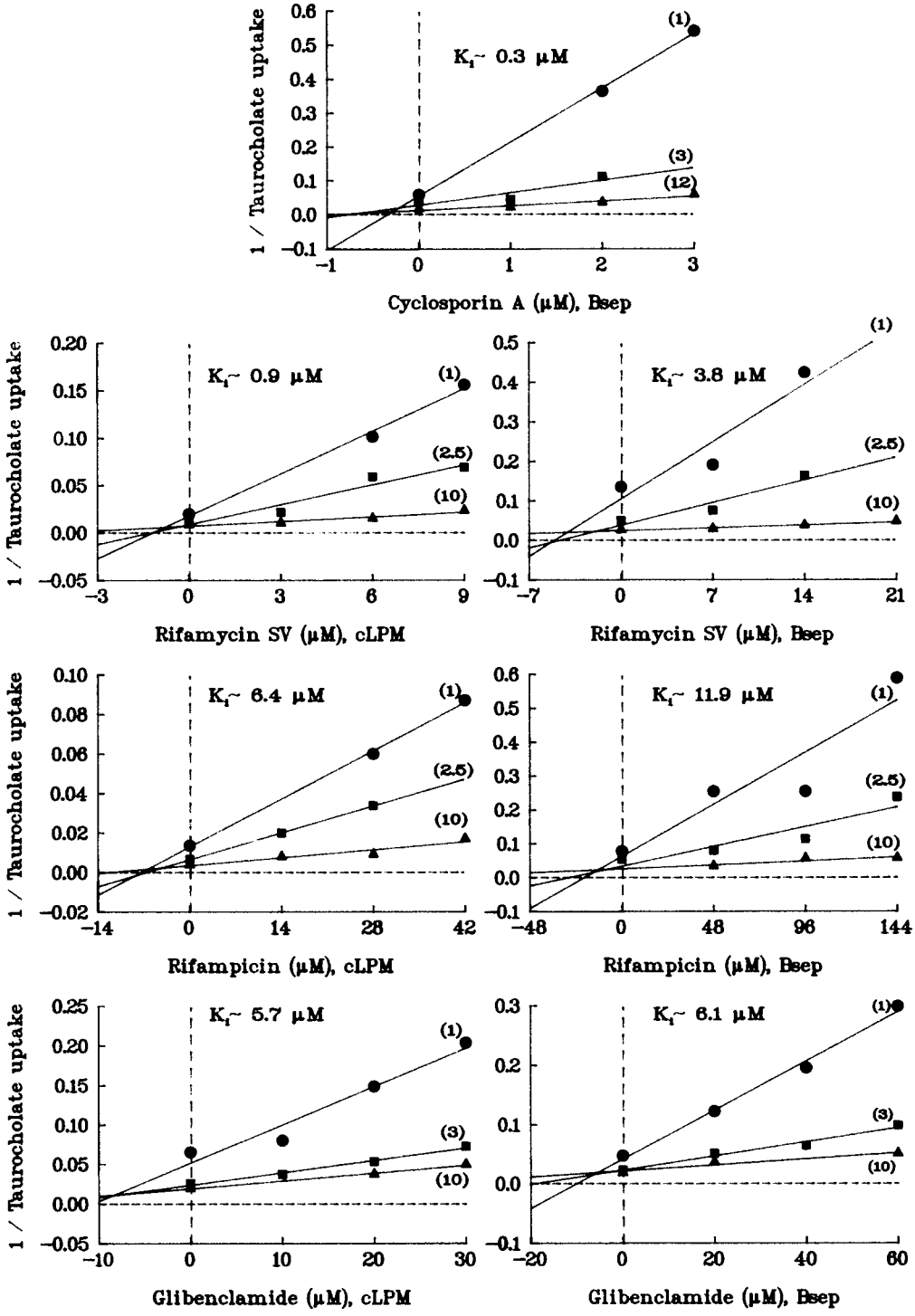

Figure 2. *cis*-Inhibition of ATP-dependent taurocholate uptake in cLPM vesicles of normal rat liver and in Bsep-expressing vesicles (Bsep) isolated from transfected Sf9 cells. ATP-dependent uptake of taurocholate (see Materials and Methods) was determined during the initial (linear) uptake phase (45 seconds) at the concentrations (µmol/L) given in parentheses and in the absence and presence of the indicated concentrations of the various drugs. Dixon plot analysis was used to evaluate the kinetics of drug-induced inhibition of ATP-dependent taurocholate uptake. The results are compatible with competitive *cis*-inhibitory effects by all drugs and indicate similar K_i values for a given drug in cLPM vesicles and Bsep-expressing Sf9 cell vesicles. Data represent the means of 6 uptake measurements in 2 of 3 separate membrane vesicle isolations.

elucidated, and several mutations predicted to disrupt BSEP gene function have been identified in patients with type 2 of PFIC (PFIC-2).[2] The phenotype of PFIC-2 is entirely consistent with an isolated defect in canalicular bile salt secretion and includes low bile acid levels in bile and elevated serum bile acids in the presence of normal serum γ-glutamyltranspeptidase and low or normal cholesterol in serum.[33] PFIC-2 invariably progresses to chronic liver disease because of the ongoing accumulation of toxic bile salts within hepatocytes. These findings suggest that a functionally defective BSEP disrupts normal canalicular bile salt secretion and induces progressive chronic cholestatic liver disease.

In the present study we have investigated whether Bsep (BSEP) might be a major target also in more frequently acquired forms of cholestasis, including drug- and estrogen-induced cholestasis. The studies have been performed with rat liver Bsep, because rat Bsep can be

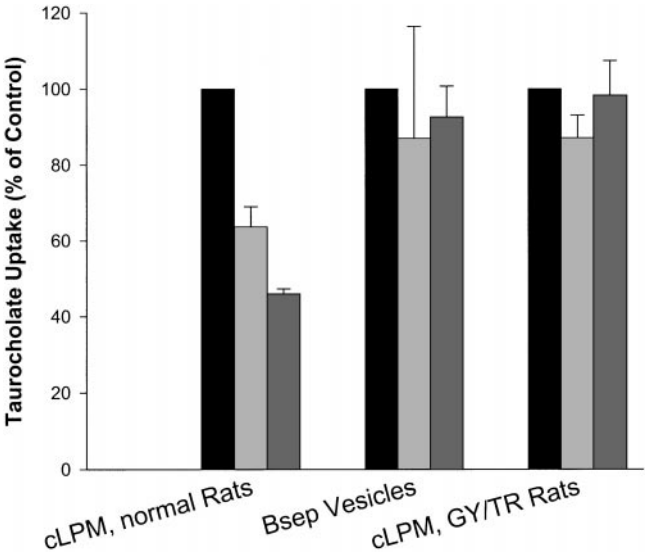

Figure 3. Effects of $E_2$17G on ATP-dependent taurocholate transport in cLPM vesicles of normal and Mrp2-deficient (GY/TR$^-$) rat liver and in Bsep-expressing Sf9 cell vesicles. Initial (45 seconds) ATP-dependent taurocholate (2 μmol/L) uptake was determined (see Materials and Methods) in the absence (control = 100%, ■) and presence of 10 (□) and 100 (▨) μmol/L $E_2$17G. Although $E_2$17G exerted a concentration-dependent inhibitory effect in cLPM vesicles of normal rat liver, this was clearly not the case in either Bsep or in GY/TR$^-$/cLPM vesicles. The results represent the means ± SD of 6 uptake determinations in 2 separate membrane vesicle isolations.

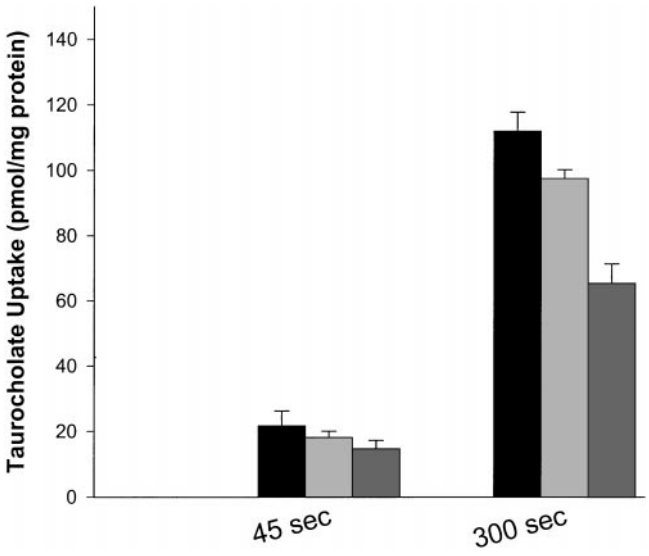

Figure 4. Inhibition of Bsep-mediated taurocholate transport by $E_2$17G in Mrp2/Bsep-coexpressing Sf9 cell vesicles. ATP-dependent uptake of taurocholate (2 μmol/L) was determined in Mrp2/Bsep vesicles in the absence (control, ■) and presence of 10 (□) and 100 (▨) μmol/L $E_2$17G. In contrast to Bsep vesicles (Figure 3), $E_2$17G exhibited a time- and concentration-dependent inhibition of ATP-dependent taurocholate uptake in Mrp2 and Bsep coexpressing vesicles, indicating *trans*-inhibition of Bsep-mediated taurocholate transport by $E_2$17G. Data represent means ± SD of triplicate determinations in 1 representative experiment.

functionally expressed in baculovirus-infected Sf9 cells (Table 1 and Figure 1),[8] and functional transport studies can be performed in parallel in isolated cLPM vesicles[14] and in Bsep-expressing Sf9 cell vesicles,[8] thus permitting the direct comparison of selective Bsep-mediated transport with ATP-dependent canalicular bile salt transport properties. The results show that (1) Bsep can mediate ATP-dependent transport of various primary and secondary conjugated monoanionic bile salts, but it does not transport Mrp2 substrates (Table 1); (2) the K_m values of ATP-dependent bile salt transport are similar in cLPM vesicles of rat liver and in Bsep-expressing vesicles (Table 2); (3) CyA, rifamycin SV, rifampicin, and glibenclamide *cis*-inhibit ATP-dependent taurocholate transport with similar K_i values in cLPM vesicles and Bsep-expressing vesicles (Figure 2); and (4) $E_2$17G is taken up into cLPM vesicles by Mrp2 (Table 1) and can *trans*-inhibit Bsep in a dose-dependent manner (Figures 3 and 4). These results provide further evidence that Bsep is the only ATP-dependent bile salt export pump in rat liver. Furthermore, they show that inhibition of Bsep by drugs and biotransformation products can account for certain acquired forms of cholestasis.

Obviously, and similar to PFIC-2, cholestasis induced by selective inhibition of Bsep (BSEP) should initially manifest itself by a predominant or even exclusive increase of bile salt concentrations within hepatocytes and in serum. Only after intracellular accumulation of toxic concentrations of bile salts within hepatocytes will biochemical signs of liver cell damage and clinical cholestasis become evident. Interestingly, CyA has been shown to preferentially increase serum bile salts in heart transplant patients with normal liver function,[18–20] sug-

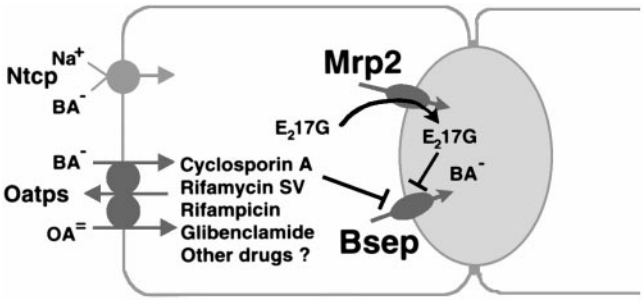

Figure 5. Scheme of the molecular pathogenesis of drug-induced cholestasis through inhibition of the canalicular bile salt export pump (Bsep). Drugs and drug metabolites can either directly *cis*-inhibit or indirectly *trans*-inhibit Bsep function at the canalicular membrane of hepatocytes. Both mechanisms lead to an intracellular accumulation of toxic bile salts that may or may not be associated with structural disintegration of hepatocytes depending on the extent of Bsep inhibition. The latter may be aggravated in humans by preexisting impairments of BSEP function caused by the existence of genetic polymorphisms in susceptible persons (see also text). For complementary reasons, the sinusoidal bile salt uptake systems are also illustrated: Ntcp, Na^+-dependent taurocholate cotransporting polypeptide[1]; Oatps, Na^+-independent and polyspecific organic anion transporting polypeptides[1]; BA$^-$, bile acids; OA$^-$, divalent organic anions.

gesting that CyA induces a decrease in hepatic excretory function in humans. Similar observations have also been made in rats[34,35] in which a single intravenous dose led to a significant but reversible decrease in bile flow and bile salt secretion.[36] This cholestatic effect of CyA has been explained by a high-affinity inhibition (K_i, ~0.2 μmol/L) of ATP-dependent taurocholate transport in rat liver canalicular LPM vesicles.[30] These previous experimental and clinical observations are very compatible with our findings of high-affinity *cis*-inhibition of Bsep by CyA (Figure 2). Hence, Bsep seems to be a primary target for CyA, and its direct *cis*-inhibition may represent the ultimate molecular basis of CyA-induced cholestasis.

In addition to CyA, the antibiotics rifamycin SV and rifampicin and the sulfonylurea antidiabetic glibenclamide competitively inhibited ATP-dependent taurocholate transport in cLPM and Bsep-expressing vesicles (Figure 2). These findings provide a molecular explanation for the reported decrease of biliary bile salt secretion by rifamycin SV[21,22] and by high doses of rifampicin[37] in rat liver. Furthermore, rifampicin has been reported to increase bile salt concentrations in serum[23,24] and induce reversible cholestasis[38] in some patients with otherwise normal liver function. For glibenclamide, no experimental data regarding its effects on canalicular bile salt secretion are yet available, but glibenclamide has also been suggested to induce dose-dependent cholestasis in a minority of patients.[25–29] Although the estimates of K_i for CyA, rifamycin SV, and rifampicin lie in the range of the corresponding therapeutic blood and serum drug concentrations,[19,39,40] the K_i for glibenclamide (~6 μmol/L) is considerably above the usual therapeutic serum concentrations of 0.05–0.2 μmol/L.[41] However, glibenclamide is concentrated up to 50-fold above serum concentrations[42] in rat liver, indicating that concentrations in hepatocytes might well reach the range of the estimated K_i. Although the ultimate significance of inhibited canalicular bile salt secretion for drug-induced cholestasis in humans remains to be investigated, the high amino acid identity (82%) between the rat and human canalicular bile salt transporters[2] as well as the reported inhibition of ATP-dependent canalicular bile salt transport by CyA in human cLPM[43] strongly indicate that the human BSEP is also a primary target for drug-induced inhibition. Therefore, the fact that only a minority of patients develop cholestasis during therapy with potentially cholestatic drugs could relate to the existence of genetic BSEP polymorphisms associated with constitutional impairments of BSEP function in susceptible patients. Furthermore, individual differences in drug metabolism[44] and/or cholehepatic shunting (i.e., recirculation of amphipathic drugs between bile ductular cells and hepatocytes)[45] could lead to the accumulation of inhibitory drug and drug metabolite concentrations within hepatocytes in predisposed patients.

In contrast to the direct *cis*-inhibitory effects of the investigated drugs (Figure 2), the cholestatic estrogen-metabolite $E_2$17G *trans*-inhibited Bsep function in cLPM vesicles of normal rat liver and in Mrp2/Bsep-coexpressing vesicles but not in cLPM of Mrp2-deficient GY/TR$^-$ rat liver or in selectively Bsep-expressing vesicles (Figures 3 and 4). These findings are fully consistent with the in vivo observations that normally cholestatic doses of $E_2$17G fail to induce cholestasis in Mrp2-deficient rat strains,[46] thus supporting the concept that Mrp2-mediated canalicular excretion is a prerequisite for $E_2$17G-induced cholestasis (Figure 5). Although $E_2$17G has been suggested to also *trans*-inhibit the canalicular Mdr1 or P-glycoprotein, the mechanism by which the interaction of $E_2$17G with Mdr1 could alter canalicular bile formation in rat liver remained unknown.[47] Our studies now indicate that *trans*-inhibition of Bsep-mediated bile salt secretion, which in contrast to Mdr1-dependent transport represents an important driving force for the generation of bile flow within bile canaliculi,[48] may be the predominant mechanism of $E_2$17G-induced cholestasis. Whether other steroid conjugates such as sulfated progesterone metabolites, which have been identified in large amounts in individuals with genetic predisposition for pregnancy-induced cholestasis,[49] exert similar effects as $E_2$17G, remains to be investigated.

In conclusion, the identification of the rat liver Bsep as a primary target for drug- and $E_2$17G-induced cholestasis provides the basis for similar future studies with the human BSEP. To account for the individual susceptibility, genetic BSEP polymorphisms will have to be looked for in clinical cohorts of patients with drug-induced cholestasis and cholestasis of pregnancy. These studies should also include the possible coexistence of a poor drug metabolizer phenotype[44] with a genetically determined impairment of BSEP function, because both mechanisms could amplify the cholestatic effects of xenobiotic substances. Finally, it will also be important to include the canalicular phosphatidylcholine translocase Mdr2 (rat) and MDR3 (human) in similar future studies because drug-induced inhibition of canalicular phospholipid secretion could account for additional forms of cholestasis that are primarily characterized by toxic damage of intrahepatic bile ductules and the vanishing bile duct syndrome.[3,4] These studies should ultimately permit a better prediction of the cholestatic potential of newly developed drugs and improvement of the individualization of drug therapy in susceptible patients with impaired liver function.

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