Toxicology in Vitro 29 (2015) 1454–1463

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Basal efflux of bile acids contributes to drug-induced bile acid-dependent hepatocyte toxicity in rat sandwich-cultured hepatocytes

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ARTICLE INFO

Article history:
Received 7 January 2015
Revised 22 April 2015
Accepted 4 June 2015
Available online 6 June 2015

Keywords:
Drug-induced liver injury
Bile acid
Sandwich-cultured hepatocyte
Bile salt export pump
Multidrug resistance-associated protein

ABSTRACT

The bile salt export pump (BSEP or Bsep) functions as an apical transporter to eliminate bile acids (BAs) from hepatocytes into the bile. BSEP or Bsep inhibitors engender BA retention, suggested as an underlying mechanism of cholestatic drug-induced liver injury. We previously reported a method to evaluate BSEP-mediated BA-dependent hepatocyte toxicity by using sandwich-cultured hepatocytes (SCHs). However, basal efflux transporters, including multidrug resistance-associated proteins (MRP or Mrp) 3 and 4, also participate in BA efflux. This study examined the contribution of basal efflux transporters to BA-dependent hepatocyte toxicity in rat SCHs. The apical efflux of [^{3}H]taurocholic acid (TC) was potently inhibited by 10 μM cyclosporine A (CsA), with later inhibition of basal [^{3}H]TC efflux, while MK571 simultaneously inhibited both apical and basal [^{3}H]TC efflux. CsA-induced BA-dependent hepatocyte toxicity was 30% at most at 10 μM CsA and ~60% at 50 μM, while MK571 exacerbated hepatocyte toxicity at concentrations of ≥50 μM. Quinidine inhibited only basal [^{3}H]TC efflux and showed BA-dependent hepatocyte toxicity in rat SCHs. Hence, inhibition of basal efflux transporters as well as Bsep may precipitate BA-dependent hepatocyte toxicity in rat SCHs.

1. Introduction

Drug-induced liver injury (DILI) is a potentially serious adverse event leading to the dropout of candidate compounds from drug development and the withdrawal of pharmaceuticals from clinical use (Kaplowitz, 2001; Schuster et al., 2005). DILI can severely damage the liver, resulting in liver transplantation in worst case scenarios. Hence, it is essential to promptly identify, remove, and/or assign alerts for possible risk compounds of DILI at all stages of the drug development process. Recently, the accumulation of bile acids (BAs) within hepatocytes was suggested as an underlying mechanism of cholestatic DILI (Byrne et al., 2002; Fattinger et al., 2001; Kostrubsky et al., 2006; Stieger et al., 2000). The bile salt export pump (human BSEP or rat Bsep), localized on the apical side of the hepatocyte plasma membrane, plays a major role in the excretion of BAs from the liver into the bile (Meier and Stieger, 2002). Therefore, control of BSEP function is an important factor for the regulation/dysregulation of hepatic BA content.

Several genetic mutations of BSEP are associated with progressive familial intrahepatic cholestasis type 2 (PFIC2) and cause severe intracellular accumulation of BAs within the liver (Strautnieks et al., 1998). Thus, BSEP dysfunction is quite likely to be related to liver injury. In fact, many researchers have reported that most drugs causing cholestatic DILI also potently inhibit BSEP (Dawson et al., 2012; Morgan et al., 2010; Pedersen et al., 2013; Warner et al., 2012). Accordingly, several methods have been developed for the determination of BSEP inhibition, including the widely used membrane vesicle assay. Nonetheless, ample experimental evidence indicates that the membrane vesicle assay might misestimate the clinical risk of cholestatic DILI, because this cell-free

Abbreviations: BA, bile acid; BEI, biliary excretion index; BSEP/Bsep, bile salt export pump; CsA, cyclosporine A; DILI, drug-induced liver injury; DMSO, dimethyl sulfoxide; $E_2$17βG, estradiol-17β-D-glucuronide; FBS, fetal bovine serum; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; IC_{50}, half-maximal inhibitory concentration; LDH, lactate dehydrogenase; MRP/Mrp, multidrug resistance-associated protein; NTCP/Ntcp, Na^+-taurocholate co-transporting polypeptide; OATP/Oatp, organic anion-transporting polypeptide; PFIC2, progressive familial intrahepatic cholestasis type 2; qPCR, quantitative polymerase chain reaction; SCH, sandwich-cultured hepatocyte; SD, standard deviation; TC, taurocholic acid; WME, Williams' Medium E.

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http://dx.doi.org/10.1016/j.tiv.2015.06.004
0887-2333/

system lacks certain molecular players related to BA disposition (e.g., metabolic enzymes and uptake transporters) (Dawson et al., 2012). To overcome this shortcoming, an alternative protocol using sandwich-cultured hepatocytes (SCHs) was established (Swift et al., 2010).

We recently utilized rat SCHs for constructing an *in vitro* BA-dependent hepatocyte toxicity assay system to mimic cholestatic DILI (Ogimura et al., 2011). We determined that recognized potent BSEP or Bsep inhibitors induced BA-dependent hepatocyte toxicity in SCHs, and successfully observed the influence of cytochrome P450-mediated drug metabolism on this toxicity. However, selected drugs (i.e., imipramine and quinidine), which were not previously regarded as BSEP inhibitors, also showed BA-dependent hepatocyte toxicity (Ogimura et al., 2011). These findings imply that BSEP-independent mechanisms might also underlie BA-dependent hepatotoxicity, at least to some extent.

Multidrug resistance-associated proteins 3 and 4 (human MRP3 or rat Mrp3 and human MRP4 or rat Mrp4) are additional BA efflux transporters. Unlike BSEP or Bsep, these transporters are localized on the basal side of the hepatocyte membrane (Akita et al., 2002; Jemnitz et al., 2010; Rius et al., 2003). Liver injury caused by bile duct ligation in mice was reportedly attenuated by the induction of Mrp3 (Teng and Piquette-Miller, 2007), and worsened by the genetic depletion of Mrp4 (Mennone et al., 2006). Therefore, Mrp3 and 4 seemingly protect hepatocytes from cellular accumulation of toxic BAs when Bsep function is abolished or compromised.

Evidence from clinical cases stresses the importance of MRP3 and 4. For example, protein expression levels of MRP3 were strongly increased in intensive care unit cholestasis patients, together with decreased protein expression levels of BSEP (Vanwijngaerden et al., 2011). On the other hand, MRP4 protein levels were induced in PFIC2 patients (Keitel et al., 2005) and primary biliary cirrhosis patients (Zollner et al., 2007). Hence, the importance of MRP3 and 4 as compensatory BA efflux transporters under cholestatic conditions is now increasingly recognized.

The current study focused on the involvement of basal BA efflux transporters, including Mrp3 and 4, on BA-dependent hepatocyte toxicity in rat SCHs. We hypothesized that Bsep-mediated BA-dependent hepatocyte toxicity might be aggravated in the face of basal efflux transporter blockade. Cyclosporine A (CsA) and MK571 were chosen as test compounds, because CsA and MK571 showed an inhibitory effect against both human BSEP and MRP3 or 4 in earlier work (Morgan et al., 2013). Moreover, we examined to demonstrate an example of BA-dependent hepatocyte toxicity caused by selective inhibitor of basal BA efflux transporters. For this study, quinidine was chosen as test compound, because quinidine did not inhibit human BSEP but inhibits human MRP4 (Morgan et al., 2013). Our results now indicate that basal efflux transporters in addition to Bsep may contribute to BA-dependent hepatocyte toxicity in rat SCHs.

2. Material and methods

2.1. Animals

Sprague Dawley rats (SLC Japan Inc., Tokyo, Japan), 7–8-weeks-old, were used throughout the study. The animals were treated humanely in accordance with the guidelines issued by the National Institutes of Health (Bethesda, MD, USA). In addition, all procedures were approved by the Animal Care Committee of Chiba University (Chiba, Japan).

2.2. Materials and cells

BAs and test compounds were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma–Aldrich (St. Louis, MO, USA), or Calbiochem (Darmstadt, Germany). Williams' Medium E (WME), antibiotic–antimycotic solution, and GlutaMAX™ were purchased from Invitrogen (Carlsbad, CA, USA). Insulin was purchased from Sigma–Aldrich. Matrigel and ITS premix culture supplement were purchased from BD Biosciences (San Jose, CA, USA). Collagenase and dexamethasone were purchased from Wako Pure Chemical Industries, Ltd. Bsep-expressing Sf9 membrane vesicles were purchased from Genomembrane (Kanagawa, Japan). [^{3}H]TC (5 Ci/mmol) was purchased from Perkin–Elmer (Waltham, MA, USA). [^{3}H]estradiol-17 β-D-glucuronide ($E_2$17βG) (50 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Sf9 cells were maintained as a suspension culture at 27 °C in serum-free EX-CELL 420 medium (JRH Biosciences, Inc., Lenexa, KS, USA). Human embryonic kidney (HEK) 293 and HEK293A cells were cultured at 37 °C with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). All other chemicals and solvents were of analytical grade, unless otherwise noted.

2.3. Preparation of membrane vesicles expressing Mrp3 or 4

Preparation of membrane vesicles from Sf9 cells infected with recombinant Mrp3 baculovirus (Akita et al., 2002) was conducted as reported previously (Ninomiya et al., 2005). Rat Mrp4 adenovirus expression vector was constructed as follows. Rat Mrp4 cDNA was amplified from the excised rat liver by using the forward primer 5′-aaaaagcaggctCCCGGG**ACC**ATGCTGCCGGTGCACACC-3′, which included the Kozak sequence (**ACC**) and the SmaI restriction site (CCCGGG), and the reverse primer 5′-agaaagctgggtGTCGACTC ACAATGCTGTTTCAAATATCG-3′, which included the SalI site (GTCGAC). The amplified fragment was inserted into the pDONR™ 221 vector (Invitrogen), and its sequence was confirmed. The vector was inserted into the pAd/CMV/V5-DEST™ vector (Invitrogen) to finally produce the adenovirus expression vector. The final expression vector was digested with PacI, transfected into 5.0×10^5 HEK293A cells by using DNA-Lipofectamine™ 2000 (Invitrogen), and amplified until the appropriate titer was reached.

Next, HEK293 cells were infected with recombinant Mrp4 adenoviral stock at a multiplicity of infection of 2 and incubated for 48 h. Preparation of membrane vesicles from the infected HEK293 cells was carried out by using the same procedures as those previously described for Sf9 membrane vesicles (Ninomiya et al., 2005). Membrane vesicles were also prepared from green fluorescent protein (GFP)-expressing Sf9 insect cells and lacZ-expressing HEK293 cells and employed as negative controls for ATP-dependent transport.

2.4. Membrane vesicle transport assay

The membrane vesicle transport study was performed by using the rapid filtration technique, as described previously (Ninomiya et al., 2005). Membrane vesicles were prepared from Sf9 insect cells (10 μg) or HEK293 cells (10 μg) and incubated for 5 min at 37 °C with transport buffer (10 mM Tris–HCl, 4 mM ATP or AMP, 250 mM sucrose, and 10 mM $MgCl_2$) containing test compounds. The test compounds (CsA, MK571, and quinidine) were dissolved in dimethyl sulfoxide (DMSO, final concentration = 1%) prior to use. [^{3}H]TC (1 μM for Bsep) and [^{3}H]$E_2$17βG (1 μM for Mrp3 and 4) were used as the substrates. The transport reaction was terminated by the addition of 1 mL of ice-cold buffer (10 mM Tris–HCl, 250 mM sucrose, and 100 mM NaCl). The terminated reaction mixture was passed through a 0.45-μm membrane filter (Advantec Mfs, Inc., Dublin, CA, USA) and then washed twice with 5 mL of stop buffer. Radioactivity of all samples was quantified using a LSC-6100 liquid scintillation counter (Hitachi Aloka Medical,

Tokyo, Japan). GFP-expressing Sf9 membrane vesicles were used as negative controls for Bsep- and Mrp3-mediated transport, and lacZ-expressing HEK293 membrane vesicles were used as negative controls for Mrp4-mediated transport. The transport activity of each negative control vesicle was subtracted from that of the Bsep-, Mrp3-, and Mrp4-expressing vesicles in the presence of ATP or AMP for normalization of the experimental data.

2.5. Hepatocyte isolation and sandwich culture

Tissue culture (24- or 96-well) plates were pre-coated with type 1 collagen (BD Biosciences) at least 1 h prior to the preparation of hepatocyte cultures. Rat hepatocytes were isolated using a two-step perfusion method, as described previously by our group (Ogimura et al., 2011). Isolated hepatocytes were seeded onto collagen (1.5 mg/mL, pH 7.4)-coated 24- or 96-well plates at a density of 8.0×10^5 or 0.48×10^5 cells/well, respectively, in plating medium consisting of WME containing 5% FBS, 0.1 μM dexamethasone, 4 mg/L insulin, 2 mM GlutaMAX™, 15 mM HEPES, pH 7.4, penicillin (100 units/mL), and streptomycin (100 μg/mL). At 1.5 h after seeding, the medium was aspirated, and fresh plating WME was added to each well. On the next day, rat SCHs were prepared as described (Ogimura et al., 2011). Briefly, 24 h after plating, hepatocytes were overlaid with matrigel (0.25 mg/mL) dissolved in ice-cold culture medium consisting of WME containing 1% ITS, 0.1 μM dexamethasone, 2 mM GlutaMAX™, penicillin (100 units/mL), and streptomycin (100 μg/mL). Thereafter, the medium (WME) was changed daily. All experiments were conducted at 4 days after cell seeding. Rat SCHs were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

2.6. Apical and basal efflux of [^{3}H]TC in rat SCHs

Apical efflux was evaluated by the biliary excretion index (BEI) method, as reported previously (Kemp et al., 2005). $Accumulation_{standard}$ and $Accumulation_{Ca2+,\ Mg2+\ -free}$ represent TC content in (cells + bile) and (cells), respectively. As above, test compounds were dissolved in DMSO (final concentration of DMSO, 1%). To determine the dose-dependency of drug-induced actions on the BEI, several concentrations of each test compound were employed. For basal efflux analysis, rat SCHs were washed twice with warm standard Hank's balanced salt solution (HBSS, 0.5 mL) and preincubated for 15 min with CsA, MK571, quinidine, or vehicle (1% DMSO) dissolved in the same buffer. Next, the medium was removed and incubated with standard HBSS (0.5 mL) containing [^{3}H]TC (1 μM) with CsA, MK571, quinidine, or vehicle (1% DMSO) for 10 min. The medium was changed to fresh standard HBSS and collected over time to measure the amount of released [^{3}H]TC. Finally, rat SCHs were washed three times with ice-cold standard HBSS and lysed with 1% (v/v) Triton X-100 (0.5 mL). All samples were quantified by using a LSC-6100 liquid scintillation counter, and the bicinchoninic acid protein assay was used to normalize protein content for each efflux assay. The apical efflux ratio was calculated by using the BEI calculation formula, as follows:

$$\text{BEI}\ (\%) = \frac{\text{accumulation}_{\text{standard}} - \text{accumulation}_{\text{Ca2+,\ Mg2+-free}}}{\text{accumulation}_{\text{standard}}} \times 100$$

The basal efflux ratio of [^{3}H]TC was calculated by dividing the ([^{3}H]TC efflux amount in 5 min) by the ([^{3}H]TC intracellular content at time zero).

2.7. Assessment of BA-dependent cell toxicity

Rat SCHs were exposed to each test compound in the presence or absence of a BA mixture containing the 12 different BAs shown in Table 1. After exposure to compounds for 24 h, cytotoxicity was assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells (LDH_{sample}) via the LDH-Cytotoxic Test (Takara Bio Inc., Shiga, Japan). The degree of LDH activity was expressed as a percentage of maximum LDH activity ($LDH_{Triton\ X\text{-}100}$), measured in the medium from 24 h-Triton X-100-treated control rat SCHs. The following equation was employed:

$$\text{Cell toxicity}\ (\%) = (LDH_{sample} - LDH_{blank})/(LDH_{TritonX\text{-}100} - LDH_{blank}) \times 100$$

LDH_{blank} was determined from the LDH sample of untreated rat SCHs.

Table 1
Concentration of BAs used in the hepatocyte toxicity assay, determined with reference to the standard BA constituents of human serum.

Bile acid	Concentration (μM)
Cholic acid (CA)	10.0
Chenodeoxy cholic acid (CDCA)	17.0
Glycochenodeoxycholic acid (GCDCA)	85.5
Deoxycholic acid (DCA)	36.5
Lithocholic acid (LCA)	1.5
Ursodeoxycholic acid (UDCA)	5.5
Glycocholic acid (GCA)	20.5
Glycodeoxycholic acid (GDCA)	19.0
Taurocholic acid (TC)	2.4
Taurochenpodeoxycholic acid (TUDCA)	10.5
Taurolithocholic acid (TLCA)	4.4
Tauroursodeoxycholic acid (TUDCA)	14.5
Total	227

2.8. RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from rat SCHs and whole liver by using RNA-Solv™ reagent (Omega Bio-tek, Inc., Norcross, GA, USA). Total RNA (1 μg) was reverse-transcribed, and the resultant cDNA (equivalent to 40 ng of total RNA) was mixed with nuclease-free water and THUNDERBIRD™ qPCR Mix (Toyobo Co., Ltd., Osaka, Japan). The mixture was subjected to qPCR by using an Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) under the following thermal cycler conditions: 1 min at 95 °C (activation), 40 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 15 s. Primer sequences used for each transporter are shown in Table 2. Relative mRNA expression levels were calculated after normalizing to β-actin mRNA levels by using Eco™ Real-Time PCR Software.

2.9. Statistical analysis

The significance of differences between conditions was determined by using the two tailed *t*-test (Student's *t*-test) for multiple

Table 2
Primers used for qPCR.

Gene	Primer sequence: 5′ → 3′	Produced fragment (bp)
Bsep	Forward: TTTTCCAGAGGCAGCTATCG	242
	Reverse: ATGGCTGCACTCAAAGATCC	
Mrp3	Forward: ACACCGAGCCAGCCATATAC	301
	Reverse: ACATTGGCTCCGATAGCAAC	
Mrp4	Forward: AAGTGATAACGGGCATGAGG	177
	Reverse: TGAAGGTCACGAACAGGATG	
Ntcp	Forward: AGGCATGATCATCACCTTCC	277
	Reverse: AAGTGGCCCAATGACTTCAG	
Oatp1a1	Forward: CGTTCTTGCCCAAATACCTG	177
	Reverse: CCGATAGGCAAAATGCTAGG	
β-actin	Forward: TTCAACACCCCAGCCATGTACG	230
	Reverse: GTGGTGGTGAAGCTGTAGCC	

qPCR, quantitative polymerase chain reaction.

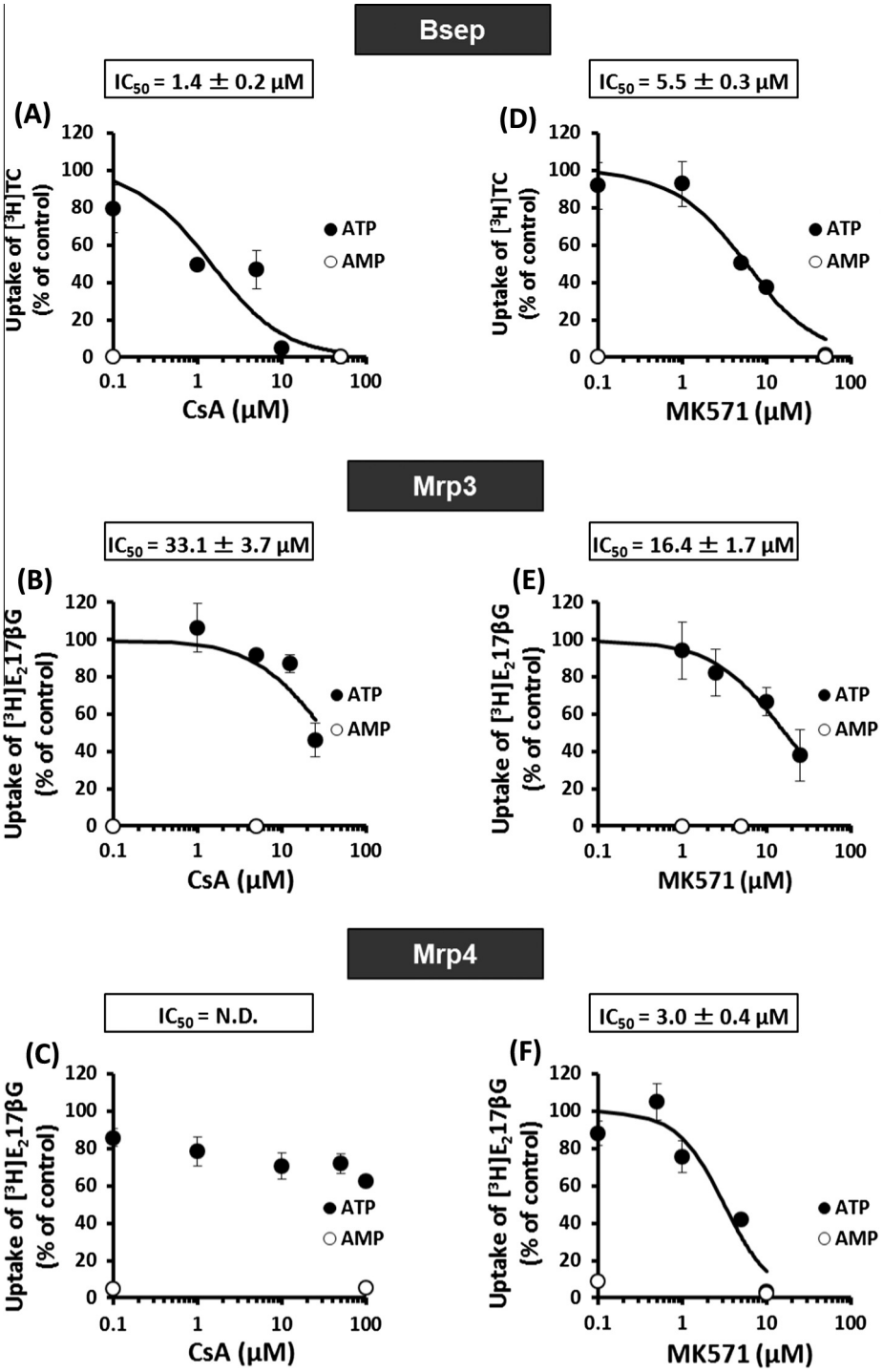

Fig. 1. Inhibition of ATP-dependent [^{3}H]TC (1 μM) or [^{3}H]$E_2$17βG (1 μM) uptake by CsA (A–C) and MK571 (D–F) in the presence of 4 mM ATP (closed circles) or AMP (open circles). Uptake transport was conducted for 5 min in Bsep (A and D)- or Mrp3 (B and E)-expressing membrane vesicles prepared from Sf9 insect cells or Mrp4 (C and F)-expressing membrane vesicles prepared from HEK293 cells, and then measured by rapid filtration. GFP-expressing Sf9 and lacZ-expressing HEK293 membrane vesicles were used as negative controls, and their transport values in the presence of ATP or AMP were subtracted from those obtained by using Bsep-, Mrp3-, or Mrp4-expressing membrane vesicles as normalization controls. Each data point represents the mean ± standard deviation (S.D.) (n = 3).

comparisons. The kinetic parameters (Figs. 1 and 2) were estimated by the following equation: $\alpha = IC_{50}/([I] + IC_{50})$, where α is the transport activity, and $[I]$ is the concentration of the inhibitor. Data were fitted to an iterative nonlinear least-squares model by using the MULTI program to obtain IC_{50} values, as previously described (Yamaoka et al., 1981). In all cases, p values of <0.05 were considered statistically significant.

3. Results

3.1. Inhibitory effects of CsA and MK571 against Bsep, Mrp3, and Mrp4

To determine the affinities of CsA and MK571 for rat Bsep, Mrp3, and Mrp4, we assessed their inhibitory effects by using Bsep- or Mrp3-expressing Sf9 membrane vesicles and Mrp4-expressing

HEK293 membrane vesicles (Fig. 1). [^{3}H]TC (1 μM) was used as a substrate for Bsep, while [^{3}H]$E_2$17βG (1 μM) was used as a substrate for Mrp3 and 4, given that glucuronidated compounds like [^{3}H]$E_2$17βG are more rapidly transported by Mrp transporters than [^{3}H]TC (Akita et al., 2002; Zelcer et al., 2003).

The strongest inhibitory activity of CsA was observed against Bsep, and [^{3}H]TC transport was completely blocked by the compound at 10 μM (Fig. 1A). Weaker inhibition was observed against Mrp3, where [^{3}H]$E_2$17βG transport in Sf9 membrane vesicles was barely decreased to <50% at 25 μM (Fig. 1B). By contrast, CsA failed to block Mrp4-mediated transport; indeed, [^{3}H]$E_2$17βG transport in HEK293 membrane vesicles was wholly unaffected by the agent, at least within the drug concentration range of 0.1–100 μM employed herein (Fig. 1C). From these results, we calculated the IC_{50} values for Bsep and Mrp3 as 1.4 ± 0.2 and 33.1 ± 3.7 μM, respectively. As for MK571, inhibitory effects of the drug were observed against Bsep and Mrp4, with similar IC_{50} values of 5.5 ± 0.3 and 3.0 ± 0.4 μM, respectively. MK571 showed weaker inhibition against Mrp3, with an IC_{50} value of 16.4 ± 1.7 μM (Fig. 1D–F).

3.2. Inhibitory effects of CsA and MK571 against apical and basal [^{3}H]TC efflux across the rat SCH plasma membrane

We next examined the inhibitory actions of CsA and MK571 against the efflux of [^{3}H]TC, a common efflux substrate of Bsep, Mrp3, and Mrp4, across the rat SCH membrane. Since both CsA and MK571 are considered to cross the plasma membrane by passive diffusion (Nakanishi et al., 2011), we set the extracellular concentration ranges from 0.1 to 100 μM covering experimentally obtained IC_{50} values in membrane vesicle study. Apical and basal [^{3}H]TC efflux rates were separately determined by using the BEI method and the standard preincubation-efflux method, respectively (Fig. 2). The efflux of [^{3}H]TC from both apical and basal sides of the membrane was reduced by CsA and MK571 in a concentration-dependent manner. The apical efflux of [^{3}H]TC was readily reduced to 16.4% at 10 μM CsA, with a calculated IC_{50} value of 4.9 ± 2.2 μM (Fig. 2A). The basal efflux of [^{3}H]TC was less susceptible to CsA and showed decreases to 58.4% at 10 μM CsA and 41.7% at 50 μM CsA. The calculated IC_{50} value for basal efflux was 22.5 ± 7.0 μM, which was significantly different from the IC_{50} value for apical efflux ($p < 0.001$). Contrarily, both apical and basal efflux were inhibited by MK571 with similar sensitivity (Fig. 2B). Apical efflux decreased to 45.7% at 10 μM MK571 and was completely inhibited at 50 μM. Likewise, basal efflux decreased to 58.3% at 10 μM MK571 and 25.9% at 50 μM MK571. Calculated IC_{50} values were 7.4 ± 3.6 μM (apical) and 15.1 ± 3.2 μM (basal), and did not significantly differ from each other ($p = 0.22$).

3.3. Estimation of CsA or MK571-induced BA-dependent hepatocyte toxicity in rat SCHs

We previously reported that 10 μM CsA readily induced BA-dependent hepatocyte toxicity in rat SCHs (Ogimura et al., 2011). In our earlier work, the concentration of the BA mixture was set at 681 μM, corresponding to 150-fold of normal human serum BA contents, to sensitize hepatocytes to BA-dependent hepatocyte toxicity. However, the difference in the contribution of each transporter to BA-dependent hepatocyte toxicity was obscured under such excessive conditions. Therefore, we reduced the total concentration of the BAs in the BA mixture to 227 μM, corresponding to 50-fold of normal human serum BA contents (Table 1). This lower BA concentration allowed ready discernment of the influence of apical vs. basal BA efflux on CsA or MK571-induced BA-dependent hepatocyte toxicity.

CsA showed no cytotoxicity at any concentration up until 10 μM in the absence of the BA mixture, while it showed considerable hepatocyte toxicity at concentrations of ≥50 μM (Fig. 3A). When the BA mixture (227 μM) was introduced, a significant increase in cell toxicity was observed at 10 μM CsA (mild toxicity); this toxicity was exacerbated at higher concentrations (≥50 μM CsA; massive toxicity). On the other hand, MK571 showed no cell toxicity at ≤50 μM in the absence of the BA mixture, but a substantial increase in BA-dependent hepatocyte toxicity was observed at 50 μM MK571 in the presence of 227 μM BAs (Fig. 3B). At a concentration of 100 μM, MK571-induced cytotoxicity was extremely strong even in the absence of BAs and almost hit the ceiling. Therefore, we were unable to clearly observe BA-dependent hepatocyte toxicity at high MK571 concentrations (100 μM).

3.4. Estimation of quinidine-induced BA-dependent hepatocyte toxicity in rat SCHs

Quinidine is a represented drug that showed an inhibitory effect against only basal BA efflux transporter (human MRP4), but not against human BSEP (Morgan et al., 2013). In our earlier work with rat SCHs, 50 μM quinidine showed BA-dependent hepatocyte toxicity, implying that might be due to Bsep-independent mechanisms (Ogimura et al., 2011). Therefore, we expected quinidine would act as a selective Mrp4 inhibitor also in rat SCHs. At first, we demonstrated the repeatability of quinidine-induced BA-dependent hepatocyte toxicity in rat SCHs (Fig. 4). Quinidine showed no cytotoxicity at 50 μM employed herein and at most 100 μM in the absence of the BA mixture (data not shown). The BA mixture (681 μM) itself induced mild cell toxicity (~50%). When quinidine (50 μM) was co-exposed, the cell toxicity was significantly exacerbated to almost 100% (massive toxicity).

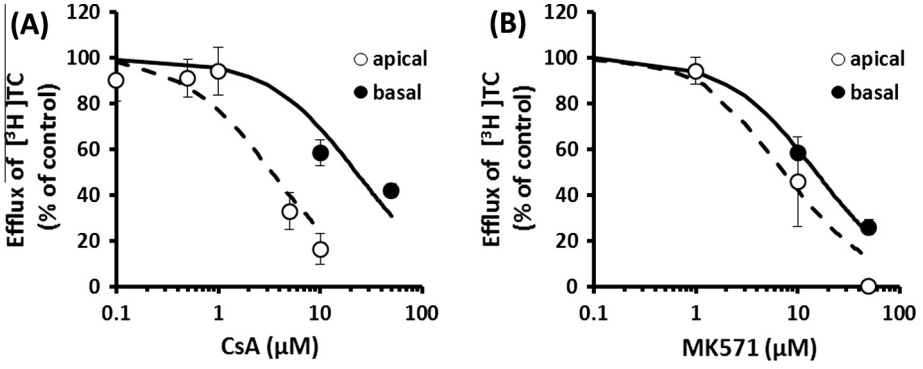

Fig. 2. Inhibitory effect of CsA (A) and MK571 (B) against [^{3}H]TC efflux from the apical (open circles) and basal (closed circles) side of the membrane in rat SCHs. Hepatocytes were preincubated with test compounds or vehicle (1% DMSO) for 15 min and then treated with [^{3}H]TC (1 μM) for 10 min at 37 °C. Apical and basal efflux rates were determined as described in Section 2. Each bar represents the mean ± S.D. (n = 3–6, doublet or triplicate samples from two livers).

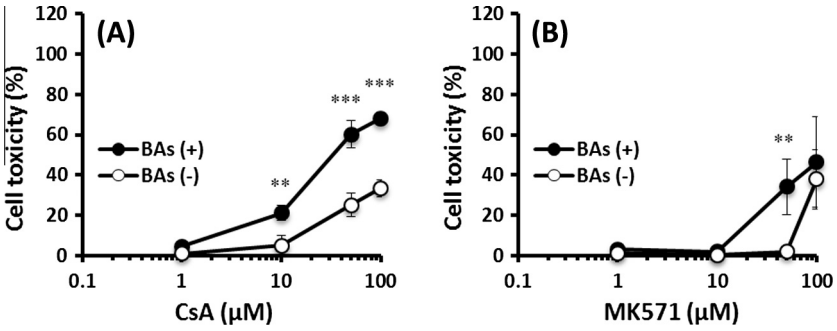

Fig. 3. BA-dependent hepatocyte toxicity in the presence of inhibitors at various concentrations with or without 227 μM of total BAs (corresponding to 50-fold of human serum BA contents). Rat SCHs were incubated with test compounds or vehicle (1% DMSO) for 24 h in the presence (closed circles) or absence (open circles) of BAs. Cell toxicity was calculated by using LDH leakage based on the equation as described in Section 2. Each data point represents the mean ± S.D. (n = 6, triplicate samples from two livers; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. absence of BAs).

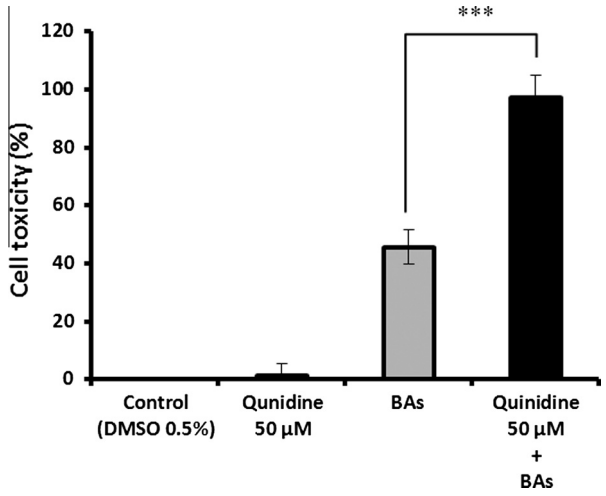

Fig. 4. BA-dependent hepatocyte toxicity in the presence of 50 μM quinidine with or without 681 μM of total BAs (corresponding to 150-fold of human serum BA contents). Rat SCHs were incubated with 50 μM or vehicle (0.5% DMSO) for 24 h in the presence (closed bar) or absence (open bar) of BAs. Cell toxicity was calculated by using LDH leakage based on the equation as described in Section 2. Each data point represents the mean ± S.D. (n = 3, triplicate sample from one liver; $^{***}p < 0.001$ vs. absence of 50 μM quinidine).

3.5. Selective inhibitory effect of quinidine against basal [^{3}H] TC efflux across the rat SCH plasma membrane

We next examined whether quinidine inhibits only basal [^{3}H]TC efflux, but not apical [^{3}H]TC efflux (Fig. 5). The apical [^{3}H]TC efflux was not affected by quinidine within the drug concentration range of 10–100 μM employed herein and at most 500 μM (data not shown). On the other hand, the basal [^{3}H]TC efflux was significantly reduced by quinidine in a concentration dependent manner; the efflux was decreased to 89.2% at 10 μM, 55.5% at 50 μM, and 60.0% at 100 μM (Fig. 5A). Moreover, we also analyzed the intracellular retention of [^{3}H]TC in rat SCHs at 1, 3, and 5 min after initiating its basal efflux (Fig. 5B). After 5 min basal [^{3}H]TC efflux, significant intracellular retention of [^{3}H]TC was observed in the presence of 10–100 μM quinidine. It was increased to approximately 1.5–2 times as much as that of control in a concentration dependent manner.

3.6. Inhibitory effects of quinidine against Bsep, Mrp3, and Mrp4

We next examined the inhibition spectrum of quinidine against rat counterparts of BA efflux transporters (Bsep, Mrp3 and Mrp4) by using Bsep- or Mrp3-expressing Sf9 membrane vesicles and Mrp4-expressing HEK293 membrane vesicles (Fig. 6). The steady state ratio between unbound intracellular concentration and extracellular medium concentration of quinidine was reported as low as 2.2 (Di Marco et al., 2003). Therefore, intracellular concentration of approximately 100 μM would be expected when 50 μM quinidine showed BA-dependent hepatocyte toxicity in rat SCHs; nevertheless 100 μM quinidine did not significantly inhibit transport activities of Bsep, Mrp3, and Mrp4.

3.7. Culture-and time-dependent mRNA expression changes of BA uptake and efflux transporters in rat SCHs

Generally, the mRNA expression levels of CYP enzymes, nuclear receptors, and transporters are downregulated in rat SCHs over time in culture, but some of these factors are instead upregulated as a compensatory mechanism (Tchaparian et al., 2011). Hence, we confirmed the mRNA expression levels of transporters that are related to BA uptake and efflux in rat SCHs vs. intact liver (Fig. 7A and B). No significant differences were distinguished in mRNA content between the intact liver (homogenized) and cultured hepatocytes (isolated on day 0). However, the mRNA expression level of Mrp3 was significantly increased by 4-fold in rat SCHs after 4 days in culture (Fig. 7A). By contrast, Bsep and Mrp4 mRNA levels were decreased to 22% and 14% of their levels on day 0, respectively. Moreover, mRNA expression levels of Na^+-taurocholate co-transporting polypeptide (Ntcp) and organic anion-transporting polypeptide 1a1 (Oatp1a1), which are jointly responsible for BA uptake in rat SCHs, were decreased to 6% and 7% of their levels on day 0, respectively, during the 4 days in culture (Fig. 7B).

4. Discussion

BSEP or Bsep is generally regarded as the most important transporter involved in BA efflux from hepatocytes. Information concerning BSEP-mediated drug inhibition is thus extremely useful in the estimation of drug-induced liver cholestasis, as reported previously (Dawson et al., 2012; Morgan et al., 2010; Pedersen et al., 2013; Warner et al., 2012). Our laboratory previously proposed an *in vitro* rat SCH-based method to evaluate Bsep-mediated BA-dependent hepatocyte toxicity of test compounds by exposing an optimized total BA mixture, which reflected the BA contents in normal human serum, but was enriched by 150-fold (Ogimura et al., 2011). However, basal efflux transporters for BAs (MRP3 or Mrp3 and MRP4 or Mrp4) also reportedly contribute to BA efflux, and expression of Mrp3 or 4 can rescue the liver from cholestatic injury in mice after the abolishment of biliary

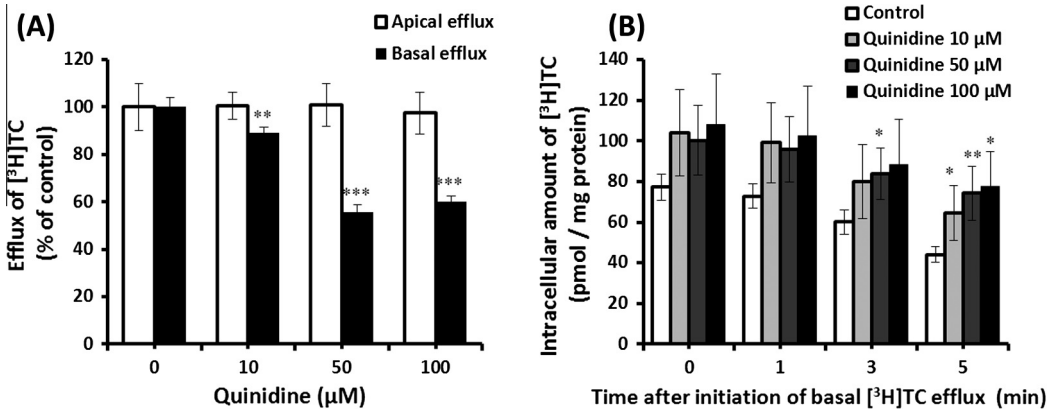

Fig. 5. (A) Inhibitory effect of quinidine against [^{3}H]TC efflux from the apical (open bars) and basal (closed bars) side of the membrane in rat SCHs. Hepatocytes were preincubated with quinidine or vehicle (1% DMSO) for 15 min and then treated with [^{3}H]TC (1 μM) for 10 min at 37 °C. Apical and basal efflux rates were determined as described in Section 2. Each bar represents the mean ± S.D. (n = 6, triplicate samples from two livers; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. absence of quinidine). (B) Intracellular accumulation of [^{3}H]TC in the presence (closed bars) or absence (open bars) of quinidine in rat SCHs. After initiation of basal [^{3}H]TC efflux, the medium was collected over time to measure the amount of released [^{3}H]TC. Each bar represents the mean ± S.D. (n = 6, triplicate samples from two livers; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control).

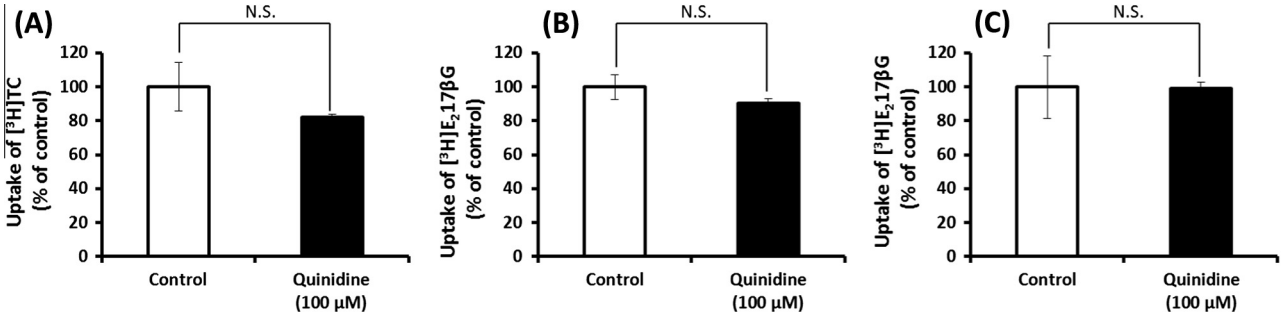

Fig. 6. ATP-dependent [^{3}H]TC (1 μM) or [^{3}H]$E_2$17βG (1 μM) uptake in the presence (closed bars) or the absence (open bars) of 100 μM quinidine. Uptake transport was conducted for 5 min in Bsep (A)- or Mrp3 (B)-expressing membrane vesicles prepared from Sf9 insect cells or Mrp4 (C)-expressing membrane vesicles prepared from HEK293 cells, and then measured by rapid filtration. GFP-expressing Sf9 and lacZ-expressing HEK293 membrane vesicles were used as negative controls, and their transport values in the presence of ATP were subtracted from those obtained by using Bsep-, Mrp3-, or Mrp4-expressing membrane vesicles as normalization controls. Each data point represents the mean ± S.D. (n = 3). N.S. represents not significant.

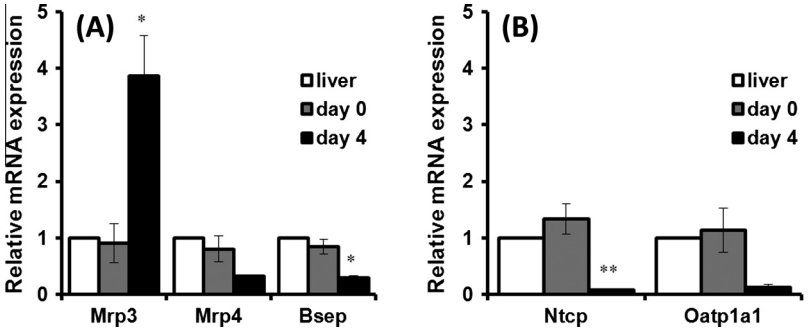

Fig. 7. Culture dependent changes of mRNA expression level of efflux (A) and uptake (B) transporters of BAs in rat SCHs on day 0 and on day 4 compared with whole liver. Total RNA was isolated from rat SCHs and whole liver as described in Section 2. Relative expressions were calculated after normalizing by β-actin. One μg of total RNA was reverse-transcribed and the resulting cDNAs were subjected to real-time PCR quantification analysis. Each bar represents the mean ± standard error (S.E.) (n = 3, from three livers, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. day 0).

excretion function (Akita et al., 2002; Jemnitz et al., 2010; Mennone et al., 2006; Rius et al., 2003; Teng and Piquette-Miller, 2007; Zelcer et al., 2003).

First part of the present study focused on prototypical rat Bsep inhibitors (CsA and MK571) that likely also inhibited rat Mrp3 or 4 to elucidate the contribution of basal efflux transporters to BA-dependent hepatocyte toxicity in our rat SCH assay system. The inhibition spectrum of CsA against the rat BA transporters (Bsep > Mrp3 ≫ Mrp4) resembled that of human transporters (Morgan et al., 2013). Consistent with this inhibition profile, the apical efflux of [^{3}H]TC from rat SCHs was potently inhibited by CsA at a concentration of 10 μM, while the basal efflux of [^{3}H]TC was only weakly affected (Fig. 2A). These findings imply that CsA is a relatively selective inhibitor of Bsep at 10 μM, and a non-selective inhibitor of both Bsep and Mrp3 at higher concentrations (≥50 μM).

Our earlier work demonstrated that 10 μM CsA induced almost 100% BA-dependent hepatocyte toxicity in the presence of the total BA mixture at 681 μM (Ogimura et al., 2011). However, at the lower BA concentration used in the current investigation (corresponding to a 50-fold enrichment of normal human serum BA contents), CsA-induced BA-dependent hepatocyte toxicity was at most 30%, and was further enhanced to ~60% at higher concentrations of CsA (≥50 μM) (Fig. 3A). These results suggest that CsA-induced BA-dependent hepatocyte toxicity in rat SCHs might be aggravated by Mrp3 inhibition in addition to Bsep inhibition.

MK571 showed a different inhibition profile from CsA. Specifically, our vesicle transport studies suggested that Mrp4 was inhibited by MK571 with similar potency as Bsep, but that MK571 was less effective against Mrp3 (Fig. 1D–F). Moreover, the IC_{50} values of both basal and apical [^{3}H]TC effluxes were similar to each other (Fig. 2B). MK571-induced BA-dependent hepatocyte toxicity was intensified at concentrations of >50 μM, but MK571 showed no harmful effects at 10 μM (Fig. 3B). Hence, the augmentation of BA-dependent hepatocyte toxicity in the presence of 50 μM MK571 was probably caused by Mrp3 and 4 inhibition in addition to Bsep blockade. Therefore, basal BA efflux transporters, including Mrp3 and 4, apparently participate in BA-dependent cytotoxicity in rat SCHs.

Second part of this study focused on the non-Bsep inhibitor (quinidine) that was previously reported as a selective MRP4 inhibitor at least in human (Morgan et al., 2013). We have reported that quinidine causes BA-dependent hepatocyte toxicity in our rat SCH assay system (Ogimura et al., 2011) and it is also observed in this study (Fig. 4). Quinidine significantly reduced basal [^{3}H]TC efflux in rat SCHs while the apical efflux of [^{3}H]TC was not affected (Fig. 5A). Moreover, quinidine significantly increased intracellular accumulation of [^{3}H]TC to almost twice as much as that of control in rat SCHs (Fig. 5B). These results suggests that quinidine-induced BA-dependent hepatocyte toxicity in rat SCHs might be aggravated by intracellular BA accumulation due to only basal BA efflux inhibition, not apical BA efflux inhibition. Although, we had expected that it would inhibit Mrp4, quinidine did not show inhibitory effect to any rat BA efflux transporters tested (Fig. 6A–C). Hence, basal efflux transporter(s) other than Mrp3 and 4 may be underlying in quinidine-induced basal BA efflux inhibition and BA-dependent hepatocyte toxicity in rat SCHs.

At present, there is a little information about comparative studies of rat Bsep, Mrp3 or Mrp4 with human counterparts. According to the previous reports, a close correlation was observed between the inhibition of human BSEP and rat Bsep activity by 85 drugs (Dawson et al., 2012). In addition, the substrate specificity of human MRP3 resembles that of rat Mrp3 (Akita et al., 2002). However, in some cases, interspecies differences exist between human and rat; 12 of the 85 compounds exhibited >2-fold more potent inhibition of human BSEP than of rat Bsep activity (Dawson et al., 2012), and the affinity of human MRP3 for methotrexate was much lower than that of rat Mrp3 (Akita et al., 2002). In contrast to these transporters, there have been no reports about comparative studies between human MRP4 and rat Mrp4. Therefore, it is not surprising even if the inhibition spectrum against the rat transporters does not resemble that of human transporters. Since we used the same substrate ($E_2$17βG) as human MRP4-expressed membrane vesicle study (Morgan et al., 2013), the result that quinidine did not inhibit rat Mrp4 might be due to interspecies difference between inhibition potency of human MRP4 and rat Mrp4 by quinidine.

In addition to Mrp3 and 4, organic solute transporter alpha/beta (Ostα/β) has been reported as basal BA efflux transporter in liver (Ballatori et al., 2005; Dawson et al., 2005). Therefore, Ostα/β may have a chance to be involved in BA-dependent hepatocyte toxicity in rat SCHs. Although inhibitory effect of some bile salts, steroids, and anionic drugs (sulfobromophtalein, bilirubin ditaurate, probenecid, and indomethacin) on human or mouse Ostα/β-mediated transport was evaluated (Seward et al., 2003), CsA, MK571 and quinidine have not been examined yet in those reports. This point is now under investigation in our laboratory by constructing Ostα/β-expressed HEK293 cell transport system.

Moreover, OATPs are well known BA uptake transporters expressed on the basolateral membrane of the hepatocytes (St-Pierre et al., 2001). Rat Oatp1 and Oatp2 took up [^{3}H]TC or [^{3}H]leukotriene C_4 in exchange of intracellular glutathione (Li et al., 1998, 2000). Given that OATPs are exchange transporters for organic anions, it might be possible that they are involved in BA efflux at least under particular condition such as cholestasis; however this concept has not been proved yet. Since both CsA and MK571 are known to inhibit OATPs-mediated hepatic uptake (Letschert et al., 2006; Shitara et al., 2003), it is possible that OATPs are involved in BA-dependent hepatocyte toxicity observed in our rat SCHs.

The rat SCH assay system described herein might be useful to evaluate whether the inhibition of Mrp3 and/or Mrp4 (and/or other efflux transporters such as OATPs and Ostα/β) indeed resulted in the worsening of BA-dependent hepatocyte toxicity *in vitro*. However, it remains to be elucidated if the selective inhibition of either of these basal efflux transporters induces BA-dependent hepatotoxicity, and which of these transporters makes the strongest contribution. Another approach, such as using a knockdown system for each transporter, will probably provide an answer to these questions in the future (Yang et al., 2014).

Abundant experimental data has demonstrated the strong upregulation of both mRNA and protein levels of MRP3 or 4 in patients suffering from cholestasis, together with decreased protein levels of BSEP (Keitel et al., 2005; Vanwijngaerden et al., 2011; Zollner et al., 2007). Induced MRP3 or 4 may therefore be indispensable for a compensatory excretion pathway of BAs from hepatocytes under cholestatic conditions. One can speculate that the inhibition of MRP3 or 4 would potentiate hepatotoxicity as a result of the enhanced accumulation of toxic BAs within hepatocytes. In support of this hypothesis, several researchers recently reported an improvement in the prediction accuracy for cholestatic DILI risk compounds when the IC_{50} values for MRP3 and 4 were taken into consideration along with that for BSEP (Kock et al., 2014; Morgan et al., 2013). Among 635 marketed or withdrawn drugs, the prediction accuracy of compounds with evidence of liver injury increased by up to 96% when MRP3 and 4 were included in the analysis (Morgan et al., 2013). Moreover, even among BSEP non-inhibitors, selective MRP4 inhibition is seemingly associated with an increased risk of cholestatic DILI (Kock et al., 2014).

The expression profile of BA transporters changes over time in culture. For example, the mRNA expression levels of BA uptake transporters (i.e., Ntcp and Oatp1a1) are dramatically diminished in both rat primary hepatocytes (Liang et al., 1993) and SCHs (Tchaparian et al., 2011). The downregulation of uptake transporters supports our rationale for employing an extremely high concentration range for total BAs (227–681 μM) compared with BA levels observed in clinical situations. Remarkably, the mRNA expression of the apical BA transporter, Bsep, decreased over time in cultured rat SCHs in an earlier investigation, while that of the basal BA transporters, Mrp3 and 4, increased (Tchaparian et al., 2011). The same tendency was observed in the current investigation (Fig. 7). Such compensatory regulation is also observed in certain *in vivo* situations, including clinical cases (Keitel et al., 2005; Vanwijngaerden et al., 2011; Zollner et al., 2007) and cholestatic animal models (e.g., Bsep knockout mice and bile duct ligated rodents) (Wang et al., 2013). In light of these observations, it is interesting to note that *in vitro* hepatocyte culture models mimic cholestatic conditions *in vivo*, where Ntcp/Oatp1a1 and Bsep

expression levels are suppressed and Mrp3 or 4 expression levels are induced. However, it is still unclear how alterations in mRNA expression profiles for specific transporters are actually translated into prediction accuracy for the cholestatic DILI risk of test compounds. This point is now under exploration in our laboratory by using rat as well as human SCHs.

In conclusion, the current study demonstrated that not only inhibition of Bsep, but also inhibition of basal efflux transporters, is involved in the onset of BA-dependent hepatocyte toxicity in rat SCHs. Therefore, the rat SCH assay system may be useful to comprehensively evaluate the detrimental potential of cholestatic DILI compounds, given that the importance of basal BA efflux transporters is gradually gaining recognition under clinical conditions.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency Document associated with this article can be found in the online version.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Numbers 24390037, 23790172.

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