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Bile salt export pump inhibitors are associated with bile acid-dependent drug-induced toxicity in sandwich-cultured hepatocytes

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ABSTRACT

Drug-induced liver injury (DILI) is a major reason for the dropout of candidate compounds from drug testing and the withdrawal of pharmaceuticals from clinical use. Among the various mechanisms of liver injury, the accumulation of bile acids (BAs) within hepatocytes is thought to be a primary mechanism for the development of DILI. Although bile salt export pump (BSEP) dysfunction is considered a susceptibility factor for DILI, little is known about the relationship between drug-induced BSEP dysfunction and BA-dependent hepatotoxicity. Furthermore, few methods are at hand for the systematic and quantitative evaluation of BA-dependent DILI. This study aimed to construct a model of DILI by employing sandwich-cultured hepatocytes (SCHs). SCHs can be used to assess functions of canalicular transporters such as BSEP and the activity of metabolic enzymes. Here, the impact of 26 test compounds (ritonavir, troglitazone, etc.) was investigated on BA-dependent cytotoxicity in SCHs. SCHs were exposed to each compound for 24 h with or without BAs (glycochenodeoxycholic acid, deoxycholic acid, etc.). As a result, BA-dependent toxicity was observed for 11 test compounds in SCHs treated in the presence of BAs, while no signs of toxicity were observed for SCHs treated in the absence of BAs. Of the 11 compounds, nine were known BSEP inhibitors. Moreover, for some compounds, an increase in the severity of BA-dependent toxicity was observed in SCHs that were co-treated with 1-aminobenzotriazole, a non-selective inhibitor of cytochrome P450 (CYP450)-mediated drug metabolism. These results indicate that the SCH-based model is likely to prove useful for the evaluation of BA-dependent DILI, including the effects of drug metabolism and BSEP inhibition on liver injury.

1. Introduction

Drug-induced liver injury (DILI) is the most common adverse event leading to the dropout of candidate compounds from drug testing and the withdrawal of pharmaceuticals from clinical use [1,2]. Therefore, elucidating the mechanisms of DILI and constructing useful evaluation methods are critically important issues. Recently, inhibition of bile acid (BA) transport was suggested to be an underlying cause of DILI due to accumulation of BAs within hepatocytes [3–7].

BAs are synthesized from cholesterol in the liver and are crucial for absorption of fat-soluble vitamins and lipids. Thus, BAs play a key role in digestion and nutritional processes. However, certain BAs, such as glycochenodeoxycholic acid, lithocholic acid, and deoxycholic acid, induce hepatotoxicity as a result of apoptosis and mitochondrial disorder [8–11]. Therefore, hepatic BA levels must be strictly regulated by various mechanisms.

The bile salt export pump (human BSEP/rat Bsep) is located on the canalicular membrane of hepatocytes and participates in the excretion of BAs from the liver into the bile [12]. BSEP is thus indispensable for the regulation of hepatic BA content. Several genetic mutations of BSEP are associated with progressive familial intrahepatic cholestasis type 2 [13], which results in liver failure stemming from hepatocytic accumulation of BAs. Therefore, BSEP dysfunction is likely related to liver injury. Morgan et al. reported that 25% of 200 benchmark compounds inhibited BSEP-mediated taurocholate excretion, with an IC_{50} of under 100 μM [7]. In addition, troglitazone (an antidiabetic drug) and nefazodone (an antidepressant drug) were withdrawn from the market due to severe liver injury; these drugs are also potent BSEP inhibitors [6,7,14,15]. As such, a number of methods for the determination of BSEP inhibition (e.g., the membrane vesicle assay) have been developed. However, few reports indicate that drug-induced BSEP dysfunction actually leads to hepatotoxicity, and the relationship between drug-induced BSEP dysfunction and liver injury risk is yet to be determined.

Abbreviations: 1-ABT, 1-aminobenzotriazole; BA, bile acid; BSEP/Bsep, human/rat bile salt export pump; CYP450, cytochrome P450; DILI, drug-induced liver injury; IC50, 50% inhibitory concentration; Ki, inhibition constant; LDH, lactate dehydrogenase; NAPQI, *N*-acetyl-p-benzoquinone imine; SCH (SCRH), sandwich-cultured (rat) hepatocytes.

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Many endogenous and exogenous substances, including BAs and drugs, undergo vectorial transport and metabolism in the liver. Drug metabolites such as *N*-acetyl-p-benzoquinone imine (NAPQI, the metabolite of acetaminophen) are integral players in the development of hepatotoxicity [16]. Therefore, the aim of this study was to develop an experimental model reflecting the hepatic functions of transport and drug metabolism for the evaluation of BSEP dysfunction-related/BA-dependent DILI. The model employed sandwich-cultured hepatocytes (SCHs), which are widely used in toxicologic and pharmacokinetic assays [17]. SCHs are distinctive in that they maintain cell polarity and the expression of transporters and metabolic enzymes [17], enabling *in vitro* studies of vectorial transport via BSEP and drug metabolism.

The current study focused on BA-dependent hepatotoxicity and explored the hypothesis that BA accumulation due to BSEP dysfunction may be a causative factor for the development of DILI. In addition, this study examined whether the SCH model could reflect the effect of drug metabolism on BA-dependent DILI.

2. Material and methods

2.1. Animals

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

2.2. Materials

BAs and test compounds were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Sigma–Aldrich (St. Louis, MO), and Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and insulin were purchased from Sigma–Aldrich. Matrigel and ITS^+ premix were purchased from BD Biosciences (San Jose, CA). Collagenase was purchased from Wako Pure Chemical Industries Ltd. All other chemicals and solvents were of analytical grade.

2.3. Preparation of culture plates

Tissue culture (12-well) plates were pre-coated with type 1 collagen (Koken collagen IAC50, Koken Co. Ltd., Tokyo, Japan) at least 1 day prior to preparation of hepatocyte cultures. Ice-cold neutralized collagen (1.5 mg/ml, pH 7.4) was spread onto the bottom of each well. Freshly coated plates were placed at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for approximately 1 h, followed by the addition of 0.5 ml DMEM to each well and storage in the humidified atmosphere.

2.4. Hepatocyte isolation and sandwich culture

Rat hepatocytes were isolated using a two-step perfusion method, as reported previously, with modifications [18]. Briefly, male rats were anesthetized with pentobarbital (64.8 mg/kg body weight, i.p) before portal vein cannulation. Rat livers were perfused with Ca^{2+}/Mg^{2+}-free perfusion buffer containing 0.6 mM EGTA, followed by perfusion with perfusion buffer containing 4 mM $CaCl_2$ and 0.3 mg/ml collagenase. Isolated hepatocytes were dispersed in DMEM, filtered through a sterile nylon mesh, and centrifuged at 50*g* for 3 min. The cell pellets were resuspended in 45% isotonic Percoll in DMEM and centrifuged at 50*g* for 15 min. The cells were then resuspended in DMEM, filtered again through a sterile nylon mesh, and washed once by centrifugation at 50*g* for 3 min. Hepatocyte viability was determined by trypan blue exclusion. Only those hepatocyte preparations with viability greater than 88% were used for experiments.

Hepatocytes were suspended in DMEM containing 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone, and 4 mg/l insulin. The hepatocyte suspensions were then added to the collagen-coated 12-well plates at a density of 1.25×10^5 cells/cm^2. Approximately 1.5 h after plating the cells, the medium was aspirated, and fresh DMEM (0.75 ml) was added to each well.

Next, sandwich-cultured rat hepatocytes (SCRHs) were prepared. Twenty-four hours after plating, hepatocytes were overlaid with matrigel (0.25 mg/ml) dissolved in 0.75 ml ice-cold DMEM containing 1% ITS^+, 0.1 μM dexamethasone, 100 units/ml penicillin, and 100 μg/ml streptomycin. Thereafter, the medium (DMEM) was changed daily. All experiments were conducted 5 days after cell seeding. SCRHs were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.5. BA composition of human serum and test compounds

Twelve different BAs (Table 1) were selected by making reference to the standard BA constituents of human serum [19]. Test compounds were chosen from an array of hepatotoxic drugs. The concentration of each compound was determined based on the 50% inhibitory concentration (IC_{50}) or the inhibition constant (Ki) value for BSEP/Bsep inhibition [7,20,21]. The maximum concentration for all test compounds was set at 50 μM (Table 2) to avoid drug-based cytotoxicity. The test compounds or BAs were added to DMEM. Stock solutions of the test compounds and BAs originally were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.5%.

2.6. Assessment of BA-dependent cytotoxicity

SCRHs 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 using the LDH-Cytotoxic Test (Wako Pure Chemical Industries Ltd.). The degree of LDH activity was expressed as a percentage of maximum LDH activity, as measured in Triton-X-100 lysates of SCRHs. The following equation was employed:

$$\text{Cytotoxicity} = (\text{LDH}_{\text{sample}} - \text{LDH}_{\text{blank}})/(\text{LDH}_{\text{Triton X-100}} - \text{LDH}_{\text{Blank}}) \times 100.$$

Table 1
Standard BA constituents of human serum.

BA	Concentration (μM)
Cholic acid	0.200
Chenodeoxy cholic acid	0.340
Glycochenodeoxycholic acid	1.710
Deoxycholic acid	0.734
Lithocholic acid	0.030
Ursodeoxycholic acid	0.110
Glycocholic acid	0.410
Glycodeoxycholic acid	0.380
Taurocholic acid	0.048
Taurochenpodeoxycholic acid	0.210
Taurolithocholic acid	0.087
Tauroursodeoxycholic acid	0.287
Total bile acid (TBA)	4.546