Ticlopidine, a Cholestatic Liver Injury-Inducible Drug, Causes Dysfunction of Bile Formation via Diminished Biliary Secretion of Phospholipids: Involvement of Biliary-Excreted Glutathione-Conjugated Ticlopidine Metabolites

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ABSTRACT

The antiplatelet drug, ticlopidine (TIC), reportedly causes cholestatic liver injuries. The present study analyzed the effect of TIC on bile formation, revealing that the biliary secretion of phospholipids was significantly decreased in TIC-administered Sprague Dawley (SD) rats. However, the effect of TIC on biliary phospholipids was not observed in SD rats pretreated with diethylaminoethyl diphenylpropylacetate that inhibits cytochrome P450s (P450), or in Eisai hyperbilirubinemic rats (EHBR) lacking functional multidrug resistance-associated protein 2 (MRP2/ABCC2). These results suggest that glutathione-conjugated TIC metabolites (TIC-SGs), which were formed in the liver after P450s-mediated metabolism and were excreted extensively into bile by MRP2, mediated the observed alterations of the bile composition. Administration of TIC caused significant liver injuries in SD rats, with decreased biliary phospholipids, but not in EHBR, consistent with the in vitro observation that phospholipid–bile acid–mixed micelles moderated the cytotoxic effects of bile acids. Further analyses revealed that TIC-SGs did not directly inhibit multidrug resistance 3 P-glycoprotein (MDR3/ABCB4)-mediated phosphatidylcholine efflux in vitro. Because the diminished biliary secretion of phospholipids with TIC administration was restored by taurocholate infusion in SD rats, the decreased biliary concentration of bile acids, due to the stimulation of bile acid–independent bile flow driven by TIC-SGs, might have indirectly attenuated phospholipid secretion. In conclusion, extensive biliary excretion of TIC-SGs decreased the biliary secretion of phospholipids, which might have increased the risk of TIC-induced cholestatic liver injury.

Introduction

Bile formation, one of the most essential functions of the liver, is mediated in bile acid–dependent and –independent manners through ATP-binding–cassette (ABC) transporters expressed on the bile canalicular membrane (Lee and Boyer, 2000). Bile acid–dependent bile flow is driven by the bile salt export pump (BSEP/ABCB11) that mediates the biliary secretion of bile acids (Gerloff et al., 1998). Any functional disturbance of BSEP can lead to an intrahepatic accumulation of potentially cytotoxic bile acids (Stieger et al., 2007). Multidrug resistance 3 P-glycoprotein (MDR3/ABCB4) regulates the biliary secretion of phospholipids, especially phosphatidylcholine (PC) (Smits et al., 1993), which are essential for the formation of biliary mixed micelles with bile acids, and for the protection of hepatocytes against the detergent effects of bile acids (Oude Elferink and Paulusma, 2007). Indeed, genetic disruptions of BSEP and MDR3 cause the severe hereditary disorders: progressive familial intrahepatic cholestasis type 2 (PFIC2) and type 3 (PFIC3), respectively (Oude Elferink et al., 2006). On the other hand, bile acid–independent bile flow is driven by other biliary osmotic solutes, such as glutathione (GSH) and its conjugates,
which are secreted mainly by the activities of multidrug resistance–associated protein 2 (MRP2/ABCC2) (Paulusma et al., 1996). Because the secretion of osmotic solutes and the subsequent attraction of biliary water affect biliary micelle formation (Pauli-Magnus and Meier, 2006), the function of MRP2 is also important for appropriate bile formation.

Given the physiologic importance of BSEP, MDR3, and MRP2, drugs that affect the functions of these canalicular transporters and, subsequently, disturb bile formation may cause cholestatic liver injury. This type of drug-induced liver injury (DILI) is diagnosed on the basis of an elevation in serum alkaline phosphatase (ALP) and alanine aminotransferase (ALT) levels, according to the criteria described in detail by Aithal et al. (2011). Previous studies have reported the inhibition of BSEP by several drugs in vitro (Stieger et al., 2000; Fattinger et al., 2001; Funk et al., 2001), although such inhibitory effects were mostly observed at much higher concentrations than those used in clinical situations. Recently, Dawson et al. showed that no clear distinction was evident between the BSEP IC50 or the unbound plasma concentration of the drugs in humans and whether the drugs caused DILI (Dawson et al., 2012). By contrast, little has been known about the drug-mediated inhibition of MDR3, presumably because of the difficulty in analyzing MDR3-mediated phospholipid transport in vitro. We recently showed that itraconazole (ITZ), a cholestatic liver injury-inducible drug, inhibited MDR3 function in vitro and in vivo at concentrations similar to those used in clinical situations (Yoshikado et al., 2011). Furthermore, the administration of estradiol-17β-D-glucuronide to rats significantly reduced bile flow by inducing the endocytic internalization of Mrp2 (Huang et al., 2000; Mottino et al., 2002) or Bsep (Croenzi et al., 2003). Accordingly, attempts to understand the molecular mechanisms of drug-induced cholestatic liver injuries must consider drug-induced alterations in the functions of bile canalicular transporters.

In the present study, we focused on ticlopidine (TIC), an antiplatelet drug that is widely used to prevent thromboembolic events but is known to cause severe cholestatic liver injury (Greany et al., 1993; Grimm and Litynskyi, 1994; Iqbal et al., 1998; Alberti and Alberti-Flor, 2002; Mambelli et al., 2007). We showed that the biliary secretion of phospholipids was significantly decreased in TIC-administered Sprague Dawley (SD) rats. Given the physiologic importance of biliary phospholipids, we further studied the mechanism of decreased biliary phospholipids and their involvement in TIC-induced hepatotoxicity, by performing a series of in vivo and in vitro experiments.

Materials and Methods

Materials. [14C]Choline (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). LLC-PK1 cells and CaCo-2 cells were purchased from American Type Culture Collection (Manassas, VA), and cultured in Medium 199 (Sigma-Aldrich, St. Louis, MO) and Minimum Essential Medium (Nacalai Tesque, Kyoto, Japan), respectively, with 10% fetal bovine serum (Gibco, Tokyo, Japan) and 1% penicillin/streptomycin (Nacalai Tesque) at 37°C in an atmosphere supplemented with 5% CO2. All other chemicals used were commercially available and of reagent grade.

In Vivo Experiments. Animal studies were performed according to the method approved by the Institutional Animal Care Committee of the University of Tokyo. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23, revised 1985).

Male SD rats (Japan SLC, Shizuoka, Japan) weighing 230–270 g and Eisai hyperbilirubinemic rats (EHBHR; Japan SLC) weighing 280–300 g were fed standard rat chow and water. On the day of the operation, the rats were anesthetized with an intraperitoneal injection of 1.25 g/kg urethane (Sigma-Aldrich). The femoral artery and vein were cannulated with an SP-21 (0.5-mm) polyethylene tube (Natsume, Tokyo, Japan), and the bile duct was cannulated with an SP-8 (0.2-mm) polyethylene tube (Natsume). To examine the effect of TIC on bile formation, the rats were intravenously administered TIC (Wako Pure Chemical, Osaka, Japan) at a bolus dose of 48 mg/kg, and were infused with TIC continuously at 13 mg/kg/h for 2 hours.

To inhibit the metabolism of TIC by cytochrome P450s (P450) in vivo, rats were intraperitoneally administered the nonspecific P450s inhibitor SKF-525A (Sigma-Aldrich) at a bolus dose of 50 mg/kg at 1 hour before TIC administration (Yang et al., 2009). To increase the biliary concentration of bile acids, taurocholic (TCA) (Sigma-Aldrich) was continuously infused, intravenously, at 65 mg/kg/h from 1 hour before TIC administration. The TIC, SKF-525A, and TCA were dissolved in 0.9% NaCl. Control rats underwent the same protocol with 0.9% NaCl.

Bile and blood specimens were taken every 30 minutes. Plasma specimens were obtained by centrifuging the blood specimens at 1,000 g for 15 minutes. To examine the bile flow rate in rats, bile specimens were weighed, and bile volume was determined by assuming a specific gravity of 1.0 g/ml (Fouassier et al., 2002). The biliary concentrations of bile acids and phospholipids were measured with the Total Bile Acids-Test kit and Phospholipids C-Test kit (Wako Pure Chemical), respectively. The biliary concentration of total GSH was determined by the enzymatic recycling method, as reported previously (Rahman et al., 2006).

To examine the hepatotoxic effect of TIC in vivo, rats were intraperitoneally administered lipopolysaccharide (LPS) (Sigma-Aldrich), which has been shown to increase susceptibility to hepatotoxic drugs in numerous in vivo experiments (Deng et al., 2009). First, rats were treated with LPS at a bolus dose of 1 mg/kg. After 18 hours, the rats were intravenously administered TIC at a bolus dose of 48 mg/kg and were infused with TIC continuously at 13 mg/kg/h. Levels of alanine aminotransferase (ALT) and direct bilirubin-in-the-plasma specimens collected after 4 hours of TIC infusion were analyzed by an outside service (SRL, Tokyo, Japan).

Isolation of GSH-Conjugated TIC Metabolites by High-Performance Liquid Chromatography. Two types of GSH-conjugated TIC metabolites (TIC-SGs), referred to as TIC-SG1 and TIC-SG2 (Supplemental Fig. S1), were isolated from the rat bile according to a previously reported process (Shimizu et al., 2009). Briefly, bile specimens collected from SD rats that were continuously infused with TIC at 13 mg/kg/h were applied to solid-phase extraction columns (Mega Bond Elut C18, Varian Inc., Palo Alto, CA). The columns were washed with 5% acetonitrile-10 mM ammonium acetate, and eluted with 20% acetonitrile-10 mM ammonium acetate. The eluted samples were subjected to preparative high-performance liquid chromatography (HPLC) with a Hitachi HPLC system, which consisted of an L-4200 UV-VIS detector, L-6300 Intelligent Pump (Hitachi, Tokyo, Japan), and TSKgel ODS-100V preparative column (TOSOH Bioscience, Tokyo, Japan). The HPLC was performed under gradient mobile-phase conditions, with a mixture of 10 mM ammonium acetate in water and 100% acetonitrile as solvents (2.0 mL/min; 0–2 minutes 85:15 v/v, 2–18 minutes 85:15 to 50:50 v/v, 18–20 minutes 50:50 v/v, 20–23 minutes 50:50 to 85:15 v/v, 23–25 minutes 85:15 v/v). Each isolated fraction of TIC-SG1 and TIC-SG2 was lyophilized. The obtained powder was weighed and prepared for liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described in the next section.
Measurement of Biliary Concentrations of GSH-Conjugated TIC Metabolites by LC–MS/MS. Bilirubin concentrations of TIC-SGs were measured by the ACQUITY Ultra-Performance LC System, which was coupled with a Quattro Premier XE electrospray ionization (ESI) MS/MS device (UPLC–MS/MS, Waters Corporation, Milford, MA). The column used was a UPLC BEH C18 Shield (3 mm; 2, 100 mm, Waters Corporation), and UPLC was performed under gradient mobile-phase conditions, with a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile as solvents (0.3 mL/min; 0–1 minutes 95:5 v/v, 1–6 minutes 95:5 to 5:95 v/v, 6–7 minutes 5:95 to 95:5 v/v, and 7–8 minutes 95:5 v/v). The UPLC eluent was introduced into the MS via positive ESI mode (+). The TIC-SGs were quantified in multiple-reactions-monitoring mode (587.20 > 257.10, Cone 22 V, Collision 32 eV for TIC-SG1; 587.20 > 183.90, Cone 20 V, and Collision 38 eV for TIC-SG2), in which metoprolol was used as the internal standard (268.27 > 158.86, Cone 39 V, and Collision 22 eV).

In Vitro Transport Studies Using MRP2-Expressing Membrane Vesicles. Transport experiments of 50 μM TIC-SGs into human MRP2-expressing and control vesicles (Genomembrane, Kanagawa, Japan) were performed according to the manufacturer’s protocol. The reaction mixture was passed through a 0.45-μm HVLP filter (Millipore, Billerica, MA), and washed twice with 5 mL of ice-cold buffer. Extraction with methanol and evaporation with a Centrifugal Filter (Millipore, Billerica, MA) were performed, and the concentrations of TIC-SGs were determined by LC–MS/MS methods. ATP-dependent transport was calculated by subtracting the transport activity in the presence of 10 mM ATP from activity in the presence of 10 mM ADP.

Measurement of PC-Efflux Mediated by MDR3. Recombinant MDR3-expressing adenoviruses (Ad-MDR3) and enhanced green fluorescence protein (EGFP)-expressing adenoviruses for control (Ad-EGFP) were prepared as described previously (Ikebuchi et al., 2009). Tet-regulatory adenoviruses (Ad-Tet) required for the expression of EGFP were prepared as described previously (Park et al., 2000). The PC efflux mediated by MDR3 was evaluated according to the method reported previously (Yoshikado et al., 2011). Briefly, LLC-PK1 cells were seeded onto 24-well plates (BD Falcon, Bedford, MA) at 0.8 × 10^4 cells/well. After 48 hours, cells were infected with 10–multiplicity-of-infection (MOI) Ad-MDR3 and 20-MOI Ad-Tet. At 24 hours after infection, cells were washed with Krebs-Henseleit (KH) buffer (118 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl2, pH 7.4) and preincubated with KH buffer containing [3H]choline for 3 hours at 37°C, under an atmosphere of 5% CO2. During this preincubation, [3H]choline was converted to [3H]PC, a well-known substrate of MDR3, and then incubated with KH buffer containing 3 mM TCA and TIC-SGs (100 μM and 1,000 μM) for an additional 3 hours. The incubated buffer was collected from each well and lyophilized (efflux specimens). The remaining cells were washed with ice-cold KH buffer, lysed with 0.1 M NaOH plus 0.1% SDS, neutralized with 0.1 M HCl, and lyophilized (cell-lysate specimens).

These specimens were dissolved in chloroform and subjected to thin-layer chromatography (TLC) on a silica 60 plate (Merck KGaA, Darmstadt, Germany) with a development solvent containing chloroform, methanol, and 30% ammonia solution (65:35:8). To determine the rate-of-flow value of PC, nonradioactive PC (Sigma-Aldrich) was similarly subjected to the TLC plate and visualized with Dragendorff’s reagent (Wagner et al., 1961). After separation by TLC, the intensities of the [3H]PC spots were measured with a Cyclone Phosphor Imager (PerkinElmer, Waltham, MA). Cellular efflux activities of PC were evaluated by using the following equation: efflux PC (cellular PC + efflux PC) × 100 (%).

Western Blot Analyses. To detect MDR3 protein, LLC-PK1 cells were seeded on 60-mm dishes (BD Falcon) at 4.0 × 10^4 cells/dish and infected with adenoviruses at the MOI described in the transport experiments. Crude membrane fractions were prepared and protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Then, the MDR3 protein and MRP2 protein expressed in membrane vesicles were size-fractionated on 7% SDS-polyacrylamide gels (10 μg/ lane for MDR3 and 2.5 μg/ lane for MRP2), and transferred onto Immobilon (Millipore). After blocking with 3% bovine serum albumin, membranes were immunoblotted with mouse PIII26 antibody (MONOSAN, Uden, the Netherlands) for MDR3 and mouse M2II16 antibody (Abcam, Cambridge, UK) for MRP2. As the secondary antibody, antimouse IgG antibody labeled with horseradish peroxidase (GE Healthcare, Piscataway, NJ) was used. These immunoblotted membranes were treated with ECL Plus (GE Healthcare), and analyzed with a Chemidoc XRS (Bio-Rad Laboratories, Richmond, CA).

Results

Biliary Secretion of Phospholipids Was Decreased in TIC-Administered SD Rats. To search for potential drugs that induce cholestasis under clinical situations, drugs associated with DILI reported in Japan were rearranged in descending order according to the number of cholestatic cases per year, according to the Manual for Drug-Induced Liver Injury from the Ministry of Health, Labor, and Welfare (Supplemental Table S1). The types of liver injury were classified by the diagnostic criteria of the Digestive Disease Week-Japan (DDW-J) scale (Takikawa and Onji, 2005). TIC was responsible for the most cases of cholestatic and mixed-type liver injury in the report, although less than half of TIC-induced liver injuries were classified as simple cholestatic cases. Considering that many previous studies have reported severe cholestatic liver injuries caused by TIC administration (Greany et al., 1993; Grimm and Litynski, 1994; Iqbal et al., 1998; Alberti and Alberti-Flor, 2002; Mambelli et al., 2007), we further analyzed the mechanism of TIC-induced cholestasis.

Bile flow is generated by bile acid–dependent and –independent mechanisms, both of which are essential for the maintenance of normal bile balance. Biliary secretions of phospholipids and bile acids, which are key components of bile acid–dependent bile flow, were analyzed in TIC-administered SD rats (Fig. 1). TIC administration for 0.5 hour, bile flow tended to be increased compared with the control condition, although the difference was not statistically significant (Fig. 1A). The biliary output of phospholipids was significantly decreased in TIC-administered rats (Fig. 1B), in contrast to the small change in the output of bile acids (Fig. 1C). Two hours after TIC administration was started, the plasma concentration of TIC was 17.6 ± 3.9 μM (Supplemental Fig. S2A), which was close to the peak plasma concentration of TIC observed in clinical situations (mean 9.8 ± 2.3 μM in patients repeatedly receiving 500 mg of TIC in tablet form) (Ebihara et al., 1978), considering interindividual differences in the pharmacokinetics of TIC.

Because TIC reportedly undergoes extensive metabolism in the liver and <1% of the parent compound is detected in urine (Shimizu et al., 2009), we examined the involvement of TIC metabolites in the altered bile composition. We pretreated SD rats with SKF-525A, a nonspecific inhibitor of P450s (Yang et al., 2009), prior to the administration of TIC. The biliary secretion of phospholipids in rats treated with SKF-525A and TIC was not significantly different from that in control rats (Fig. 1B), suggesting that TIC metabolites, rather than parent TIC, might have caused the decrease in the biliary secretion of phospholipids observed in rats treated with TIC alone.
GSH and GSH-Conjugated TIC Metabolites Were Markedly Excreted into Bile in TIC-Administered SD Rats. We examined the effect of TIC on bile acid–independent bile flow driven by the biliary output of osmotic solutes, such as GSH and its conjugates. Bile analysis in SD rats revealed that the biliary excretion of GSH was dramatically increased to approximately 2 μmol/kg body weight (BW) at 0.5–1.0 hour after TIC administration (Fig. 2A).

Because two types of GSH-conjugated TIC metabolites were previously detected in the bile of TIC-administered rats (Shimizu et al., 2009), we isolated these metabolites (TIC-SG1 and TIC-SG2) from rat bile and constructed quantification methods by LC-MS/MS (Supplemental Fig. S1). The biliary excretions of TIC-SG1 and TIC-SG2 were approximately 20 and 5 μmol/kg BW at 0.5–1.0 hour after TIC administration, respectively (Fig. 2B), and were 10- and 2.5-fold higher, respectively, than that of the parent GSH. The biliary output of TIC-SGs in rats treated with SKF-525A and TIC was much less than that in rats treated with TIC alone (Fig. 2B), consistent with the previous report that TIC-SGs were formed in the liver after P450s-mediated metabolism (Shimizu et al., 2009).

TIC Administration Did Not Affect Bile Composition in Mrp2-Deficient Rats. Administration of TIC to SD rats stimulated the biliary excretion of GSH and TIC-SGs, and significantly reduced the biliary phospholipids. We examined whether such changes in bile composition would be observed in EHBR lacking functional MRP2, which mediates the

Effect of Ticlopidine on Bile Formation

Fig. 1. Effect of TIC on the bile flow rate and biliary secretion of phospholipids and bile acids in SD rats. Bile formations in SD rats treated with TIC alone, TIC and SKF-525A, or control solution were analyzed. The value at 0.5 hour in each panel is defined as 100%. (A) Bile flow rates. Values at 0.5 hour were 2.03 ± 0.42 ml/h/kg body weight (BW) for TIC-treated rats and 2.19 ± 0.28 ml/h/kg BW for control rats. (B) Biliary output of phospholipids. Values at 0.5 hour were 118 ± 10 nmol/min/kg BW (TIC) and 107 ± 10 nmol/min/kg BW (control). (C) Biliary output of bile acids. Values at 0.5 hour were 0.57 ± 0.14 μmol/min/kg BW (TIC) and 0.39 ± 0.04 μmol/min/kg BW (control). Data are shown as the mean ± S.E. of four to five independent experiments. *P < 0.05 versus control by Student’s t test.

Fig. 2. Biliary excretion of GSH and TIC-SGs in SD rats. TIC was administered to SD rats. (A) Biliary excretion of GSH at indicated periods. (B) Biliary excretion of TIC-SGs at indicated periods. Data are shown as the mean ± S.E. of four to five independent experiments. **P < 0.01 and *P < 0.05 versus TIC-administered SD rats by Student’s t test.
biliary excretion of osmotic solutes like GSH and its conjugates (Paulusma et al., 1996). TIC was administered to EHBR in the same condition as SD rats, and the plasma concentration of TIC was 19.2 ± 8.4 μM (Supplemental Fig. S2A). Although the plasma concentrations of TIC-SGs in SD rats were below the limits of quantification (<0.1 μM), the concentrations in EHBR could be measured: The plasma concentration of TIC-SG1 2 hours after starting TIC administration was 0.33 μM and that of TIC-SG2 was 2.3 μM (Supplemental Fig. S2A). Remarkably, no significant change in the bile flow or the biliary output of phospholipids or bile acids was observed in TIC-administered EHBR (Fig. 3, A-C). As expected, the biliary output of GSH in EHBR was under the detection limit, and the biliary outputs of TIC-SG1 and TIC-SG2 were <1% of outputs in SD rats (Fig. 3D). These results suggested that Mrp2 might be responsible for the biliary excretion of TIC-SGs, and for the diminished biliary phospholipids observed in TIC-administered SD rats.

**TIC-Induced Liver Injuries in SD Rats.** Because TIC administration caused significant changes in the bile composition of SD rats, we examined whether TIC had a hepatotoxic effect in vivo. Plasma ALT, a representative marker of liver injury, was significantly elevated after 4 hours of TIC infusion in LPS-pretreated SD rats, but not in LPS-pretreated EHBR (Fig. 4A). Additionally, the plasma level of direct bilirubin, a cholestatic marker, was dramatically increased in LPS-pretreated SD rats after TIC administration (Fig. 4B). These results suggest that Mrp2 function was required for the TIC-mediated hepatotoxicity. No significant hepatotoxicity in rats treated with TIC alone was observed, which suggests that LPS pretreatment increased susceptibility to the TIC-induced hepatotoxicity.

Because biliary phospholipids play essential roles in protecting the bile canalicular membranes from the detergent effect of free bile acids by forming phospholipid–bile acid–mixed micelles, the reduction of biliary phospholipids observed in TIC-administered SD rats, but not in EHBR, might induce cholestatic liver injury. Indeed, because the extent of the decrease in the biliary concentration of phospholipids (Supplemental Fig. S3A) was larger than that of bile acids (Supplemental Fig. S3B), the phospholipid:bile acid ratio (mM:mM) was significantly decreased after TIC administration to SD rats (Fig. 4C). This effect might have induced damage to the bile canalicular membranes. The cytoprotective effects of phospholipids in phospholipid–bile acid–mixed micelles were studied previously in vitro (Moschetta et al., 2000). The release of lactate dehydrogenase (LDH), a marker of cellular membrane damage, from cells treated with phospholipid–bile acid–mixed micelles was gradually decreased as the phospholipid:bile acid ratio in mixed micelles was increased. By using CaCo-2 cells, we could confirm these previous results (unpublished observations).

**GSH-Conjugated TIC Metabolites Were Transported by MRp2.** The above results suggested that TIC-induced alterations in the bile composition might be a factor in cholestatic liver injury. Therefore, we investigated the involvement of transporters in the TIC-induced changes in biliary composition through in vitro experiments using transporter-expressing cells and membrane vesicles. Because the biliary output of TIC-SGs in EHBR lacking functional Mrp2 was much lower (Fig. 3D) than that in SD rats (Fig. 2B), we supposed that TIC-SGs might be excreted into bile by Mrp2.
Mrp2. To examine this possibility, we performed transport assays using MRP2-expressing membrane vesicles (Fig. 5A). The ATP-dependent transports of TIC-SG1 and TIC-SG2 by MRP2-expressing vesicles were significantly higher than those by control vesicles (Fig. 5, B and C), which demonstrated that TIC-SGs were actively transported by MRP2.

**GSH-Conjugated TIC Metabolites Showed No Inhibitory Effect on MDR3-Mediated PC Efflux.** In Mrp2-deficient rats (EHBR), TIC administration did not affect the biliary secretion of phospholipids (Fig. 3B). This result suggests that the Mrp2-mediated biliary excretion of TIC-SGs was necessary for the TIC-induced alteration in bile composition. Therefore, TIC-SGs would inhibit the MDR3 activity from the outside of the canalicular membranes. Indeed, although the hepatic concentrations of TIC-SG1 and TIC-SG2 in EHBR were higher than those in SD rats (Supplemental Fig. S2B), we observed no effect of TIC-SGs on the biliary secretion of phospholipids in EHBR. Using LLC-PK1 cells expressing MDR3 (Fig. 6A), we examined the inhibitory effects of TIC-SGs on the function of MDR3 by measuring PC efflux from the cells. The efflux of [14C]PC from MDR3-expressing cells was more significant than that from control

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**Fig. 4.** Evaluation of toxicity caused by TIC. Hepatotoxic effects of TIC were examined in SD rats and EHBR pretreated with lipopolysaccharide (LPS). Plasma levels of alanine aminotransferase (ALT) (A) and direct bilirubin (B) were determined. Data are shown as the mean ± S.E. of four to six independent experiments. **P < 0.01 and *P < 0.05 versus values at 0 hour by Student’s t test. (C) Phospholipids:bile acids ratio (mM:mM) in SD rats treated with TIC or control solution. Data are shown as the mean ± S.E. of four to five independent experiments. *P < 0.05 versus control by Student’s t test.
cells (Fig. 6B). Neither TIC-SG1 nor TIC-SG2 inhibited the MDR3-mediated efflux of PC. By contrast, verapamil, a well-established inhibitor of MDR3 (van Helvoort et al., 1996; Morita et al., 2007), significantly inhibited MDR3 (Fig. 6B). Therefore, the decreased biliary secretion of phospholipids observed in TIC-administered SD rats might not have resulted from a direct inhibition of MDR3 but from an indirect mechanism, presumably due to the disruption of normal bile formation.

Decreased Biliary Secretion of Phospholipids in TIC-Administered SD Rats Was Restored by TCA Infusion. We attempted to clarify the indirect mechanism of decreased biliary phospholipids in TIC-administered SD rats. Because the significant biliary excretion of GSH and TIC-SGs (Fig. 2, A and B) might stimulate bile acid–independent bile flow in an osmotic manner and, consequently, reduce the biliary concentration of bile acids (Supplemental Fig. S3B), the formation of biliary phospholipid–bile acid–micelles for the protection from the detergent effect of bile acids might be diminished in TIC-administered SD rats. To investigate this point, we supplied rats with bile acids by TCA infusion and analyzed the effect of TIC administration on the biliary secretion of phospholipids (Fig. 7). Even if TIC was administered, the biliary concentration of bile acids was kept at a normal level by TCA infusion (Supplemental Fig. S3D), and the biliary output of phospholipids was maintained to a similar extent as that in control rats (Fig. 7B). Therefore, we concluded that TCA infusion maintained the biliary concentration of bile acids sufficiently to allow for normal biliary secretion of phospholipids.

Fig. 5. MRP2-mediated transport of GSH-conjugated TIC metabolites. Transport of TIC-SGs was examined with MRP2-expressing membrane vesicles. (A) Western blot analysis was performed to verify the expression of MRP2 protein in MRP2-expressing membrane vesicles. (B) Transports of TIC-SG1 (50 μM) and (C) TIC-SG2 (50 μM) by MRP2-expressing vesicles were examined. The incubation condition was 37°C for 2.5 or 5.0 minutes. ATP-dependent transport was calculated by subtracting the transport activity in the presence of 10 mM AMP from the activity in the presence of 10 mM ATP. Data are presented as mean ± S.E. of three independent experiments. **P < 0.01 versus control vesicles by Student’s t test.

Fig. 6. Effect of GSH-conjugated TIC metabolites on MDR3-mediated PC efflux. Effects of TIC-SGs on the function of MDR3 were examined with MDR3-expressing LLC-PK1 cells. (A) Western blot analysis was performed to verify the expression of MDR3 protein in MDR3-expressing LLC-PK1 cells. (B) Efflux of PC from MDR3-expressing cells was examined at 37°C for 3 hours. Cells were incubated with TIC-SG1, TIC-SG2, or verapamil at indicated concentrations. Data are presented as mean ± S.E. of three to four independent experiments. **P < 0.01 versus untreated MDR3-expressing cells by ANOVA (analysis of variance) followed by Dunnett’s test.

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acids at a sufficient level for normal formation of the biliary phospholipid–bile acid micelles.

The effects of TIC on bile formation are summarized in Fig. 8. In the normal condition, bile acids secreted into bile form mixed micelles with phospholipids and cholesterol (Fig. 8A). When TIC is administered to SD rats (Fig. 8B), TIC undergoes P450s-mediated metabolism and is conjugated with GSH. These GSH-conjugated TIC metabolites (TIC-SGs) and GSH are excreted into bile by MRP2 and stimulate bile acid–independent bile flow in an osmotic manner, which leads to a decrease in the biliary concentration of bile acids. These changes in bile composition might affect the normal formation of biliary phospholipid–bile acid micelles and indirectly inhibit the biliary secretion of phospholipids. Such a reduction in biliary phospholipids might lead to the accumulation of damage on the canalicular membranes and contribute to the risk of TIC-induced cholestatic liver injury. On the other hand, in TIC-administered Mrp2-deficient EHBR (Fig. 8C), the biliary outputs of TIC-SGs are much less than those in SD rats, and the bile compositions are little affected.

**Discussion**

In the present study, we analyzed the effects of TIC on bile formation to identify the mechanism of TIC-induced cholestasis. The biliary output of phospholipids, which protect bile canaliculi from the detergent effect of biliary bile acids, was significantly decreased in TIC-administered SD rats (Fig. 1), but not in TIC-administered EHBR lacking Mrp2 function (Fig. 3). Moreover, stimulation of the Mrp2-mediated biliary excretion of GSH and TIC-SGs in TIC-administered SD rats (Fig. 2) affected the formation of biliary phospholipid–bile acid micelles and indirectly inhibited the biliary secretion of phospholipids. These effects might have led to the accumulation of damage on the canalicular membranes, contributing to the risk of TIC-induced cholestatic liver injuries observed in SD rats (Fig. 4).

Because genetic disruptions of BSEP and MDR3 are known to cause PFIC2 and PFIC3, respectively, drugs that have the potential to affect the function of these transporters may induce cholestasis. BSEP-related drug-induced cholestasis has been suggested in several in vitro studies (Stieger et al., 2000; Fattinger et al., 2001; Funk et al., 2001; Horikawa et al., 2003), although at much higher concentrations than those observed in clinical conditions. Recently, we revealed the mechanism of ITZ-induced cholestasis caused by the direct inhibition of MDR3 (Yoshikado et al., 2011). We identified two patients suffering from ITZ-induced cholestatic liver injuries and observed that biliary phospholipids were significantly decreased in ITZ-administered rats. In addition, the MDR3-mediated efflux of PC was inhibited in the presence of clinically relevant concentrations of ITZ.

Although the administration of TIC to SD rats significantly reduced the biliary secretion of phospholipids (Fig. 1B), a direct inhibition of MDR3 was not likely to be the major cause of decreased biliary phospholipids because TCA infusion recovered the biliary phospholipid secretion (Fig. 7B). TIC-induced stimulation of the MRP2-mediated biliary excretion of GSH and TIC-SGs (Fig. 2, A and B), which drove bile acid–independent bile flow by an osmotic mechanism, decreased the biliary concentration of bile acids (Supplemental Fig. 3B). Because the maintenance of biliary bile acids is necessary for the proper formation of biliary phospholipid–bile acid micelles via the extraction of phospholipids from canalicular membranes (Oude Elferink and Paulusma, 2007), a TIC-induced decrease in the biliary concentration of bile acids might cause the diminished biliary secretion of phospholipids. To discuss whether these phenomena occur in clinical conditions, we should consider the possibility of
species differences between human MDR3/ABCB4 and rat Mdr2/Abcb4 in terms of the susceptibility to TIC-SGs. From this perspective, studies on the effects of TIC-SGs by using sandwich-cultured human/rat hepatocytes or Mdr2 knockout mice may be helpful for further understanding.

Previous reports have suggested some other drugs that may stimulate bile acid–independent bile flow. Bosentan, a dual endothelin ET receptor antagonist, has been shown to cause liver injuries in some clinical cases. In bosentan-administered rats, the biliary output of GSH was increased, but that of phospholipids was decreased; these changes were not observed in Mrp2-deficient rats (Fouassier et al., 2002). Similar phenomena were observed in rats administered ampicillin (Verkade et al., 1990), cefmetazole (Cava et al., 1991), bromosulfophthalein, and cefpiramide (Takikawa et al., 1993), although the doses of these drugs were much higher than those used in clinical conditions.

On the other hand, in the present study we revealed the mechanism of cholestatic liver injury caused by a clinically relevant concentration of TIC, by comprehensively analyzing bile formation in rats, hepatotoxicity, and effects on canalicular transporters in vitro. The GSH-conjugated TIC metabolites were markedly excreted into bile, where they affected the formation of biliary micelles and, consequently, reduced the biliary phospholipids, which might have damaged the bile canalicular membranes. Because many drugs and drug metabolites are reported to be substrates of canalicular transporters (MRP2, BCRP, and MDR1), close attention should be
paid to the extent of biliary excretion of drugs and metabolites, given the possibility of alteration in bile composition.

Similar to most drug-induced liver injury cases, TIC-induced cholestatic liver injuries often occur in an idiosyncratic manner. Therefore, in addition to the damage on bile canalicular membranes caused by the diminished biliary secretion of phospholipids, other factors might affect the occurrence and aggravation of TIC-induced cholestatic liver injuries. Genetic variations in human leukocyte antigen (HLA) have been shown to be involved in TIC-induced idiosyncratic liver injuries. HLA-A*3303 alleles were found in 68% of TIC-induced liver injury cases in Japan, but only in 14% of TIC-tolerant subjects (Hirata et al., 2008). Because the allele frequency of HLA-A*3303 was reportedly higher in Japanese than in other ethnic groups (Ariyoshi et al., 2010), this allele may partly account for the higher frequency of TIC-induced liver injuries in Japan. In addition to the mutation in HLA, MDR3 mutations that cause the diminished expression and function of the MDR3 protein (Oude Elferink and Paulusma, 2007) may increase susceptibility to TIC-induced cholestatic liver injury. Additionally, Dawson et al. reported TIC-mediated inhibition of human BSEP (IC50: 74 μM) and rat Bsep (IC50: 49 μM) (Dawson et al., 2012), which might be another factor contributing to TIC-induced cholestatic liver injury, particularly when combined with lower BSEP expression or activity. Furthermore, the cooperative function of MDR3 and ATP8B1 to generate lipid asymmetry in the bile canalicular membrane is considered to be important for protection of the plasma membrane (Groen et al., 2011). ATP8B1 is a P-type ATPase that is responsible for PFIC1 disease. It mediates the translocation of phosphatidylserine from the outer to the inner leaflet of the bile canalicular membrane. These findings suggest that alterations in the function of ATP8B1 may also affect the susceptibility to TIC-induced cholestatic liver injury. Further pharmacogenomic studies of bile canalicular transporters, including MDR3, BSEP, ATP8B1 and MRP2, may be helpful for understanding the appropriate use of cholestatic drugs in clinical situations.

In conclusion, the present study reported a new mechanism of TIC-induced cholestatic liver injury. Administration of TIC stimulated bile acid–dependent bile flow, driven by the biliary excretion of GSH and TIC-SGs, which affected the bile composition balance and indirectly inhibited the biliary secretion of phospholipids. To reveal the mechanisms of cholestatic liver injuries induced by various kinds of clinically used drugs, one should consider the alterations in bile composition induced by the significant biliary excretion of drugs and drug metabolites, as well as the direct effects on the function of canalicular transporters.

Authorship Contributions
Participated in research design: Yoshikado, Takada, Ito, Suzuki.
Conducted experiments: Yoshikado, Yamamoto, Tan.
Contributed new reagents or analytic tools: Santa.
Performed data analysis: Yoshikado, Takada.
Wrote or contributed to the writing of the manuscript: Yoshikado, Takada, Suzuki.

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