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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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Running Title: Effect of ticlopidine on bile formation

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Abbreviations:

ABC, ATP-binding cassette; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BSEP, bile salt export pump; CYP, cytochrome P-450; DILI, drug-induced liver injury; EGFP, enhanced green fluorescent protein; EHBR, Eisai hyperbilirubinemic rat; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MDR3, multidrug resistance 3 P-glycoprotein; MOI, multiplicity of infection; MRP2, multidrug resistance-associated protein 2; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; TCA, taurocholate; TIC, ticlopidine; TIC-SG, GSH-conjugated TIC metabolite; TLC, thin-layer chromatography

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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 SKF-525A that inhibits cytochrome P-450s (CYPs), or in Eisai hyperbilirubinemic rats (EHBR) lacking functional multidrug resistance-associated protein 2 (MRP2/ABCC2). These results suggest that glutathione (GSH)-conjugated TIC metabolites (TIC-SGs), which were formed in the liver after CYPs-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.

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Introduction

Bile formation, one of the most essential functions of the liver, is mediated by 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) (Smit 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 physiological importance of BSEP, MDR3, and MRP2, drugs that affect the functions of these canalicular transporters and, subsequently, disturb bile formation may

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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. (Aithal et al., 2011). Previous studies have reported the inhibition of BSEP by several drugs *in vitro* (Fattinger et al., 2001; Funk et al., 2001; Stieger et al., 2000), 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 IC_{50} 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 (Crocenzi 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 (Alberti and Alberti-Flor, 2002; Greany et al., 1993; Grimm and Litynski, 1994; Iqbal et al.,

1998; Mambelli et al., 2007). We showed that the biliary secretion of phospholipids was significantly decreased in TIC-administered Sprague-Dawley (SD) rats. Given the physiological 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.

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Materials and Methods

Materials. [^{14}C]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 MEM (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% CO_2. 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 Institute of Health (NIH publication 86–23 revised 1985).

Male SD rats (Japan SLC, Shizuoka, Japan) weighing 230–270 g and Eisai hyperbilirubinemic rats (EHBR; Japan SLC) weighing 260–300 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-31 (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 h.

To inhibit the metabolism of TIC by cytochrome P-450s (CYPs) *in vivo*, rats were intraperitoneally administered the nonspecific CYPs inhibitor SKF-525A (Sigma Aldrich) at a bolus dose of 50 mg/kg at 1 h before TIC administration (Yang et al., 2009). To increase the biliary concentration of bile acids, taurocholate (TCA) (Sigma Aldrich) was continuously infused, intravenously, at 65 mg/kg/h from 1 h 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 min. Plasma specimens were obtained by centrifuging the blood specimens at 1,000 *g* for 15 min. 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 h, the rats were intravenously administered with TIC at a bolus dose of 48 mg/kg and were infused with TIC continuously

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at 13 mg/kg/h. Levels of alanine aminotransferase (ALT) and direct bilirubin in the plasma specimens collected after 4 h of TIC infusion were analyzed by an outside service (SRL, Tokyo, Japan).

Isolation of GSH-conjugated TIC metabolites by high performance liquid chromatography (HPLC). Two types of GSH-conjugated TIC metabolites, 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 C_{18}, 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 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 (2.8 mL/min; 0–2 min 85:15 v/v, 2–18 min 85:15 to 50:50 v/v, 18–20 min 50:50 v/v, 20–23 min 50:50 to 85:15 v/v, 23–25 min 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 below.

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Measurement of biliary concentrations of GSH-conjugated TIC metabolites by LC-MS/MS. Biliary 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 C_{18} Shield (3 μm; 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 min 95:5 v/v, 1–6 min 95:5 to 5:95 v/v, 6–7 min 5:95 to 95:5 v/v, and 7–8 min 95:5 v/v). The UPLC eluent was introduced into the MS via positive ESI mode (ESI+). The TIC-SGs were quantified in multiple reactions monitoring (MRM) 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 Evaporator EC-57C3 (SAKUMA, Tokyo, Japan) 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 AMP from activity in the presence of 10 mM ATP.

Measurement of PC-efflux mediated by MDR3. Recombinant MDR3-expressing adenoviruses (Ad-MDR3) and enhanced green fluorescent 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 target proteins were included in an Adeno-X Tet-Off Expression System 1 kit (Clontech/Takara-Bio, Mountain View, CA).

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) at 0.8×10^5 cells/well. After 48 h, cells were infected with 10 multiplicity of infection (MOI) Ad-MDR3 and 20 MOI Ad-Tet. At 24 h after infection, cells were washed with Krebs-Henseleit (KH) buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4) and preincubated with KH buffer containing [^{14}C]choline for 3 h at 37 °C, under an atmosphere of 5% CO_2. During this preincubation, [^{14}C]choline was converted to [^{14}C]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 h. 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.l% SDS, neutralized with 0.1

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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 (Rf) 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 [^{14}C]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 × 10^5 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 P3II26 antibody (MONOSAN, Uden, the Netherlands) for MDR3 and mouse M2III6 antibody (Abcam, Cambridge, UK) for MRP2. As the secondary antibody, anti-mouse 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).

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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 DDW-J scale (Takikawa et al., 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 (Alberti and Alberti-Flor, 2002; Greany et al., 1993; Grimm and Litynski, 1994; Iqbal et al., 1998; 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). After TIC administration for 0.5 h, bile flow tended to be increased compared to 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

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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 CYPs (Yang et al., 2009), prior to the administration of TIC. The biliary output 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 h 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

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(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 h 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 CYPs-mediated metabolism (Shimizu et al., 2009).

TIC administration did not affect bile composition in Mrp2-deficient rats (EHBR).

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 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 h 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

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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 h 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. 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,

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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 [^{14}C]PC from MDR3-expressing cells was more significant than that from control 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 (Morita et al., 2007; van Helvoort et al., 1996), 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

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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 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 CYPs-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.

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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 (Fattinger et al., 2001; Funk et al., 2001; Horikawa et al., 2003; Stieger et al., 2000), 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 $ET_{A/B}$ 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 idiosyncratic manners. 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

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(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 (IC_{50}: 74 μM) and rat Bsep (IC_{50}: 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-independent 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, and Suzuki.

Conducted experiments: Yoshikado, Yamamoto and Tan.

Contributed new reagents or analytic tools: Santa.

Performed data analysis: Yoshikado and Takada.

Wrote or contributed to the writing of the manuscript: Yoshikado, Takada and Suzuki.

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Footnotes

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Legends for Figures

Figure 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 ticlopidine (TIC) alone, TIC and SKF-525A, or control solution were analyzed. The value at 0.5 h in each panel is defined as 100%. (A) Bile flow rates. Values at 0.5 h 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 h 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 h 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. *$p < 0.05$ *vs.* control by Student's *t*-test.

Figure 2. **Biliary excretion of GSH and TIC-SGs in SD rats.** Ticlopidine (TIC) was administered to SD rats. (A) Biliary excretion of GSH at indicated periods. (B) Biliary excretion of GSH-conjugated TIC metabolites (TIC-SGs) at indicated periods. Data are shown as the mean ± S.E. of four to five independent experiments. **$p < 0.01$ and *$p < 0.05$ *vs.* TIC-administered SD rats by Student's *t*-test.

Figure 3. **Effect of TIC on bile composition in Mrp2-deficient rats (EHBR).** Ticlopidine (TIC) was administered to Eisai hyperbilirubinemic rats (EHBR). The value at 0.5 h in each panel is defined as 100%. (A) Bile flow rate. Values at 0.5 h were 1.35 ± 0.07 mL/h/kg body weight (BW) for TIC-treated rats and 1.54 ± 0.20 mL/h/kg BW for control rats. (B) Biliary

output of phospholipids. Values at 0.5 h were 203 ± 25 nmol/min/kg BW (TIC) and 195 ± 22 nmol/min/kg BW (control). (C) Biliary output of bile acids. Values at 0.5 h were 0.56 ± 0.06 μmol/min/kg BW (TIC) and 0.53 ± 0.11 μmol/min/kg BW (control). (D) Biliary excretion of GSH-conjugated TIC metabolites (TIC-SGs) at indicated periods. Data are shown as the mean ± S.E. of four to five independent experiments.

Figure 4. **Evaluation of toxicity caused by TIC.** Hepatotoxic effects of ticlopidine (TIC) were examined in SD rats and Eisai hyperbilirubinemic rats (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 $^{*}p < 0.05$ *vs.* values at 0 h by Student's *t*-test. (C) Phospholipids / bile acids ratio (mM / mM) in SD rats treated with ticlopidine (TIC) or control solution. Data are shown as the mean ± S.E. of four to five independent experiments. $^{*}p < 0.05$ *vs*. control by Student's *t*-test.

Figure 5. **MRP2-mediated transport of GSH-conjugated TIC metabolites.** Transport of GSH-conjugated ticlopidine metabolites (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 min. 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. $^{**}p < 0.01$ *vs.* control vesicles by Student's *t*-test.

Figure 6. **Effect of GSH-conjugated TIC metabolites on MDR3-mediated PC efflux.** Effects of GSH-conjugated ticlopidine metabolites (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 [^{14}C]phosphatidylcholine (PC) from MDR3-expressing cells was examined at 37 °C for 3 h. 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. $^{**}p < 0.01$ *vs.* untreated MDR3-expressing cells by ANOVA followed by Dunnett's test.

Figure 7. **Effect of TIC on bile formation in SD rats with taurocholate-infusion.** Ticlopidine (TIC) was administered to SD rats with taurocholate (TCA)-infusion, which was started 1 h before TIC administration. Value at 0.5 h in each panel is defined as 100%. (A) Bile flow rate. Values at 0.5 h were 2.63 ± 0.34 mL/h/kg body weight (BW) for TIC-treated rats and 3.30 ± 0.25 mL/h/kg BW for control rats. (B) Biliary output of phospholipids. Values at 0.5 h were 299 ± 38 nmol/min/kg BW (TIC) and 330 ± 28 nmol/min/kg BW (control). (C) Biliary output of bile acids. Values at 0.5 h were 1.98 ± 0.24 μmol/min/kg BW (TIC) and 1.91 ± 0.28 μmol/min/kg BW (control). Data are shown as the mean ± S.E. of five

independent experiments.

Figure 8. Effects of TIC on bile formation. (A) Bile formation in SD rats without ticlopidine (TIC), (B) in TIC-administered SD rats and (C) in TIC-administered Mrp2-deficient rats (Eisai hyperbilirubinemic rats [EHBR]). In the normal condition, bile acids secreted into bile form mixed micelles with phospholipids and cholesterol (A). When TIC is administered to SD rats (B), TIC undergoes CYPs-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 (C), biliary outputs of TIC-SGs are much less than those in SD rats and bile composition might not be affected.

Fig. 1

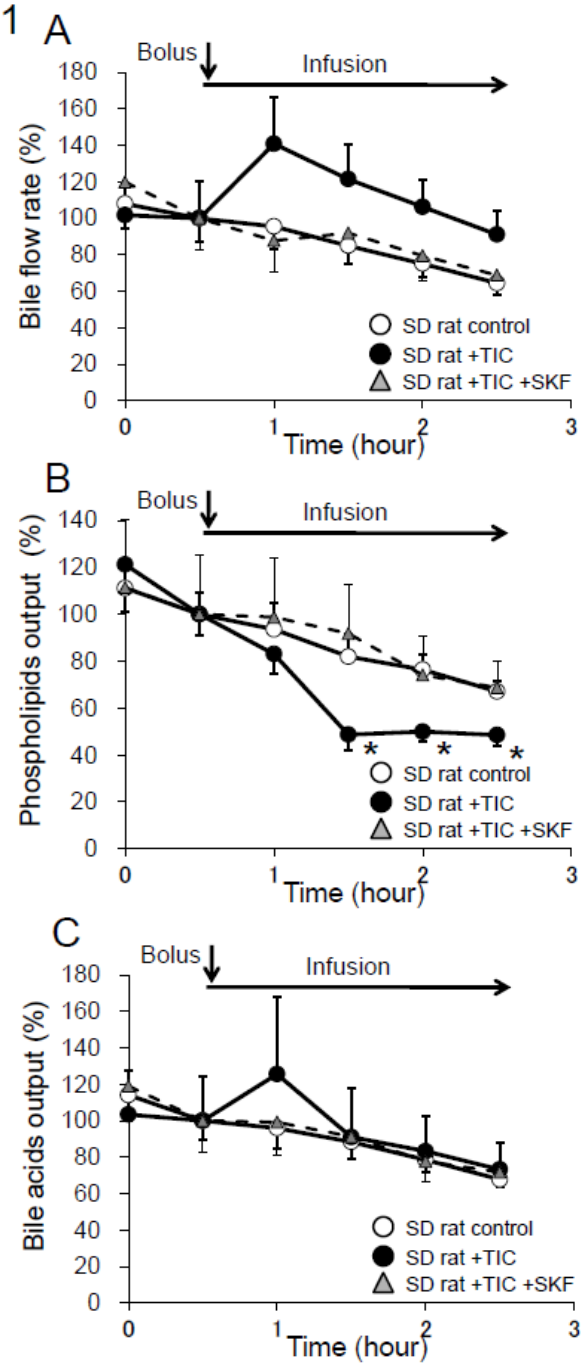
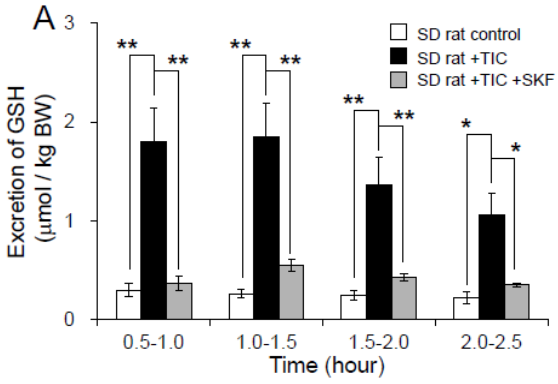
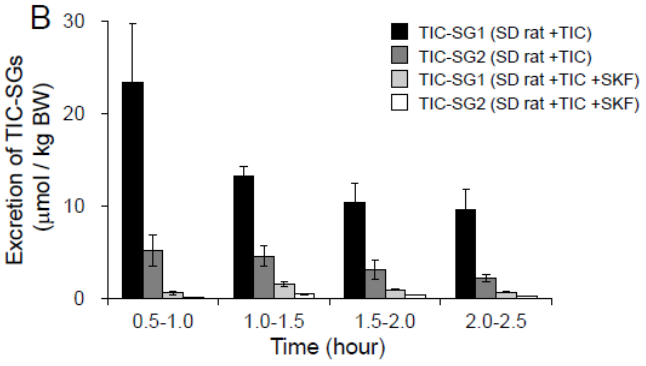

A
Bolus
Infusion
Bile flow rate (%)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
SD rat control
SD rat +TIC
SD rat +TIC +SKF
B
Bolus
Infusion
Phospholipids output (%)
140
120
100
80
60
40
20
0
*
*
*
0
1
2
3
Time (hour)
SD rat control
SD rat +TIC
SD rat +TIC +SKF
C
Bolus
Infusion
Bile acids output (%)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
SD rat control
SD rat +TIC
SD rat +TIC +SKF

Fig. 2


A
SD rat control
SD rat +TIC
SD rat +TIC +SKF
Excretion of GSH (μmol / kg BW)
0
1
2
3
**
**
**
**
**
**
*
*
0.5-1.0
1.0-1.5
1.5-2.0
2.0-2.5
Time (hour)


B
TIC-SG1 (SD rat +TIC)
TIC-SG2 (SD rat +TIC)
TIC-SG1 (SD rat +TIC +SKF)
TIC-SG2 (SD rat +TIC +SKF)
Excretion of TIC-SGs (μmol / kg BW)
0
10
20
30
0.5-1.0
1.0-1.5
1.5-2.0
2.0-2.5
Time (hour)

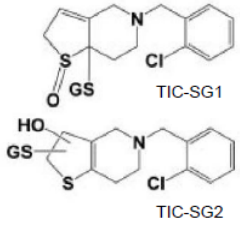

N
S
GS
O
Cl
TIC-SG1
HO
GS
N
S
Cl
TIC-SG2

Fig. 3

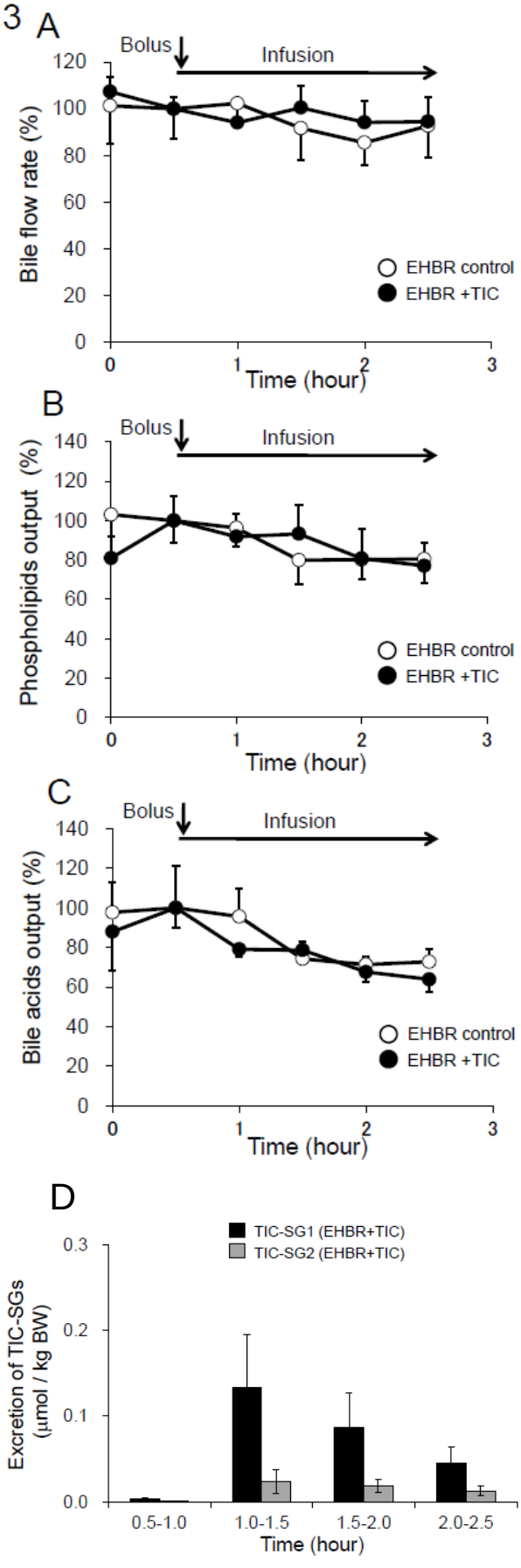

A
Bolus
Infusion
Bile flow rate (%)
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
EHBR control
EHBR +TIC
B
Bolus
Infusion
Phospholipids output (%)
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
EHBR control
EHBR +TIC
C
Bolus
Infusion
Bile acids output (%)
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
EHBR control
EHBR +TIC
D
TIC-SG1 (EHBR+TIC)
TIC-SG2 (EHBR+TIC)
Excretion of TIC-SGs (μmol / kg BW)
0.3
0.2
0.1
0.0
0.5-1.0
1.0-1.5
1.5-2.0
2.0-2.5
Time (hour)

Fig. 4

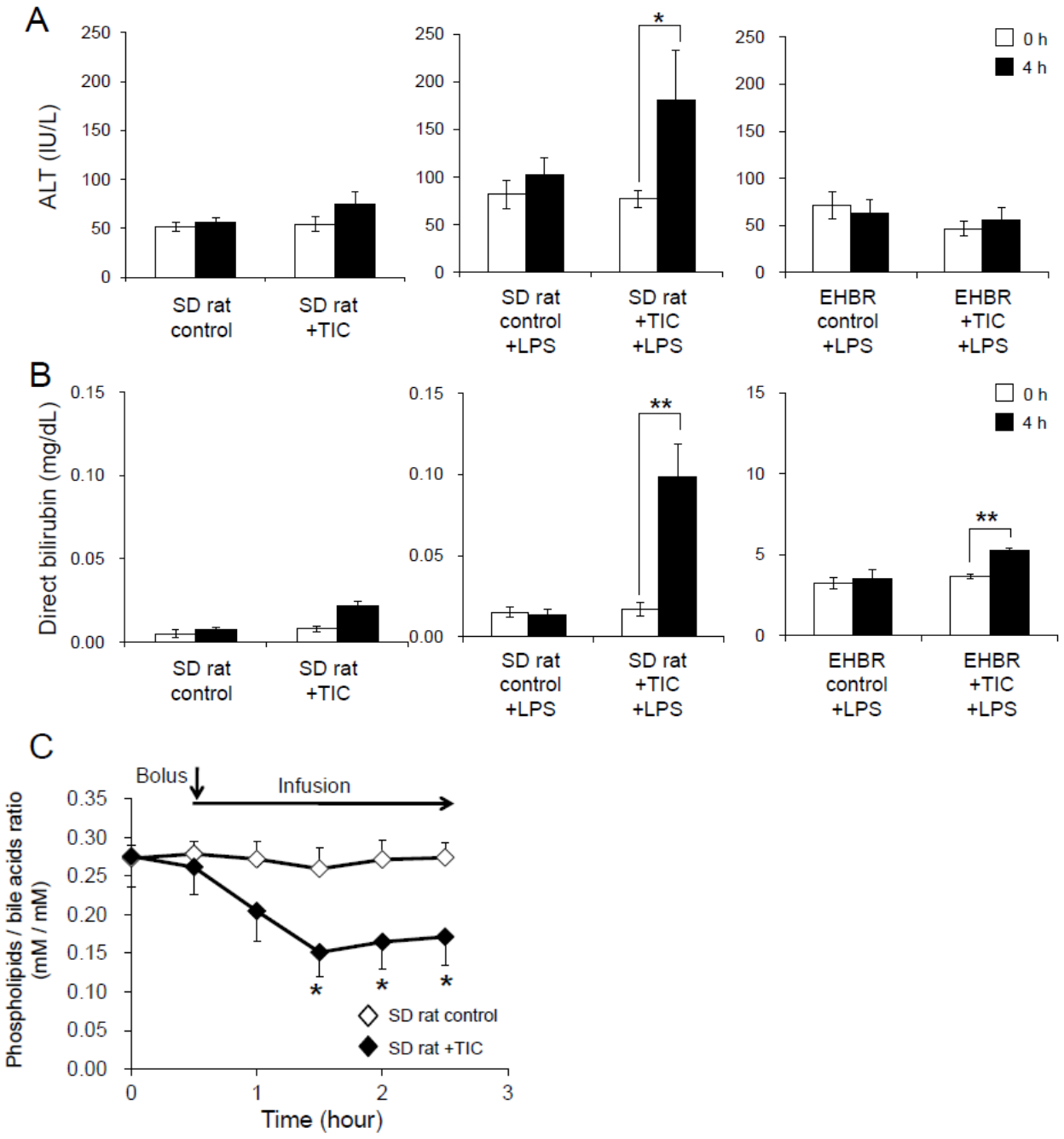

A
ALT (IU/L)
250
200
150
100
50
0
*
0 h
4 h
SD rat control
SD rat +TIC
SD rat control +LPS
SD rat +TIC +LPS
EHBR control +LPS
EHBR +TIC +LPS
B
Direct bilirubin (mg/dL)
0.15
0.10
0.05
0.00
15
10
5
**
C
Bolus
Infusion
Phospholipids / bile acids ratio (mM / mM)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
SD rat control
SD rat +TIC
Time (hour)
0
1
2
3

Fig. 5

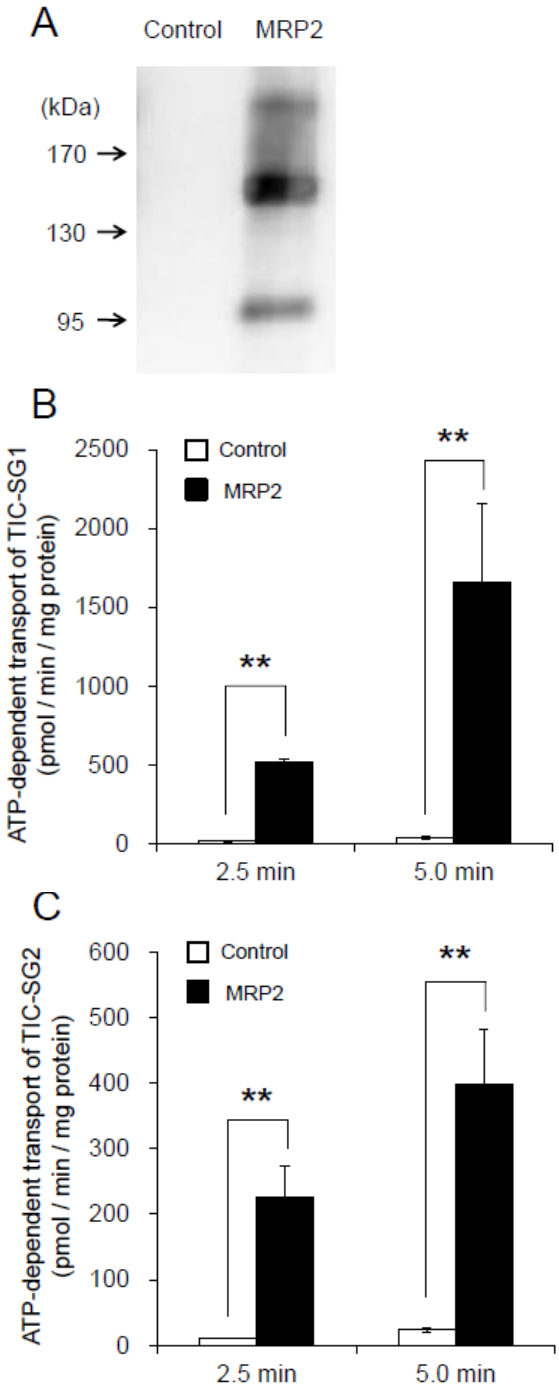

A
Control
MRP2
(kDa)
170
130
95
B
ATP-dependent transport of TIC-SG1
(pmol / min / mg protein)
Control
MRP2
2500
2000
1500
1000
500
0
**
**
2.5 min
5.0 min
C
ATP-dependent transport of TIC-SG2
(pmol / min / mg protein)
Control
MRP2
600
500
400
300
200
100
0
**
**
2.5 min
5.0 min

Fig. 6

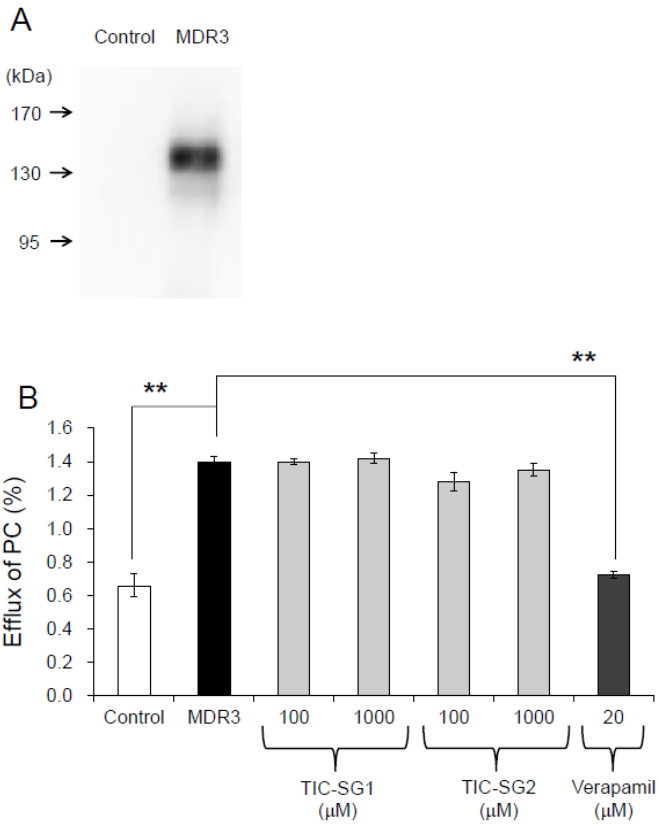

A
Control
MDR3
(kDa)
170
130
95
B
**
**
Efflux of PC (%)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
MDR3
100
1000
100
1000
20
TIC-SG1 (μM)
TIC-SG2 (μM)
Verapamil (μM)

Fig. 7

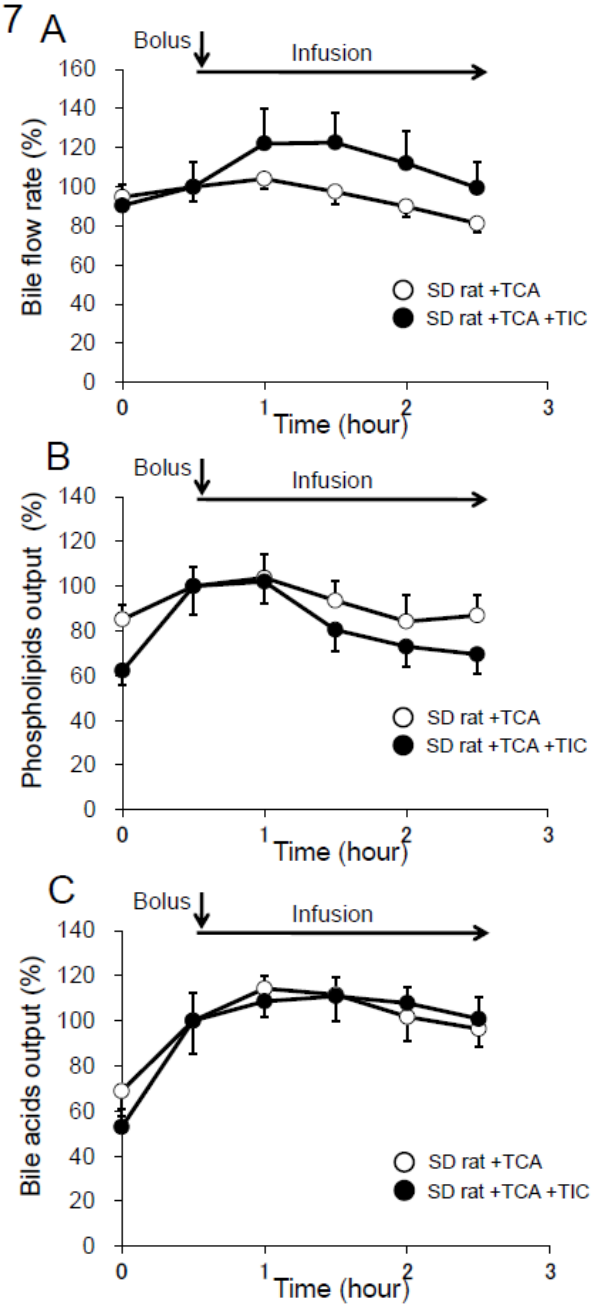
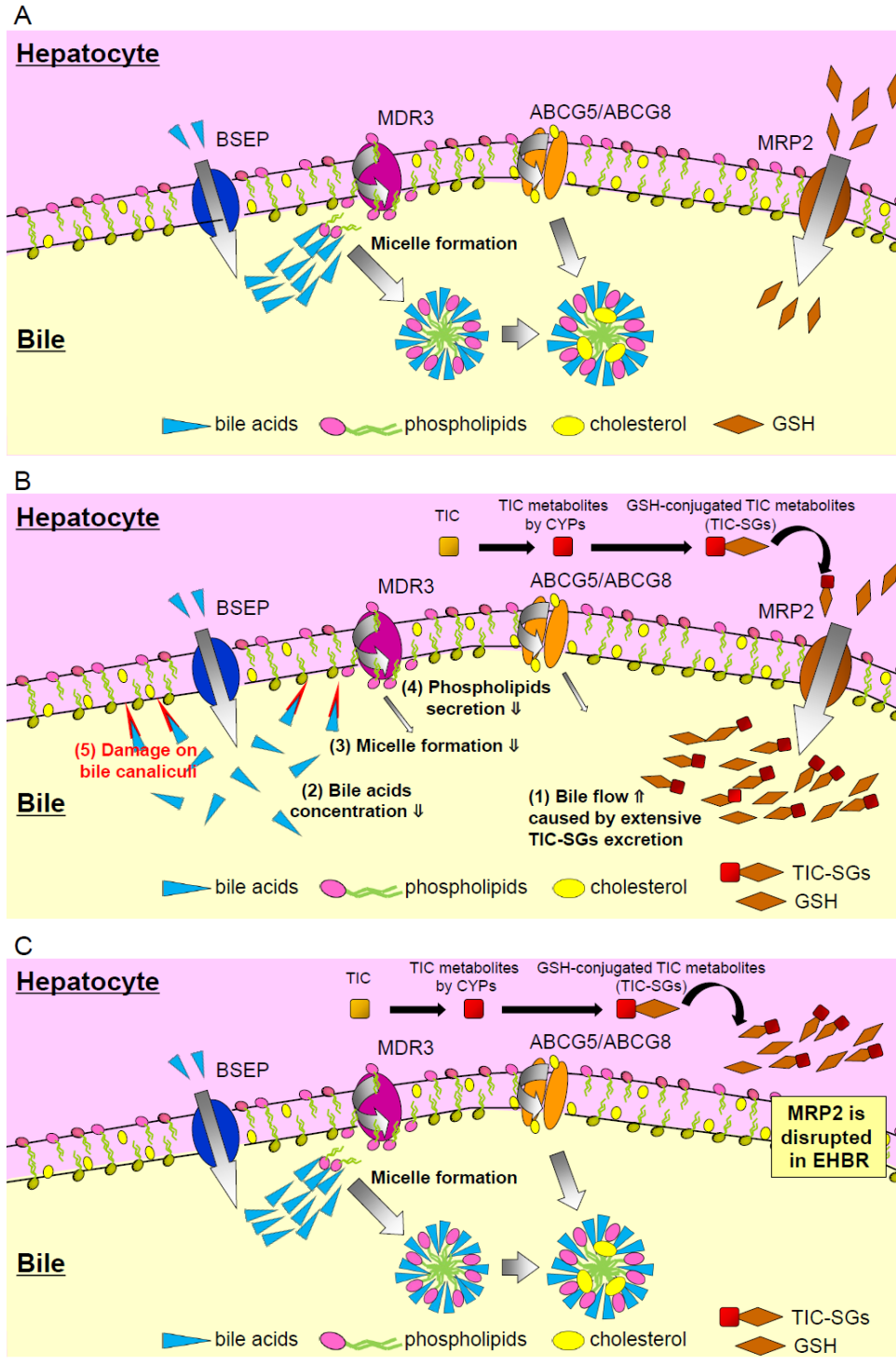
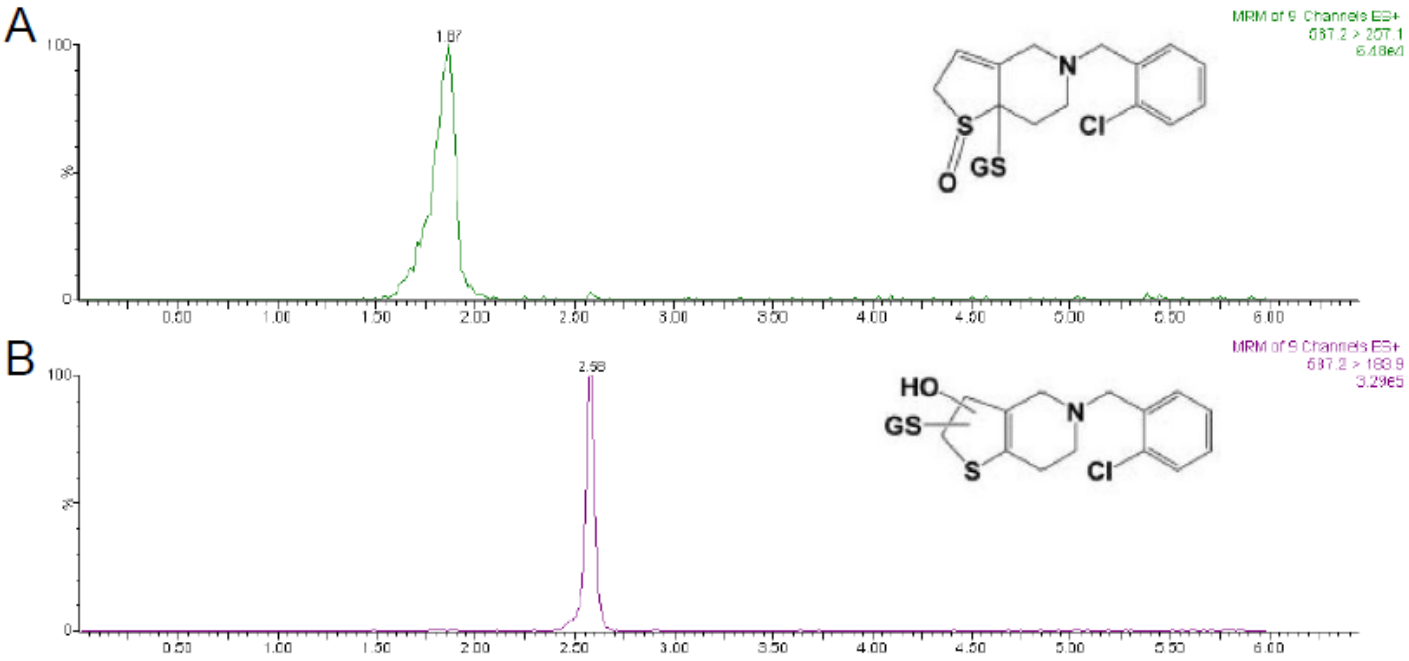

A
Bolus
Infusion
Bile flow rate (%)
160
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
SD rat +TCA
SD rat +TCA +TIC
B
Bolus
Infusion
Phospholipids output (%)
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
SD rat +TCA
SD rat +TCA +TIC
C
Bolus
Infusion
Bile acids output (%)
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)
SD rat +TCA
SD rat +TCA +TIC

Fig. 8


A
Hepatocyte
BSEP
MDR3
ABCG5/ABCG8
MRP2
Micelle formation
Bile
bile acids
phospholipids
cholesterol
GSH
B
Hepatocyte
TIC
TIC metabolites by CYPs
GSH-conjugated TIC metabolites (TIC-SGs)
BSEP
MDR3
ABCG5/ABCG8
MRP2
(4) Phospholipids secretion ⇓
(5) Damage on bile canaliculi
(3) Micelle formation ⇓
(2) Bile acids concentration ⇓
(1) Bile flow ⇑ caused by extensive TIC-SGs excretion
Bile
bile acids
phospholipids
cholesterol
TIC-SGs
GSH
C
Hepatocyte
TIC
TIC metabolites by CYPs
GSH-conjugated TIC metabolites (TIC-SGs)
BSEP
MDR3
ABCG5/ABCG8
MRP2 is disrupted in EHBR
Micelle formation
Bile
bile acids
phospholipids
cholesterol
TIC-SGs
GSH

Title: 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
Authors: Takashi Yoshikado, Tappei Takada, Hideaki Yamamoto, Jeng Kae Tan, Kousei Ito, Tomofumi Santa and Hiroshi Suzuki
Journal: Molecular Pharmacology



Supplemental Figure S1 ----- LC-MS/MS charts of GSH-conjugated ticlopidine metabolites (TIC-SG1 and TIC-SG2). (A) LC-MS/MS chart of TIC-SG1 (587.20 > 257.10). (B) LC-MS/MS chart of TIC-SG2 (587.20 > 183.90).

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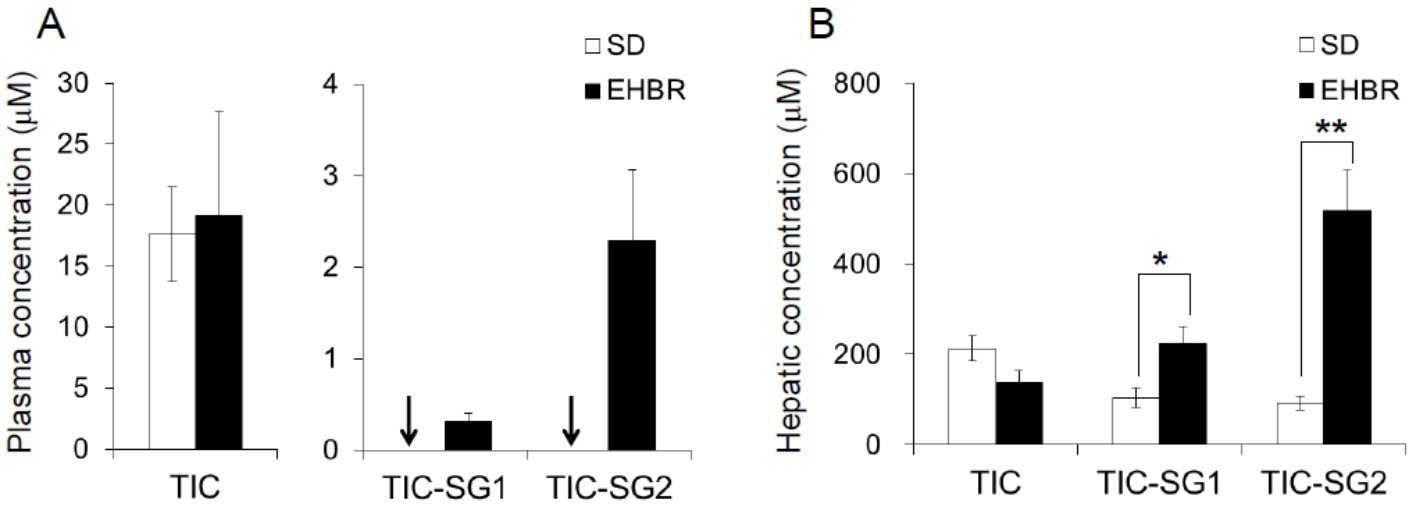
Supplemental Figure S2



Supplemental Figure S2 ----- Plasma and hepatic concentrations of ticlopidine (TIC) and GSH-conjugated TIC metabolites (TIC-SG1, TIC-SG2). Ticlopidine (TIC) was administered to SD rats and Eisai hyperbilirubinemic rats (EHBR). (A) Plasma concentrations of TIC, TIC-SG1 and TIC-SG2 2 h after starting TIC-administration. The arrowed lines mean that the plasma concentrations of TIC-SG1 and TIC-SG2 in SD rats were below the limits of quantification (< 0.1 μM). (B) Hepatic concentrations of TIC, TIC-SG1 and TIC-SG2. Data are shown as the mean ± S.E. of four independent experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. SD rats by Student's t-test.

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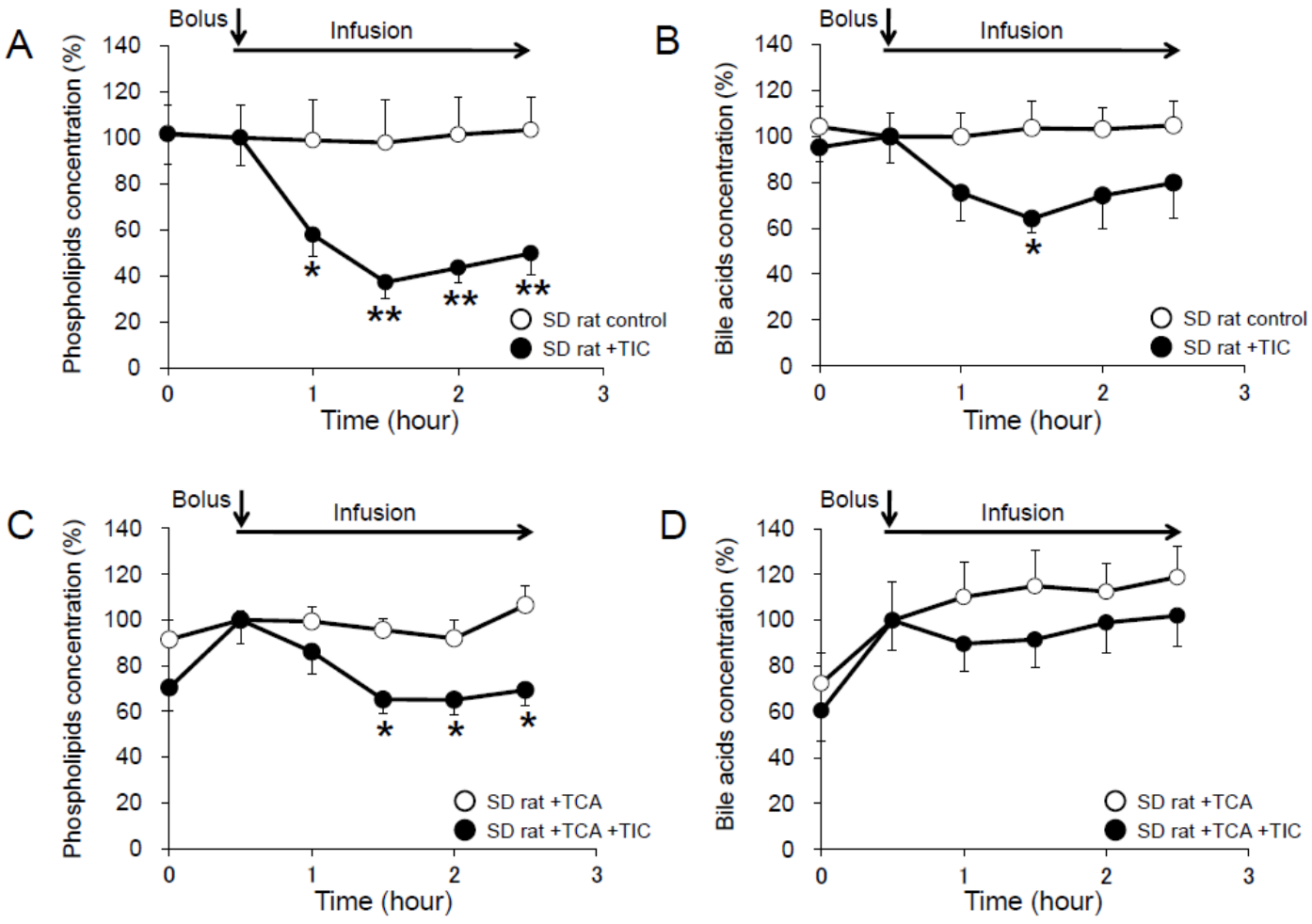
Supplemental Figure S3



Supplemental Figure S3 ----- Effect of TIC on the biliary concentration of phospholipids and bile acids in SD rats and TCA-infused SD rats. Ticlopidine (TIC) was administered to SD rats. The value at 0.5 h in each panel is defined as 100%. (A) Biliary concentration of phospholipids in SD rats. (B) Biliary concentration of bile acids in SD rats. (C) Biliary concentration of phospholipids in taurocholate (TCA)-infused SD rats. (D) Biliary concentration of bile acids in TCA-infused SD rats. Data are shown as the mean ± S.E. of four to five independent experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. SD rats without TIC by Student's t-test.

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Supplemental Table S1 ----- Reports of drug-induced liver injury cases

The types of liver injury were classified by the diagnostic criteria of the DDW-J scale, which was proposed in Japan by modifying the International Consensus Meeting (ICM) scale based on clinical chemistry (ALP and ALT) in the items concerning chronologic criteria, concomitant drugs and extrahepatic manifestations (Takikawa et al., 2005).

Drug	Number of cases				Cholestatic + Mixed (%)	Hepatocellular (%)
	All	Cholestatic	Mixed	Hepatocellular		
ticlopidine	52	19	19	14	73	27
tiopronin	44	19	13	12	73	27
diclofenac	41	9	16	16	61	39
thiamazole	21	8	6	7	67	33
oxatomide	20	7	9	4	80	20
aprindine	14	5	5	4	71	29
ajmaline	10	4	5	1	90	10
tranilast	19	4	10	5	74	26
famotidine	13	4	2	7	46	54
rifampicin	26	4	6	16	38	62
sulfamethoxazole	6	3	1	2	67	33
norfloxacin	8	3	2	3	63	38
ofloxacin	16	3	5	8	50	50
levofloxavin	16	3	4	7	44	44
chlorpromazine	10	2	6	2	80	20
indomethacin	8	2	3	3	63	38
nifedipine	8	2	3	3	63	38
carbamazepine	18	2	9	6	61	33
methyldopa	5	2	1	2	60	40
glibenclamide	5	2	1	2	60	40
epalrestat	5	2	1	2	60	40
chlormadinone acetate	5	2	1	2	60	40
troglitazone	15	2	5	8	47	53
griseofulvin	5	1	2	2	60	40
valproic acid	9	1	4	4	56	44
isoniazid	17	1	7	9	47	53
acarbose	11	1	4	6	45	55
allopurinol	7	1	2	2	43	29
cimetidine	6	1	1	4	33	67
phenytoin	19	1	4	14	26	74