Hepatocellular Exposure of Troglitazone Metabolites in Rat Sandwich-Cultured Hepatocytes Lacking Bcrp and Mrp2: Interplay between Formation and Excretion

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ABSTRACT

Inhibition of bile acid transport by troglitazone (TGZ) and its major metabolite, TGZ sulfate (TS), may lead to hepatocellular accumulation of toxic bile acids; TS accumulation and hepatotoxicity may be associated with impaired TS biliary excretion. This study evaluated the impact of impaired transport of breast cancer resistance protein (Bcrp) and multidrug resistance–associated protein 2 (Mrp2) on the hepatobiliary disposition of generated metabolites, TS and TGZ glucuronide (TG). Sandwich-cultured hepatocytes (SCH) from Mrp2-deficient (TR[−]) rats in combination with Bcrp knockdown using RNA interference were employed. The biliary excretion index (BEI) of generated TS was not significantly altered by impaired Bcrp (20.9 to 21.1%) and/or Mrp2 function (24.4% and 17.5% in WT and TR[−] rat SCH, respectively). Thus, loss-of-function of Mrp2 and/or Bcrp do not appear to be risk factors

for increased hepatocellular TS accumulation in rats, potentially because of a compensatory transporter(s) that excretes TS into bile. Further investigations revealed that the compensatory TS biliary transporter was not the bile salt export pump (Bsep) or P-glycoprotein (P-gp). Interestingly, TGZ sulfation was significantly decreased in TR[−] compared with WT rat SCH (total recovery: 2.8 versus 5.0% of TGZ dose), resulting in decreased hepatocellular TS accumulation, even though sulfotransferase activity in TR[−] rat hepatocyte S9 fraction was similar. Hepatocellular TG accumulation was significantly increased in TR[−] compared with WT rat SCH due to increased glucuronidation and negligible TG biliary excretion. These data emphasize that the interplay between metabolite formation and excretion determines hepatocellular exposure to generated metabolites such as TS and TG.

Introduction

Drug-induced liver injury (DILI) is one of the primary reasons for withdrawal of approved drugs from the market (Temple and Himmel, 2002); however, the ability to accurately predict a drug's propensity for DILI is limited due to a lack of understanding of the underlying mechanism(s). One important proposed mechanism of DILI is inhibition of bile salt export pump (BSEP)–mediated excretion of bile acids, which may increase hepatic exposure to bile acids, ultimately leading to necrotic and/or apoptotic cell death (Perez and Briz, 2009; Maillette de Buy Wenniger and Beuers, 2010; Morgan et al., 2010; Dawson et al., 2012). Many drugs that cause either cholestatic or mixed hepatocellular/cholestatic liver injury [e.g., troglitazone (TGZ), bosentan, cyclosporine,

rifampin, sulindac, and glibenclamide] inhibit BSEP/Bsep-mediated biliary excretion of bile acids (Bohme et al., 1994; Fattinger et al., 2001; Funk et al., 2001a; Mano et al., 2007; Kis et al., 2009; Lee et al., 2010b). However, only a small fraction of patients treated with BSEP inhibitors develop DILI, suggesting that injury is driven by a combination of drug- and patient-specific risk factors. For drugs with a rare incidence of DILI, hepatic exposure to the causative drugs/metabolites may be high in the subset of patients who develop DILI. It is also possible that toxic reactions triggered by drug exposure may develop into severe liver injury in a subset of susceptible patients. Potential patient-specific risk factors that may influence drug disposition and/or toxicity include age, gender, activation of the innate immune system, comedications, underlying disease, and/or genetic predisposition.

TGZ was the first marketed thiazolidinedione approved for the treatment of type II noninsulin-dependent diabetes. However, 2% of TGZ-treated patients developed serum alanine aminotransferase (ALT) elevations more than 3-fold greater than the upper limit of normal (Watkins and Whitcomb, 1998; St Peter et al., 2001). Subsequent to reports of lethal DILI in humans, TGZ was withdrawn from worldwide markets. Several mechanisms have been proposed to explain TGZ-mediated DILI, including formation of reactive metabolites, mitochondrial toxicity, apoptosis, and inhibition of bile acid transport (Smith,

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ABBREVIATIONS: ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; LC-MS/MS, liquid chromatography–tandem mass spectrometry; BCRP/Bcrp, breast cancer resistance protein; BEI, biliary excretion index; BSEP/Bsep, bile salt export pump; DILI, drug-induced liver injury; f_u , unbound fraction; GF120918, elacridar; MRP/Mrp, multidrug resistance–associated protein; P-gp, P-glycoprotein; SCH, sandwich-cultured hepatocytes; SULT/Sult, sulfotransferase; TG, troglitazone glucuronide; TGZ, troglitazone; TQ, troglitazone quinone; TR[−] rats, Mrp2-deficient rats; TS, troglitazone sulfate; TSB, Tris-sucrose buffer; UGT, UDP-glucuronosyltransferase.

2003; Chojkier, 2005). Clinical observations and in vivo studies in rats have implicated a cholestatic mechanism in TGZ-induced hepatotoxicity (Gitlin et al., 1998; Fukano et al., 2000; Funk et al., 2001a, b). In vitro vesicular transport assays demonstrated that TGZ and its major metabolite, TGZ sulfate (TS), are potent inhibitors of bile acid efflux transporters, supporting the involvement of cholestasis in TGZ-induced hepatotoxicity; TGZ inhibits human BSEP- and rat Bsep-mediated bile acid transport with IC₅₀ values of 2.7–5.9 and 3.9–10.6 μ M, respectively (Funk et al., 2001a; Morgan et al., 2010; Dawson et al., 2012). TS is a 10-fold more potent inhibitor of ATP-dependent transport of taurocholate in rat liver canalicular membrane vesicles compared with TGZ (Funk et al., 2001a). Data from our laboratory demonstrated that TS also inhibits the hepatic basolateral efflux transport protein multidrug resistance-associated protein 4 (MRP4) (Yang et al., 2011).

In humans and male rats, TGZ is metabolized extensively in the liver, primarily by sulfation, with glucuronidation and oxidation as successively less prominent metabolic pathways; TS exhibited 10-fold higher plasma concentrations than TGZ, and was excreted primarily into bile, suggesting that TGZ metabolism and excretion were similar in humans and male rats (Kawai et al., 1997; Loi et al., 1997; Loi et al., 1999). Previously published data suggested that TS is excreted into bile predominantly via breast cancer resistance protein (Bcrp) and multidrug resistance-associated protein 2 (Mrp2) (Kostrubsky et al., 2001; Enokizono et al., 2007). Extensive hepatic accumulation of TS (Kawai et al., 1997; Lee et al., 2010a), coupled with the finding that TS potently inhibits bile acid transporters (e.g., BSEP, MRP4), led to the hypothesis that TS is primarily responsible for altered bile acid disposition and subsequent hepatotoxicity; impaired function of Bcrp and Mrp2 (e.g., attributable to underlying disease, genetic variations, or drug-drug interactions) would increase hepatocellular TS accumulation and enhance inhibition of bile acid transport (Fig. 1).

In this study, the hepatobiliary disposition of TGZ and generated metabolites, TS and TG, was examined in rat sandwich-cultured hepatocytes (SCH) in the setting of impaired Bcrp and Mrp2 function. A novel experimental system was employed to quantitatively assess the effects of impaired function of Bcrp (using RNA interference technique) in combination with Mrp2-deficient TR⁻ rats (Yang et al., 2014). Differential metabolism of TGZ also was investigated using S9 fractions prepared from liver tissues of WT and TR⁻ rats. This work highlights the importance of considering both metabolic and transport pathways when predicting hepatocellular exposure to generated metabolites.

Materials and Methods

TGZ was purchased from Cayman Chemical Company (Ann Arbor, MI). TS, TGZ glucuronide (TG), and TGZ quinone (TQ) were kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). TS also was synthesized from TGZ in-house (Saha et al., 2010). [³H]Taurocholate (5 Ci/mmol; purity >97%) was purchased from PerkinElmer (Waltham, MA). [³H]Rosuvastatin (10 Ci/mmol; purity >99%) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Non-radiolabeled rosuvastatin was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Taurocholate, Triton X-100, Hanks' balanced salt solution (HBSS) premix, HBSS modified (with no calcium chloride, magnesium sulfate, phenol red, and sodium bicarbonate) premix, dexamethasone, penicillin-streptomycin solution, and collagenase (type IV) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was obtained from Thermo Fisher Scientific (Waltham, MA). Gibco brand fetal bovine serum, recombinant human insulin, and Dulbecco's modified Eagle's medium (DMEM), and membrane vesicles prepared from rat Bsep-overexpressing Sf9 cells (Sf9-Bsep) and control Sf9 cells (Sf9-control) were purchased from Life Technologies (Carlsbad, CA). Insulin/transferrin/selenium (ITS) culture supplement, BioCoat culture plates, and Matrigel extracellular matrix were purchased from BD Biosciences Discovery Labware (Bedford, MA). GF120918 (elacridar) was a generous gift from GlaxoSmith-Kline (Research Triangle Park, NC). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Hepatocyte Isolation and Culture in a Sandwich Configuration. Hepatocytes were isolated from male Wistar rats (270–300 g; Charles River Laboratories, Inc., Wilmington, MA) and TR⁻ rats (220–300 g, bred in-house; breeding stock obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) using a two-step collagenase perfusion method previously described (LeCluyse et al., 1996). Animals had free access to water and food before surgery and were allowed to acclimate for at least 5 days. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina, Chapel Hill, NC). Hepatocytes were seeded onto 24-well BioCoat culture plates at a density of 0.35×10^6 cells/well in seeding medium (DMEM containing 5% fetal bovine serum, 10 μ M insulin, 1 μ M dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 IU penicillin G sodium, and 100 μ g of streptomycin) as described previously (Swift et al., 2010). Hepatocytes were incubated for 1 hour at 37°C in a humidified incubator (95% O₂, 5% CO₂) and allowed to attach to the collagen substratum, after which time the medium was aspirated to remove unattached cells, and replaced with fresh medium. On the next day, cells were overlaid with BD Matrigel at a concentration of 0.25 mg/ml in ice-cold feeding medium (DMEM supplemented with 0.1 μ M dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 IU penicillin G sodium, 100 μ g of streptomycin, and 1% insulin/transferrin/selenium culture supplement). The culture medium was changed daily until experiments were performed on day 4.

Knockdown of Bcrp Protein in WT and TR⁻ Rat SCH. Adenoviral vectors expressing short hairpin RNA targeting Bcrp (Ad-siBcrp) or a nontarget control (Ad-siNT) were prepared as described previously (Yue et al., 2009). One hour after seeding on day 0, hepatocytes were infected with Ad-siBcrp or Ad-siNT at multiplicity of infection of 5 by replacing the seeding medium with fresh seeding medium containing virus. On the next day, medium including viruses were removed, and cells were overlaid with Matrigel as described earlier.

Accumulation of TGZ and Generated Metabolites in WT and TR⁻ Rat SCH in the Absence or Presence of Bcrp Knockdown. Following 30-minute exposure to 0.5 ml of feeding medium containing 10 μ M TGZ, 0.2-ml aliquots of medium were collected from day 4 WT and TR⁻ rat SCH in the absence or presence of Bcrp knockdown. The remaining culture medium was completely aspirated, and SCH were rinsed with 0.5 ml/well of warm standard (Ca²⁺-containing) or Ca²⁺-free HBSS buffers. After rinses, HBSS buffers were aspirated, and SCH were incubated with 0.5 ml of HBSS buffers (standard or Ca²⁺-free) at 37°C for 5 minutes. After incubation, the HBSS buffers were aspirated from all wells. Plates were sealed and stored at –80°C until analysis.

Membrane Vesicle Assay. Membrane vesicles (Sf9-control and Sf9-Bsep, 10 and 25 μ g/reaction for taurocholate and TS uptake, respectively) were incubated at 37°C with the substrate (5 μ M taurocholate or 100 μ M TS) in Tris-sucrose buffer (TSB) containing MgCl₂ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μ g/ml), adenosine triphosphate (ATP) or adenosine

Fig. 1. Proposed mechanism of troglitazone (TGZ)-mediated hepatotoxicity. (1) Impaired BCRP and/or MRP2 function (2) causes accumulation of TGZ sulfate (TS) (the major TGZ metabolite and a potent inhibitor of bile acid transport proteins) in hepatocytes, (3) which inhibits bile acid excretion leading to bile acid accumulation and (4) hepatotoxicity.

monophosphate (AMP) (4 mM) in a final volume of 50 μ l. The assays were performed in three separate experiments in triplicate. After incubation for 5 minutes, the reaction was stopped by addition of 800 μ l of ice-cold TSB and immediately filtered using Type A/E glass fiber filter (Pall Corporation, Port Washington, NY) presoaked in TSB overnight. Under aspiration, the filters were washed twice with ice-cold TSB using a vacuum filtration system. Glass filters were transferred to glass vials, and 1 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) was added before counting radioactivity of taurocholate using the Tri-Carb 3100TR liquid scintillation analyzer (PerkinElmer, Waltham, MA). For liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of TS, glass filters were dissolved with 1 ml of methanol including 1 nM ethyl warfarin (internal standard). Following vortex mixing and sonication, the supernatant was transferred to microcentrifuge tubes and centrifuged for 20 minutes at 4°C. After centrifugation, 500 μ l of supernatant was transferred to a 96-well LC-MS/MS plate, evaporated using SPE Dry 96 (Biotage, Uppsala, Sweden), and reconstituted with 100 μ l 1:1 mixture of methanol and DMSO. The ATP-dependent uptake of substrate was calculated by subtracting substrate uptake in the presence of AMP from substrate uptake in the presence of ATP.

Effects of GF120918 on the Biliary Excretion of TS in WT and TR⁻ Rat SCH. WT and TR⁻ rat SCH were preincubated with 0.5 μ M GF120918 or vehicle control for 10 minutes followed by 30-minute exposure to 0.5 ml of feeding medium containing 10 μ M TGZ and 0.5 μ M GF120918 (or vehicle control). After incubation, the culture medium was completely aspirated, and SCH were rinsed with 0.5 ml/well of warm standard or Ca²⁺-free HBSS buffers. After the second rinse, HBSS buffers were aspirated, and SCH were incubated with 0.5 ml of HBSS buffers (standard or Ca²⁺-free) at 37°C for 5 minutes. After incubation, the HBSS buffers were aspirated from all wells. Plates were sealed and stored at -80°C until analysis.

In Vitro Metabolism of TGZ in S9 Fraction. S9 fractions were prepared from liver tissues of WT and TR⁻ rats ($n = 3$, respectively) according to standard procedures and stored at -80°C until used (Hill, 2004). Protein concentration was determined by the BCA protein assay (Pierce Biotechnology/Thermo Scientific, Rockford, IL) using human serum albumin (standard solution; Sigma-Aldrich) as a standard. Reaction mixture [95 μ l of Tris-HCl buffer (pH 7.5, 0.1 M) containing 3'-phosphoadenosine-5'-phosphosulfate (PAPS; final concentration 0.1 mM), uridine 5'-diphosphoglucuronic acid (UDPGA; final concentration 2 mM), nicotinamide adenine dinucleotide phosphate (reduced, NADPH; final concentration 1 mM), alamethicin (final concentration 25 μ l/ml), and TGZ (final concentrations of 1 or 10 μ M)] was preincubated for 5 minutes at 37°C. TGZ was dissolved in DMSO; the final concentration of DMSO in the reaction mixture was 0.2%. TGZ concentrations of 1 and 10 μ M were selected for investigation because they yielded unbound concentrations of TGZ in the S9 fraction that were similar to the unbound concentrations in hepatocytes. Intracellular total concentrations of TGZ ranged from 100 to 250 μ M after a 30-minute incubation of WT and TR⁻ rat SCH with 10 μ M TGZ (Lee et al., 2010a). Assuming that the intracellular unbound fraction (f_u) is equal to the plasma f_u of 0.000921 (Izumi et al., 1996), intracellular concentrations of unbound TGZ would be in the range of 0.09–0.23 μ M. TGZ also is highly bound to microsomal and cytosolic protein (TGZ f_u was 0.01 and 0.03 in 2 mg/ml of rat liver microsomes and 0.5 mg/ml of rat liver cytosol, respectively) (Izumi et al., 1997); therefore, 1 and 10 μ M total TGZ in S9 fraction would result in TGZ unbound concentrations of 0.018–0.18 μ M, which are similar to expected intracellular unbound TGZ concentrations in rat SCH. The reaction was started by adding 5 μ l of the S9 fraction (final concentration 1 mg/ml). The reaction was stopped after 3 minutes by mixing with 200 μ l of ice-cold acetonitrile containing 15 nM ethyl warfarin (analytical internal standard). The 3-minute time point was chosen on the basis of the reported linear range to calculate the initial velocity of TS and TG formation (Izumi et al., 1997). The reaction mixture was centrifuged for 20 minutes at 4°C, and the supernatant was transferred to 96-well LC-MS/MS plates for analysis. To determine desulfation of TS, 5 μ l of TS (final concentration of 100 μ M) was incubated with 95 μ l of 0.1M Tris-HCl buffer (pH 7.5) containing S9 fraction (final concentration 1 mg/ml) for 30 minutes, and the same procedure was followed as detailed above.

LC-MS/MS Analysis. TGZ and generated metabolites were analyzed by LC-MS/MS as described previously (Lee et al., 2010a). Briefly, the medium and cells or cells+bile lysate samples were centrifuged at 12,000g for 10 minutes

at 4°C, and the supernatant was diluted 1:6 (v/v) with 79%:21% (v/v) methanol/water containing the internal standard (ethyl warfarin). An Applied Biosystems API 4000 triple quadrupole mass spectrometer with a TurbolonSpray Ion Source (Applied Biosystems, Foster City, CA) was used for analysis in negative ionization mode. The ranges of the standard curves were extended by adding additional points to the previously reported range (Lee et al., 2010a); eleven-point (5–10000 nM; TGZ) or ten-point (5–5000 nM; TS, TG, and TQ) calibration curves were constructed as composites of TGZ (440.0→397.1), TS (520.2→440.1), TG (616.2→440.1), and TQ (456.1→413.1) by using peak area ratios of analyte and ethyl warfarin (320.8→160.9). All points on the curves back-calculated to within 15% of the nominal value. Care was taken to minimize the light-sensitive degradation of TGZ during all experimental procedures.

Data Analysis and Statistics. TGZ accumulation was corrected for nonspecific binding to the BioCoat plate without cells, and normalized to protein concentration measured by the BCA protein assay (Pierce Biotechnology). To account for the incompatibility of the protein assay with methanol, the average protein concentration for standard HBSS or Ca²⁺-free HBSS incubations in a representative plate from the same liver preparation was used to normalize accumulation. The biliary excretion index (BEI; %), defined as the percentage of accumulated substrate residing within the bile canaliculi, was calculated using B-CLEAR[®] technology (Qualyst Transporter Solutions, Durham, NC) according to the following equation (Liu et al., 1999):

$$\text{BEI}(\%) = \frac{\text{Accumulation}_{\text{Standard HBSS}} - \text{Accumulation}_{\text{Ca}^{2+} - \text{free HBSS}}}{\text{Accumulation}_{\text{Standard HBSS}}} \times 100 \quad (1)$$

Effects of viral infection (noninfected versus Ad-siNT versus Ad-siBcrp) and rat type (WT versus TR⁻) on hepatobiliary disposition (accumulation in cells+bile, cellular accumulation, and BEI) and recovery of TGZ and its metabolites were evaluated using a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Two-way ANOVA also was used to evaluate the effects of GF120918 and rat type (WT versus TR⁻) on hepatobiliary disposition of TS and TG. Student's *t* test was used to compare ATP-dependent substrate transport in Sf9-Bsep versus Sf9-control, and the initial velocity of TS/TG formation in S9 fractions prepared from livers of WT and TR⁻ rats. In all cases, $P < 0.05$ was considered statistically significant. All analyses were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA).

Results

Hepatobiliary Disposition of TGZ and Generated Metabolites in WT and TR⁻ Rat SCH in the Absence or Presence of Bcrp.

Loss of function of Mrp2 and Bcrp was validated in this experiment using rosuvastatin, a dual substrate of Mrp2 and Bcrp; rosuvastatin BEI was significantly decreased by impaired function of Mrp2 or Bcrp, and biliary excretion was almost ablated in Bcrp knockdown TR⁻ rat SCH (Yang et al., 2014). Accumulation of TGZ and generated metabolites in cells+bile, cells, and medium after a 30-minute incubation of WT and TR⁻ rat SCH with 10 μ M TGZ is shown in Figs. 2 and 3. BEI values of TS and TG in noninfected WT hepatocytes were 24 and 49%, respectively (Fig. 2, B and C); biliary excretion of TGZ (Fig. 2A) and TQ (data not shown) was negligible, consistent with previously published data (Lee et al., 2010a). The hepatobiliary disposition of TGZ and generated metabolites was not affected by infection of the nontarget control adenoviral vector (Ad-siNT), confirming that off-target effects of viral infection were negligible in these studies. Unexpectedly, the BEI of TS was not altered by impaired Mrp2 (TR⁻) and/or Bcrp function (Ad-siBcrp), suggesting that other biliary transporters may compensate for the impaired function of Mrp2 and Bcrp (Fig. 2B). Interestingly, TS accumulation in cells+bile, cells, and medium was decreased in TR⁻ rat SCH compared with WT rat SCH (Figs. 2B and 3B). Bcrp knockdown did not significantly alter TS accumulation in cells+bile, cells, or

Fig. 2. Intracellular accumulation of troglitazone (TGZ) and generated metabolites [TGZ sulfate (TS) and TGZ glucuronide (TG)] in WT and TR⁻ rat SCH in the absence or presence of Bcrp knockdown. WT and TR⁻ rat SCH were treated with 10 μ M TGZ for 30 minutes in the absence [CTL (noninfected cells) and Ad-siNT (nontarget control)] or presence (Ad-siBcrp) of Bcrp knockdown. Hepatobiliary disposition of (A) TGZ, (B) TS, and (C) TG were measured in cells+bile (solid bars) and in cells (open bars). Effects of Bcrp knockdown (CTL versus Ad-siNT versus Ad-siBcrp) and rat type (WT versus TR⁻) on accumulation of TGZ and generated metabolites and biliary excretion index (BEI; pink diamonds) of generated metabolites were evaluated using two-way ANOVA. Data represent mean $\pm$ S.E.M. (n=3 in triplicate); [†]*P* < 0.05, significantly different from WT (cells+bile); [‡]*P* < 0.05, significantly different from WT (cell); **P* < 0.05, significantly different from WT (BEI).

medium (Figs. 2B and 3B). The BEI of TG was significantly decreased in TR⁻ rat SCH compared with WT rat SCH but was not influenced by knockdown of Bcrp (Fig. 2C). TG cellular accumulation was significantly increased in TR⁻ rat SCH compared

with WT rat SCH, whereas TG accumulation in cells+bile was not altered (Fig. 2C). In TR⁻ rat SCH, TG medium concentrations were significantly higher compared with WT rat SCH (Fig. 3C). TG accumulation in cells+bile, cells, and medium was not significantly

Fig. 3. Accumulation of troglitazone (TGZ) and generated metabolites [TGZ sulfate (TS) and TGZ glucuronide (TG)] in the medium of WT and TR⁻ rat SCH in the absence or presence of Bcrp knockdown. WT and TR⁻ rat SCH were treated with 10 μ M TGZ for 30 minutes in the absence [CTL (noninfected cells) and Ad-siNT (nontarget control)] or presence (Ad-siBcrp) of Bcrp knockdown. Concentrations of (A) TGZ, (B) TS, and (C) TG were measured in medium. Effects of Bcrp knockdown (CTL versus Ad-siNT versus Ad-siBcrp) and Mrp2 deficiency (WT versus TR⁻) on media concentrations of TGZ and generated metabolites were evaluated using two-way ANOVA. Data represent mean $\pm$ S.E.M. (n=3 in triplicate); **P* < 0.05, significantly different from WT.

altered by Bcrp knockdown (Figs. 2C and 3C). TQ accumulation in cells and cells+bile was significantly increased in TR⁻ rat SCH in the absence and presence of Bcrp knockdown compared with WT rat SCH, but medium concentrations were not significantly different (data not shown).

Differential Metabolism of TGZ in WT and TR⁻ Rat SCH. Cumulative recovery of TGZ and generated metabolites from medium, hepatocytes, and bile was comparable among all groups (92.3–99.6%) (Fig. 4A). After a 30-minute incubation, more than 80% of the TGZ dose remained as parent compound in both WT and TR⁻ SCH. Among three generated metabolites, TS was predominant (4.8% of the dose recovered in cells, bile, and medium), followed by TQ (1.5% of the dose) and TG (0.9% of the dose) in noninfected WT rat SCH (Fig. 4A). In TR⁻ rat SCH, total recovery (medium, hepatocytes, and bile) of TS was significantly lower compared with WT rat SCH (Fig. 4A). Total recovery of TG was significantly greater in TR⁻ compared with WT rat SCH (Fig. 4A). TS was recovered primarily from hepatocytes, whereas TG was recovered primarily from the medium (Figs. 4, B and C).

Transport of TS in Membrane Vesicles from Bsep-Overexpressing Sf9 Cells. To investigate the transport protein(s) involved in the compensatory biliary excretion of TS in the absence of Mrp2 and Bcrp, ATP-dependent uptake of 10 μ M taurocholate (positive control) and 100 μ M TS was determined in Sf9-control and Sf9-Bsep vesicles. ATP-dependent taurocholate transport was 60-fold greater in Sf9-Bsep vesicles compared with Sf9-control vesicles (Fig. 5). However, ATP-dependent TS transport was comparable between Sf9-control and Sf9-Bsep vesicles, indicating that TS is not transported by Bsep.

Effects of GF120918 on the Hepatobiliary Disposition of TGZ and Generated Metabolites in WT and TR⁻ Rat SCH. The BEI of

Fig. 5. Bsep-mediated transport of taurocholate (TC) and troglitazone sulfate (TS) in membrane vesicles. ATP-dependent transport of TC and TS in membrane vesicles prepared from Sf9 cells overexpressing Bsep (Sf9-Bsep; open bars) or mock controls (Sf9-control; solid bars). The data represent mean $\pm$ S.E.M. ($n = 3$ in triplicate). * $P < 0.05$, significantly different from mock controls.

TS was not significantly altered by GF120918, a potent inhibitor of Bcrp and P-glycoprotein (P-gp), nor by rat type (WT versus TR⁻) (Fig. 6A). The accumulation of TS in cells+bile and cells was significantly decreased in TR⁻ rat SCH compared with WT rat SCH as shown in Fig. 2B, but was not altered by the presence of GF120918. The BEI of TG was significantly decreased in TR⁻ rat SCH compared with WT rat SCH, consistent with Fig. 2C, but was not altered by the presence of GF120918 (Fig. 6B). TG accumulation in cells+bile and cells was not influenced significantly by GF120918 or by rat type, but there was a trend toward an increase in cellular TG accumulation in TR⁻ compared with WT rat SCH (Fig. 6B).

Fig. 4. Recovery of troglitazone (TGZ) and generated metabolites [TGZ sulfate (TS), TGZ glucuronide (TG), and TGZ quinone (TQ)] in WT and TR⁻ rat SCH in the absence or presence of Bcrp knockdown. WT and TR⁻ rat SCH were treated with 10 μ M TGZ for 30 minutes in the absence (CTL and Ad-siNT) or presence (Ad-siBcrp) of Bcrp knockdown. (A) Total recovery of TGZ and generated metabolites in cells, bile, and medium are presented as % of the TGZ dose. Effects of Bcrp knockdown (CTL versus Ad-siNT versus Ad-siBcrp) and Mrp2 deficiency (WT versus TR⁻) on total recovery of TGZ and generated metabolites were evaluated using two-way ANOVA. * $P < 0.05$, significantly different from WT. Recovery of TS (B) and TG (C) in cells, bile, and medium are presented as % of the TGZ dose.

Fig. 6. Hepatobiliary disposition of troglitazone (TGZ) and generated metabolites [TGZ sulfate (TS) and TGZ glucuronide (TG)] in WT and TR⁻ rat SCH in the absence or presence of GF120918. WT and TR⁻ rat SCH were incubated with 10 μ M TGZ for 30 minutes in the absence or presence of GF120918. Hepatobiliary disposition of (A) TS and (B) TG were measured in cells+bile (solid bars) and in cells (open bars). Effects of GF120918 and rat type (WT versus TR⁻) on accumulation and the biliary excretion index (BEI; pink diamonds) of TS and TG were evaluated using two-way ANOVA. Data represent mean $\pm$ S.E.M. (n=3 in triplicate). [†]*P* < 0.05, significantly different from WT (cells+bile); [‡]*P* < 0.05, significantly different from WT (cell); **P* < 0.05, significantly different from WT (BEI).

TGZ Metabolism in S9 Fraction Prepared from Liver Tissues of WT and TR⁻ Rats. To investigate the differential metabolism (i.e., sulfation and glucuronidation) of TGZ in WT and TR⁻ rats, formation of TS and TG was quantified after incubating 1 or 10 μ M TGZ with S9 fraction prepared from liver tissues of WT and TR⁻ rats for 3 minutes. These concentrations were selected for investigation because they yielded unbound concentrations of TGZ in the S9 fraction that were similar to the unbound concentrations in hepatocytes (see *Materials and Methods* for details). The initial velocity of TS formation in S9 fraction from WT and TR⁻ rat liver tissue was comparable after incubation with 1 and 10 μ M TGZ, indicating that the activity of sulfotransferases was not altered in TR⁻ rats (Fig. 7). The initial velocity of TG formation was increased by 15- and 6.5-fold in TR⁻ rats after incubation with 1 and 10 μ M TGZ, respectively, consistent with the increased recovery of TG in TR⁻ rat SCH (Fig. 7). TS did not undergo de-sulfation after incubation of 100 μ M TS with S9 fractions over a 30-minute period (data not shown).

Discussion

This study examined the effects of impaired function of Bcrp and/or Mrp2 on the hepatobiliary disposition of TGZ and generated metabolites, TS and TG; although TQ was measured, this investigation did not focus on TQ because it is a minor metabolite that also is formed by photo-oxidation (Fu et al., 1996). A recently established novel in vitro system, rat SCH lacking Mrp2 (using Mrp2-deficient TR⁻ rat) and Bcrp (using adenoviral vectors expressing shRNA targeting Bcrp), was employed (Yang et al., 2014). In addition, differential metabolism of TGZ in WT and TR⁻ rats was investigated by in vitro metabolism studies using hepatic S9 fractions.

TGZ is metabolized by Phase I and II enzymes and the generated metabolites are eliminated by multiple transport proteins. Consequently, defects in relevant metabolic enzymes and/or transport proteins attributable to genetic polymorphisms, underlying disease, or coadministered drugs may predispose certain patients to TGZ-induced hepatotoxicity. Hewitt et al. (2002) demonstrated that low CYP3A4 and UDP-glucuronosyltransferase (UGT) activity combined with high sulfotransferase (SULT) activity was associated with TGZ toxicity in human hepatocytes. Pharmacokinetic modeling and Monte Carlo simulations in rat SCH revealed that intracellular concentrations of TS may increase up to 5.7-fold when biliary excretion of TS is decreased 10-fold (Lee et al., 2010a), but effects of altered function of biliary transporters on TS disposition have not been evaluated experimentally. TS, a potent inhibitor of bile acid efflux transporters, is extensively excreted into bile via Mrp2 and Bcrp (Kostrubsky et al., 2001; Enokizono et al., 2007). Thus, we hypothesized that loss-of-function of Bcrp and/or Mrp2 would result in decreased biliary excretion and increased cellular accumulation of TS, which may lead to further inhibition of bile acid transport and hepatotoxicity.

Unexpectedly, biliary excretion of TS was not altered by loss-of-function of Bcrp and Mrp2, potentially attributable to the presence of a compensatory canalicular protein that transports TS in the absence of Mrp2 and Bcrp. It is not probable that residual Bcrp expression played a role in TS transport, because loss-of-function of Mrp2 and Bcrp in this experimental system was validated using rosuvastatin, a dual substrate of Mrp2 and Bcrp. Biliary excretion of rosuvastatin was almost ablated in Bcrp knockdown TR⁻ rat SCH. Thus, Bsep and P-gp were investigated as potential compensatory transporters mediating the biliary excretion of TS. Although TS is a potent Bsep inhibitor (Funk et al., 2001b), results from membrane

Fig. 7. Metabolism of troglitazone (TGZ) in S9 fraction prepared from liver tissues of WT and TR⁻ rats. The initial velocity of TGZ sulfate (TS) and TGZ glucuronide (TG) formation was obtained after incubation of 1 and 10 μ M TGZ with S9 fractions prepared from liver tissues of WT (solid bars) and TR⁻ (open bars) rats. **P* < 0.05, significantly different from WT.

vesicle studies demonstrated that TS is not transported by Bsep. P-gp is probably not involved in TS biliary excretion because excretion of TS into bile was not altered in the presence of GF120918, a potent inhibitor of P-gp and Bcrp (Mao and Unadkat, 2005; Rautio et al., 2006), in WT and TR⁻ rat SCH. Other hepatic canalicular transporters, such as multidrug and toxin extrusion protein 1 (MATE1) or multidrug resistance P-glycoprotein 2 (MDR2), might be involved in compensatory TS biliary excretion. MATE1 typically transports hydrophilic organic cations with low molecular weight (e.g., metformin, tetraethylammonium, 1-methyl-4-phenylpyridinium, oxaliplatin), although transport of anionic compounds (e.g., acyclovir, gancyclovir, estrone sulfate) and zwitterions (e.g., cephalexin, cephadrine) has been reported (Klaassen and Aleksunes, 2010; Hillgren et al., 2013). MDR2 is the rodent ortholog of human MDR3, a phosphatidylcholine flippase, which also transports paclitaxel, digoxin, and verapamil (Smith et al., 2000).

Even though the BEI of TS remained the same, the total recovery of TS was decreased in TR⁻ compared with WT rat SCH on the basis of decreased TS cellular accumulation and medium concentrations. These data suggested that TGZ sulfation was decreased, or desulfation of TS was increased in TR⁻ rat SCH. TGZ is metabolized to TS primarily by SULT1A1 and also by SULT1E1 in humans (Honma et al., 2002). In rats, TS formation is four times faster in male rats compared with female rats, which is consistent with greater expression of Sult1a1 and Sult1e1 in male rats (Demian et al., 1992; Liu and Klaassen, 1996). Interestingly, protein levels of hepatic Sult1a1 and Sult1e1 were comparable between WT and TR⁻ rats (Johnson et al., 2006; Sun et al., 2010), and our in vitro metabolism study using S9 fractions from livers of WT and TR⁻ rats revealed that TS formation rates were comparable. A decrease in net sulfation of estradiol-17 β -glucuronide (E₂17G) reported in TR⁻ rats was attributed to increased desulfation of estradiol-3-sulfate-17 β -glucuronide (Sun et al., 2010). However, desulfation of TS to TGZ was not observed in S9 fraction from livers of WT or TR⁻ rats. It is possible that the availability of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is less in TR⁻ compared with WT rat SCH. In rats and humans, sulfation can be limited by the availability of the cofactor PAPS. PAPS is synthesized rapidly by ATP-sulfurylase and adenosine-5'-phosphosulfate (APS)-kinase, but its availability is limited by hepatic sulfate concentrations, which are largely dependent on circulating levels of inorganic sulfate (Klaassen and Boles, 1997). TR⁻ rat SCH may have lower levels of PAPS precursors or lower activity of enzymes involved in PAPS synthesis. These differences would not be detected in the current experimental design, in which adequate PAPS was added exogenously to the in vitro metabolism system. TR⁻ rat SCH also may have higher cellular concentrations of endogenous substrates (e.g., bile acids) that deplete PAPS, leading to decreased recovery of TS. Compared with WT rats, hepatic sulfation activities in TR⁻ rats have been reported to be modestly increased (i.e., acetaminophen, 4-methylumbelliferone), similar (i.e., harmol, E₂17G), or decreased (i.e., resveratrol) in isolated perfused liver studies (Xiong et al., 2000; Zamek-Gliszczynski et al., 2005; Zamek-Gliszczynski et al., 2006; Maier-Salamon et al., 2008; Zamek-Gliszczynski et al., 2008; Sun et al., 2010). However, with the exception of the harmol and E₂17G studies, recovery was measured only in perfusate and bile, and mass balance did not account for recovery in the liver tissue. Further studies are warranted to investigate the mechanisms of altered sulfation in TR⁻ rats and the underlying reason for decreased recovery of TS in TR⁻ rat SCH.

As expected, TG biliary excretion was significantly decreased in TR⁻ rat SCH compared with WT rat SCH, but was not altered by Bcrp knockdown, consistent with previous data that Mrp2 plays a major role in the biliary excretion of TG (Kostrubsky et al., 2001). The

increase in total recovery of TG in cells, bile, and medium of TR⁻ rat SCH compared with WT rat SCH can be explained by increased activity of UGT enzymes, as shown in the in vitro S9 metabolism study. Species differences exist between humans and rats in UGT isozymes responsible for TGZ glucuronidation. TG formation is mediated primarily by UGT1A1 in humans, whereas Ugt2b2 is the major isozyme responsible for TGZ glucuronidation in rats (Yoshigae et al., 2000; Watanabe et al., 2002). Expression of hepatic Ugt1a protein was increased in TR⁻ rats (Johnson et al., 2006). Although expression of hepatic Ugt2b2 in TR⁻ rats has not been characterized, the present in vitro S9 metabolism data suggest that expression and/or activity of Ugt2b2 is increased in TR⁻ rats. Overall, decreased biliary excretion combined with increased glucuronidation led to significantly increased cellular accumulation of TG in TR⁻ compared with WT rat SCH. Medium concentrations of TG also were increased in TR⁻ rat SCH, consistent with increased glucuronidation and increased expression of the basolateral efflux transporter Mrp3 (Johnson et al., 2006; Sun et al., 2010).

In conclusion, the present study revealed that loss-of-function of Mrp2 and Bcrp is not a risk factor for increased hepatocellular TS accumulation in rats. Whether these findings can be directly translated to humans remains to be determined. Species differences in abundance, substrate specificity, and/or affinity for canalicular transporters may exist. For example, Mrp2 protein expression in rat liver tissue and fresh hepatocytes was about 10-fold higher compared with humans (Li et al., 2009). Mrp2 contributes to TS and TG biliary excretion in rats (Kostrubsky et al., 2001), but the contribution of human MRP2 to TS and TG biliary excretion has not been evaluated. To identify DILI risk factors in humans, the results from the current study need to be confirmed in primary human hepatocytes or human-derived cell systems. In addition, this study demonstrated for the first time that in TR⁻ rat SCH, TGZ sulfation was decreased, but these changes were not attributable to altered sulfotransferase activity. Evaluation of hepatocellular exposure of a drug may be important in predicting efficacy and/or toxicity if the liver is the site of action, or the parent drug and/or generated metabolites elicit hepatotoxic effects. Altogether, the results from the present study suggest that altered hepatocellular exposure to hepatically-generated metabolites is determined by both formation and excretion, and that compensatory mechanisms may play important roles when elimination pathways are impaired.

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Authorship Contributions

Participated in research design: Yang, Brouwer.
Conducted experiments: Yang.
Contributed new reagents or analytic tools: Yang.
Performed data analysis: Yang.
Wrote or contributed to writing the manuscript: Yang, Brouwer.

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