Involvement of Breast Cancer Resistance Protein (BCRP/ABCG2) in the Biliary Excretion and Intestinal Efflux of Troglitazone Sulfate, the Major Metabolite of Troglitazone with a Cholestatic Effect

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ABSTRACT:

Troglitazone sulfate (TGZS) is the major metabolite of troglitazone (TGZ), an antidiabetic agent, and thought to be a cause of the cholestasis induced by TGZ. The aim of the present study is to elucidate the involvement of breast cancer resistance protein (BCRP/ABCG2) in the hepatic disposition of TGZS. The basal-to-apical transport of TGZS was enhanced in organic anion transporting polypeptide 1B1-expressing Madin-Darby canine kidney II cells by infection of recombinant adenovirus harboring human BCRP and mouse Bcrp cDNA. TGZS was given to wild-type and Bcrp (−/−) mice by infection of recombinant adenovirus harboring human BCRP and mouse Bcrp cDNA. TGZS was given to wild-type and Bcrp (−/−) mice by constant infusion. Biliary excretion is the predominant elimination pathway of TGZS in wild-type mice, and the biliary excretion clearance of TGZS with regard to the hepatic concentration was reduced to 30% of the control in Bcrp (−/−) mice. However, plasma and hepatic concentrations were unchanged, suggesting induction of compensatory mechanisms in Bcrp (−/−) mice for the elimination of TGZS. Involvement of BCRP in the intestinal efflux transport of TGZS was examined using everted sacs. The mucosal efflux clearance of TGZS showed only a slight reduction (15% reduction) in Bcrp (−/−) mice. Our results suggest that BCRP plays a major role in the biliary excretion but a minor role in the intestinal transport of TGZS.

Troglitazone (TGZ) (Fig. 1a) was the first marketed thiazolidinedione, and it has been used for the treatment of type 2 hyperglycemia. It can sensitize tissues to insulin by activating peroxisome proliferator-activated receptor-γ, thereby inhibiting glucose release from hepatocytes and enhancing the insulin-dependent glucose metabolism in adipose tissue and skeletal muscle (reviewed in Chen, 1998). However, TGZ was withdrawn from the market in 2000 because of idiosyncratic severe hepatotoxicity. A great deal of research was carried out on the hepatotoxicity of TGZ, and multiple mechanisms were proposed, such as the production of reactive intermediates and direct mitochondrial injury (reviewed in Smith, 2003). An alternative mechanism has been proposed is cholestasis. Cholestasis was observed in patients with severe hepatotoxicity (Fukano et al., 2000; Menon et al., 2001), and Funk et al. (2001b) showed that a single bolus administration of TGZ increased the plasma bile acid concentration in rats. Because troglitazone sulfate (TGZS) (Fig. 1b), the major metabolite of TGZ, is a more potent inhibitor of the bile salt export pump (BSEP) than TGZ, and the hepatic concentration of TGZS was much greater than that of TGZ (Funk et al., 2001b), TGZS has been hypothesized to account for the cholestatic effect of TGZ. A gender difference in the cholestatic effect of TGZ in rats was related to the sex-dependent formation of TGZS: the formation of TGZS was greater in male rats, which exhibit more severe cholestasis (Funk et al., 2001a). Based on these findings, it has been speculated that TGZS increases the likelihood of hepatotoxicity induced by TGZ. In addition to the conjugation rate, the hepatic elimination mechanism of TGZS will be important for hepatotoxicity. Elucidation of the molecular mechanism of the hepatic disposition of TGZS is also important to obtain a clue to account for the idiosyncratic hepatotoxicity of TGZ. Polymorphisms or mutations of the enzymes and transporters may be associated with this idiosyncrasy.

TGZS is produced mainly by sulfotransferase (SULT) 1A1 in the liver and is predominantly excreted into the bile (Kawai et al., 1997; Honma et al., 2002). Almost 50% was recovered in the bile as unchanged form after intraduodenal administration of TGZS in rats (Kawai et al., 2000). As far as hepatic uptake is concerned, Nozawa et al. (2004) showed that TGZS is transported by organic anion transporting polypeptide (OATP) 1B1 and OATP1B3, and OATP1B1 transported TGZS more efficiently than OATP1B3 (Nozawa et al., 2004). OATP1B1 will play a major role in the biliary uptake of TGZS. As far as the biliary excretion process is concerned, Kostrubsky et al. (2001) investigated the involvement of multigrid resistance-

ABBREVIATIONS: TGZ, troglitazone; TGZS, troglitazone sulfate; BSEP, bile salt excrete pump; SULT, sulfotransferase; OATP, organic anion transporting polypeptide; MRP2/Mrp2, multidrug resistance-associated protein 2; BCRP/Bcrp, breast cancer resistance protein; ME3277, sodium hydrogen [4-[[4,5,6,7-tetrahydrothieno [3,2-c] pyridin-2-yl] carbonylamino] acetyl-o-phenylene] dioxydiacetate; MDCKII, Madin-Darby canine kidney II; GFP, green fluorescent protein; mBcrp, mouse Bcrp; hBCRP, human BCRP; PBS, phosphate-buffered saline; LC/MS, liquid chromatography/mass spectrometry; BSA-KRB, bovine serum albumin/Krebs-Ringer bicarbonate; SNP, single nucleotide polymorphism(s).
associated protein 2 (MRP2/ABCC2) in the biliary excretion of TGZS using TR− rats, a mutant strain with an inherited deficiency in MRP2. The biliary excretion of TGZS was delayed in TR− rats compared with normal rats; however, the amount of TGZS excreted into the bile was not markedly reduced in TR− rats (Kostubsky et al., 2001). One explanation of this is that other transporters are also involved in the biliary excretion of TGZS. The transport system involved in the intestinal reabsorption of TGZS remains to be identified.

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette transporter family. BCRP is ubiquitously expressed in normal tissues, and cumulative studies using Bcrp−/− mice have shown the importance of BCRP in the urinary excretion, secretion into milk and tissue, and fetal distribution of xenobiotics (Malpepaard et al., 2001; Jonker et al., 2002, 2005). In the liver and intestine, BCRP is localized in the canalicular and brush-border membranes, respectively, and it mediates the biliary excretion of nitrofurantoin and pitavastatin and limits the oral absorption of topotecan, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, and ME3277.

Materials and Methods

Materials and Animals. TGZ and TGZS were gifts from Sankyo Co. Ltd. (Tokyo, Japan). All the other chemicals were commercially available and of reagent grade. A 24-well Transwell (6.5-mm diameter, 0.4-µm pore size) was purchased from Corning Costar (Bodenheim, Germany). Male Bcrp−/− mice (Jonker et al., 2002) and wild-type FVB mice used in the present study were 9 to 16 weeks old and weighed 25 to 33 g. The animals were maintained under controlled temperature with a light/dark cycle of 12 h. Food and water were available ad libitum.

Transcellular Transport Study. The transcellular transport study was performed as previously reported with minor modifications (Matsushima et al., 2005). In brief, Madin-Darby canine kidney II (MDCKII) cells expressing human OATP1B1 (MDCKII/OATP1B1) were grown on the Transwell membrane for 3 days in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). The cells were infected with the recombinant adenovirus harboring green fluorescent protein (GFP), mouse Bcrp (mBcrp), or human BCRP (hBCRP) expression vector at an infection multiplicity of 200. The details of the construction of these recombinant adenoviruses were described in a previous report (Kondo et al., 2004). After 2 days of culture, the cells were used for transport studies. The cells were preincubated in Krebs-Henseleit buffer (142 mM NaCl, 23.8 mM Na2CO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2, pH 7.4) for 30 min, and then transport experiments were initiated by replacing the medium on one side of the cell monolayer with Krebs-Henseleit buffer containing 3 µM TGZS. At appropriate times (1, 2, and 3 h), 100-µl aliquots were taken from the opposite side of the cell monolayer and replaced with 100 µl of buffer. After the last sampling, the cell monolayers were solubilized with 500 µl of 0.2 M NaOH and then neutralized with 100 µl of 1 M HCl. The protein concentration was measured by the Lowry method.

The flux of TGZS across cell monolayers was calculated as follows: Flux (µmol/mg protein) = (C acceptor × V acceptor)/C donor × protein amount, where C acceptor is the TGZS concentration in the acceptor solution, V acceptor is the volume of the acceptor solution, and C donor is the initial TGZS concentration in the donor solution (3 µM). The flux was plotted against time, and the efflux clearances (µl/h/mg protein) were calculated from the slopes.

Determination of Biliary Excretion of TGZS in Wild-Type and Bcrp−/− Mice. Under urethane anesthesia (1.25 g/kg, i.p.), the right jugular vein was cannulated with a polyethylene tube (PE-10; Becton Dickinson, Franklin Lakes, NJ) for injection of TGZS. After abdominal dissection, the gallbladder was ligated, and the bile duct was cannulated with a polyethylene tube (UT-3; Unique Medical, Tokyo, Japan) to collect bile. TGZS was injected at a dose of 0.5 µmol/kg, followed by continuous infusion at a dose rate of 0.1 µmol/h/kg. Blood samples were collected from the left jugular vein at 60, 80, 100, and 120 min. The blood samples were heparinized and centrifuged to obtain plasma samples. Bile samples were collected at 20-min intervals between 60 and 120 min postdosage. Immediately after the last blood and bile sampling, mice were sacrificed, and the liver was removed. The liver was homogenized with a 9-fold volume of phosphate-buffered saline (PBS) to obtain a 10% liver homogenate.

The plasma samples (5 µl) were mixed with 20 µl of PBS and 75 µl of acetonitrile; the bile samples (1 µl) were mixed with 49 µl of PBS and 100 µl of acetonitrile; and the liver homogenates (10 µl) were mixed with 40 µl of PBS and 100 µl of acetonitrile. All these mixed solutions were centrifuged at 15,000g for 10 min. The supernatants (plasma sample, 80 µl; bile and liver sample, 10 µl) were evaporated, and the pellets were reconstituted with 20% acetonitrile (plasma sample, 80 µl; bile and liver sample, 200 µl) and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis.

Determination of Urinary Excretion of TGZS in Wild-Type and Bcrp−/− Mice. Under urethane anesthesia (1.25 g/kg, i.p.), the right jugular vein was cannulated with a polyethylene tube (PE-10) for injection of TGZS, and the urinary bladder was cannulated with two polyethylene tubes (PE-50; Becton Dickinson). One cannula was fitted with a syringe filled with saline to wash the inside of the urinary bladder, and the other cannula was used for the collection of urine and wash solution. TGZS was injected at a dose of 0.5 µmol/kg followed by continuous infusion at a dose rate of 0.1 µmol/h/kg. Mannitol was concomitantly infused at a dose rate of 160 mg/h/kg to increase the urine volume. Urine samples were collected at 20-min intervals between 60 and 120 min. After every collection of urine, the bladder was flushed with about 300 µl of saline, and the wash solution was added to the urine. The urine samples (50 µl) were mixed with 150 µl of acetonitrile and centrifuged at 15,000g for 10 min. The supernatants (100 µl) were evaporated, and the pellets were reconstituted in 20% acetonitrile (100 µl) and subjected to LC/MS analysis.

Everted Sac Study. Mice were anesthetized with ether and sacrificed by exsanguination from the femoral artery and vein. Immediately after sacrifice, the ileum was dissected. The ileum was ligated at one end and then everted. The open end of the everted ileum was ligated after the insertion of a polyethylene tube (SP-45; Natsume, Tokyo, Japan) to make a 5-cm-long sac. Krebs-Ringer buffer (350 µl) containing 0.3% bovine serum albumin (BSA-KRB, pH 6.4) was added to the serosal side of the everted sac via the cannula, and the everted sacs were incubated at 37°C in 10 ml of BSA-KRB. After a 10-min preincubation, the everted sacs were placed in BSA-KRB containing

![Fig. 1. Chemical structures of TGZ (A) and TGZS (B).](image)
10 μM TGZ and incubated again at 37°C. Aliquots (200 μl) were collected from the mucosal side at 15, 30, 45, and 60 min after incubation with TGZ. After the last sampling, the serosal solution was removed, and the everted sacs were rinsed with ice-cold PBS. Throughout the entire procedure, the mucosal solution was bubbled with O₂/CO₂ (95:5) gas.

The aliquots (75 μl) from the mucosal solution were mixed with acetonitrile (75 μl) and centrifuged at 15,000g for 10 min. The supernatants were subjected to LC/MS analysis. The tissue samples were weighed and homogenized with a 9-fold volume of PBS to give 10% tissue homogenates. The tissue homogenates (50 μl) were mixed with acetonitrile (75 μl) and centrifuged at 15,000g for 10 min, and the supernatants were subjected to LC/MS analysis.

**LC/MS Analysis.** An LC/MS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. Samples were separated on a CAPCELL PAK C18 MGII column (3 μm, 2 × 50 mm; Shiseido, Tokyo, Japan) in binary gradient mode. For the mobile phase, 10 mM acetic ammonium and acetonitrile were used. The acetonitrile concentration was initially 23%, then linearly increased up to 70% over 1.5 min, and kept at 70% for a further 3 min. Finally, the column was re-equilibrated at an acetonitrile concentration of 23% for 2.5 min. The total run time was 5 min. TGZS was eluted at 3 min using this method. In the mass analysis, TGZS was detected at a mass-to-charge ratio of 520 under negative electron spray ionization conditions. The interface voltage was −3.5 kV, and the nebulizer gas (N₂) flow was 1.5 l/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

**Data Analysis of Biliary Excretion.** Because the plasma and biliary excretion was almost constant between 60 and 120 min (Fig. 3), the plasma and liver concentrations at 120 min were assumed to be steady-state concentrations. Total clearance (CL tot) was calculated by dividing the infusion rate by the plasma concentration at 120 min. The biliary excretion clearance based on the plasma concentration (CL bili, plasma) was calculated by dividing the biliary excretion rate in the last time segment (100–120 min) by the plasma concentration at 120 min. The biliary excretion clearance based on the liver concentration (CL bili, liver) was calculated by dividing the biliary excretion rate in the last time segment by the liver concentration at 120 min. The fraction of the biliary excretion was calculated by dividing the biliary excretion rate in the last time segment by the infusion rate.

**Efflux Transport in Everted Sac Study.** The mucosally excreted amounts of TGZS per unit length of tissue were calculated as follows: Mucosal efflux = (C mucosal × V mucosal)/tissue length, where C mucosal is the TGZS concentration in the mucosal solution, V mucosal is the volume of the mucosal solution (10 ml), and the tissue length was 5 cm. The excreted amounts of TGZS in the mucosal side were plotted against the incubation time (Fig. 4a), and the mucosal efflux rates were estimated from the slope. Mucosal efflux clearances (CL mucosal) were calculated by dividing the mucosal efflux rates by the tissue concentrations assuming 1 g of intestine = 1 ml.

**Statistical Analysis.** Statistical analysis for significant differences was performed using the two-tailed Student’s t test or one-way analysis of variance, followed by the Tukey multiple comparison test. A probability of <0.05 was considered to be statistically significant.

**Results**

**In Vitro Transport Study of TGZS in BCRP-Expressed Cells.** BCRP-mediated transport of TGZS was examined using GFP-, mBCRP-, and hBCRP-expressed MDCKII/OATP1B1 cells. Figure 2 shows the time courses of the transcellular transport of TGZS across the monolayers of MDCKII/OATP1B1 cells expressing GFP, mBCRP, and hBCRP. The transport in the apical-to-basal direction is represented by closed circles and that in the basolateral direction by open circles. Data are represented by mean values ± S.E. of triplicate experiments.
below the lower limit of quantification (10 nM). Because the volume of the urine sample (urine volume) was less than 0.45 mmol/kg. This means that the urinary excretion of TGZS is less than 1% of the dose in Bcrp (-/-) mice. Therefore, induction of BCRP is the predominant elimination pathway in wild-type mice. The biliary excretion of TGZS was markedly reduced in Bcrp (-/-) mice, and CLmucosal, liver, which represents the intrinsic efflux ability at the canalicular membrane, was reduced in Bcrp (-/-) mice to 29% of that in wild-type mice. Therefore, BCRP is the major transporter involved in the biliary excretion of TGZS. Cumulative studies using Mrp2-deficient mutant rats have shown that MRP2 accounts for the biliary excretion of some sulfate conjugates, such as tauro-conjugated bile acid sulfate, phenolphthalein sulfate, and acetaminophen sulfate (Takikawa et al., 1991; Akimoto et al., 2001; Tanaka et al., 2003; Zamek-Gliszczynski et al., 2005). Very recently, Zamek-Gliszczynski et al. (2006) showed that BCRP is also involved in the biliary excretion of sulfate conjugates, including 4-methylumbellifereone sulfate, acetaminophen sulfate, and harmol sulfate, using Bcrp (-/-) mice. These results suggest that BCRP and MRP2 are two major transporters responsible for the biliary excretion of sulfate conjugates, and BCRP makes a major contribution to the biliary excretion of xenobiotic sulfate conjugates. In humans, the biliary excretion of TGZS may be mediated by BCRP because hBCRP transports TGZS more efficiently than mBCRP.

The total clearance and hepatic concentration were not changed in Bcrp (-/-) mice, although biliary excretion is the predominant elimination pathway in wild-type mice. At steady state, the biliary excretion accounts for, at most, 30% of the total body clearance in Bcrp (-/-) mice, and the remaining fraction could be explained by other elimination mechanisms that were induced to compensate for the loss of biliary excretion. Urinary excretion could be an alternative elimination pathway of TGZS. However, the urinary excretion of TGZS was negligible in Bcrp (-/-) mice. Therefore, induction of hepatic metabolic enzymes is a likely mechanism. Currently, there is no information regarding the enzymes that are induced in Bcrp (-/-) mice, and further studies are necessary to identify them.

The effect of BCRP on the intestinal reabsorption was examined using everted ileum sacs. The mucosal efflux clearance (CLmucosal) of TGZS represents the intrinsic efflux ability of TGZS in the brush-border membrane of the intestinal epithelial cells, and the effect of BCRP on the mucosal excretion of TGZS can be measured by a comparison of CLmucosal between wild-type and Bcrp (-/-) mice. The CLmucosal of TGZS showed only a slight reduction in Bcrp (-/-) mice.
mice. This result suggests that BCRP plays only a limited role in preventing oral absorption of TGZS.

Figure 5 summarizes the molecular mechanism involved in the hepatic disposition of TGZS. TGZS is produced by SULT1A1 from TGZ and is excreted into the bile mainly by BCRP. TGZS excreted into the intestinal lumen undergoes reabsorption into the circulating blood (Kawai et al., 2000). SULT1A1, BCRP, and OATP1B1 are the important molecules controlling the hepatic disposition of TGZS. BCRP has some functional single nucleotide polymorphisms (SNP), such as C376T (Q126stop), C421A (Q141K), and G1322A (S441N). The C376T mutation introduces a stop codon, resulting in the production of truncated BCRP (Imai et al., 2002). The C421A allele is associated with a reduction in the protein level (Imai et al., 2002; Kondo et al., 2004; Kobayashi et al., 2005). The G1322A allele affects both the protein level and localization of BCRP (Kondo et al., 2004). Among these SNP, C421A exhibits the highest frequency (about 10 and 30% in Caucasian and Japanese, respectively) (Kobayashi et al., 2005). Clinical studies revealed that the plasma exposure of diflomotecan, topotecan, and rosuvastatin was higher in subjects carrying the C421A allele (Sparreboom et al., 2004, 2005; Zhang et al., 2006). These functional SNP of BCRP may increase the liver and/or plasma exposure of TGZS, affecting the susceptibility to TGZS if any induc-
tional transport of TGZS in everted ileum sacs. a, the time courses of the mucosal efflux of TGZS, b, the tissue concentrations of TGZS after a 60-min incubation. c, the mucosal efflux clearances of TGZS. The data in wild-type mice are shown by closed circles or columns and those in Bcrp (−/−) mice by open circles or columns. All the data are represented by mean values ± S.E. of four everted sacs independently prepared from four mice. Asterisks represent statically significant differences between wild-type and Bcrp (−/−) mice. *, P < 0.05 and **, P < 0.001.

References
Kostrubsky VE, Vore M, Kindt E, Burleigh J, Rogers K, Peter G, Altrogge D, and Sinz MW FIG. 4. The effect of BCRP on the intestin-

FIG. 5. Schematic representation of the molecular mechanism involved in the hepatic disposition of TGZS.


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