Interplay of Phase II Enzymes and Transporters in Futile Cycling: Influence of Multidrug Resistance-Associated Protein 2-Mediated Excretion of Estradiol 17β-β-D-Glucuronide and Its 3-Sulfate Metabolite on Net Sulfation in Perfused TR− and Wistar Rat Liver Preparations

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ABSTRACT:
The hepatic disposition of estradiol 17β-β-D-glucuronide (E217G), a substrate of the organic anion-transporting polypeptides Oatp1a1, Oatp1a4, and Oatp1b2, was investigated in Wistar and TR− [multidrug resistance-associated protein (Mrp) 2-mutant] rats to elucidate how absence of Mrp2, the major excretory transporter for both E217G and its 3-sulfate metabolite (E23S17G), affected the net sulfation. With absence of Mrp2, lower microsomal desulfation activity and higher Mrp3 but unchanged immunoreactive protein expression of other transporters (Oatps and Mrp4) and estrogen sulfotransferase were found in TR− rats. In recirculating, perfused liver preparations, the rapid decay of E217G and sluggish appearance of low levels of E23S17G in perfusate for Wistar livers were replaced by a protracted, biexponential decay of E217G and E23S17G (11 × 104) in liver and reduced net sulfation (40 ± 6 from 77 ± 6% dose, P < 0.05) were observed at 2 h for the TR− versus the Wistar rats. With use of a physiologically based pharmacokinetic model, analytical solutions for the areas under the curve for the precursor and metabolite were obtained to reveal how enzyme- and transporter-mediated processes affected the hepatic disposition of the precursor and metabolite in futile cycling. The analytical solutions were useful to explain transporter-enzyme interplay in futile cycling and predicted that a shutdown of Mrp2 function led to decreased net sulfation of E217G by raising the intracellular concentration of the metabolite, E23S17G, which readily refurnished E217G via desulfation.

The liver is the most important drug eliminatory organ and involves enzymes and basolateral influx/efflux and canalicular transporters for entry and elimination. The basolateral influx transporters [organic anion-transporting polypeptides (Oatps), sodium-dependent taurocholate-cotransporting polypeptide, organic anion transporter 2, and organic cation transporter 1] regulate the entry of substrate into the cell to undergo metabolism by phase I and/or phase II enzymes. Then both the parent drug and phase II metabolites are subject to canalicular transport via P-glycoprotein, multidrug resistance-associated protein (MRP) 2, the bile salt export pump, and breast cancer resistance protein, whereas basolateral efflux occurs via MRPs and MRP4 into sinusoidal blood. Although metabolism is normally deemed to be irreversible, the metabolite, on occasion, may re-form the parent drug. This phenomenon, known as “reversible metabolism” or “futile cycling,” describes the interconversion between the parent drug and its metabolite and can occur between a precursor and its phase I (Meffin et al., 1983; Baillie et al., 2001) or phase II (Hansel and Morris, 1996; Grubb et al., 1999) metabolites. For example, the sulfated metabolite formed via sulfotransferases (SULTs) in the cytosol may access the arylsulfatases in the endoplasmic reticulum to become desulfated to re-form the parent drug (Ratna et al., 1993; Kauffman et al., 1998). Acinar heterogeneity of the SULTs further complicates the scenario (Xu et al., 1993; Tan et al., 2001).

A well-stirred liver model with membrane barriers has been shown to be useful to relate the physiological and biochemical factors to hepatic drug and metabolite processing (Sirianni and Pang, 1997; Liu and Pang, 2005; Sun and Pang, 2010). In brief, transporter or enzyme activity is denoted by the intrinsic clearance or the ratio of Vmax, the maximum rate of the enzyme or transporter-mediated process, and Km, the Michaelis-Menten constant, under linear conditions. CLin and...
CLint,sec is the canalicular, secretory transporter activity. Solutions rat liver preparations. In the rat liver, E217G is taken up efficiently by estrogen sulfotransferase (Sult1e1) to form E23S17G, which is de- 
metabolized. The Groningen Yellow/transport-deficient (TR gene, whereby the resultant changes in drug processing can be eval-
interplay in the presence of futile cycling remains unknown.

CLint,met (Liu and Pang, 2005). However, the transporter-enzyme 
changes in the apparent clearances would result with changes in Mrp2 (Morikawa et al., 2000). Mrp3 and Mrp4 are expressed at low 
levels under physiological conditions and, when up-regulated, would 
increase in the CLliver,ex and CLliver,tot but would result in decreased CLliver,met the compensatory pathway of drug elimination. The op- 
potent trend would arise when CLint,sec is decreased. Analogous changes in the apparent clearances would result with changes in CLint,met (Liu and Pang, 2005). However, the transporter-enzyme 
interplay in the presence of futile cycling remains unknown.

Transporter or enzyme activity can be perturbed with use of a 
pecific inhibitor or inducer and knockdown or knockdown of the target 
gene, whereby the resultant changes in drug processing can be eval- 
uated. The Groningen Yellow/transport-deficient (TR-) rats are de- 
ived from Wistar rats but lack functional Mrp2 because of a single 
nucleotide substitution in the Mrp2 gene (Jansen et al., 1985; Müller 
et al., 1996; Ito et al., 1997). This renders the TR- rat as a useful 
imal model to assess the role of Mrp2 in drug biliary excretion as 
well as the interplay of Mrp2 and other transporters and enzymes 
(Xiong et al., 2000). In this article, the effect of Mrp2 on net sulfation 
and futile cycling of estradiol 17β-glucuronide (E217G) and its 3-sulfated metabolite (E23S17G) was assessed by comparison of their 
osition in recirculating, perfused TR- (lacking Mrp2) and Wistar 
rat liver preparations. In the rat liver, E217G is taken up efficiently by Oatpl1a1, Oatpl1a4, and Oatpl1b2 at the basolateral membrane (Cattori et al., 2001). In rat hepatocytes, E217G is sulfated mainly by the 
estrogen sulfotransferase (Sult1e1) to form E23S17G, which is de- 
sulfated back to re-form E217G by arylsulfatase C (Sun et al., 2006); 
both E217G and E23S17G are secreted into bile, predominantly via 
Mrp2 (Morikawa et al., 2000). Mrp3 and Mrp4 are expressed at low 
levels under physiological conditions and, when up-regulated, would 
play an important role by enhancing the efflux of E217G and 
E23S17G back to the blood.

Materials and Methods

Materials and Animals. [6,7-3H]E217G (specific activity 46.9 Ci/mmol) was purchased from PerkinElmer and Analytical Life Sciences (Waltham, MA). [6,7-3H]E23S17G was biosynthesized by recirculation of [6,7-3H]E217G (~100,000 dpm/ml) in blood perfusate at 12 ml/min through the rat liver. The bile was pooled and lyophilized and then reconstituted and injected into a high-performance liquid chromatograph (see HPLC Methods); the eluant 
ctions corresponding to [6,7-3H]E217G (retention time 12–13 min) were 
poled and desalted by a reverse-phase Sep-Pak cartridge (Waters, Milford, MA), and the purified content was concentrated and stored at ~20°C. 
The radiochemical purities of [6,7-3H]E217G and [6,7-3H]E23S17G were measured by HPLC as 98 and 97%, respectively. Unlabeled E217G, 4-methylumbel- 
liferyl sulfate (4-MUS), and bovine serum albumin in Tyrode’s solution were 
pooled and desalted by a reverse-phase Sep-Pak cartridge (Waters, Milford, MA). 

The Mrp2 antibody (M2III-6) was obtained from Enzo Life 
and Dr. John D. Schuetz (St. Jude Children’s Research Hospital, Memphis, TN), respectively. All reagents were of HPLC grade, and deionized distilled water was used in all experiments.

Male Wistar rats were purchased from Charles River (St. Constant, QC, Canada). TR- rats were generously provided by Dr. Kim L. R. Brouwer (University of North Carolina, Chapel Hill, NC) and bred in the Division of Comparative Medicine, University of Toronto. All the rats were maintained under a 12:12 h dark/light cycle in the housing facility (two rats per cage) and 
given access to animal chow and water ad libitum. The procedures used for the animal studies were conducted in accordance with the protocols approved by the animal committee at the University of Toronto.

Analytical Solutions of AUCs and Cumulative Biliary Excretion (from Time = 0 to Infinity, A∞). A physiologically based pharmacokinetic (PBPK) model (Fig. 1) was used to describe the transport and metabolism of a precursor and metabolite pair that undergoes futile cycling. The model consists of the reservoir, sinusoidal blood, hepatocyte, and bile compartments, denoted by subscripts R, LB (or B), L, and bile, respectively. D and Mi represent the precursor and the interconversion metabolite, respectively. The precursor (D0) and metabolite (Mi0) in the reservoir are delivered at a flow rate of Q (12 ml/min) to the liver. The concentrations of drug (Df0) and metabolite (Mif0) in sinusoidal blood are identical to those in the hepatocyte venous blood. The unbound fractions that describe the binding of the precursor and metabolite in sinusoidal blood are denoted by fD0 and fM0, respectively, and are identi- 
cal to those in perfusate blood, fD and fM [respectively, the unbound precursor and metabolite in sinusoid that rapidly equilibrate with the species bound to albumin (D LB-BSA, Mi LB-BSA) and red blood cells (D LB-rbc, Mi LB-rbc) are taken up into the hepatocyte with the influx clearances, CLin and CLin [respectively].

Within the hepatocyte, the precursor drug is metabolized to its interconver- 
sion metabolite with the metabolic intrinsic clearance, CLD→Mi [respectively, and to other metabolites with the metabolic intrinsic clearance, C\textsubscript{Mi}→\textsubscript{Mi}\textsuperscript{ entert}]. Likewise, the metabolite re-forms the precursor drug with the metabolic intrinsic clearance of the metabolite, CLMi→\textsubscript{D} [respectively, or forms other secondary metabolites with the intrinsic clearance, CLMi→\textsubscript{Mi}]. Both the precursor and metabolite in the hepatocyte may be effluxed back to the sinusoid with CL\textsubscript{out} and CL\textsubscript{out} [respectively, or excreted into bile with CL\textsubscript{int,sec} and CL\textsubscript{int,sec}]. The bile flow rate is Q\textsubscript{bile}.

The AUCs (AUC\textsubscript{D} and AUC\textsubscript{Mi}) and cumulative biliary excretion of drug and metabolite were solved analytically by the inversion of matrices derived for the model (Fig. 1) and the associated mass balance equations, shown in the 

Preparation of Subcellular Fractions. Rat livers at the end of perfusion were used for the preparation of crude membranes and cytosolic fractions for immunoblotting. As described previously (Sun et al., 2006), the liver tissue was homogenized in buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Tris-base, pH 7.4), and centrifuged at 3000g for 10 min; the resultant super- natant was centrifuged at 33,000g for 1 h. The pellet (crude membrane) was reconstituted in resuspension buffer (50 mM mannitol and 20 mM HEPES in 20 mM Tris base, pH 7.5). For preparation of the cytosolic fraction, the liver tissue was homogenized in SET buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 3000g for 20 min and the resultant supernatant was further centrifuged at 100,000g for 1 h to yield the microsomal and cytosolic fractions that were used for Western blotting. All buffers were freshly prepared and supplemented with protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada). The microsomal fraction, prepared as follows, was used for the assay of sulfatase activities. A piece of liver tissue was homogenized with Tris-HCl (25 mM, pH 7.4). The homogenate was centrifuged at 9000g for 20 min. The resultant supernatant
(S9) was centrifuged at 100,000g for 1 h; the pellet was resuspended in Tris-HCl (25 mM, pH 7.4) to yield the microsomal fraction. All of the procedures were performed at 4°C, and subcellular fractions were stored at −80°C until analyses. Protein concentration was determined by the method of Lowry et al. (1951).

**Immunoblot Analysis.** Protein expression of the Oatps, Mrps, and Sults in the perfused rat livers was determined semiquantitatively by immunoblotting. In brief, 20 μg of crude membrane (for Oatps and Mrps) or cytosolic (for Sults) proteins were resolved by SDS-polyacrylamide gel electrophoresis in 7.5% and 12% (for Sults) gels and electrophoretically transferred onto nitrocellulose membranes. The blots were blocked with Tris-buffered saline/Tween 20 (1%) buffer containing 5% nonfat milk for 15 min and then were incubated with primary antibodies (rabbit-anti-Oatp1a1 at 1:2500, rabbit-anti-Oatp1a3 at 1:10,000, mouse-anti-Mrp2 at 1:750, rabbit-anti-Mrp3 at 1:5000, rabbit-anti-Mrp4 at 1:2000, rabbit-anti-Sult1a1, and mouse-anti-Sult1e1 at 1:10,000) overnight at 4°C. Finally, goat-anti-rabbit (for Oatps, Mrps, and Sults) or goat-anti-mouse (for Sult1e1 and Mrp2) IgG (H + L)-horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA) was incubated with the blots at room temperature for 1 h. Upon reaction with ECL reagent, the blots were scanned by the FluorChem Imaging System (Alpha Innotech, San Leandro, CA) and the scan time was controlled to ensure linearity of the chemiluminescent signal. The autoradiograms obtained were quantified densitometrically with Scion Image (Scion Corporation, Frederick, MD). Because the molecular weights of Oatps and Mrps (>75 kDa) are distinctly different from that of GAPDH (~37 kDa), the loading control, the blot was cut into two portions after transfer: the upper portion was used for the detection of the target protein, and the lower portion was used for the detection of GAPDH. Because the Sults have molecular weights similar to that of GAPDH, the blot was stripped with Restore Western Blot Stripping Buffer (Pierce Biotechnology Inc., Rockford, IL) and re-probed with the antibody for GAPDH (Abcam Inc., Cambridge, MA), after the target protein was quantified. Protein expression of the target protein was presented as the ratio of densitometric measurements of the target protein to that of GAPDH.

**In Vitro Sulfatase Activity in Subcellular Fractions.** As described previously (Sun et al., 2006), a relatively higher microsomal protein concentration (4 mg/ml) was used for the incubation study because of the low sulfatase activity toward E23S17G. [3H]E23S17G (~50,000 dpm) and Tris-HCl (25 mM, pH 7.4) were separately preincubated at 37°C for 5 min and then were mixed (1:1, v/v) and incubated in a rotating incubator at 37°C for 10 min. Samples were retrieved at various times between 0 and 10 min to determine the linearity in [3H]E23S17G formation versus time. The reaction was terminated by the addition of 150 μl of ice-cold acetonitrile that contained 4-MUS (50 μg/ml) as an internal standard. After centrifugation at 6000g for 10 min, the resultant supernatant was dried under a gentle stream of nitrogen. The residue was reconstituted in the buffer (NH₄Ac-acetonitrile, 85:15, v/v) and then the radioactivities in outflow eluants that corresponded to peaks of the radiochromatogram were collected. A calibration curve was constructed by mixing boiled microsomal fractions with the various known concentrations of [3H]E217G for quantification of the [3H]E217G formed in microsomes. The in vitro desulfation intrinsic clearance (CLint,met mi) in vivo in microliters per second per milligram of microsomal protein, estimated as the formation rate of [3H]E217G divided by the concentration of [3H]E23S17G, was only an apparent value because the estimate did not consider the binding of E23S17G to microsomal proteins.

**Recircculating Rat Liver Perfusion.** The surgical procedure and the perfusion apparatus were described previously (Sun et al., 2006). In brief, the male Wistar or TR rats serving as liver donors was anesthetized by an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). Cannulae were placed in the portal vein, hepatic vein, and bile duct, and the temperature of the liver preparation was maintained close to 37°C under a heating lamp. Blank perfusate was oxygenated (95% O₂-5% CO₂) with Scion Image (Scion Corporation, Frederick, MD). The surgical procedure and the perfusion apparatus were described previously (Sun et al., 2006). In brief, the male Wistar or TR rat serving as liver donors was anesthetized by an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). Cannulae were placed in the portal vein, hepatic vein, and bile duct, and the temperature of the liver preparation was maintained close to 37°C under a heating lamp. Blank perfusate was oxygenated (95% O₂-5% CO₂). Krebs-Henseleit bicarbonate solution (pH 7.4) that was supplemented with washed bovine erythrocytes (~20%) (a kind gift of Ryding-Regency Meat...
various concentrations of \([3H]E217G\) or \([3H]E23S17G\) with boiled liver homogenate followed by the addition of 10 ml of 30% hydrogen peroxide. After the addition of 30% hydrogen peroxide, the liver homogenate was spiked with 50 nM \([3H]E217G\) and 45 nM \([3H]E23S17G\) radioactivities in the eluant fractions were counted to determine the fractions of \([3H]E217G\) and \([3H]E23S17G\) in each sample, and these were multiplied by the total disintegrations per minute to provide the disintegrations per minute of \([3H]E217G\) and \([3H]E23S17G\) in the sample. The PBPK model was also used for fitting and simulations. Inasmuch as the model for futile cycling was extremely complicated, the likelihood of success for data fitting was low. As alternate measures, simulations were used. Various assumptions, highlighted under Results, were taken for parameter estimates. The influx clearances for \(E217G\) and \(E23S17G\) were taken to be the same as those of Sun et al. (2006). In addition, the attainment of steady-state conditions for \(E217G\) and \(E23S17G\) in the TR allowed estimation of CLint,ClSt,ClL,Clm,Clm2, and the sulfation and desulfation intrinsic clearances, as described later under Results. It was further assumed that the unbound fractions \(f_{1}\) and \(f_{2}\) equaled the observed \(f_{0}\) (Sun et al., 2006).

Parameter estimates of the interconversion and secretory intrinsic clearances for the Wistar rat were obtained from fitting upon fixing the values of CLint,CLSt,CLL,CLm,CLm2, and CLm3. The relative intensities of the immunoblots obtained from Wistar and TR livers were then compared to provide estimates of relative transporter activities for all sinusoidal influx (Oatps), basolateral efflux (Mrp3), and relative sulfation (Sult1e1) activities, keeping in mind that the ex vivo desulfation activity could be obtained by scale-up of the in vitro microsomal activity. These optimized values were used for simulation of the concentrations/amounts of \(E217G\) and \(E23S17G\) in perfusate, liver, and bile according to the differential equations shown in the Appendix. The estimates were also used to simulate the AUCs and clearances with various extents of reduction of Mrp2 activity.

Statistical Analyses. The data are presented as mean values ± S.D. A two-tailed Student’s t test was used to compare the means, and \(P < 0.05\) was viewed as significant.

Results

Solved Analytical Solution for AUC and A \(_{\text{cum}}\). As shown in Table 1, the solutions for \(\text{AUC}_R\) and \(\text{AUC}_R\{mi,P\}\) were similar to those in the absence of futile cycling (de Lannoy et al., 1993; Sirianin and Pang, 1997), with the exception that the \(e_{\text{fun}}\) and \(e_{\text{fum}}\) terms are now present. Both terms, \(e_{\text{fun}}\) and \(e_{\text{fum}}\), were introduced to describe modulation of the backward and forward processes in futile cycling, respectively, on the net metabolism of the precursor and metabolite. In these solutions, \(e_{\text{fun}}\) or \(e_{\text{fum}}\) effective coefficient for metabolism formation, was associated with CLint,met or the metabolism formation intrinsic clearance. The term \(e_{\text{fun}}\) or \(e_{\text{fum}}\) consisted of intrinsic clearance terms associated with the metabolite: the intrinsic clearance denoting re-formation of the precursor (CLint,met{mi}) as well as other terms for secretion (CLint,sec{mi}) and sequential metabolism (CLint,met{mi}) of the metabolite. Conversely, \(e_{\text{fun}}\) or the effective recycling coefficient,
was associated with the metabolic intrinsic clearance of metabolite to re-form the precursor, $CL_{\text{int,sec}^{\text{other}}}^{\text{mi}}$ that appeared as in the denominator of the solution of $AUC_{\text{P}}^{\text{mi},\text{P}}$ and $AUC_{\text{L}}^{\text{mi},\text{P}}$ in the presence of futile cycling (Table 1).

The area under the curve for the formed metabolite in the reservoir, $A_{\text{L}^{\text{mi}},\text{P}}$, was found to depend on all the intrinsic clearances of the precursor, including basolateral influx and efflux and metabolic $CL_{\text{int,sec}^{\text{other}}}^{\text{mi}}$ and secretory $CL_{\text{int,sec}^{\text{int}}}^{\text{mi}}$ clearances. The net metabolite formation intrinsic clearance of the precursor ($ef_m^{\text{efm}}\left(\frac{CL_{\text{int,sec}^{\text{int}}}}{CL_{\text{int,sec}^{\text{int}}}^{\text{mi}} + CL_{\text{int,sec}^{\text{int}}}^{\text{other}}\text{mi}}\right)$ was reduced by the factor of $(1 - ef_m^{\text{efm}})$ or

$$\frac{CL_{\text{int,sec}^{\text{other}^{\text{mi}}}}^{\text{mi}}}{CL_{\text{int,sec}^{\text{int}}^{\text{mi}}} + CL_{\text{int,sec}^{\text{int}}^{\text{other}}\text{mi}} + CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}}$$

The area under the curve for the formed metabolite in the reservoir, $A_{\text{L}^{\text{mi}},\text{P}}$, was found to depend on parameters relating to metabolite handling ($CL_{\text{int,sec}^{\text{other}}}^{\text{mi}}$, $CL_{\text{int,sec}}^{\text{mi}}$, $CL_{\text{int,sec}}^{\text{other}}$, $CL_{\text{int,sec}^{\text{other}}^{\text{mi}}}$) and metabolic $\left(\frac{CL_{\text{int,sec}^{\text{other}}^{\text{mi}}}}{CL_{\text{int,sec}^{\text{int}}^{\text{mi}}} + CL_{\text{int,sec}^{\text{int}}^{\text{other}}\text{mi}} + CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}}\right)$ and the secretory intrinsic clearance ($CL_{\text{int,sec}^{\text{int}}}^{\text{mi}}$), metabolic intrinsic clearance for alternate metabolism ($CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}$), and the total intrinsic clearance of the precursor ($CL_{\text{int,sec}}^{\text{int}} + CL_{\text{int,sec}}^{\text{other}} + CL_{\text{int,sec}}^{\text{other}^{\text{mi}}}$). Analogously, $A_{\text{L}^{\text{mi}},\text{P}}$ and $A_{\text{L}^{\text{mi}},\text{P}}$ and the cumulative excretion of the precursor ($A_{\text{L}^{\text{mi}},\text{P}}$) and metabolite ($A_{\text{L}^{\text{mi}},\text{P}}$) were modified by $ef_m^{\text{efm}}$ or $ef_m^{\text{ecm}}$, accordingly, when futile cycling existed. The apparent total ($CL_{\text{int,met}^{\text{total}}}^{\text{total}}$) and excretory ($CL_{\text{int,met}^{\text{ex}}}^{\text{total}}$) clearances of the liver were estimated as dose/$AUC_{\text{P}}$ and $A_{\text{L}^{\text{mi}},\text{P}}$ respectively. The apparent metabolic clearance ($CL_{\text{int,met}^{\text{met}}}^{\text{total}}$) was obtained from the difference between $CL_{\text{int,met}^{\text{met}}}^{\text{total}}$ and $CL_{\text{int,met}^{\text{met}}}^{\text{ex}}$ (Table 2). In absence of a membrane barrier, the basolateral uptake and efflux clearances ($CL_{\text{int,met}^{\text{total}}}^{\text{total}}$ were set to greatly exceed $CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}$ and $CL_{\text{int,met}^{\text{total}}}^{\text{met}}$). As noted, $ef_m^{\text{efm}}$, together with $ef_m^{\text{ecm}}$, appeared in the solutions of all the apparent clearances (Table 2).

### Relative Protein Expression from Immunoblotting

As shown in Fig. 2, Mrp3 protein expression was significantly increased in the TR liver compared with that in the Wistar liver (4.26 ± 1.08 versus 1.00 ± 0.65), and Mrp2 protein expression was absent in the TR rat. However, protein expression of other transporters (Oatpl1a1, Oatpl1a4, Oatpl1b2, and Mrp4) and enzymes (Sult1e1 and Sult1a1) was not significantly different between TR and Wistar livers (Fig. 2).

### TABLE 2

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<th>Terms</th>
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<td>$A_{\text{L}^{\text{mi}},\text{P}}$</td>
<td>$\text{Dose} \frac{f_{\text{L}^{\text{mi}}}}{L_{\text{CL}<em>{\text{int,sec}^{\text{int}}^{\text{mi}}} + CL</em>{\text{int,sec}^{\text{int}}^{\text{other}}\text{mi}} + CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}}$</td>
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* $ef_m^{\text{efm}}$, effective coefficient for metabolite formation, = $\text{Dose} \frac{CL_{\text{int,sec}^{\text{int}}^{\text{mi}}} + CL_{\text{int,sec}^{\text{int}}^{\text{other}}\text{mi}} + CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}}{CL_{\text{int,sec}^{\text{int}}^{\text{mi}}} + CL_{\text{int,sec}^{\text{int}}^{\text{other}}\text{mi}} + CL_{\text{int,sec}^{\text{other}}}^{\text{other}^{\text{mi}}}}$
Desulfation Activity of Microsomal Fractions. The apparent desulfation activity toward E23S17G in microsomal fractions prepared from TR/H11002 rat livers (CLint,met Mi Dmin vitro), estimated as rate of E217G formation/E23S17G concentration, was slightly but significantly lower than those from Wistar rat livers (0.07 ± 0.02 versus 0.11 ± 0.02 μl/s/mg microsomal protein; n = 3). These represented apparent values because the binding of E23S17G to microsomal proteins of Wistar and TR⁻ livers was not considered.

Recirculating Liver Perfusion. In perfusate, E17G rapidly decayed monoexponentially for up to 45 min in the Wistar liver (Fig. 3A). Beyond that time, the radioactivity in the perfusate was too low to be measured accurately. The metabolite, E23S17G, was present at very low levels (<3% dose) in the reservoir perfusate for Wistar livers, and no apparent trend was observed with time. Almost all of the dose (92 ± 6%) was recovered in bile for the Wistar group at 2 h, with 75 ± 5% dose as the E23S17G metabolite and 17 ± 4% dose as the parent drug (Fig. 3B). Very low counts were found for the liver tissue of the Wistar rat at 2 h (E217G of 1.4 ± 0.7% dose and E23S17G of 2.5 ± 2.1% dose) (Table 3).

In contrast, perfusate E17G of the TR⁻ liver preparation decayed rapidly in the early time points, and then the decay became protracted and finally reached a plateau after 1 h. The amount of E23S17G in the perfusate appeared at much higher levels than that for the Wistar preparation and also reached a plateau after 1 h (Fig. 3C). The bile flow rate in the TR⁻ group was much lower than that in the Wistar group (2.4 ± 0.5 versus 6.3 ± 0.7 l/min), as expected for rats lacking Mrp2 (Jansen et al., 1985). As shown in Fig. 3D, almost no

FIG. 3. Time-dependent profile of E17G and E3S17G in the perfusate and liver (A and C) and bile (B and D) of recirculating perfused Wistar (A and B) and TR⁻ (C and D) rat liver preparations (n = 4 for both groups) based on parameters shown in Table 4 and differential equations in the Appendix. For experimental data, E17G is denoted by • in perfusate and bile and ○ in liver; E3S17G is denoted by ▲ in perfusate and bile and △ in liver. For prediction, E17G is denoted by —— in perfusate and bile and -- in liver; E3S17G is denoted by --- in perfusate and bile and – – – in liver.
radioactivity (1.8 ± 0.4% dose at 2 h) was observed in the bile of the TR group. Both E217G and E23S17G were minimally excreted into bile, although E217G was present in slightly higher proportions (1.3 ± 0.3% dose) than E23S17G (0.4 ± 0.1% dose). Much radioactivity was trapped in the TR rat liver, with 39 ± 9% dose at 2 h as E217G and 28 ± 5% dose at 2 h as E23S17G. At 2 h, the fractional dose of E23S17G for the TR group was significantly lower than that in the Wistar group (40 ± 6 versus 77 ± 6%) (Table 3).

**PBPK Modeling and Simulations.** The volume of the reservoir (\(V_R\)) was taken as the average value between the initial volume (200 ml) and the volume obtained at the end of the experiment, yielding values of 182 and 180 ml for the Wistar and TR livers, respectively (Table 4). As shown in Appendix, the \(f_B\) value of E217G was calculated from hematocrit of the perfusate, unbound fractions in plasma (\(f_u\)), and the drug partitioning coefficient between red cells and plasma; the latter two were obtained in previous binding experiments (Sun et al., 2006). Recognizing that it was difficult to accurately determine the unbound fractions in liver tissue (\(f_u\) and \(f_{mi}[mi]\)) because of the presence of metabolism during incubation studies, we further assumed that unbound fractions were all equal (\(f_B = f_u = f_{mi}[mi] = f_{mi}[mi]\)) for the sake of simplicity. The unbound fractions in liver would not materially alter the AUCs or clearances (Tables 1 and 2), and the value of the unbound fraction would not greatly affect the clearance of highly cleared drugs.

Because E217G and E23S17G levels reached a plateau 1 h after onset of perfusion among the TR liver (Fig. 3C), rates of change for the precursor and metabolite became zero at steady state, and the left sides of all the equations in the Appendix were zero. This condition allowed simplification and estimation of various parameters. At steady state, it was deduced in eq. A3 of the Appendix that the ratio of the observed drug concentrations in perfusate and in liver (\(D_v/D_h\) or 0.011; eq. 1) equaled the ratio of the net efflux to influx clearances because the drug concentration in reservoir equaled drug concentration in liver blood (see eq. A1):
3) (Table 4). The discrepancy was most likely due to the assumption of a steady-state strategy in perfusion studies (0.155 or 4.81/31.1 using eq. 19/12.1) that differed from the ratio estimated from fitting and the corresponding CL\text{int,met}\text{mi} resulted in poor prediction of the data (results not shown). (Slikker et al., 1983). Simulations conducted with low values of CL\text{int}[mi] were used to estimate CL\text{int,met}\text{mi} for the TR group. The value of efm\text{D} in vitro was 0.11 and 0.07 μmol/mg of microsomal protein, after multiplication by the scale-up factor (45 mg of microsomal protein/g of liver tissue) (Houston and Carlile, 1997), yielding an in vitro Wistar/TR− desulfation activity ratio of 1.57 (0.11/0.07 or 19/12.1) that differed from the ratio estimated from fitting and the steady-state strategy in perfusion studies (0.155 or 4.81/31.1 using eq. 3) (Table 4). The discrepancy was most likely due to the assumption that f\text{H} = f\text{H}[mi] = f\text{L} = f\text{L}[mi] in eqs. 1, 2, and 3 and that values reported in Table 4 for the transporter and metabolic intrinsic clearances were only the “effective” or “apparent” intrinsic clearances that could be modified by the unbound fractions. Values of CL\text{int,sec} and CL\text{int,sec}[mi] for the TR− rat were obtained by trial and error simulations and found to be 0.02 and 0.008 ml/min, respectively (Table 4). These values were very low and almost zero, as expected for the TR− rat.

The optimized parameters summarized in Table 4 were used to simulate E217G and E23S17G data in reservoir perfusate, liver, and bile based on the PBPK model for the Wistar and TR− livers with the program Scientist (Micromath, St. Louis, MO). As shown in Fig. 3, C and D (blood and bile), the experimental data for the TR− group was well predicted by the simulations based on the rationally chosen set of model parameters (Table 4). The parameter e\text{fW,mut}, effective coefficient for metabolite formation that denotes the effect of futile cycling on the disposition of the precursor, decreased from 0.346 in the Wistar liver to almost zero in the TR− liver (Table 4), suggesting that alteration in secretory intrinsic clearance of the metabolite would greatly affect the disposition of the precursor in the presence of the futile cycling. The value of e\text{fW,mut}, the effective recycling coefficient, was reduced from 0.078 in the Wistar liver to 0.0009 in the TR− rat liver (Table 4), suggesting that, again, reduction of the secretory intrinsic clearance of drug would affect the disposition of the metabolite in the presence of futile cycling. The low value of e\text{fW,mut} was due to the high value of CL\text{int,sec}[mi] (22.4 ml/min) in relation to CL\text{other}\text{int,met} = 0 and CL\text{int,sec} in both Wistar (1.9 ml/min) and TR− (0.02 ml/min) rats. The reduction in e\text{fW,mut} and e\text{fW,mut} in the TR− livers to those in the Wistar livers in futile cycling was attributed to reduced secretion upon reduction of Mrp2 activity.

Simulations Based on Solve Solutions for AUCs and Clearances. As shown in Fig. 4, AUC\text{R} (Fig. 4A) and AUC\text{L} (Fig. 4B) for E217G and E23S17G were both low due to rapid excretion into bile. The AUC\text{R} and AUC\text{L} values for both the precursor and formed metabolite increased gradually upon the loss of Mrp2 activity for E217G and E23S17G. When the majority of Mrp2 activity was blunted (>80%), values of the AUC\text{R} and AUC\text{L} for both E217G and E23S17G were elevated precipitously toward infinity (Fig. 4, A and B). Correspondingly, all the liver clearances were decreased upon the reduction of Mrp2 activity, and the values approached zero (Fig. 4C). These scenarios were expected to occur when a potent Mrp2 inhibitor was present or when Mrp2 was genetically knocked-out as for the mutant (TR−) rat. The simulation predicted observations in this com-
munication: when Mrp2 activity was almost abolished in TR−/− rat livers, a steady state would be reached for E217G and E23S17G, the precursor-metabolite pair that undergoes futile cycling, with the AUCR and AUCR{mi,P} approaching infinity (Fig. 4A) and clearances nearing toward zero (Fig. 4C).

Other Simulations for AUCs and Clearances. Two hypothetical simulations were conducted to mimic more general scenarios in which the parent drug and the metabolite do not share the same canalicular transporter. Again, the parameter values for the Wistar rat liver were used for the simulation (Table 4). As shown in Fig. 5 (case 1), AUCR and AUCR{mi,P} (Fig. 5A) and AUCR and AUCR{mi,P} (Fig. 5B) for the precursor and metabolite were increased when the secretory transporter activity of the metabolite but not the parent drug was reduced. The extent of change was not as dramatic as when both the precursor and metabolite secretory transporter activities were blunted (Fig. 4, A and B). With the loss of biliary secretion of the metabolite, the net metabolic clearance was decreased because of increased futile cycling; this led to an apparent increase in the excretory clearance and decrease in the net metabolic clearance for the precursor. The total liver clearance was decreased.

In the second simulation (case 2), in which there was loss of secretory transporter activity for the parent drug and not the metabolite, the AUCR was increased only very slightly, but the AUCR was evidently elevated, as shown in Fig. 6, A and B. The excretory clearance of the precursor was decreased, and the metabolic clearance was increased, as expected of competing pathways (Liu and Pang, 2005); the total liver clearance was decreased (Fig. 6C). These changes were not as dramatic as case 1, because the inhibited pathway (excretion of precursor) was not a major elimination pathway relative to that for sulfation, and the CLint,sec of the precursor was less than 1/10 that of CLint,met.

Discussion

The futile cycling between a precursor-metabolite is a complex relationship. Previous examples of interconversion had revealed parallel decay half-lives of drug and metabolite, and AUC relationships that allowed interconversion metabolic intrinsic clearances to be estimated (Wagner et al., 1981; Ebling and Jusko, 1986; Ferry and Wagner, 1986; Aarons, 1987; Cheng and Jusko, 1990a,b,c, 1991, 1993a,b). These solutions mainly pertain to flow-limited substrates and lack consideration of transporters. Later modeling of 4-methylumbelliferone and its sulfate (Ratna et al., 1993; Chiba et al., 1998) and estrone and estrone sulfate, other compounds that undergo futile cycling, has evoked consideration of sinusoidal and canalicular transporters and revealed the influence of lack of substrate equilibration on futile cycling kinetics (Tan et al., 2001).

With intentions of defining the role of membrane transporters and enzymes, we had previously used the PBPK model that describes transporter- and enzyme-mediated processes, such as basolateral influx, efflux, canalicular secretion, and metabolism and allows one to appraise the significance of rate-limited pathways and transporter-enzyme interplay (de Lannoy et al., 1993; Liu and Pang, 2005; Shitara et al., 2006; Sun et al., 2006; Sun and Pang, 2010). However, most of the focus was on precursor-metabolite pairs that undergo irreversible metabolism, and AUCR{mi,P} of the formed metabolite was found to be modulated by eliminatory parameters of the precursor and not vice versa (de Lannoy et al., 1993; Pang et al., 2008) (Tables 1 and 2 when CLint,met = 0). Upon incorporation of interconversion between the precursor and metabolite and alternate metabolic pathways in present PBPK model, we provided analytical solutions and showed that, in the presence of futile cycling, the eliminatory parameters of the metabolite would affect the disposition of the precursor (Table 2). We unveiled two coefficients that can modulate the AUCR and AUCR{mi,P} in futile cycling. These are the effective metabolism coefficient, efm, and the effective recycling coefficient, em', which
serve as modulators of $CL_{\text{int.met}}^{\text{met}}$ and $CL_{\text{int.met}}^{\text{met}}$, respectively. When futile cycling is absent ($CL_{\text{int.met}}^{\text{met}}(\text{ml}) = 0$), $ef_m$ equals unity. With futile cycling, the $ef_m$ value is less than unity, and a lower $ef_m$ renders a more pronounced effect on precursor disposition. The value of $ef_m$' or

$$\frac{CL_{\text{int.met}}^{\text{met}} + CL_{\text{other}}}{CL_{\text{int.met}}^{\text{met}} + CL_{\text{other}}}$$

is not affected by the presence or absence of futile cycling. In the presence of futile cycling, $ef_m$ modifies $CL_{\text{int.met}}^{\text{met}}(\text{ml})$ and thus affects the disposition of the metabolite: a lower AUC(\text{mi},P) arises from a higher $ef_m$.

Experimentally, we illustrated the interplay between phase II excretion and sulfation and desulfation enzymes with data from Wistar rats as controls and TR rats as the Mrp2-deficient livers in liver perfusion studies. First, we identified changes in hepatic Mrp2 and Mrp3 as well as microsomal, desulfation activities but not in the protein expression of other transporters and sulfotransferases between the two rat strains (Fig. 2). The compensatory increase of Mrp3 protein in TR versus Wistar rats (Fig. 2) is consistent with other reports (Johnson et al., 2006), although the change was slightly lower. Changes of similar orders of magnitude were also observed for TR rats originating from the same laboratory, with Mrp3 ratios for TR versus Wistar rats varying from 6 to 12 (Johnson et al., 2006; Zamek-Gliszczynski et al., 2006). Our slightly lower ratio of 4.2 could conceivably be due to differences in sample handling and preparation or the variability inherent in the semiquantitative nature of Western blotting. Dramatic changes in perfusate decay and biliary excretion profiles of Wistar versus TR perfusion studies were observed (Fig. 3). As expected, biliary excretion of E217G and E23S17G was almost abolished in TR rats (Fig. 3, B and D; Table 3), confirming that Mrp2 is the predominant transporter for biliary excretion of both species (Morikawa et al., 2000). In the TR liver, absence of Mrp2 for the excretion of E217G and E23S17G that undergo futile cycling, together with the compensatory increase of Mrp3, diverted both E217G and E23S17G into reservoir perfusate that reached near plateau levels (Fig. 3C). Because of the absence of Mrp2 and abolition of the excretion pathway, most of the radioactivities associated with [3H]E217G and [3H]E23S17G were trapped in the TR rat liver (Table 3).

Modeling of the data proved to be challenging. Fitting of the model to the Wistar data was not that successful because of the complexity of futile cycling, unless the basolateral influx and efflux clearances were assigned. As shown by previous perfusion studies (Sun et al., 2006), the uptake mechanism of E217G was rapid, and the same was assumed for E23S17G because it was documented that 80% radioactivity of [3H]E23S17G appeared rapidly in bile within 15 min after intravenous dosing (Slikker et al., 1983) and simulations based on low $CL_{\text{in}}^{\text{met}}$ for E23S17G resulted in poor predictions. Moreover, no inhibition was found for E23S17G on the uptake of E217G in basolateral membrane vesicles prepared from male Sprague-Dawley rat livers (Vore and Hoffman, 1994).

The relationships extracted from the steady state (eqs. 1–3), together with the comparison of immunoblots, proved to be a useful strategy to provide parameters for the simulation of E217G and E23S17G profiles in perfusate, liver, and bile to match the experimental data (Fig. 3) for the extremely complex PBPK model (Fig. 1). The approach, although not ideal, offers an alternative to data fitting, which failed to converge or resulted in parameters associated with high S.D.s. The estimated parameters, when compared to those of Sun et al. (2006), revealed strain differences. Higher sulfation extents in Wistar versus Wag/Rij rat livers at 2 h (77 ± 6 versus 37 ± 5% dose; Table 4) (Sun et al., 2006) were noted. This may be explained by enhanced sulfation coupled with faster excretion of E23S17G by Mrp2.
in the Wistar versus Wag/Rij rat. The CL_{ef} values for Wistar and TR−
rat livers were estimated to be low and that for the Wag/Rij liver was
almost zero (Sun et al., 2006). These values were again very low
compared with the extremely rapid influx (CL_{in} values) assigned for
E_{217G} (Table 4). Data from the TR− rat model, however, supported
the notion that CL_{ef}(mi) was appreciable owing to the elevated Mrp3,
because accumulation of E_{3S17G} in perfusate was observed. A
discrepancy in the desulfation intrinsic clearances existed, estimated as
1.57 in vitro and 0.155 ex vivo (Table 4). This may be due to the
arbitrary assumption that the unbound fractions of E_{217G} and
E_{3S17G} in liver tissue are equal to those in perfusate and sinusoidal
blood. The actual binding could also differ between the Wistar and
TR− strains, affecting the apparent estimates of the secretory, meta-
bolic, and efflux clearances. The initial decay of E_{217G} in the per-
 fusate was slightly underestimated (Fig. 3A). This may be due to the
assumption in PBPK modeling that the tubing volume (∼20 ml or
∼10% reservoir volume) of the perfusion apparatus was negligible,
with the drug concentration in the tubing being identical to that in the
reservoir. However, for a highly cleared compound such as E_{217G},
the drug in the tubing may decay much faster that that in the reservoir
due to the lack of mixing with the returning perfusate. Hence, the
PBPK model that does not consider the tubing as a separate compart-
ment may slightly underestimate the decay.

The analytical solutions, the experimental data, and model simula-
tions do support the view that a reduction in the excretion of E_{217G}
and E_{3S17G} by Mrp2 affects futile cycling kinetics and reduces the
net metabolism of E_{217G}. Ordinarily, metabolite parameters would
not have an impact on parent drug processing (Pang et al., 2008).
However, metabolite and drug parameters do affect the net metabo-
limism, via the coefficient, e_{in}^−, when futile cycling exists (Table 2).
Moreover, with a reduction in excretory activities toward the meta-
bolite and drug, both e_{in}^− and e_{in} would be reduced (cf. Wistar and
TR− rats) (Table 4). Under these conditions, both drug and metabolite
AUC values would increase. In fact, a pseudo-steady state emerges
with futile cycling. The shutdown of biliary excretion of both E_{217G}
and E_{3S17G} in the absence of Mrp2 reduced both excretory clear-
ance to almost zero and the total liver clearance (∼0); the net forma-
tion of E_{3S17G} from E_{217G} was reduced. This first demonstration
allows a deeper understanding of the transporter-enzyme interplay of
precursor-product relationships and how metabolite excretion exerts
an impact on the net metabolism of the parent drug. The interplay
between transporter and enzyme in drug and metabolite processing
that was described as a see-saw phenomenon for irreversible meta-
bolic events (Liu and Pang, 2005) has now been extended for futile
cycling.

Appendix

Mass Balance Equations for a PBPK Model

In eqs. A1 to A10, D and Mi denote concentrations of the parent
drug ( representing E_{17G}) and its formed metabolite (representing
E_{3S17G}, respectively. Q_{lt}, V, and f denote the blood flow rate, the
volume in each compartment, and unbound fraction, respectively. The
subscripts R, LB, L, and bile denote reservoir blood, liver blood/
sinusoids, liver tissue, and canicular bile, respectively. Other param-
eters (intrinsic clearances) have been defined under Materials and
Methods (see also Fig. 1).

For rates of change of E_{17G} and E_{3S17G} in reservoir blood,
\[ \frac{dD_{R}}{dt} = Q_{lt}(D_{LB} - D_{R}) \] (A1)

For rates of change of E_{217G} and E_{3S17G} in liver blood or the
sinusoid,
\[ \frac{dM_{LB}}{dt} = Q_{lt}(D_{R} - D_{LB}) + f_{1i}CL_{ef}(D_{L} - f_{1a}CL_{in}D_{LB}) \] (A3)

where,
\[ f_{1a} = f_{p} \frac{1}{Hct} + \frac{D_{RB}/D_{P} \times Hct}{1 - Hct} \]

Het is the hematocrit, f_{p} is the unbound fraction of the parent
drug in plasma. D_{RB}/D_{P} is the drug concentration ratio between red blood
cells (RBC) and plasma (p);
\[ V_{LB} \frac{dM_{LB}}{dt} = Q_{lt}(D_{R} - D_{LB}) + f_{1i}[Mi]CL_{ef}(Mi) \]
\[ - f_{1a}[Mi]CL_{in}(Mi)M_{LB} \] (A4)

For rates of change of E_{217G} and E_{3S17G} in liver tissue,
\[ V_{L} \frac{dD_{L}}{dt} = f_{1i}CL_{in}D_{LB} + f_{1}[Mi]CL_{in}^{mi}D_{L} \]
\[ - f_{1i}[Mi]CL_{in}^{mi}D_{L} \] (A5)

\[ V_{L} \frac{dM_{L}}{dt} = f_{1}[Mi]CL_{in}^{mi}D_{LB} + f_{1}[Mi]CL_{in}^{mi}D_{L} \]
\[ + CL_{int,sec}^{mi}[Mi] \] (A6)

For rates of change of E_{17G} and E_{3S17G} in canicular bile
(subscript bile),
\[ V_{bile} \frac{dD_{bile}}{dt} = f_{L}CL_{int,sec}D_{L} - Q_{bile}D_{bile} \] (A7)

\[ V_{bile} \frac{dM_{bile}}{dt} = f_{L}[Mi]CL_{int,sec}[Mi] - Q_{bile}M_{bile} \] (A8)

For rates of change of the amounts of E_{217G} and E_{3S17G} in
collected bile samples (subscript e),
\[ \frac{dA_{e}}{dt} = Q_{bile}D_{bile} \] (A9)

\[ \frac{dA_{e}[mi,P]}{dt} = Q_{bile}M_{bile} \] (A10)

where dA_{e} and dA_{e}[mi,P] denote the cumulative secreted amount
of parent drug and the formed metabolite, respectively.

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