Altered Hepatobiliary Disposition of Acetaminophen Glucuronide in Isolated Perfused Livers from Multidrug Resistance-Associated Protein 2-Deficient TR− Rats

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Accepted for publication July 24, 2000 This paper is available online at http://www.jpet.org

ABSTRACT
Previous studies have demonstrated that phenobarbital treatment impairs the biliary excretion of acetaminophen glucuronide (AG), although the transport system(s) responsible for AG excretion into bile has not been identified. Initial studies in rat canalicular liver plasma membrane vesicles indicated that AG uptake was stimulated modestly by ATP, but not by membrane potential, HCO3−, or pH gradients. To examine the role of the ATP-dependent canalicular transporter multidrug resistance-associated protein 2 (Mrp2)/canalicular multispecific organic anion transporter (cMOAT) in the biliary excretion of AG, the hepatobiliary disposition of acetaminophen, AG, and acetaminophen sulfate (AS) was examined in isolated perfused livers from control and TR− (Mrp2-deficient) Wistar rats. Mean bile flow in TR− livers was ~0.3 μl/min/g of liver (~4-fold lower than control), AG biliary excretion was decreased (>300-fold) to negligible levels in TR− rat livers, indicating that AG is an Mrp2 substrate. Similarly, AS biliary excretion in TR− rats was decreased (~5-fold); however, concentrations were still measurable, suggesting that multiple mechanisms, including Mrp2-mediated active transport, may be involved in AS biliary excretion. AG and AS perfusate concentrations were significantly higher in livers from TR− compared with control rats. Pharmacokinetic modeling of the data revealed that the rate constant for basolateral egress of AG increased significantly from 0.028 to 0.206 min−1, consistent with up-regulation of a basolateral organic anion transporter in Mrp2-deficient rat livers. In conclusion, these data indicate that AG biliary excretion is mediated by Mrp2, and clearly demonstrate that substrate disposition may be influenced by alterations in complementary transport systems in transport-deficient animals.

Acetaminophen glucuronide (AG), a monovalent organic anion formed in hepatocytes, is excreted into bile and undergoes basolateral egress from the hepatocyte into blood. AG excretion in bile accounts for ~7% of the administered acetaminophen (APAP) dose (100 mg/kg) in rats in vivo (Brouwer and Jones, 1990) and ~10% in the isolated perfused rat liver at equivalent APAP concentrations (Studenberg and Brouwer, 1992). Approximately 50% of AG formed in hepatocytes is excreted in bile; the remainder traverses the basolateral membrane and undergoes renal elimination. Phenobarbital, a common enzyme-inducing agent, significantly increases AG formation, but impairs AG biliary excretion ~3–6-fold in rats in vivo (Brouwer and Jones, 1990) and in the isolated perfused rat liver (Studenberg and Brouwer, 1992). Phenobarbital (or a phenobarbital metabolite) may impair AG biliary excretion by interacting with the carrier system responsible for AG transport across the canalicular membrane. However, the transport mechanism(s) for AG biliary excretion has not been identified.

Canalicular transport systems are responsible for the concentrative biliary excretion of many organic anions. In general, these transporters are temperature dependent, saturable, exhibit cis-inhibition and trans-stimulation, and may be inhibited by competing substrates for the carrier systems. At least one distinct ATP-dependent canalicular carrier for non-

ABBREVIATIONS: AG, acetaminophen glucuronide; APAP, acetaminophen; Mrp2, multidrug resistance-associated protein 2; cMOAT, canalicular multispecific organic anion transporter; bLPM, basolateral liver plasma membrane; cLPM, canalicular liver plasma membrane; AS, acetaminophen sulfate; TC, taurocholate; KHCO3, potassium bicarbonate; KpAG, rate constant for the basolateral egress of AG; KOTHER, first-order rate constant for all elimination pathways other than the formation of AG and AS; KMAS, Michaelis-Menten constant for AS formation; K2AG, rate constant for the canalicular egress of AG; K2AS, rate constant for the canalicular egress of AS; VmaxAS, maximum velocity for AS formation; CLAS, clearance of AS from perfusate to hepatocytes; kaoAG, first-order rate constant for AG formation; V, apparent volume of distribution of APAP; KpAS, rate constant for the basolateral egress AS; Vp, volume of perfusate; Mrp3, multidrug resistance-associated protein 3.
bile acid organic anions [multidrug resistance-associated protein 2 (Mrp2); canalicular multispecific organic anion transporter (cMOAT)] has been identified. The functional significance of Mrp2/cMOAT was characterized initially as a hereditary deficiency in the biliary excretion of conjugated bilirubin in mutant TR− Wistar rats (Jansen et al., 1985). Subsequently, TR− rats, which are deficient in Mrp2, have been used to identify many Mrp2 substrates (Oude Elferink et al., 1995). The prevailing hypothesis regarding Mrp2 substrates is that they must be di- or multivalent organic anions. However, two monovalent organic anions, 1-naphthol-glucuronide and estradiol 17-β-D-glucuronide, have been characterized as Mrp2 substrates (de Vries et al., 1989; Cui et al., 1999). Kobayashi et al. (1991) suggested that p-nitrophenyl glucuronide, another monovalent organic anion, may be an Mrp2 substrate. Several ATP-independent canalicular transport systems also have been suggested for nonbile acid organic anions. Some substrates such as dinitrophenyl glutathione and bilirubin diglucuronide may be transported by both ATP-dependent and membrane potential-dependent transport systems (Inoue et al., 1984; Kobayashi et al., 1990; Nishida et al., 1992; Ballatori and Truong, 1995). Meier et al. (1985) postulated that canalicular translocation of organic anions may be facilitated by exchange with bicarbonate ion. In addition, pH gradient-sensitive transport has been reported in basolateral liver plasma membrane (bLPM) (Hugentobler and Meier, 1986) and canalicular liver plasma membrane (cLPM) vesicles (Ziegler et al., 1994).

The use of animals that are deficient in a specific transport protein has been growing in popularity for drug disposition studies. An underlying assumption with this approach is that altered disposition is related directly to the defective transport protein. However, data interpretation may be confounded by indirect alterations in complementary transport systems. Pharmacokinetic modeling may be a useful tool to identify alterations in discrete transport processes that may not be evident based on mass balance analysis.

The main purpose of the present investigation was to examine the role of Mrp2 in the biliary excretion of AG based on initial studies that examined the effects of ATP, membrane potential, HCO3−, and pH gradients on the uptake of AG into cLPM vesicles. Basolateral translocation and biliary excretion of AG and AS were examined in isolated perfused livers from Mrp2-deficient TR− rats. A pharmacokinetic model was developed to describe alterations in the discrete processes associated with the hepatobiliary transport of AG and AS.

Materials and Methods

Chemicals. APAP and taurocholate (TC) were purchased from Sigma Chemical Co. (St. Louis, MO). AG and AS were gifts from McNeil Pharmaceuticals (Fl. Washington, PA). [3H]AG (12.4 Ci/mmol) was synthesized by Amersham Life Sciences (Buckinghamshire, England) and was >99% pure as determined by HPLC. [3H]TC (2.4 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA). All other chemicals were of analytical reagent grade.

Animals. Male Sprague-Dawley rats (~250 g; Charles River Laboratories; Raleigh, NC) were used to prepare liver plasma membrane vesicles. For isolated perfused liver studies, male Wistar rats (240–255 g; Charles River Laboratories) and TR− rats (240–265 g; breeding colony of the Academic Medical Center, Amsterdam, The Netherlands) were used as liver donors. Male retired breeders (Wistar; Charles River Laboratories) were used as blood donors. Rats were maintained on a 12-h light/dark cycle. Access to rat chow and water was allowed ad libitum. Rats were allowed to acclimate for at least 5 days before experimentation. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures.

Preparation of Rat Liver Plasma Membrane Vesicles. Livers (~40 g) from four rats were minced and homogenized in 720 ml of 1 M sodium bicarbonate/0.1 M phenylmethylsulfonyl fluoride (pH 7.4) with a loose-fitting Dounce tissue grinder. The homogenate was filtered through cheesecloth and centrifuged at 1500g for 15 min. The resulting pellet was mixed with 2.2 volumes of 70% sucrose. The mixture was overlaid with discontinuous sucrose gradients (44%/36.5%; 8 ml/9 ml) and centrifuged at 39,500g for 1.5 h. The mixed LPM at the 44%/36.5% interface was collected. Separation of cLPM and bLPM subfractions was performed as described by Meier and Boyer (1990). The resulting purified cLPM and bLPM fractions were resuspended in standard membrane suspension buffer [250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), and 0.2 mM CaCl2], homogenized, and stored at ~80°C. The specific activities of Na+,K+ ATPase, Mg2+-ATPase, Mg2+-ATPas, and alkaline phosphatase were determined for each preparation according to Schoner et al. (1967) and Bessey et al. (1946). Glucose-6-phosphatase (microsomal marker) was assessed by monitoring production of inorganic phosphate (Fiske and Subbarow, 1925). Mitochondrial contamination (sucinate dehydrogenase) was determined by the method of Shephard and Hubscher (1969). Vesicle orientation was assessed by measuring sialic acid liberation from membrane vesicles (Warren, 1959; Steck and Kant, 1974).

Measurement of Solute Transport in cLPM Vesicles. Frozen membrane suspensions were thawed quickly in a 37°C water bath, and passed repeatedly (15 times) through a 27-gauge needle. [3H]TC was used as a positive control to verify integrity of ATP-dependent and membrane potential-dependent solute uptake by cLPM vesicles. In studies where preloading of the vesicles with potassium thiocyanate or potassium bicarbonate (KHC03) was required, the compound was added before revesiculation of the thawed membranes. Uptake of [3H]TC and [3H]AG into cLPM vesicles was measured by a rapid Millipore filtration system (Millipore Corp.; Bedford, MA). Aliquots of membrane suspensions (20 µl; 30–60 µl of protein for TC; 60–100 µg of protein for AG) were preincubated for 5 min at 37°C, and uptake was initiated by the addition of 80 µl of prewarmed incubation medium to the membrane suspensions. The incubation buffer for AG transport consisted of 250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), and 0.2 mM CaCl2. In studies examining the effects of ATP on solute transport, the incubation buffer also contained an ATP-regenerating system (10 mM phosphocreatine, 100 µg/ml creatine phosphokinase, and 10 mM MgCl2) with or without ATP. Nonspecific binding of [3H]TC and [3H]AG was determined in each experiment. These values were subtracted from all determinations.

Isolated Perfused Liver Experiments. Livers were isolated and perfused by standard techniques (Brouwer and Thurman, 1996). After anesthesia (ketamine, 60 mg/kg, and xylazine, 12 mg/kg i.p.), the bile duct and portal vein were cannulated and the liver was perfused in situ with oxygenated Krebs-Ringer bicarbonate buffer maintained at 37°C. After the liver was transferred to a 37°C perfusion chamber, perfusion was continued with 80 ml of recirculating oxygenated Krebs-Ringer bicarbonate buffer containing 1% dextrose (w/v) and 20% (v/v) heparinized (1000 U/ml) male rat blood at a constant flow rate of 20 ml/min. A constant infusion of sodium taurocholate (30 µmol/h) was delivered into the perfusate reservoir at a rate of 2 ml/h. The liver and perfusate were allowed to equilibrate for ~15 min before administration of APAP (10 mg in 1 ml). The liver was perfused for 120 min. Bile was collected continuously and 0.5 ml of perfusate samples was collected at 15-min intervals. After the 120-min perfusion period, livers were removed from the chamber, blotted, and weighed. All samples were frozen at ~20°C until assayed. Liver viability was determined by bile flow rate (<0.8
and >0.13 μL/min/g of liver for control and TR rats, respectively), constant inflow perfusion pressure (<15 cm of H2O), and the release of lactate dehydrogenase from the liver (<0.1 I.U./g of liver/h; assayed with Sigma lactate dehydrogenase diagnostic kit, catalog 500).

**Assay Methodology.** APAP, AG, and AS concentrations in perfusate and bile were quantitated by the HPLC method of Brouwer and Jones (1990). Standard curves for APAP, AG, and AS were prepared in perfusate (5–500 μg/ml) and bile (25–1000 μg/ml) linear (r ≥ 0.997) and were prepared daily. The lower limit of quantitation for APAP, AG, and AS was 2 μg/ml. AG and AS concentrations were expressed as APAP equivalents by determining the conversion factors (0.374 for AG and 0.544 for AS) as described by Brouwer and Jones (1990).

**Pharmacokinetic Modeling and Simulations.** A compartmental modeling approach was used to describe the hepatobiliary disposition of APAP, AG, and AS in the isolated perfused rat liver. The perfusate concentration versus time data were analyzed by a one-compartment model; the elimination rate constant for APAP initially estimated was used. The slope of the terminal phase of the log-concentration versus time profile. APAP was assumed to be eliminated from the system via the formation of AG, AS, and a non-AG/AS pathway, including the biliary excretion of APAP. Rate constants for the formation of AG and AS, as well as the non-AG/AS pathway, were estimated based on the amount of each metabolite recovered at each end of the perfusion. AG and AS biliary excretion rates were calculated as the product of bile flow and biliary concentration during each collection period. To describe the disposition of AG and AS, the liver, perfusate, and bile were considered as three distinct compartments. Differential equations based on the mass balance of APAP, AG, and AS in each compartment were resolved simultaneously by nonlinear least-squares regression (WinNonlin 1.1; Pharsight Corporation, Mountain View, CA). A weighting scheme of 1/(Y(predicted)^2) was used. The apparent volume of distribution for APAP was restricted within a physiologically meaningful range (85–110 ml) and was based on the perfusate volume plus the estimated distribution volume of APAP in the liver assuming a liver/body partition coefficient of ~1.5 (Ara and Ahmad, 1980). Various models using linear and saturable processes were fit to the data. The goodness of fit of each model was assessed by visual examination of the distribution of residuals, the condition number, and Akaike's information criterion (Akaide, 1976).

The cumulative excretion of AG in bile, and perfusate AG concentration versus time profiles, were simulated with the model that best described the hepatobiliary disposition of APAP, AG, and AS in isolated perfused livers from control rats to examine the possibility impaired biliary excretion of AG in isolated perfused TR rat livers was due solely to an increase in the basolateral egress of AG, instead of to a deficiency in Mrp2-mediated biliary excretion. The basolateral egress rate constant for AG (K_PAG) was increased until the simulated cumulative biliary excretion of AG approximated the actual mass excreted in bile, and the impact on the appearance of AG in perfusate was examined. Simulations of the perfusate AG concentration versus time profiles with the model that best described the hepatobiliary disposition of APAP, AG, and AS in isolated perfused TR rat livers were conducted to test the hypothesis that the increased basolateral egress of AG in isolated perfused TR rat livers was due to up-regulation of the rate process and not due solely to an increased driving force because AG biliary excretion was impaired. In these simulations, K_PAG was fixed at the mean value estimated for control livers, and the impact on the appearance of AG in perfusate was assessed.

**Statistics.** Two-way ANOVA tables were constructed to examine the effects of time and ATP concentration on AG uptake in rat cLPM vesicles. When significant effects were noted with no interactions between independent variables, one-way ANOVA was performed to examine the effects of a single factor on AG uptake. The Student's t test was used to determine statistically significant differences between the control and TR livers with respect to the total amount of each metabolite recovered in perfusate and bile, and the model parameter estimates. In all cases, data were presented as mean ± S.D. and the criterion for statistical significance was P < .05.

**Results**

**cLPM Vesicle Uptake Studies.** cLPM fractions were enriched ~37- and ~59-fold in the canalicular membrane markers alkaline phosphatase and Mg2+-ATPase, respectively; enrichment of the basolateral membrane marker Na+,K+-ATPase was ~4-fold. The low enrichment of marker enzymes glucose-6-phosphatase (~0.7) and succinate dehydrogenase (~0.03) indicated minimal microsomal and mitochondrial contamination of the cLPM fractions, respectively. Assessment of sialic acid liberation from canalicular membranes indicated that 81.9 ± 6.4% of cLPM vesicles were oriented right-side out.

Uptake of 1 μM [3H]TC into cLPM vesicles was stimulated markedly by 1 mM ATP; uptake exceeded equilibrium values at early time points in the presence of ATP (data not shown). These data demonstrate functional integrity of ATP-dependent solute transport in cLPM vesicles. Accumulation of [3H]AG in cLPM vesicles was temperature dependent, but did not exceed equilibrium values in the absence and presence of ATP. [3H]AG uptake increased significantly with both duration of uptake (P < .0001) and ATP (P < .0012; two-way ANOVA). The effect of ATP on [3H]AG uptake was most evident at 5 and 8 min (P = .0290, P = .0189, respectively; one-way ANOVA) (Fig. 1). Uptake of 1 μM [3H]TC in cLPM vesicles was stimulated by an inside-positive diffusion potential generated by preloading the vesicles with potassium thiocyanate followed by incubation of the vesicles with potassium gluconate (data not shown). [3H]TC uptake at 20 s was significantly greater than equilibrium values (60 min) (P < .0337; one-way ANOVA). In contrast, uptake of 5 μM [3H]AG in the same cLPM preparations was not stimulated by an inside-positive diffusion potential. To determine whether [3H]AG uptake was facilitated by exchange with bicarbonate, cLPM vesicles were preloaded with KHCO3, or either KHCO3 (control) or potassium gluconate (treated) was added to the incubation medium with [3H]AG. Uptake of 5 μM [3H]AG in cLPM vesicles was not stimulated by an outwardly directed bicarbonate gradient (Table 1). In additional stud-

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*Fig. 1. Time course and temperature dependence of [3H]AG uptake in rat cLPM vesicles incubated with 5 μM [3H]AG in the absence (open symbols) or presence (closed symbols) of 1 mM ATP in incubation medium containing an ATP-regenerating system at either 4°C [●, ■] or 37°C [●, ○]. [3H]AG uptake was influenced significantly by time (P < .0001) and by treatment (P < .0012). Values represent mean ± S.D. (n = 3); *P < .05.
between the control and TR. Total recovery of AG and AS was not significantly different per fusion was below the limit of quantitation. Although the concentration of APAP in the perfusate at the end of the 2-h amount of APAP recovered in bile was very low, and the concentration of APAP in the perfusate at the end of the 2-h perfusion is similar. The bile flow rate in TR rats was significantly higher, and the percentage of the APAP dose recovered as AG and AS in bile was significantly lower in TR rats. The percentage of the APAP dose recovered as APAP, AG, and AS in perfusate and bile during the 2-h perfusion is best described the disposition of APAP, AG, and AS in isolated perfused rat liver. Processes characterized by \( K_{\text{OTHER}} \) and \( K_{\text{BAG}} \), were excluded in isolated perfused rat livers. Parameters are designated as follows: \( K_{\text{AG}} \), first order rate constant for AG formation; \( K_{\text{AS}} \) and \( V_{\text{max AS}} \), Michaelis-Menten constant and maximum velocity for AS formation; \( K_{\text{OTHER}} \), first order rate constant for all elimination pathways other than the formation of AG and AS; \( K_{\text{PAG}} \) and \( K_{\text{PAS}} \) rate constants for the basolateral egress of AG and AS, respectively; \( K_{\text{BAG}} \) and \( K_{\text{BAS}} \) rate constants for the canalicular egress of AG and AS, respectively; CL\_AS, clearance of AS from perfusate to hepatocytes; \( V \), apparent volume of distribution of APAP; and \( V_R \), volume of perfusate.

**TABLE 1**

Influence of bicarbonate and pH gradients on [3H]AG uptake in cLPM vesicles

<table>
<thead>
<tr>
<th>Bicarbonate gradient ( ^* )</th>
<th>AG Uptake (pmol/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>20 s</td>
</tr>
<tr>
<td>Control</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>Treated</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>Inside/outside pH ( ^b )</td>
<td>7.4/6.0</td>
</tr>
<tr>
<td></td>
<td>7.4/7.4</td>
</tr>
<tr>
<td></td>
<td>7.4/8.0</td>
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</tbody>
</table>

\( ^* \) Rat cLPM vesicles were preloaded with standard membrane suspension buffer and 100 mM KHCO\(_3\). Standard incubation buffer contained 100 mM KHCO\(_3\) (control) or 100 mM potassium gluconate (treated). Stop solution contained 250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), 0.2 mM CaCl\(_2\), and 100 mM potassium gluconate. 

\( ^b \) Rat cLPM vesicles were preloaded with standard membrane suspension buffer (pH 7.4) and incubated with standard incubation buffer at pH 6.0, 7.4, or 8.0.

**TABLE 2**

Hepatobiliary disposition of APAP, AG, and AS in the isolated perfused rat liver

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TR ( ^* )</th>
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<tbody>
<tr>
<td>Bile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% AG</td>
<td>13.5 ± 0.9</td>
<td>0.04 ± 0.01 ( ^* )</td>
</tr>
<tr>
<td>% AS</td>
<td>4.43 ± 0.31</td>
<td>0.85 ± 0.24 ( ^* )</td>
</tr>
<tr>
<td>% APAP</td>
<td>1.21 ± 0.92</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Per fusate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% AG</td>
<td>15.3 ± 5.5</td>
<td>26.2 ± 3.6 ( ^* )</td>
</tr>
<tr>
<td>% AS</td>
<td>47.1 ± 7.1</td>
<td>99.8 ± 7.8 ( ^* )</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% AG</td>
<td>28.7 ± 5.0</td>
<td>26.2 ± 3.6</td>
</tr>
<tr>
<td>% AS</td>
<td>52.2 ± 7.4</td>
<td>60.7 ± 7.9</td>
</tr>
</tbody>
</table>

\( ^* \) Mean ± S.D., \( n = 4 \) for control group, \( n = 5 \) for TR \( ^* \) group.

\( ^b \) The amount of APAP in perfusate was below the limit of detection at 2 h.

\( ^* \) \( P < 0.05 \), control vs. TR.

**Fig. 2.** Model scheme depicting the disposition of APAP, AG, and AS in the isolated perfused rat liver. Processes characterized by \( K_{\text{OTHER}} \) and \( K_{\text{BAG}} \), were excluded in isolated perfused rat livers. Parameters are designated as follows: \( K_{\text{AG}} \), first order rate constant for AG formation; \( K_{\text{AS}} \) and \( V_{\text{max AS}} \), Michaelis-Menten constant and maximum velocity for AS formation; \( K_{\text{OTHER}} \), first order rate constant for all elimination pathways other than the formation of AG and AS; \( K_{\text{PAG}} \) and \( K_{\text{PAS}} \) rate constants for the basolateral egress of AG and AS, respectively; \( K_{\text{BAG}} \) and \( K_{\text{BAS}} \) rate constants for the canalicular egress of AG and AS, respectively; CL\_AS, clearance of AS from perfusate to hepatocytes; \( V \), apparent volume of distribution of APAP; and \( V_R \), volume of perfusate.

**Fig. 3.** Mean simulated perfusate concentration versus time profiles for AG in isolated perfused livers from control rats. The mean data well-described the mean data well (Fig. 4, A and B). When \( K_{\text{VAG}} \) was increased 10-, 100- and 1000-fold, the cumulative biliary excretion of AG decreased from 13% to 2.5%, 0.26% and way (\( K_{\text{OTHER}} \)) were described best as first order processes, and AS formation was described best as a saturable process. The Michaelis-Menten constant for AS formation (\( K_{\text{PAS}} \)) was assigned a value of 17 \( \mu \)g/ml based on previous work (Watari et al., 1983; Lin and Levy, 1986; Tone et al., 1990). Data from TR rats were best described by a model that excluded both the non-AG/AS elimination pathway for APAP (characterized by \( K_{\text{OTHER}} \)) and AG biliary excretion (\( K_{\text{BAG}} \)), because the biliary excretion of unchanged APAP and the formation of other metabolites, as well as the amount of AG excreted in bile, were negligible. Observed data points and model-generated best fit curves of APAP, AG, and AS perfusate concentration versus time profiles, and AG and AS biliary excretion rate versus time profiles from representative control and TR rats, are shown in Fig. 3. APAP perfusate concentration versus time profiles in control and TR rats were similar. However, AG and AS perfusate concentrations were higher, and AS biliary excretion rates were markedly lower, in livers from TR rats.

The pharmacokinetic parameters describing the hepatobiliary disposition of APAP, AG, and AS in control and TR rats are provided in Table 3. In TR rats, the rate constant for the basolateral egress of AG (\( K_{\text{PAG}} \)) was increased ~7-fold, and the rate constant for the biliary excretion of AS (\( K_{\text{BAS}} \)) was decreased ~10-fold. The \( V_{\text{max AS}} \) for AS formation (\( V_{\text{max AS}} \)) and the clearance of AS from perfusate to hepatocytes (CL\_AS) tended to increase in TR rats, but these trends were not statistically significant.

Based on the model depicted in Fig. 2, and using the mean values of the parameter estimates shown in Table 3, the simulated perfusate concentration versus time profiles for AG in isolated perfused livers from control and TR rats described the mean data well (Fig. 4, A and B). When \( K_{\text{VAG}} \) was increased 10-, 100- and 1000-fold, the cumulative biliary excretion of AG decreased from 13% to 2.5%, 0.26% and
Pharmacokinetic parameters describing the hepatobiliary disposition of APAP, AG, and AS were expressed as APAP equivalents. The amount of AG in bile was negligible in TR− rats.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TR−</th>
</tr>
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<tbody>
<tr>
<td>( K_{\text{OTHER}} ) (min−1)</td>
<td>0.003 ± 0.001</td>
<td>N/A</td>
</tr>
<tr>
<td>( K_{\text{AG}} ) (min−1)</td>
<td>0.007 ± 0.001</td>
<td>0.010 ± 0.003</td>
</tr>
<tr>
<td>( V_{\text{max,AG}} ) (μg/ml/min/g of liver)</td>
<td>0.106 ± 0.007</td>
<td>0.143 ± 0.036</td>
</tr>
<tr>
<td>( K_{\text{BAG}} ) (min−1)</td>
<td>0.028 ± 0.004</td>
<td>0.206 ± 0.095*</td>
</tr>
<tr>
<td>( K_{\text{BAG}} ) (min−1)</td>
<td>0.027 ± 0.016</td>
<td>N/A</td>
</tr>
<tr>
<td>( K_{\text{PARAS}} ) (min−1)</td>
<td>0.115 ± 0.051</td>
<td>0.108 ± 0.058</td>
</tr>
<tr>
<td>( K_{\text{PARAS}} ) (min−1)</td>
<td>0.007 ± 0.003</td>
<td>0.0005 ± 0.0002*</td>
</tr>
<tr>
<td>( V_{\text{max,CL}} ) (ml/min/g of liver)</td>
<td>0.045 ± 0.013</td>
<td>0.132 ± 0.073</td>
</tr>
<tr>
<td>( V_{\text{m}} ) (ml)</td>
<td>92.8 ± 5.7</td>
<td>95.3 ± 8.7</td>
</tr>
</tbody>
</table>

### Discussion

Initial studies were conducted in rat cLPM vesicles. The specific activity and relative enrichment of membrane marker enzymes in cLPM preparations were consistent with literature values (Meier et al., 1984b). Taurocholate uptake was stimulated markedly by ATP in cLPM vesicles, consistent with previously published data (Müller et al., 1991; Nishida et al., 1991). AG uptake in cLPM vesicles was temperature dependent, and enhanced modestly by ATP (significant differences in AG uptake were noted at 5 and 8 min); however, AG uptake did not exceed equilibrium values at any time through 60 min. Similar results were reported for S-ethylglutathione (Ballatori and Truong, 1995). S-ethylglutathione uptake in cLPM vesicles exhibited minimal stimulation by ATP (10–20% above control values), and the stimulatory effects of ATP were evident only at later time points. Typical Mrp2 substrates such as dinitrophenylglutathione and bilirubin digluconide exhibit rapid initial uptake and extensive accumulation above equilibrium values in the presence of ATP at early time points; uptake declines at later times as ATP concentrations in the incubation medium decrease (Kobayashi et al., 1990; Nishida et al., 1992).

Temperature-dependent transport of AG in the absence of ATP in cLPM vesicles suggested that an ATP-independent canalicular transport mechanism also might be involved. Taurocholate was used as a positive control for ATP-independent canalicular transport mechanisms. As expected, taurocholate uptake in cLPM vesicles was stimulated by an inside-positive diffusion potential (Meier et al., 1984a). In contrast, AG uptake in cLPM vesicles was not stimulated by an inwardly directed positive diffusion potential. Alternate mechanisms for AG transport also were examined. Meier et al. (1985) reported electroneutral chloride/bicarbonate exchange in rat cLPM vesicles, and postulated that canalicular translocation of organic anions may be facilitated by a secondary exchange process with bicarbonate. However, AG uptake in cLPM vesicles was not facilitated by the presence of an outwardly directed bicarbonate gradient. Furthermore, changes in extravesicular pH over a range of 6.0 to 8.0 had no effect on AG uptake into cLPM vesicles with an intravesicular pH of 7.4.

Modest stimulation of AG uptake in cLPM vesicles by ATP was consistent with the hypothesis that AG is a low-affinity substrate for Mrp2, an ATP-dependent organic anion trans-
porter on the canalicular membrane. Rat cLPM vesicles may not be a practical system, from an animal consumption standpoint, for investigating transport mechanisms for low-affinity substrates due to a relatively low intra-to extravesicular concentration ratio. The TR− Wistar rat, an Mrp2/cMOAT-deficient mutant strain that exhibits an autosomal recessive defect in the biliary excretion of many multivalent organic anions (Jansen et al., 1985), was used to test the hypothesis that AG is an Mrp2 substrate. Molecular cloning of rat Mrp2 revealed that a one base-pair deletion at amino acid 393 introduces a premature stop codon in TR− rats, which results in decreased mRNA levels and complete absence of the protein from the canalicular membrane in the liver (Paulusma et al., 1996). Mrp2 is responsible for the biliary excretion of a variety of endogenous and exogenous organic anions, including many glucuronide conjugates, some glutathione conjugates and a few sulfate conjugates (Oude Elferink et al., 1995). The majority of Mrp2 substrates identified to date have at least two negative charges (Oude Elferink et al., 1995). AG is an organic anion with one negative charge at physiological pH. Although the formation of AG was not altered (Tables 2 and 3), the biliary excretion of AG in isolated perfused TR− rat livers decreased over 300-fold (from 13.5% to only 0.04% of the dose). Simulation studies were consistent with the hypothesis that this decrease in the biliary excretion of AG could not result solely from increased basolateral egress of AG. For the biliary excretion of AG to be reduced to the observed level (0.04% of the dose), the rate constant for the basolateral egress of AG (K_{PAG}) would need to be increased by three orders of magnitude. Not only is this increase in the rate constant unrealistic but also the simulated perfusate AG concentrations markedly overestimated the observed data (Fig. 4A). The negligible biliary excretion of AG in TR− rat livers, in conjunction with the simulation studies, clearly demonstrated that AG is an Mrp2 substrate, consistent with previous reports that multiple negative charges are not an absolute requirement for Mrp2 substrates (de Vries et al., 1989; Cui et al., 1999).

AS biliary excretion was significantly decreased in livers from TR− rats, whereas AS formation remained unchanged (Tables 2 and 3). The amount of AS recovered in bile was decreased ~5-fold, and the estimated rate constant for AS biliary excretion was decreased ~10-fold, in TR− rat livers, suggesting that AS also may be an Mrp2 substrate. AS has two negative charges and a molecular weight of 269, similar to other Mrp2 substrates. The fact that measurable concentrations of AS were detected in bile from TR− rat livers suggests that AS may traverse the hepatic canalicular membrane by multiple mechanisms.

The majority of the APAP dose administered in the present study was accounted for as glucuronide and sulfate conjugates; a small fraction is metabolized by P450 enzymes and further conjugated with glutathione (Jollow et al., 1974, Miner and Kissinger, 1979). Glucuronidation and sulfation are both saturable processes at low doses. In vivo studies in rats have revealed that the K_{m} and V_{max} for APAP glucuronidation are ~138 μg/ml and 417 μg/min/kg, respectively (Watari et al., 1983). The V_{max}/K_{m} ratio (3.02 ml/min/kg) is consistent with our estimate for the formation clearance of AG in control livers (2.64 ml/min/kg) based on K_{m,AG} and V values in Table 3 and the average body weight of control rats (246 ± 4 g). The K_{m} for in vivo APAP sulfation has been estimated independently by Watari et al. (1983), Lin and Levy (1986), and Tone et al. (1990), with mean values ranging from 15.5 μg/ml to 16.9 μg/ml. In the present single-dose study, the K_{m} for AS formation was assigned a value of 17 μg/ml, and V_{max,AS} was estimated based on the data.

Impaired AG and AS biliary excretion due to the absence of canalicular Mrp2 was not the only difference in the hepatobiliary disposition of APAP and metabolites between control and TR− rat livers. Increased basolateral egress of AG from the liver to perfusate (~7-fold increase in K_{PAG}) in TR− rat hepatocytes was indicative of elevated levels of an AG transporter on the basolateral membrane. Further simulations demonstrated that impaired AG biliary excretion alone could not account for the increased basolateral egress of AG in isolated perfused TR− rat livers. Although the final AG concentration in perfusate was similar, the simulated curves with K_{PAG} fixed at the control value significantly underestimated AG perfusate concentrations at early time points (Fig. 4B). Hirohashi et al. (1998) reported that Mrp3 was up-regulated in Mrp2-deficient Eisai hyperbilirubinemic rats and by bile duct ligation in Sprague-Dawley rats. König et al. (1999) have reported up-regulation of a basolateral MRp isofrom (MRP3) in patients who are deficient in MRp2. Mrp3 shares some substrates with Mrp2, such as E3040 glucuronide, 4-methylumbelliferone glucuronide, 1-naphthol-β-glucuronide, and methotrexate (Hirohashi et al., 1999). In addition, Ogawa et al. (2000) reported a 10.6-fold increase in the expression of Mrp3 protein in Eisai hyperbilirubinemic rat livers, which is in close agreement with the 7-fold increase in K_{PAG} observed in the present study. Increased basolateral egress of AG in TR− rat livers would be consistent with the hypothesis that AG is a substrate for basolateral Mrp3.

The application of pharmacokinetic modeling in this study provided important insights regarding the hepatobiliary disposition of APAP, AG, and AS that were not evident by mass balance analysis. By comparing the percentage of the APAP dose recovered as AG and AS in perfusate and bile (Table 2), it was not possible to determine whether differences in AG and AS recovery in control and TR− rat livers were due to changes in transporter function in the canalicular membrane alone or due to changes in transporter function in both basolateral and canalicular membrane domains. Pharmacokinetic modeling provided estimates of rate constants for the formation and egress of AG and AS across the basolateral and canalicular membrane domains. Pharmacokinetic modeling and simulation studies emphasize the importance of recognizing potential alterations in substrate disposition at other sites when evaluating data generated in transport-deficient animal models.
Acknowledgment

We thank Dr. Gary Pollack for insightful suggestions regarding the pharmacokinetic modeling and simulations, and for constructive critique of this manuscript.

References


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