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Mechanisms of harmol sulfate biliary excretion in mice and rats

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Nonstandard abbreviations: harmol sulfate (HS), multidrug resistance-associated protein (Mrp, *Abcc*), breast cancer resistance protein (Bcrp, *Abcg2*), isolated perfused liver (IPL).

Abstract:

Previous experiments demonstrated that the biliary excretion of harmol sulfate (HS) was mediated by Bcrp, and not by Mrp2 or P-glycoprotein in mice. However, recent reports suggested that species differences in hepatic canalicular transport mechanisms for a given substrate exist between mice and rats. In the present study, biliary excretion of HS was examined in perfused livers from mice and rats in the absence or presence of the P-glycoprotein and Bcrp inhibitor, GF120918. As expected, in mouse liver perfusions the biliary excretion of HS was decreased ~3.5-fold by GF120918, consistent with previous reports of Bcrp-mediated HS biliary excretion. However, despite sufficient hepatic unbound concentrations of GF120918 to achieve extensive inhibition of Bcrp, the biliary excretion of HS was not decreased significantly in rats (50 ± 12 vs. 41 ± 6 %). In summary, biliary excretion of HS was mediated by a GF120918-sensitive mechanism in mice, previously elucidated as Bcrp. In contrast the pathway responsible for HS biliary excretion in rats was not impaired by GF120918. Thus, transport mechanism(s) responsible for harmol sulfate biliary excretion appear to differ between mice and rats.

Introduction:

Sulfate conjugates formed in the liver subsequently undergo excretion into sinusoidal blood or bile. These hydrophilic conjugates require carrier-mediated transport to traverse the hepatic plasma membrane. Transport processes responsible for hepatic excretion of metabolites can be critically important to drug disposition, toxicity, and pharmacologic effect, because these events dictate the rate and directionality (blood vs. bile) of hepatic excretion. For example, formation of troglitazone sulfate exposes the hepatocyte to a potent inhibitor of the bile salt export pump, resulting in increased hepatic concentrations of toxic bile acids (Funk et al., 2001). In rats deficient in the multidrug resistance-associated protein (Mrp) 2, biliary excretion of troglitazone sulfate is impaired, with a consequent increase in serum bilirubin (Kostrubsky et al., 2001). Likewise, modulation of transport proteins responsible for the hepatic excretion of pharmacologically-active metabolites may have pharmacodynamic implications. In mice deficient in basolateral Mrp3, for example, systemic concentrations of morphine-6-glucuronide were reduced, resulting in a decreased antinociceptive response (Zelcer et al., 2005). Thus, transport mechanisms responsible for hepatic excretion of metabolites are important not only for drug elimination, but in certain cases they also are an important determinant of therapeutic or toxic effects.

Biliary excretion of metabolites has been investigated most extensively in naturally-occurring Mrp2-deficient rats [reviewed in Zamek-Gliszczynski et al. (2006b)]. In this animal model, biliary excretion of glucuronide and glutathione conjugates was reduced to negligible levels, implicating a major role for Mrp2 in their disposition. In contrast, biliary excretion of many sulfate metabolites was impaired only partially in Mrp2-deficient rats. The non-Mrp2-mediated component of sulfate metabolite biliary excretion in rats for several

compounds was mediated by breast cancer resistance protein (Bcrp). Therefore, in rats, Mrp2 is primarily responsible for the biliary excretion of phase II conjugates, whereas Bcrp also participates in transporting sulfate metabolites across the hepatic canalicular membrane. Suzuki et al. (2003) reported that recombinant human BCRP efficiently transported sulfate metabolites, and to a lesser extent glucuronide and glutathione conjugates. Bcrp-knockout mice did not excrete the sulfate metabolites of acetaminophen, 4-methylumbelliferone, and harmol into bile, and exhibited impaired biliary excretion of the glucuronide conjugates (Zamek-Gliszczynski et al., 2006c). In contrast Mrp2-knockout mice did not exhibit impaired biliary excretion of sulfate conjugates, and the biliary excretion of only some glucuronide conjugates was decreased modestly.

In the present study, species differences in the biliary excretion of harmol sulfate (HS) were examined in wild-type and TR^- Wistar rats, as well as in C57BL/6 mice in the absence or presence of the P-glycoprotein and Bcrp inhibitor, GF120918 (de Bruin et al., 1999). Harmol, a plant β-carboline alkaloid, is extracted efficiently by the liver and undergoes direct sulfation; subsequently, HS is excreted into bile and sinusoidal blood (Pang and Terrell, 1981; de Vries et al., 1985). Harmol is fairly lipophilic at physiologic pH ($logD_{pH7.4}$ = +1.1), and therefore is capable of diffusing passively into hepatocytes (Pang et al., 1994). In contrast, HS is hydrophilic ($logD_{pH7.4}$ = -1.0) and requires active transport out of the liver (de Vries et al., 1985; Zamek-Gliszczynski et al., 2006c).

Materials and Methods:

Harmol, taurocholate, and Krebs-Henseleit buffer packets were purchased from Sigma Chemical Co. (St. Louis, MO). Harmol sulfate was a kind gift of Dr. K. Sandy Pang (University of Toronto, Toronto, Canada). GF120918 [*N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] was donated by GlaxoSmithKline (Research Triangle Park, NC). All other chemicals were of reagent grade and were readily available from commercial sources.

Male C57BL/6 mice (26-34 g) were obtained from Taconic Farms (Germantown, NY). Male wild-type Wistar rats (280-315 g; Charles River Laboratories, Raleigh, NC) and male TR$^-$ Wistar rats (280-300 g; in-house breeding colony; breeding pairs obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used as liver donors. Male wild-type Wistar rats (> 400 g) were used as blood donors. All experimental procedures were performed under full anesthesia induced with ketamine/xylazine (rats: 60/12 mg/kg, mice: 140/8 mg/kg, i.p.). Rodents were maintained on a 12-hour light/dark cycle with free access to water and chow. The Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill approved all animal procedures.

Mouse livers were perfused in a single-pass manner *in situ* due to technical difficulties associated with mouse liver isolation for recirculating perfusions. Perfusions were conducted by cannulating the gallbladder, portal vein, and inferior vena cava above the liver. The liver was perfused in a single-pass manner through the portal vein (5 mL/min, continually-oxygenated Krebs-Henseleit buffer containing 5 μM taurocholate) with outflow through the inferior vena cava. Following an ~15-min pre-perfusion period for equilibration of liver temperature and bile flow, the liver was perfused with buffer containing 0.5 μM

harmol and 10 μM GF120918 for 60 min. Bile was collected at 10-min intervals; outflow perfusate was collected at 10-min (0-30 min) and 5-min (30-60 min) intervals. At the end of the perfusion, livers were isolated and frozen.

Recirculating isolated perfused rat liver (IPL) experiments were conducted by cannulating the bile duct and portal vein, and isolating the liver. Recirculating liver perfusion was conducted *ex situ* in a temperature-controlled chamber with 80 mL of Krebs-Henseleit buffer containing 20% (v:v) whole rat blood. Taurocholate was infused (30 μmol/hr) into the perfusate reservoir in order to maintain bile flow. GF120918 (8 μmol, 0.5 mL dimethylsulfoxide) or vehicle was added to the perfusate reservoir 5 min prior to the commencement of the harmol infusion (65 nmol/min, 30 min; infusion rate corrected for ~35% harmol loss to infusion tubing/IPL apparatus). The perfusion was continued for an additional 60 min after the end of the harmol infusion. Bile was collected continuously and sampled together with perfusate at 10-min intervals. Livers were frozen at the end of the perfusion.

Bile, perfusate, and liver homogenate samples were analyzed for harmol and HS concentrations by liquid chromatography with detection by tandem mass spectrometry (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface, MDS Sciex, Concord, ON, Canada). Harmol and HS in rat IPL samples, as well as the internal standard, cimetidine, were eluted from a Phenomenex Primesphere C18 column (2.0 x 30 mm, d_p = 5 μm) using a mobile phase gradient (A: 0.05% formic acid in 1% methanol, B: 0.05% formic acid in 99% methanol; 0-1 min hold at 0% B, 1-3.5 min linear gradient to 70% B, 3.5-4 min hold at 70% B, 4-4.1 min linear gradient to 0% B, 4.1-6.0 min linear gradient to 0% B, 3.1-4 min hold at 0% B; flow rate = 0.65 mL/min; 1-5 min directed to mass spectrometer) and were

detected in positive ion mode using multiple reaction monitoring: harmol: 199 → 131 m/z, HS: 279 → 199 m/z, cimetidine: 253 → 117 m/z. Analytes in mouse samples were eluted from an Aquasil C18 column (2.1 x 50 mm, d_p = 5 μm, Thermo Electron Corporation, Waltham, MA) using a mobile phase gradient (A: 0.05% formic acid, B: 0.05% formic acid in methanol; 0-0.75 min hold at 0% B, 0.75-2 min linear gradient to 70% B, 2-3.5 min hold at 70% B, 3.5-3.6 min linear gradient to 0% B, 3.6-4 min hold at 0% B; flow rate = 0.75 mL/min; 0.8-4 min directed to mass spectrometer) and were detected in positive ion mode: harmol: 199 → 131 m/z, cimetidine: 253 → 117 m/z, or negative ion mode: HS: 277 → 197 m/z, cimetidine: 251 → 157 m/z.

All data are reported as mean ± S.D., n = 3-5 per condition. Statistical significance was assessed by analysis of variance with Tukey's post-hoc test, except in those cases for which the groups being compared had unequal variances or a data set failed the normality test, when analysis of variance on ranks was used. In all cases, $p < 0.05$ was the criterion for statistical significance.

Results/Discussion:

Bile flow in mouse livers (0.85 ± 0.17 μL/min/g liver), as well as wild-type (0.99 ± 0.37 μL/min/g liver) and TR^- (0.34 ± 0.10 μL/min/g liver) Wistar rat livers was not affected by GF120918. The hepatic extraction ratio of harmol in mouse livers was high (> 0.94) and was unaffected by the presence of GF120918. Steady-state conditions were attained after 30 min as evidenced by the plateau in the outflow perfusate HS concentrations (Fig. 1A inset) and biliary excretion rates (not shown). Recovery of HS in mouse bile was decreased ~3.5-fold by GF120918 (7.5 ± 0.6 vs. 26 ± 7 nmol; Fig. 1A). A compensatory increase in the basolateral excretion of HS into perfusate was noted in the presence of GF120918 (Table 1). In contrast, co-administration of GF120918 did not significantly decrease the biliary excretion of HS from either wild-type or TR^- Wistar rat livers (Fig. 1B, Table 1B). Differences between mice and rats in hepatic HS content at the end of the perfusion (Table 1) may be attributed to differences in experimental design (see *Materials and Methods*).

Previous studies indicated that the biliary excretion of HS was unaffected in Mrp2- and P-glycoprotein-knockout mice but was reduced significantly (~10-100-fold) in Bcrp-knockout mice (Zamek-Gliszczynski et al., 2006c). In the present studies, GF120918, a potent inhibitor of P-glycoprotein and Bcrp, impaired HS biliary excretion in mice. In contrast, GF120918 did not significantly decrease the biliary recovery of HS in rats. These data suggest that fundamental differences exist in the transport proteins responsible for the biliary excretion of HS between rats and mice. The biliary excretion of HS was mediated primarily by GF120918-sensitive Bcrp in mice. In contrast, GF120918 co-administration to rat IPLs had no apparent effect on the recovery of these metabolites in bile, although GF120918 hepatic concentrations were adequate to inhibit both Bcrp and P-gp in rat IPLs

even at the end of the 90-min perfusion (de Bruin et al., 1999), when hepatic unbound inhibitor concentrations were ~3 μM (data not shown).

Mrp2, Bcrp, and P-glycoprotein, traditionally have been considered relevant to the excretion of xenobiotics and their metabolites (Zamek-Gliszczynski et al., 2006b). Functional activity of all three transport proteins should have been absent or impaired during GF120918 co-infusion in TR^- rat liver (Hoffmaster et al., 2004; Zamek-Gliszczynski et al., 2006a). Thus, the residual biliary excretion of HS in rats could be attributed to other transport processes.

In addition to species differences in biliary transport, the current studies also indicated differences in harmol metabolism between rats and mice. At a similar liver-mass-normalized exposure to harmol, ~70% of the dose was recovered as HS in rats; in mice, HS accounted only for ~35% of the dose. These data are consistent with harmol metabolism *in vivo*; following a 20 μmol/kg IV dose, the ratio of sulfate-to-glucuronide recovery is 2.7 in rats but is only 0.8 in mice (Mulder and Bleeker, 1975). Likewise, at the same body mass-normalized dose, the ratio of acetaminophen sulfate-to-glucuronide formation was lower in mice than in rats (Gregus et al., 1988).

In conclusion, the mechanisms of biliary excretion of HS differ between rats and mice. In mice, HS was excreted into bile primarily by GF120918-sensitive Bcrp. However, in rats, GF120918 had no effect on HS biliary excretion. Fundamental species differences in the mechanisms of hepatic canalicular transport merit careful consideration in the selection of pre-clinical species for mechanistic biliary excretion studies.

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Footnotes:

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Reprint requests

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Figure 1. (A) Cumulative biliary excretion and outflow perfusate concentrations (inset) of harmol sulfate in *in situ* wild-type C57BL/6 mouse liver perfusions in the absence (open symbols) or presence (closed symbols) of GF120918. Mean ± S.D., n = 3/group. (B) Cumulative biliary excretion and perfusate concentrations (inset) of harmol sulfate in isolated perfused livers from wild-type (○) and TR^- Wistar (Δ) rats in the absence (open symbols) or presence (closed symbols) of GF120918. Mean ± S.D., n = 4-5/group.

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Table 1A Percentage (mean ± S.D., n = 3/group) of the harmol dose (150 nmol; ~1.8 nmol/min/g liver) recovered as HS at the end of the 60-min mouse liver perfusion.

	Wild-Type C57BL/6 Mouse *in situ* Liver Perfusion					
	Control			GF120918		
(%)	Bile	Perfusate	Liver	Bile	Perfusate	Liver
HS	18 ± 5	8 ± 1	10 ± 2	5 ± 0.4*	19 ± 3*	12.6 ± 0.8
Recovery		36 ± 5			37 ± 3	

* $p < 0.05$, GF120918 vs. control

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Table 1B Percentage (mean ± S.D., n = 4-5/group) of the harmol dose (1950 nmol, ~2.0 nmol/min/g liver) recovered as HS at the end of the 90-min rat liver perfusion.

	Wild-Type Wistar Rat IPLs					
	Control			GF120918		
(%)	Bile	Perfusate	Liver	Bile	Perfusate	Liver
HS	50 ± 12	22 ± 6	BQL[a]	41 ± 6	53 ± 5‡	BQL
Recovery		73 ± 10			95 ± 4‡	
	Mrp2-Deficient (TR^-) Wistar Rat IPLs					
	Control			GF120918		
(%)	Bile	Perfusate	Liver	Bile	Perfusate	Liver
HS	18 ± 2*	53 ± 7*	BQL	16 ± 3*	61 ± 10	BQL
Recovery		71 ± 7			77 ± 11*	

* $p < 0.05$, TR^- vs. wild-type

‡ $p < 0.05$, GF120918 vs. control

[a] BQL, below quantification limit

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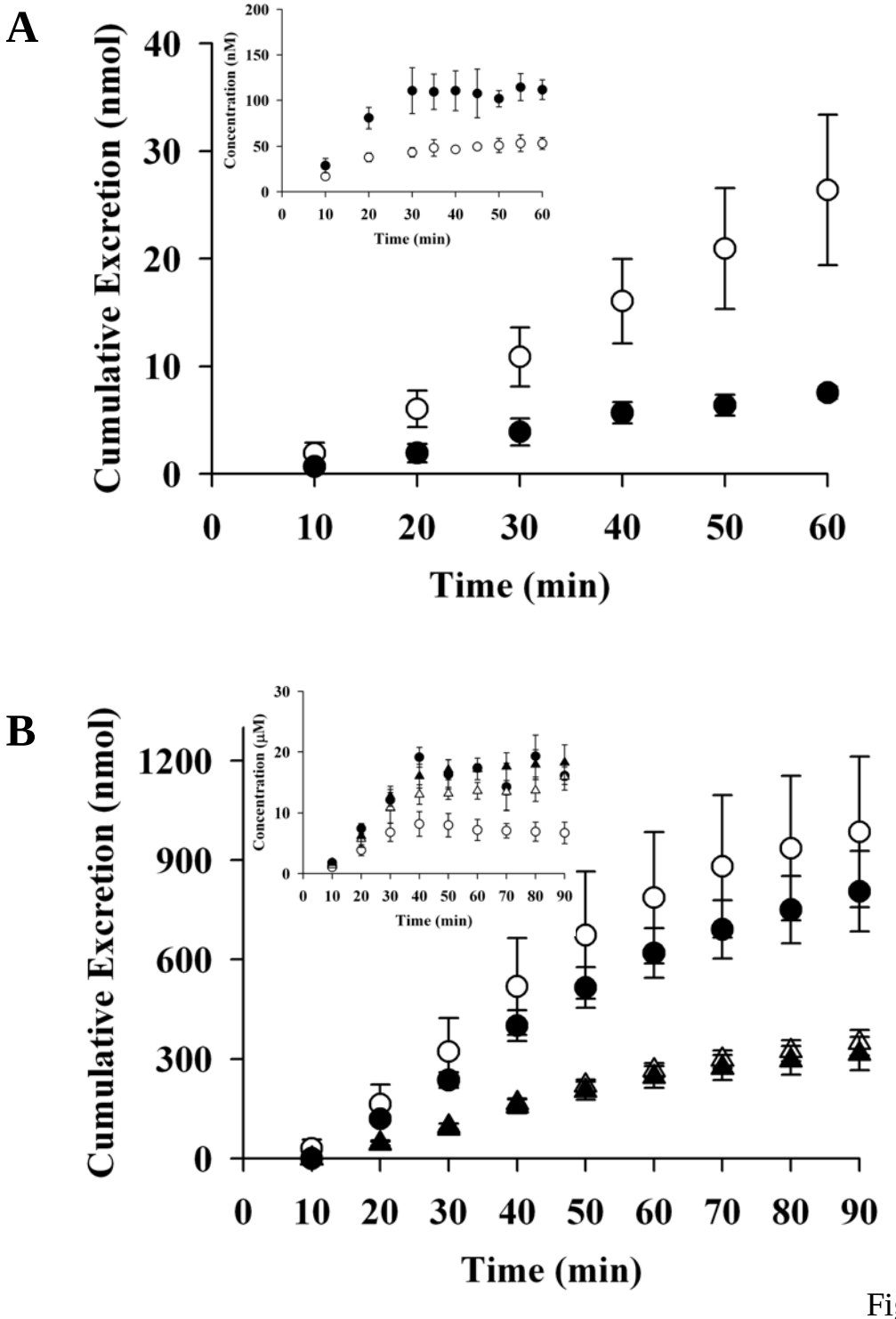

Figure 1