

# The Important Role of Bcrp (Abcg2) in the Biliary Excretion of Sulfate and Glucuronide Metabolites of Acetaminophen, 4-Methylumbelliferone, and Harmol in Mice

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## ABSTRACT

The role of Mrp2, Bcrp, and P-glycoprotein in the biliary excretion of acetaminophen sulfate (AS) and glucuronide (AG), 4-methylumbelliferyl sulfate (4MUS) and glucuronide (4MUG), and harmol sulfate (HS) and glucuronide (HG) was studied in *Abcc2*( $-/-$ ), *Abcg2*( $-/-$ ), and *Abcb1a*( $-/-$ )/*Abcb1b*( $-/-$ ) mouse livers perfused with the respective parent compounds using a cassette dosing approach. Biliary clearance of the sulfate conjugates was significantly decreased in Bcrp-deficient mouse livers, resulting in negligible biliary excretion of AS, 4MUS, and HS. It is noteworthy that the most profound decrease in the biliary clearance of the glucuronide conjugates was observed in Bcrp-deficient mouse livers, although the biliary clearance of 4MUG was also  $\sim 35\%$  lower in Mrp2-deficient mouse livers. As expected, biliary excretion of conjugates was not impaired in P-glycoprotein-deficient livers. An appreciable increase in perfusate recovery due to a shift in the directionality of metabolite excretion, from bile to perfusate, was noted in knockout mice only for conjugates whose biliary clearance constituted an appreciable ( $\geq 37\%$ ) fraction of total hepatic excretory clearance (i.e., 4MUS, HG, and HS). Biliary clearance of AG, AS, and 4MUG constituted a small fraction of total hepatic excretory clearance, so an appreciable increase in perfusate recovery of these metabolites was not observed in knockout mice despite markedly decreased biliary excretion. Unlike in rats, where sulfate and glucuronide conjugates were excreted into bile predominantly by Mrp2, mouse Bcrp mediated the biliary excretion of sulfate metabolites and also played a major role in the biliary excretion of the glucuronide metabolites, with some minor contribution from mouse Mrp2.

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Phase II metabolism, including sulfation and glucuronidation, occurs primarily in the liver. Conjugation of a substrate with a sulfate or glucuronide moiety increases its hydrophilicity to promote excretion from the body. These conjugates are typically too polar to undergo passive diffusion from hepatocytes after their intracellular formation and therefore

require carrier-mediated transport for excretion across the hepatic canalicular (apical) membrane into bile and across the basolateral membrane into sinusoidal blood. Most sulfate and glucuronide metabolites are inactive; notable exceptions include morphine-6-glucuronide, minoxidil sulfate, SN-38-glucuronide, and troglitazone sulfate (Zamek-Gliszczyński et al., 2006b). Altered hepatic export of pharmacologically and toxicologically active sulfate and glucuronide metabolites formed in the liver can have profound pharmacodynamic and toxic implications, underscoring the importance of understanding the mechanisms of metabolite excretion (Meisheri et al., 1993; Funk et al., 2001; Horikawa et al., 2002; Zelcer et al., 2005). Despite the importance of active transport in hepatic excretion of phase II metabolites, mechanisms responsible for excretion of sulfate and glucuronide conjugates have not been elucidated fully.

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**ABBREVIATIONS:** SN-38, 7-ethyl-10-hydroxycamptothecin; AG, acetaminophen glucuronide; 4MUS, 4-methylumbelliferyl sulfate; 4MUG, 4-methylumbelliferyl glucuronide; HS, harmol sulfate; AS, acetaminophen sulfate; HG, harmol glucuronide; P-gp, P-glycoprotein; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole;  $Cl_{\text{basolateral, SS}}$ , steady-state basolateral excretory unbound intrinsic clearance;  $Cl_{\text{bile, SS}}$ , steady-state biliary unbound intrinsic clearance; Mrp, multidrug resistance-associated protein; Bcrp, breast cancer resistance protein.

Previous whole-organ and in vivo studies have demonstrated the role of multidrug resistance-associated protein (Mrp) 2 in the biliary excretion of phase II conjugates in rats. Glucuronide conjugates of acetaminophen, hydroxyphenobarbital, phenolphthalein, and 4-methylumbelliferone were not excreted in the bile of Mrp2-deficient rat livers (Xiong et al., 2000; Ogasawara and Takikawa, 2001; Patel et al., 2003; Tanaka et al., 2003; Zamek-Gliszczyński et al., 2006a). In contrast, biliary recovery of sulfate conjugates was appreciable in Mrp2-deficient rat livers [phenolphthalein sulfate (~15% of wild type biliary recovery), acetaminophen sulfate (~20%), hydroxyphenobarbital sulfate (~30%), and 4-methylumbelliferyl sulfate (~40%)]. Subsequent studies demonstrated that breast cancer resistance protein (Bcrp) mediated the Mrp2-independent component of biliary excretion of the sulfate metabolites (Suzuki et al., 2003; Zamek-Gliszczyński et al., 2005, 2006a).

Recent reports have suggested that Mrp2 may play a greater role in biliary excretion of phase II conjugates in rats compared with other species because of the relatively high hepatic expression of Mrp2 in rats (Ishizuka et al., 1999; Ninomiya et al., 2005). Therefore, mechanistic biliary excretion studies using in vivo rat models may overpredict the importance of Mrp2 in other species.

The purpose of the present studies was to elucidate the functional importance of Mrp2, Bcrp, and P-glycoprotein (P-gp) in the biliary excretion of phase II metabolites in mice. The hepatobiliary disposition of sulfate and glucuronide conjugates of acetaminophen (AS and AG, respectively), 4-methylumbelliferone (4MUS and 4MUG, respectively), and harmol (HS and HG, respectively) was evaluated using in situ liver perfusions in *Abcc2*( $-/-$ ) and *Abcg2*( $-/-$ ) C57BL/6 mice and *Abcb1a*( $-/-$ )/*Abcb1b*( $-/-$ ) FVB mice. To maximize experimental throughput and conserve gene knockout mice, a cassette dosing approach, in which the livers were simultaneously perfused with the three parent compounds at nanomolar concentrations, was evaluated. Results demonstrated that Bcrp was predominantly responsible for the biliary excretion of sulfate conjugates, whereas both Bcrp and, to a lesser extent, Mrp2, transported the glucuronide conjugates in mice.

## Materials and Methods

**Chemicals.** Acetaminophen, acetaminophen glucuronide (AG), 4-methylumbelliferone, 4-methylumbelliferyl sulfate (4MUS), 4-methylumbelliferyl glucuronide (4MUG), harmol, cimetidine, taurocholate, and Krebs-Henseleit buffer packets were purchased from Sigma Chemical Co. (St. Louis, MO). Acetaminophen sulfate (AS) was purchased from Ultrafine (Manchester, UK). Harmol sulfate (HS) was a kind gift of Dr. K. Sandy Pang (University of Toronto, Toronto, ON, Canada). All other chemicals were of reagent grade and were readily available from commercial sources.

**Mice.** Male C57BL/6 wild-type, *Abcc2*( $-/-$ ), and *Abcg2*( $-/-$ ) mice (23–29 g) were a gift from Eli Lilly and Co. Embryonic stem cells derived from the 129/OlaHsd mouse substrain were used to generate chimeric mice containing full-length cDNA for either *Abcc2* (AF227274) with an 1886- to 1897-bp deletion or *Abcg2* (NM011920) with a 263- to 279-bp deletion. F1 mice were generated by breeding with C57BL/6 female mice (Deltagen, Inc., San Carlos, CA) and these were backcrossed five generations with heterozygous C57BL/6 mice before obtaining F2 homozygous mutant mice (Taconic Farms, Germantown, NY). Male FVB wild-type and *Abcb1a*( $-/-$ )/*Abcb1b*( $-/-$ ) mice (23–29 g) were purchased from Taconic farms (Germantown,

NY). Mice were maintained on a 12-h light/dark cycle with free access to water and rodent chow. All experimental procedures were performed under full anesthesia induced with ketamine/xylazine (140/8 mg/kg i.p.). The Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill approved all animal procedures.

**In Situ Liver Perfusion Experiments.** The abdominal cavity of anesthetized mice was opened to expose the intestines, liver, and the gallbladder. The common bile duct was ligated above the duodenum to prevent bile from entering the intestine, and the gallbladder was cannulated with PE-10 tubing (BD Biosciences, Franklin Lakes, NJ). A loose suture was placed around the inferior vena cava below the liver. The portal vein was cannulated with a 20-gauge catheter (B. Braun Medical, Inc., Bethlehem, PA), and the liver was perfused (5 ml/min, drug-free Krebs-Henseleit buffer containing 5  $\mu$ M taurocholate, continually oxygenated). The abdominal vena cava below the liver was immediately severed by incision below the loose suture, and the inferior vena cava above the liver was cannulated with a 20-gauge catheter. Thereafter, the loose suture around the inferior vena cava was tied off to direct all perfusate outflow through the cannula inside the inferior vena cava above the liver. After a 15-min preperfusion period for equilibration of liver temperature and bile flow, the liver was perfused with buffer containing 500 nM each acetaminophen, 4-methylumbelliferone, and harmol for 60 min. In a separate set of experiments, livers of wild-type C57BL/6 mice ( $n = 1$ /compound) were perfused individually with 500 nM acetaminophen, 4-methylumbelliferone, or harmol for 60 min to validate the cassette-dosing approach. Bile was collected in 10-min intervals; outflow perfusate was collected in 10-min intervals (0–30 min) and 5-min intervals (30–60 min). At the end of the perfusion, livers were isolated and snap frozen.

**Unbound Fraction Determination.** Livers were homogenized in 3 volumes (v/w) of 0.1 M phosphate buffer, pH 7.4. Homogenates were dialyzed to equilibrium against buffer using Spectra-Por2 membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA) in a 96-well plate dialysis apparatus (HTDialysis, Gales Ferry, CT) according to the manufacturer's instructions. Equilibrium was achieved over the course of the 4.5-h incubation with shaking (Maurer et al., 2005). Preliminary results indicated that 4-methylumbelliferyl sulfate was unstable at 37°C, so equilibrium dialysis was conducted at 4°C. Unbound concentrations determined for the stable metabolites were comparable at 37°C and 4°C. Metabolite concentrations were measured in dialyzed liver homogenate and buffer, as well as in homogenate stored at 4°C for 4.5 h and freshly prepared homogenate with immediate protein precipitation after homogenization. Unbound fractions were corrected for dilution (Kalvass and Maurer, 2002).

**Analytical Methods.** Bile, perfusate, liver homogenate, and dialysate samples were analyzed by liquid chromatography with detection by tandem mass spectrometry (API 4000 triple quadrupole with TurboIonSpray interface; Applied Biosystems/MDS Sciex, Concord, ON, Canada). AS, AG, 4-methylumbelliferone, 4MUS, 4MUG, HS, HG, and the internal standard, cimetidine, were eluted from an Aquasil C18 column (2.1  $\times$  50 mm,  $d_p = 5 \mu$ m; 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 negative ion mode using multiple reaction monitoring: AS, 230  $\rightarrow$  150  $m/z$ ; AG, 326  $\rightarrow$  150  $m/z$ ; 4-methylumbelliferone, 175  $\rightarrow$  133  $m/z$ ; 4MUG, 351  $\rightarrow$  175  $m/z$ ; 4MUS, 255  $\rightarrow$  175  $m/z$ ; HS, 277  $\rightarrow$  197  $m/z$ ; HG, 374  $\rightarrow$  198  $m/z$ ; cimetidine, 251  $\rightarrow$  157  $m/z$ . Concentrations of acetaminophen, harmol, and cimetidine were analyzed using the chromatography conditions detailed above, but were detected in positive ion mode using multiple reaction monitoring: acetaminophen: 152  $\rightarrow$  110  $m/z$ , harmol: 199  $\rightarrow$  131  $m/z$ , cimetidine: 253  $\rightarrow$  117  $m/z$ . All analytes were quantified with standard curves

(1–1000 ng/ml) prepared in the appropriate matrix, except HG, for which a pure standard was not available. Therefore, HG concentrations are expressed as the ratio of the analyte and internal standard peak areas. The lower limit of detection was 0.1 ng/ml for all analytes; inter- and intraday CVs were <15%.

**Data Analysis.** Metabolite formation clearance values were calculated as the ratio of the total metabolite recovery (sum of recovery in bile, outflow perfusate, and the liver at the end of the perfusion) and the area under the concentration-time curve of the parent compound in perfusate. Steady-state biliary and basolateral excretory unbound intrinsic clearance ( $Cl_{bile, SS}$  and  $Cl_{basolateral, SS}$ , respectively) values were calculated as the ratio of the steady-state (20 min and thereafter) biliary or basolateral excretion rate and the steady-state hepatic unbound metabolite concentration. All clearance values were normalized for liver mass.

All data are reported as mean  $\pm$  S.D.,  $n = 3$ –4 per condition. Statistical significance was assessed by analysis of variance with Tukey's post hoc test, except where the groups being compared had unequal variances or a data set failed the normality test, in which case analysis of variance on ranks was used. In all cases,  $p < 0.05$  was considered statistically significant.

## Results

Steady-state conditions were attained after 10 to 20 min of perfusion for all metabolites, as evidenced by metabolite concentrations in outflow perfusate and biliary excretion rates reaching a plateau. Biliary excretion and outflow perfusate concentrations of the sulfate and glucuronide metabolites in wild-type C57BL/6 mouse livers perfused individually with acetaminophen, 4-methylumbelliferone, or harmol were similar to that observed in cassette perfusion of the three parent compounds. Steady-state hepatic extraction ratios of acetaminophen ( $0.3 \pm 0.1$ ), 4-methylumbelliferone ( $>0.95$ ), and harmol ( $>0.94$ ) were comparable between mouse groups, as well as during cassette and individual perfusion. In all cases, formation clearances of metabolites (C57BL/6: AS =  $0.10 \pm 0.02$ , AG =  $0.9 \pm 0.1$ , 4MUS =  $1.1 \pm 0.2$ , 4MUG =  $2.4 \pm 0.5$ , HS =  $1.3 \pm 0.1$ ; FVB: AS =  $0.17 \pm 0.01$ , AG =  $0.9 \pm 0.4$ , 4MUS =  $1.6 \pm 0.3$ , 4MUG =  $2.5 \pm 0.9$ , HS =  $1.7 \pm 0.4$  ml/min/g of liver) were comparable between the knockout mice and relevant background strain controls, as well as during cassette and individual perfusion. Liver weight and bile flow were comparable between wild-type and knockout mice (Tables 1 and 2).

Biliary excretion of AS was unaffected in livers from *Abcc2*( $-/-$ ) mice but was decreased 85% in *Abcg2*( $-/-$ ) mice (Fig. 1A). The steady-state biliary unbound intrinsic clearance ( $Cl_{bile, SS}$ ) of AS was an order of magnitude lower in the absence of Bcrp (Table 1). The steady-state basolateral excretory unbound intrinsic clearance ( $Cl_{basolateral, SS}$ ) of AS was comparable between wild-type and knockout C57BL/6 mouse livers (Table 1). AS biliary excretion was not impaired in P-gp-deficient livers; in fact, it was higher than in wild-type outflow perfusate (Fig. 1A, Table 2). The appearance of AS in outflow perfusate was not significantly altered in *Abcb1a*( $-/-$ )/*Abcb1b*( $-/-$ ) mouse livers (Fig. 1B, Table 2).

Biliary recovery of AG in Mrp2-deficient mouse livers was ~60% decreased relative to wild-type (Fig. 1C). It is noteworthy that  $Cl_{bile, SS}$  of AG was not altered significantly in the absence of Mrp2 (Table 1). Thus, decreased biliary excretion of AG was due to the decreased steady-state AG hepatic concentrations in *Abcc2*( $-/-$ ) mouse livers, which were a function of an ~3-fold higher  $Cl_{basolateral, SS}$  of AG in

*Abcc2*( $-/-$ ) mice resulting in increased AG recovery in outflow perfusate (Fig. 1D, Table 1). AG biliary excretion was impaired in the absence of Bcrp (Fig. 1C). Biliary excretion of

TABLE 1

Steady-state hepatic unbound concentrations and excretory unbound intrinsic clearances (mean  $\pm$  S.D.,  $n = 3$ –4/group) of sulfate and glucuronide conjugates formed in the liver from the respective parent compounds

	C57BL/6	<i>Abcc2</i> ( $-/-$ )	<i>Abcg2</i> ( $-/-$ )
Liver mass (g)	1.18 $\pm$ 0.02	1.4 $\pm$ 0.2	1.2 $\pm$ 0.2
Bile flow ( $\mu$ l/min/g liver)	1.0 $\pm$ 0.3	0.7 $\pm$ 0.1	0.9 $\pm$ 0.2
Acetaminophen sulfate			
$Cl_{liver\ unbound, SS}$ (nM)	171 $\pm$ 32	140 $\pm$ 64	222 $\pm$ 49
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	18 $\pm$ 5	26 $\pm$ 6	2.3 $\pm$ 0.2*
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	289 $\pm$ 23	272 $\pm$ 63	189 $\pm$ 42
Acetaminophen glucuronide			
$Cl_{liver\ unbound, SS}$ (nM)	2917 $\pm$ 1257	972 $\pm$ 548	3628 $\pm$ 530
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	2 $\pm$ 1	1.8 $\pm$ 0.6	0.29 $\pm$ 0.09
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	175 $\pm$ 97	554 $\pm$ 313	99 $\pm$ 18
4-Methylumbelliferyl sulfate			
$Cl_{liver\ unbound, SS}$ (nM)	104 $\pm$ 22	55 $\pm$ 18*	162 $\pm$ 22*
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	2199 $\pm$ 495	4096 $\pm$ 254*	2 $\pm$ 1*
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	3669 $\pm$ 1888	6272 $\pm$ 1964	3296 $\pm$ 773
4-Methylumbelliferyl glucuronide			
$Cl_{liver\ unbound, SS}$ (nM)	1717 $\pm$ 402	1210 $\pm$ 547	2824 $\pm$ 552*
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	88 $\pm$ 43	59 $\pm$ 28	24 $\pm$ 1
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	703 $\pm$ 387	1152 $\pm$ 892	398 $\pm$ 45
Harmol sulfate			
$Cl_{liver\ unbound, SS}$ (nM)	399 $\pm$ 103	342 $\pm$ 79	511 $\pm$ 100
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	1081 $\pm$ 417	1211 $\pm$ 246	12 $\pm$ 1*
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	562 $\pm$ 257	497 $\pm$ 152	1254 $\pm$ 149*
Harmol glucuronide			
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	315 $\pm$ 205	248 $\pm$ 157	14 $\pm$ 2
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	219 $\pm$ 80	204 $\pm$ 95	396 $\pm$ 51*

\*  $p < 0.05$ , knockout vs. wild type.

TABLE 2

Steady-state hepatic unbound concentrations and excretory unbound intrinsic clearances (mean  $\pm$  S.D.,  $n = 3$ –4/group) of sulfate and glucuronide conjugates formed in the liver from the respective parent compounds

	FVB	<i>Abcb1a</i> ( $-/-$ )/ <i>Abcb1b</i> ( $-/-$ )
Liver mass (g)	1.3 $\pm$ 0.2	1.34 $\pm$ 0.08
Bile flow ( $\mu$ l/min/g liver)	0.9 $\pm$ 0.2	1.14 $\pm$ 0.06
Acetaminophen sulfate		
$Cl_{liver\ unbound, SS}$ (nM)	294 $\pm$ 183	234 $\pm$ 78
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	19 $\pm$ 10	29 $\pm$ 6
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	388 $\pm$ 239	388 $\pm$ 102
Acetaminophen glucuronide		
$Cl_{liver\ unbound, SS}$ (nM)	971 $\pm$ 352	1025 $\pm$ 362
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	2 $\pm$ 1	2.8 $\pm$ 0.6
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	545 $\pm$ 370	467 $\pm$ 133
4-Methylumbelliferyl sulfate		
$Cl_{liver\ unbound, SS}$ (nM)	77 $\pm$ 39	56 $\pm$ 13
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	4631 $\pm$ 2134	5449 $\pm$ 758
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	8309 $\pm$ 3857	10216 $\pm$ 1795
4-Methylumbelliferyl glucuronide		
$Cl_{liver\ unbound, SS}$ (nM)	1989 $\pm$ 675	1213 $\pm$ 244
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	37 $\pm$ 20	66 $\pm$ 20
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	683 $\pm$ 414	1096 $\pm$ 391
Harmol sulfate		
$Cl_{liver\ unbound, SS}$ (nM)	433 $\pm$ 88	317 $\pm$ 83
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	1164 $\pm$ 114	1609 $\pm$ 240*
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	702 $\pm$ 38	796 $\pm$ 200
Harmol glucuronide		
$Cl_{bile, SS}$ ( $\mu$ l/min/g liver)	138 $\pm$ 57	126 $\pm$ 35
$Cl_{basolateral, SS}$ ( $\mu$ l/min/g liver)	252 $\pm$ 157	178 $\pm$ 91

\*  $p < 0.05$ , knockout vs. wild type.

AG was slightly higher in *Abcb1a*(-/-)/*Abcb1b*(-/-) mouse livers (Fig. 1C, Table 2). Hepatic basolateral excretion of AG in P-gp-deficient mouse livers was comparable with FVB wild-type mice (Fig. 1D).

$Cl_{\text{bile, SS}}$  of 4MUS was significantly increased in *Abcc2*(-/-) mouse livers. No change in 4MUS biliary excretion was noted because hepatic unbound concentrations of 4MUS were decreased as a result of increased  $Cl_{\text{bile, SS}}$  and  $Cl_{\text{basolateral, SS}}$  (Fig. 2A, Table 1). 4MUS biliary excretion was negligible in *Bcrp*-deficient livers because of an ~1000-fold decrease in  $Cl_{\text{bile, SS}}$  (Fig. 2A, Table 1). Recovery of 4MUS in bile and perfusate, as well as  $Cl_{\text{bile, SS}}$  and  $Cl_{\text{basolateral, SS}}$ , were not altered in *Abcb1a*(-/-)/*Abcb1b*(-/-) mouse livers (Fig. 2A-B, Table 2).

Recovery of 4MUG in bile was ~50% and ~55% lower in livers from *Abcc2*(-/-) and *Abcg2*(-/-) mice, respectively (Fig. 2C).  $Cl_{\text{bile, SS}}$  of 4MUG was decreased ~35% and ~75% in the absence of *Mrp2* and *Bcrp*, respectively (Table 1). Hepatic unbound concentrations of 4MUG were significantly increased in *Abcg2*(-/-) mice because of a decrease in both  $Cl_{\text{bile, SS}}$  and  $Cl_{\text{basolateral, SS}}$  of 4MUG. The biliary and basolateral excretion of 4MUG were not altered in P-gp-deficient mouse livers (Fig. 2, C and D, Table 2).

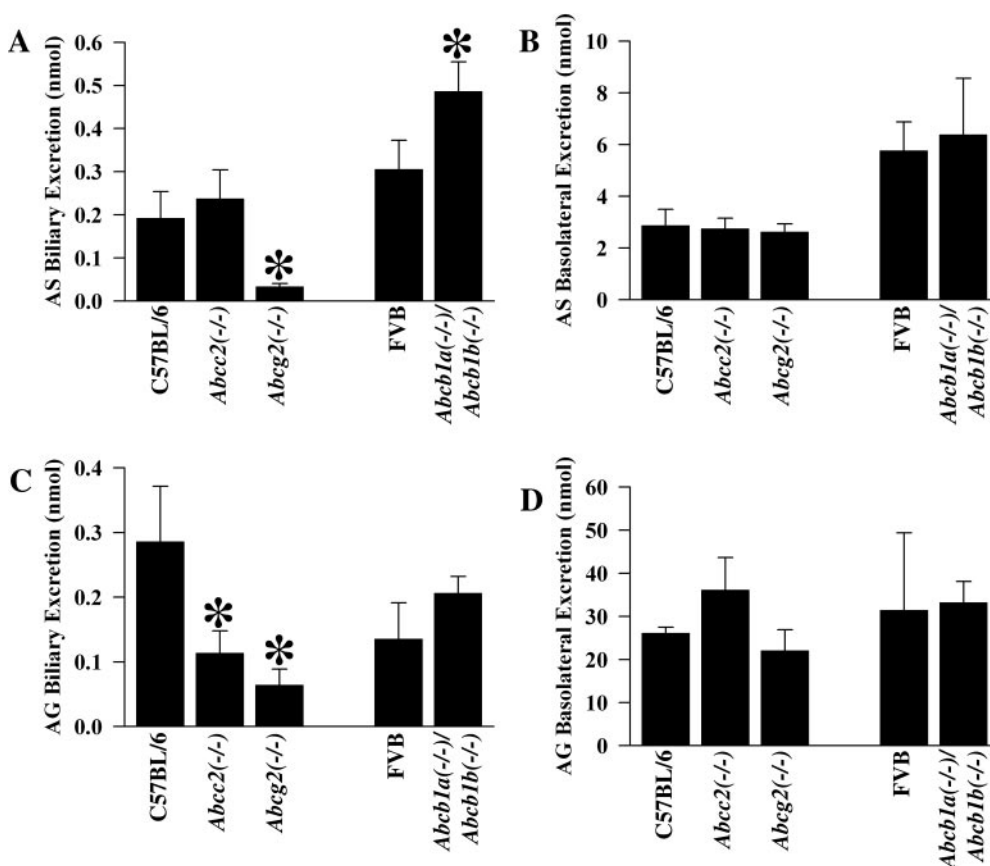
Biliary excretion of HS was unaltered in *Mrp2*-deficient livers, but was obliterated in livers deficient in *Bcrp* (Fig. 3A).  $Cl_{\text{bile, SS}}$  of HS was decreased by 2 orders of magnitude in *Abcc2*(-/-) mice (Table 1). Recovery of HS in outflow perfusate was similar in livers from *Abcc2*(-/-) mice but was significantly increased in livers from *Abcg2*(-/-) mice (Fig. 3B), as a result of an ~2-fold increase in  $Cl_{\text{basolateral, SS}}$  (Table 1).  $Cl_{\text{bile, SS}}$  of HS was significantly increased in P-gp-deficient

mouse livers, but no change in  $Cl_{\text{basolateral, SS}}$  of HS was noted (Table 2).

$Cl_{\text{bile, SS}}$  of HG was not altered in livers deficient in *Mrp2* but was ~95% lower in *Bcrp*-deficient livers (Table 1).  $Cl_{\text{basolateral, SS}}$  of HG was ~2-fold increased in livers from *Abcc2*(-/-) mice relative to C57BL/6 or *Abcc2*(-/-) mouse livers (Table 1).  $Cl_{\text{bile, SS}}$  and  $Cl_{\text{basolateral, SS}}$  of HG were not altered in P-gp-deficient livers (Table 2).

## Discussion

This research is the first comprehensive evaluation of the mechanisms of biliary excretion of glucuronide and sulfate conjugates in mice. These data provide clear evidence that species differences exist between mice and rats in the mechanisms of biliary excretion of these phase II conjugates. First, the results of the current studies indicate that different transport proteins contribute to the biliary excretion of sulfate and glucuronide conjugates in mice. In particular, *Bcrp* mediated the biliary excretion of sulfate conjugates in mice with no apparent contribution from *Mrp2* or P-gp; primarily, *Bcrp* and, to a lesser extent, *Mrp2*, contribute to the biliary excretion of the glucuronide conjugates in mice. In contrast, in *Mrp2*-deficient rats, the biliary excretion of sulfate conjugates was partially impaired (Takenaka et al., 1995; Xiong et al., 2000; Ogasawara and Takikawa, 2001; Zamek-Gliszczyński et al., 2006a), although *Bcrp* also contributed to the biliary excretion of AS and 4MUS in rats (Zamek-Gliszczyński et al., 2005, 2006a). In contrast to mice, *Mrp2* appears to be solely responsible for the biliary excretion of the glucuronide conjugates of acetaminophen, 4-methylumbelliferone,



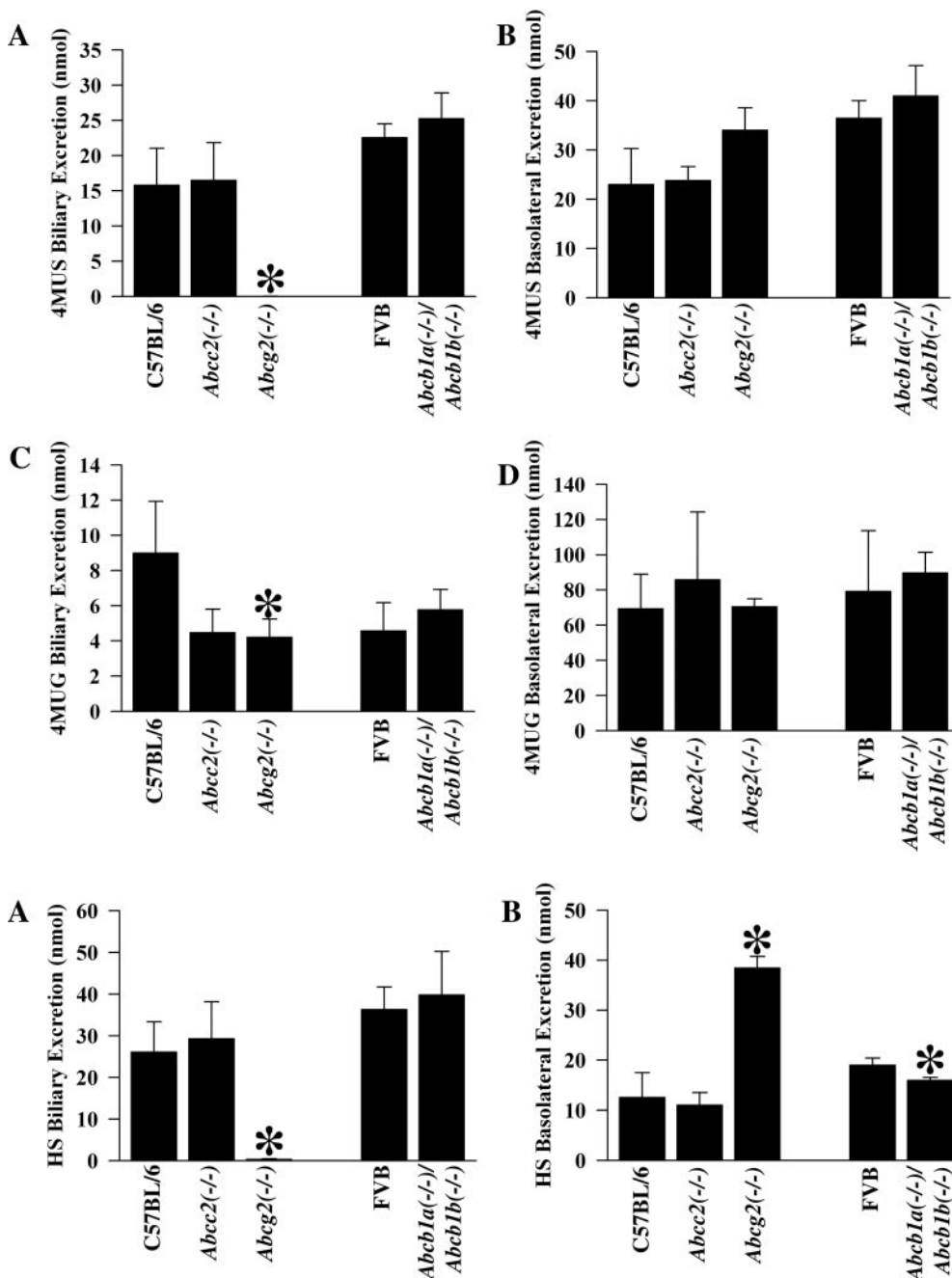
**Fig. 1.** Cumulative 60-min biliary and hepatic basolateral excretion of AS (A and B) and AG (C and D) in wild-type, *Abcc2*(-/-), and *Abcg2*(-/-) C57BL/6, as well as wild-type and *Abcb1a*(-/-)/*Abcb1b*(-/-) FVB mouse liver perfusions. Mean  $\pm$  S.D.,  $n = 3-4$ /group.

phenolphthalein, and E3040 in rats (Takenaka et al., 1995; Xiong et al., 2000; Ogasawara and Takikawa, 2001; Zamek-Gliszczynski et al., 2006a).

Although rats traditionally have been the preclinical species of choice for hepatobiliary disposition studies (Zamek-Gliszczynski and Brouwer, 2004), a better understanding of the species differences between rats and mice is important to establish the appropriate animal models for prediction of biliary excretion of sulfate and glucuronide conjugates in other species. Expression of Mrp2 protein levels in rat livers is an order of magnitude greater than in dog, and the hepatic transport capacity and/or intrinsic clearance of prototypical Mrp2 substrates (temocaprilat and dinitrophenyl-*S*-glutathione) is much higher in rats than in other species such as mouse, guinea pig, rabbit, dog, and human (Ishizuka et al.,

1999; Ninomiya et al., 2005). Thus, the high hepatic expression level of Mrp2 in rats may result in an overestimation of the contribution of this pathway to biliary excretion in other species. In addition, Suzuki et al. (2003) demonstrated that recombinant human BCRP transported 4MUS and 4MUG, a finding more consistent with mouse than rat mechanistic biliary excretion studies. Therefore, mice may better predict human canalicular transport mechanisms than rats, and the increasing availability of many gene knockout mouse models makes them a valuable tool for mechanistic studies.

The current studies provide a comprehensive evaluation of the role of Bcrp in the biliary excretion of phase II conjugates in whole liver experiments. In vitro, human BCRP overexpressed in plasma membrane vesicles prepared from P388 cells demonstrated efficient transport of sulfate and to a



**Fig. 2.** Cumulative 60-min biliary and hepatic basolateral excretion of 4MUS (A and B) and 4MUG (C and D) in wild-type, *Abcc2*(-/-), and *Abcg2*(-/-) C57BL/6, as well as wild-type and *Abcb1a*(-/-)/*Abcb1b*(-/-) FVB mouse liver perfusions. Mean  $\pm$  S.D.,  $n = 3-4$ /group.

**Fig. 3.** Cumulative 60-min biliary and hepatic basolateral excretion of HS in wild-type, *Abcc2*(-/-), and *Abcg2*(-/-) C57BL/6, as well as wild-type and *Abcb1a*(-/-)/*Abcb1b*(-/-) FVB mouse liver perfusions. Mean  $\pm$  S.D.,  $n = 3-4$ /group.

lesser extent glucuronide conjugates (Suzuki et al., 2003). Mrp2 was determined to be more important in the biliary excretion of glucuronide and sulfate metabolites in whole rat liver experiments, calling into question the relevance of the recombinant BCRP studies (Takenaka et al., 1995; Xiong et al., 2000; Ogasawara and Takikawa, 2001; Tanaka et al., 2003; Zamek-Gliszczyński et al., 2006a). The physiological importance of Bcrp in the excretion of phase II metabolites was reestablished in intestinal perfusion and renal clearance studies in *Abcg2*( $-/-$ ) mice. Intestinal secretion of 4MUS, 4MUG, and E3040 glucuronide was impaired in the absence of Bcrp in situ intestinal perfusion experiments (Adachi et al., 2005). In addition, E3040 sulfate renal clearance was decreased and renal concentrations were increased ~2- to 3-fold in *Abcg2*( $-/-$ ) mice, implicating Bcrp in the renal transport of E3040 sulfate; renal transport of 4MUS, however, was unaffected by Bcrp (Mizuno et al., 2004). A further understanding of the physiological role of Bcrp was gained in the current studies, which demonstrated that Bcrp is responsible for the biliary excretion of AS, 4MUS, and HS, and that Bcrp plays an important role in the biliary excretion of the respective glucuronide conjugates of these compounds in mice.

P-gp-deficient livers did not exhibit impaired biliary excretion of any phase II metabolites. These findings are consistent with previous studies in which the biliary excretion of AG and 4MUG was not impaired by the P-gp inhibitor, GF120918, and AS and 4MUS did not interact with P-gp in vitro (Zamek-Gliszczyński et al., 2005, 2006a). Furthermore, even though recombinant P-gp transported estradiol-17 $\beta$ -( $\beta$ -D-glucuronide) in vitro, the biliary excretion of this endogenous glucuronide conjugate in P-gp-deficient mice was not impaired (Huang et al., 1998, 2000).

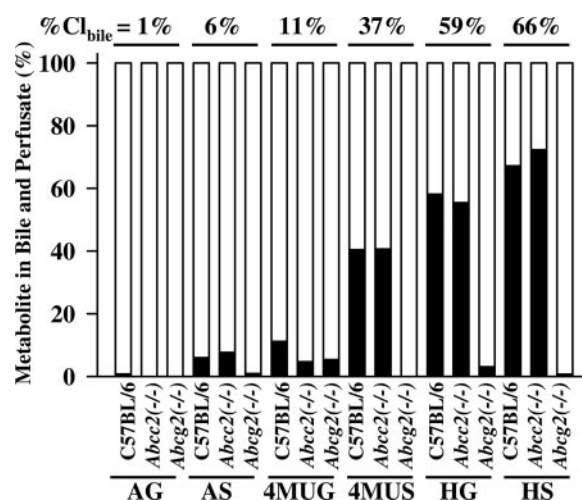
It is noteworthy that kinetic strain differences were observed in the hepatobiliary disposition of metabolites between C57BL/6 and FVB mice. Although differences in hepatic Bcrp and/or Mrp 2, 3, and/or 4 expression probably are the reason, not enough is known about mouse strain differences in transport proteins to determine the exact mechanism(s). C57BL/6 mice express Mrp2 protein at the blood-brain barrier, whereas Mrp2 expression cannot be detected in the brains of FVB mice (Soontornmalai et al., 2006). Additional strain differences in transport proteins undoubtedly exist between C57BL/6 and FVB mice.

The percentage of metabolite recovery in bile and perfusate in wild-type, Mrp2-deficient, and Bcrp-deficient C57BL/6 mice is plotted in Fig. 4. For the knockout of a biliary excretion mechanism to cause an appreciable increase in metabolite recovery in perfusate, and increase systemic exposure to a metabolite in vivo, biliary clearance must constitute an appreciable fraction of total hepatic excretory clearance (i.e., the sum of biliary and basolateral clearances). Biliary clearance of AG, AS, and 4MUG constitutes a small fraction of total hepatic excretory clearance; therefore, livers deficient in Bcrp or Mrp2 do not show an appreciable increase in perfusate recovery of these metabolites despite a marked decrease in biliary excretion. In contrast, biliary clearance of 4MUS, HG, and HS is large enough ( $\geq 37\%$ ) to cause a profound increase in perfusate recovery and a shift in the directionality of metabolite excretion, from bile to perfusate, when the mechanism responsible for the biliary excretion of the metabolite (i.e., Bcrp) is knocked out. Thus, in order for the

knockout of a canalicular transport mechanism to have an appreciable effect on systemic exposure to the metabolite, an important consideration for active phase II conjugates, the knocked-out pathway must be predominantly responsible for the biliary excretion of the metabolite, and biliary clearance must be a sizeable fraction of total hepatic excretory clearance.

$Cl_{\text{basolateral, SS}}$  values of AG were ~3-fold higher in livers from *Abcc2*( $-/-$ ) mice. AG is a substrate of recombinant rat Mrp3, and the hepatic basolateral excretion of AG is negligible in *Abcc3*( $-/-$ ) mouse livers (Xiong et al., 2002; Manautou et al., 2005). *Abcc2*( $-/-$ ) mouse livers express Mrp3 protein at ~60% higher levels and the rate constant governing the hepatic basolateral excretion of the Mrp3 substrate, carboxy-dichlorofluorescein, was elevated ~4-fold in these mice (Nezasa et al., 2006). Although the observed increase in the basolateral excretory clearance of AG may not be explained entirely by the 60% induction of Mrp3, it seems to be the most likely candidate. Nonetheless, induction of other hepatic basolateral transport proteins, such as Mrp4 (Chu et al., 2006), may also be responsible for the observed increase.

The present studies used a cassette-dosing approach to simultaneously study the hepatobiliary disposition of the metabolites of three compounds. At the nanomolar concentrations of parent compounds used in these mouse liver perfusions, the appearance of sulfate and glucuronide conjugates in bile and outflow perfusate was similar in cassette and individual dosing. Provided adequately low limits of quantification ( $\leq 1$  ng/ml), which allow the use of sufficiently low concentrations to avoid noticeable interactions between substrates, cassette dosing may be a viable approach to increase the experimental throughput of notoriously labor-intensive whole-organ experiments while minimizing the use of precious gene-knockout mice and animals. In addition, an analytical method with high selectivity, such as liquid chromatography/tandem mass spectrometry, allows the simultaneous quantification of concentrations of multiple analytes, thus further increasing experimental throughput. Although



**Fig. 4.** Percentage of total metabolite recovery (cumulative at 60 min) in bile (closed lower portion of bar) and perfusate (open upper portion of bar). Metabolites are plotted in order of increasing fractional biliary clearance (i.e., biliary clearance expressed as a percentage of total hepatic excretory clearance) in wild-type C57BL/6 mice. FVB and *Abcb1a*( $-/-$ )/*Abcb1b*( $-/-$ ) mouse percentages were similar to wild-type C57BL/6 mice and are not plotted for clarity.

the cassette dosing approach was suitable for the compounds used in this study, this approach should not be used for potent modulators of metabolism and/or transport. As detailed under *Materials and Methods*, cassette dosing experiments should be validated against individual dosing at the same concentration to ensure that the pharmacokinetic properties of each compound are not altered by the coadministered agents.

In summary, mechanisms of biliary excretion of the sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol were examined using in situ perfused livers from *Abcc2*(-/-), *Abcg2*(-/-), and *Abcb1a*(-/-)/*Abcb1b*(-/-) mice. The studies revealed that in mice, Bcrp was predominantly responsible for the biliary excretion of the examined metabolites. Mrp2 played only a minor role in the biliary excretion of some glucuronide conjugates, whereas P-gp was not relevant to the biliary excretion of these phase II metabolites. Previous reports using rat models indicated that Mrp2 was responsible for the biliary excretion of these sulfate and glucuronide metabolites, with some Bcrp-mediated biliary excretion of the sulfate conjugates. These findings demonstrate fundamental mechanistic species differences in biliary excretion between mice and rats.

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