Molecular Pharmacology Fast Forward. Published on September 7, 2006 as doi:10.1124/mol.106.026955
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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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Molecular Pharmacology Fast Forward. Published on September 7, 2006 as DOI: 10.1124/mol.106.026955
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Running Title: Biliary excretion of phase II conjugates in mice. (56/60)

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Number of text pages: 26
Number of tables: 2
Number of figures: 4
Number of references: 27/40
Number of words (Abstract): 248/250
Number of words (Introduction): 512/750
Number of words (Discussion): 1473/1500

Nonstandard abbreviations: acetaminophen sulfate (AS), acetaminophen glucuronide (AG), 4-methylumbelliferyl sulfate (4MUS), 4-methylumbelliferyl glucuronide (4MUG), harmol sulfate (HS), harmol glucuronide (HG), multidrug resistance-associated protein (Mrp, *Abcc*), breast cancer resistance protein (Bcrp, *Abcg2*), P-glycoprotein (P-gp, *Abcb1*), steady-state basolateral excretory unbound intrinsic clearance ($Cl_{basolateral, SS}$), steady-state biliary unbound intrinsic clearance ($Cl_{bile, SS}$)

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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. Interestingly, 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 ~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 (≥ 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 following 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 rendered pharmacologically inactive; however, notable exceptions include morphine-6-glucuronide and minoxidil sulfate, as well as the toxicologically-active SN-38-glucuronide and troglitazone sulfate (Zamek-Gliszczynski 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 from hepatocytes following their formation (Funk et al., 2001; Horikawa et al., 2002; Meisheri et al., 1993; 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.

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 (Ogasawara and Takikawa, 2001; Patel et al., 2003; Tanaka et al., 2003; Xiong et al., 2000; Zamek-Gliszczynski 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-Gliszczynski et al., 2006a; Zamek-Gliszczynski et al., 2005).

Recent reports have suggested that Mrp2 may play a greater role in biliary excretion of phase II conjugates in rats compared to other species due to 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 nM 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.

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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, 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. Embryonicstem cells derived from the 129/OlaHsd mouse sub-strain were used to generate chimeric mice containing full-length cDNA for either *Abcc2* (AF227274) with an 1886-1897 bp deletion, or *Abcg2* (NM011920) with a 263-279 bp deletion. F1 mice were generated by breeding with C57BL/6 females (Deltagen, Inc., San Carlos, CA) and these were backcrossed five generations with heterozygous C57BL/6 mice prior to 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-hour 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 in order to prevent bile from entering the intestine, and the gallbladder was cannulated with PE-10 tubing (Becton Dickinson, Parsippany, NJ). A loose suture was placed around the inferior vena cava below the liver. The portal vein was cannulated with a 20G catheter (B. Braun Medical, Inc., Bethlehem, PA) and the liver was perfused (5 mL/min, drug-free Krebs-Henseleit buffer containing 5 μM 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 20G catheter. Subsequently, 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. Following a 15-min pre-perfusion period for equilibration of liver temperature and bile flow, the liver was perfused with buffer containing 500 nM each of 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 three 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

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of the 4.5-hr incubation with shaking (Maurer et al., 2005). Preliminary results indicated that 4-methylumbelliferyl sulfate was unstable at 37ºC, so equilibrium dialysis was conducted at 4ºC. Unbound concentrations determined for the stable metabolites were comparable at 37ºC and 4ºC. Metabolite concentrations were measured in dialyzed liver homogenate and buffer, as well as in homogenate stored at 4ºC for 4.5 hr and freshly-prepared homogenate with immediate protein precipitation following 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 (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface, 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 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 negative ion mode using multiple reaction monitoring: AS: 230 → 150 m/z, AG: 326 → 150 m/z, 4-methylumbelliferone: 175 → 133 m/z, 4MUG: 351 → 175 m/z, 4MUS: 255 → 175 m/z, HS: 277 → 197 m/z, HG: 374 → 198 m/z, cimetidine: 251 → 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 → 110 m/z, harmol: 199 → 131 m/z, cimetidine: 253 → 117 m/z. All analytes were

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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 intra-day 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 ± 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 to be statistically significant.

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Results

Steady-state conditions were attained after 10-20 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 ± 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 ± 0.02, AG = 0.9 ± 0.1, 4MUS = 1.1 ± 0.2, 4MUG = 2.4 ± 0.5, HS = 1.3 ± 0.1; FVB: AS = 0.17 ± 0.01, AG = 0.9 ± 0.4, 4MUS = 1.6 ± 0.3, 4MUG = 2.5 ± 0.9, HS = 1.7 ± 0.4 mL/min/g 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 (Table 1).

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 1A). 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 1A). AS biliary excretion was not impaired in P-gp-deficient livers, and in fact was higher than in wild-type FVB mice (Fig. 1A, Table 1B). The appearance of AS in outflow

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perfusate was not significantly altered in *Abcb1a(-/-)/Abcb1b(-/-)* mouse livers (Fig. 1B, Table 1B).

Biliary recovery of AG in Mrp2-deficient mouse livers was ~60% decreased relative to wild type (Fig. 1C). Interestingly, $Cl_{bile, SS}$ of AG was not altered significantly in the absence of Mrp2 (Table 1A). 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 1A). AG biliary excretion was impaired in the absence of Bcrp (Fig. 1C). Biliary excretion of AG was slightly higher in *Abcb1a(-/-)/Abcb1b(-/-)* mouse livers (Fig. 1C, Table 1B). Hepatic basolateral excretion of AG in P-gp-deficient mouse livers was comparable to FVB wild-type mice (Fig. 1D).

$Cl_{bile, SS}$ 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 due to increased $Cl_{bile, SS}$ and $Cl_{basolateral, SS}$ (Fig. 2A, Table 1A). 4MUS biliary excretion was negligible in Bcrp-deficient livers due to an ~1000-fold decrease in $Cl_{bile, SS}$ (Fig. 2A, Table 1A). Recovery of 4MUS in bile and perfusate, as well as $Cl_{bile, SS}$ and $Cl_{basolateral, SS}$, were not altered in *Abcb1a(-/-)/Abcb1b(-/-)* mouse livers (Fig. 2A-B, Table 1B).

Recovery of 4MUG in bile was ~50% and ~55% lower in livers from *Abcc2(-/-)* and *Abcg2(-/-)* mice, respectively (Fig 2C). $Cl_{bile, SS}$ of 4MUG biliary excretion was decreased ~35% and ~75% in the absence of Mrp2 and Bcrp, respectively (Table 1A). Hepatic unbound concentrations of 4MUG were significantly increased in *Abcg2(-/-)*

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mice due to a decrease in both $Cl_{bile, SS}$ and $Cl_{basolateral, SS}$ of 4MUG. The biliary and basolateral excretion of 4MUG were not altered in P-gp-deficient mouse livers (Fig. 2C-D, Table 1B).

Biliary excretion of HS was unaltered in Mrp2-deficient livers, but was obliterated in livers deficient in Bcrp (Fig. 3A). $Cl_{bile, SS}$ of HS was decreased by two orders of magnitude in *Abcg2(-/-)* mice (Table 1A). Recovery of HS in outflow perfusate was similar in livers from *Abcc2(-/-)* mice, but was significantly increased in livers from *Abcg2(-/-)* mice (Fig. 3B), due to an ~2-fold increase in $Cl_{basolateral, SS}$ (Table 1A). $Cl_{bile, SS}$ of HS was significantly increased in P-gp-deficient mouse livers, but no change in $Cl_{basolateral, SS}$ of HS was noted (Table 1B).

$Cl_{bile, SS}$ of HG was not altered in livers deficient in Mrp2, but was ~95% lower in Bcrp-deficient livers (Table 1A). $Cl_{basolateral, SS}$ of HG was ~2-fold increased in livers from *Abcg2(-/-)* mice relative to C57BL/6 or *Abcc2(-/-)* mouse livers (Table 1A). $Cl_{bile, SS}$ and $Cl_{basolateral, SS}$ of HG were not altered in P-gp-deficient livers (Table 1B).

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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. Specifically, 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 (Ogasawara and Takikawa, 2001; Takenaka et al., 1995; Xiong et al., 2000; Zamek-Gliszczynski et al., 2006a), although Bcrp also contributed to the biliary excretion of AS and 4MUS in rats (Zamek-Gliszczynski et al., 2006a; Zamek-Gliszczynski et al., 2005). In contrast to mice, Mrp2 appears to be solely responsible for the biliary excretion of the glucuronide conjugates of acetaminophen, 4-methylumbelliferone, phenolphthalein, and E3040 in rats (Ogasawara and Takikawa, 2001; Takenaka et al., 1995; Xiong et al., 2000; Zamek-Gliszczynski et al., 2006a).

Although rats traditionally have been the pre-clinical 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 in order 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 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 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 (Ogasawara and Takikawa, 2001; Takenaka et al., 1995; Tanaka et al., 2003; Xiong et al., 2000; Zamek-Gliszczynski et al., 2006a). The physiological importance of Bcrp in the excretion of phase II metabolites was reestablished in intestinal and renal perfusion studies of *Abcg2(-/-)* mice. Intestinal secretion of 4MUS, 4MUG, and E3040 glucuronide was impaired in the absence of Bcrp in *in situ* intestinal perfusion experiments (Adachi et al., 2005). In addition, E3040 sulfate renal clearance was decreased and renal concentrations were

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increased ~2-3-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-Gliszczynski et al., 2006a; Zamek-Gliszczynski et al., 2005). Furthermore, even though recombinant P-gp transported estradiol-17β-(β-D-glucuronide) *in vitro*, the biliary excretion of this endogenous glucuronide conjugate in P-gp-deficient mice was not impaired (Huang et al., 1998; Huang et al., 2000).

Interestingly, kinetic strain differences were observed in the hepatobiliary disposition of metabolites between C57BL/6 and FVB mice. Although differences in hepatic Bcrp and/or Mrp2-4 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.

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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. In order 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 Mrp2 or Bcrp 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 (≥ 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 into bile, and biliary clearance must be a sizeable fraction of total hepatic excretory clearance.

$Cl_{basolateral, SS}$ 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 (Manautou et al., 2005; Xiong et al., 2002). *Abcc2(-/-)* mouse livers express Mrp3 protein at ~60% higher levels and the rate constant governing the hepatic basolateral excretion of the Mrp3 substrate, carboxydichlorofluorescein, was elevated ~4-fold in these mice (Nezasa et al., 2006).

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Although the observed increase in the basolateral excretory clearance of AG may not be explained entirely by the 60% induction of Mrp3, it appears to be the most likely candidate. Nonetheless, induction of other hepatic transport proteins for basolateral excretion of AG in *Abcc2(-/-)* mice, such as Mrp4 (Chu et al., 2006), also may be responsible for the observed increase.

The present studies utilized a cassette-dosing approach to simultaneously study the hepatobiliary disposition of the metabolites of three compounds. At the nanomolar concentrations of parent compounds utilized in these mouse liver perfusions, the appearance of sulfate and glucuronide conjugates in bile and outflow perfusate was similar between 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 LC-MS/MS, allows the simultaneous quantification of concentrations of multiple analytes, thus further increasing experimental throughput. Although the cassette dosing approach was suitable for the compounds utilized in this study, this approach should not be used for potent modulators of metabolism and/or transport. As detailed in the Methods of the present study, 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 co-administered agents.

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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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Acknowledgment

The authors thank Dr. Arlene S. Bridges for her analytical expertise and outstanding work with LC-MS/MS sample analysis.

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Footnotes

This work was funded by grant R01 GM41935 from the National Institutes of Health. Maciej J. Zamek-Gliszczynski was supported by an Eli Lilly and Company Foundation Predoctoral Fellowship in Pharmacokinetics and Drug Disposition. Findings were presented in part at the 2005 American Association of Pharmaceutical Scientists (AAPS) meeting in Memphis, TN.

Molecular Pharmacology Fast Forward. Published on September 7, 2006 as DOI: 10.1124/mol.106.026955
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Figure Legends

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

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

Figure 3 Cumulative 60-min biliary and hepatic basolateral excretion of harmol sulfate (HS) in wild-type, *Abcc2(-/-)*, and *Abcg2(-/-)* C57BL/6, as well as wild-type and *Abcb1a(-/-)/Abcb1b(-/-)* FVB mouse liver perfusions. Mean ± SD, n = 3-4/group.

Figure 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 the order of increasing fractional biliary clearance (i.e. biliary clearance expressed as the 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. AG, acetaminophen glucuronide; AS, acetaminophen sulfate; 4MUG, 4-methylumbelliferyl glucuronide; 4MUS, 4-methylumbelliferyl sulfate; HG, harmol glucuronide; HS, harmol sulfate.

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TABLE 1A

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

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

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

Molecular Pharmacology Fast Forward. Published on September 7, 2006 as DOI: 10.1124/mol.106.026955
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TABLE 1B

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

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

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

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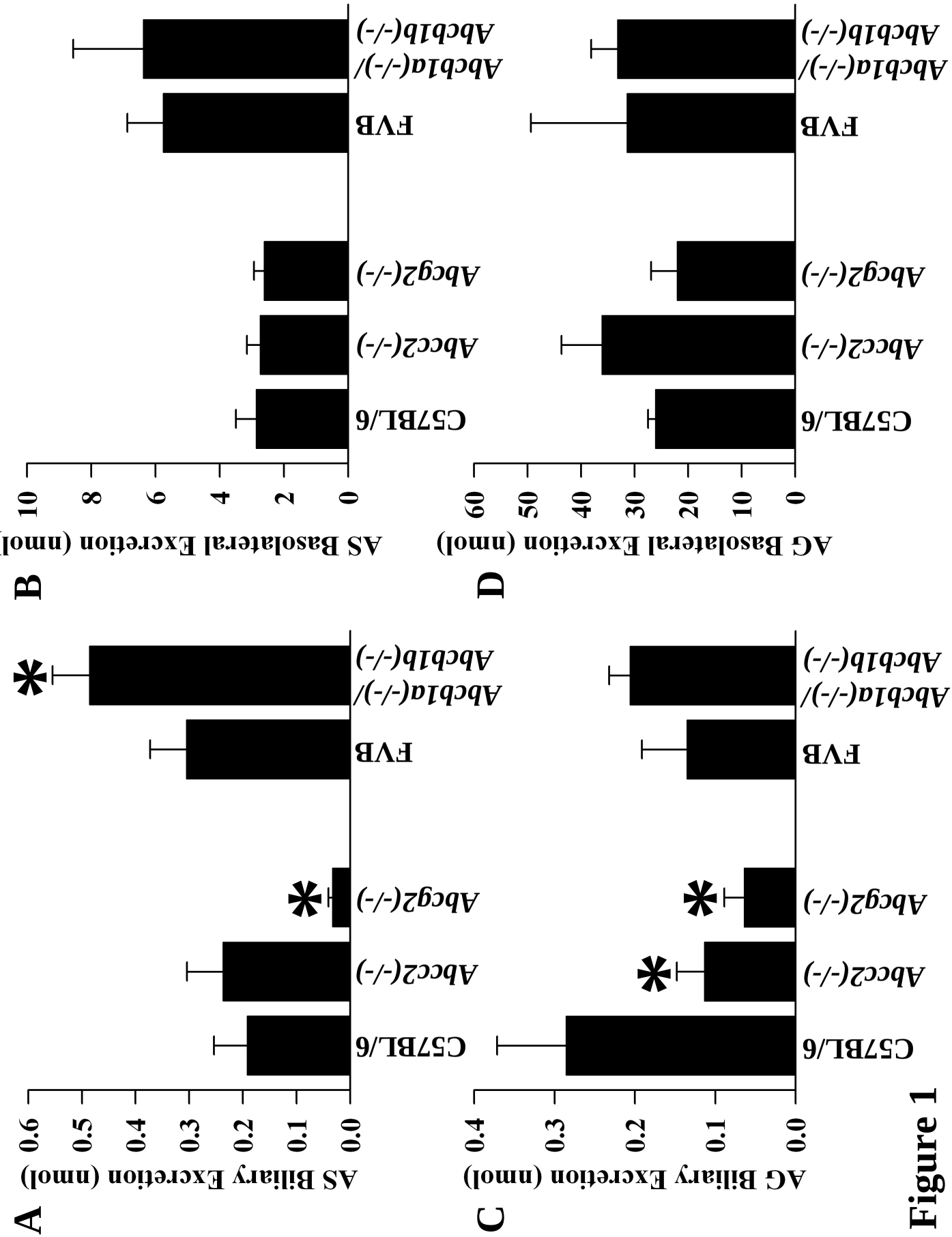

Figure 1

Figure 2

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Figure 3

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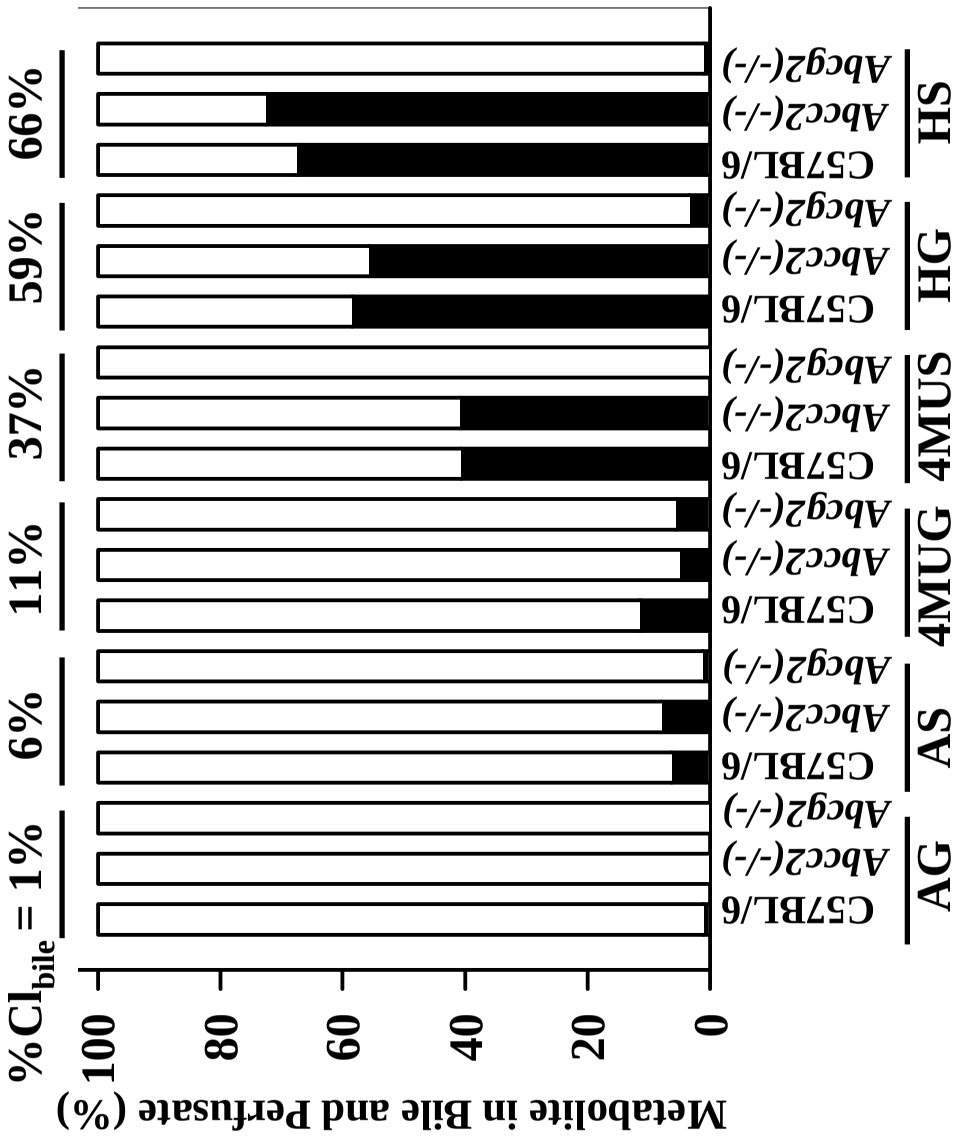

Figure 4