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Altered Hepatobiliary Disposition of 5 (and 6)-carboxy-2',7'-dichlorofluorescein in *Abcg2* (Bcrp1) and *Abcc2* (Mrp2) Knockout Mice

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Running Title: Altered Hepatic Transport in *Abcg2* and *Abcc2* Knockout Mice

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Abbreviations: ABC, ATP-binding cassette; CDF, 5 (and 6)-carboxy-2',7'-dichlorofluorescein; CDFDA, CDF diacetate; MDR/Mdr, multidrug resistance; MRP/Mrp, multidrug resistance-associated protein; BCRP/Bcrp, breast cancer resistance protein; BSEP/Bsep, bile salt export pump; GY/TR$^-$, Groningen Yellow/Transport-deficient; EHBR, Eisai hyperbilirubinemia rat

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Abstract

This study characterized the hepatobiliary disposition of 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF), a model Abcc2/Mrp2 (canalicular) and Abcc3/Mrp3 (basolateral) substrate, in perfused livers from male C57BL/6 wild-type, *Abcg2*-/-, and *Abcc2*-/- mice. After single-pass liver perfusion with 1 μM CDF diacetate for 30 min and an additional 30-min perfusion with CDF-free buffer, cumulative biliary excretion of CDF in *Abcg2*-/- mice was significantly higher than in wild-type mice (65 ± 6% and 47 ± 15% of dose, respectively, $p < 0.05$), whereas CDF was not excreted into bile of *Abcc2*-/- mice. Cumulative recovery of CDF in perfusate was significantly higher in *Abcc2*-/- (90 ± 8% of dose) relative to wild-type mice (35 ± 11% of dose). Compartmental pharmacokinetic analysis revealed that the rate constant for CDF biliary excretion was significantly increased in *Abcg2*-/- (0.061 ± 0.005 min^{-1}) compared to wild-type mice (0.039 ± 0.011 min^{-1}). The rate constant governing the basolateral excretion of CDF was ~4-fold higher in *Abcc2*-/- (0.12 ± 0.02 min^{-1}) relative to wild-type mice (0.030 ± 0.011 min^{-1}), but was not altered in *Abcg2*-/- mice (0.031 ± 0.004 min^{-1}). Hepatic Abcc3 protein levels, determined by immunoblot analysis, were ~60% higher in *Abcc2*-/- mice than in wild-type mice. In contrast, neither Abcc3 protein levels nor Abcc2 mRNA levels were altered in *Abcg2*-/- relative to wild-type mice. These data in knockout mouse models demonstrate that loss of expression and function of one canalicular transport protein may change the route and/or extent of excretion into bile or perfusate due to alterations in the function of other basolateral or canalicular transport proteins.

Introduction

Hepatocytes, the functional units of the liver, have a polarized cellular structure with unique basolateral (sinusoidal) and apical (canalicular) plasma membrane domains. Two opposing transport processes occur on the hepatic basolateral membrane: compounds are taken up into the liver and are excreted into sinusoidal blood by uni- or bi-directional transporters. In contrast, hepatic canalicular excretion is mediated by uni-directional ATP-binding cassette (ABC) transport proteins, such as Abcc2 [multidrug resistance-associated protein 2 (Mrp2)], Abcg2 [breast cancer resistance protein 1 (Bcrp1)], Abcb1 [P-glycoprotein (P-gp, MDR1)], and Abcb11 [bile salt export pump (Bsep)], which are responsible for biliary excretion of xenobiotics, endobiotics, and their metabolites. Hepatic transport, including uptake, sinusoidal and biliary excretion, plays an important role in determining systemic drug pharmacokinetics by influencing oral bioavailability, hepatic drug metabolism, and biliary elimination.

ABCC2/Abcc2 (MRP2/Mrp2) is one of the most extensively studied hepatic proteins in the MRP/Mrp family and is responsible for the biliary excretion of organic anions, including numerous antibiotics, anticancer drugs, and various phase II conjugates (Fardel et al., 2005; Leslie et al., 2005). Moreover, ABCC2/Abcc2 transports conjugates of endogenous molecules, such as bile salts and steroids, and deficiency in ABCC2 results in the clinical disorder, Dubin-Johnson syndrome (Iyanagi et al., 1998). Animals genetically deficient in Abcc2 have been used to characterize the pharmacokinetic and pharmacodynamic implications of Abcc2 loss-of-function. Groningen Yellow/Transport-deficient (GY/TR$^-$) Wistar rats (Jansen et al., 1987; Paulusma et al., 1996) and Eisai hyperbilirubinemic Sprague-Dawley rats (EHBR) (Ito et al., 1997) are used most commonly as pre-clinical models of cholestatic patients with Dubin-Johnson syndrome. To date, many studies have been performed to characterize drug disposition in Abcc2-deficient rats (Jager et al., 2003; Hanawa et al., 2004; Takayanagi et al., 2005). Additionally, it has been reported that

in Abcc2-deficient rats, expression of the basolateral membrane transporter, Abcc3, is up-regulated, which results in increased excretion of organic anions across the sinusoidal membrane (Xiong et al., 2002; Kuroda et al., 2004); ABCC3 expression is also up-regulated in patients with Dubin-Johnson syndrome (Konig et al., 1999).

ABCG2/Abcg2, an ABC half-transporter, is the most recently identified hepatic canalicular transporter that is responsible for biliary excretion of various organic anions and cations, as well as their phase II conjugates (Staud and Pavek, 2005). ABCG2/Abcg2 can also efficiently export anticancer drugs (e.g., mitoxantrone and topotecan) out of cancer cells, thus causing multidrug resistance (Doyle et al., 1998; Allen et al., 1999; Miyake et al., 1999). *Abcg2*-/- mice have been used to examine the physiological and pharmacological importance of Abcg2 and to investigate alterations in drug disposition in the absence of this transporter (Jonker et al., 2002; van Herwaarden et al., 2003; Mizuno et al., 2004; Merino et al., 2005). However, livers of *Abcg2*-/- mice may exhibit changes in the expression and function of compensatory transport mechanisms, similar to Abcc3 up-regulation in Abcc2-deficient rats, which emphasizes the need to elucidate the mechanisms of alteration in order to properly interpret the data obtained in this knockout model.

Previously, we reported that the fluorescent Abcc2 probe utilized in the current study, 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF), is an Abcc3 substrate, and that basolateral excretion of CDF is increased in livers from TR^- relative to wild-type rats (Zamek-Gliszczynski et al., 2003). The molecular weight of CDF (~445) places it in the molecular weight range of small molecule drugs that undergo appreciable biliary excretion. CDF fluorescence, which can be measured directly in bile and perfusate matrices, simplifies quantification of this transport probe. Non-fluorescent CDF diacetate (CDFDA), the diacetate promoiety used for efficient CDF delivery to cells, is taken up into liver by passive diffusion, where it is instantaneously hydrolyzed to CDF by intracellular esterases (Breeuwer et al., 1995; Zamek-Gliszczynski et al., 2003). CDF is a

useful probe for characterization of hepatic Abcc2 and Abcc3 function.

In contrast to Abcc2-deficient rats, which have been extensively characterized, there are no reports on the phenotype of *Abcc2*-/- mice. Similar to up-regulation of Abcc3 in Abcc2-deficient rats, it is possible that expression and activity of other transporter(s) could compensate for the loss of Abcc2 and Abcg2 in gene knockout mice. Therefore, in order to use genetically deficient animals, including *Abcc2*-/- and *Abcg2*-/- mice, for investigation of the role of specific proteins in drug disposition, it is necessary to characterize the phenotypes of these animals. The present studies characterized the hepatic transport phenotypes of *Abcc2*-/- and *Abcg2*-/- mice. Analysis of changes in the expression of hepatic ABC proteins, as well as mouse liver perfusion studies using CDFDA to investigate potential alterations in CDF hepatobiliary disposition, revealed significant changes in the expression and function of other basolateral and canalicular transport proteins.

Materials and Methods

Chemicals and Reagents

CDF and CDFDA were purchased from Molecular Probes (Eugene, OR). Anti-Bcrp (Abcg2) antibody (BXP-53) and anti-P-glycoprotein (Abcb1a/1b) antibody (C219) were purchased from Alexis Biochemicals (San Diego, CA). Anti-Mrp3 (Abcc3) antibody was a kind gift from Dr. Yuichi Sugiyama, University of Tokyo, Tokyo, Japan. Anti-Bsep (Abcb11) antibody was kindly provided by Dr. Peter J. Meier, University Hospital Zurich, Zurich, Switzerland. Anti-β-actin antibody (MAB1501) was purchased from Chemicon International (Temecula, CA). All other reagents were of analytical grade or the highest grade commercially available.

Animals

Male C57BL/6 wild-type (age-matched heterozygotes), *Abcc2*-/-, and *Abcg2*-/- mice (23-34 g) were a gift from Eli Lilly and Company. Embryonic stem cells derived from the 129/OlaHsd mouse sub strain were used to generate chimeric mice containing full-length cDNA for either Abcg2 (NM 011920) with a 263-279 bp deletion or Abcc2 (AF227274) with an 1886-1897 bp deletion (Deltagen, Inc., San Carlos, CA). F1 mice were generated by breeding with C57BL/6 females and these were backcrossed five generations with heterozygous C57BL/6 mice prior to obtaining F2 homozygous mutant mice (Taconic Farms, Germantown, NY). Mice were maintained on a 12-hour light/dark cycle with access to water and rodent chow *ad libitum*. 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.

Western Blotting

Mouse livers were perfused with phosphate-buffered saline solution (Sigma-Aldrich, St. Louis, MO) and were promptly removed and frozen. Livers were homogenized with 5 volumes of

Tris-HCl solution (pH 7.4) containing complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 1500 g for 10 min, and the supernatant was subsequently ultracentrifuged at 100000 g for 30 min. The resulting pellet was dissolved in Tris-HCl solution (pH 7.4) containing complete protease inhibitor cocktail, 1% sodium dodecyl sulfate and 1 mM ethylenediaminetetraacetic acid. Protein concentration of the solution was determined with the BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL). Approximately 50 μg of total protein per lane was resolved by electrophoresis on NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and was transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were incubated with primary and secondary antibodies (Amersham, Piscataway, NJ). Protein bands of interest were detected by chemiluminescence with SuperSignal West Dura (Pierce Biotechnology, Rockford, IL) and visualized using a VersaDoc 1000 molecular imager (Bio-Rad Laboratories, Hercules, CA). Protein bands were quantified by densitometry using Quantity One v.4.1 (Bio-Rad Laboratories).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total liver RNA was extracted with Trizol reagent (GIBCO BRL, Grand Island, NY) as follows. Liver tissues (ca. 100 μg) were homogenized in 1 mL Trizol, and the homogenates were incubated at room temperature for 5 min to dissociate nucleoprotein complexes. Following the addition of chloroform (1/5 volume of Trizol), the mixture was incubated at room temperature for 2-3 min and was centrifuged at 12000 g for 15 min. RNA in the upper aqueous phase was precipitated with ethanol; the purity and quantity were determined with a Lambda 35 UV/Vis Spectrometer (PerkinElmer, Wellesley, MA). For RT-PCR, the first strand cDNA was synthesized with 5 μg total RNA using Superscript™ First Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's instructions. Multiplex PCR was carried out with two primer pairs for Abcc2 and actin; Abcc2 forward (5'cttatggttctcctggtcc3') and Abcc2 reverse

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(5'cgaatggcagacaagtcc3') amplified a product of 480 bp from Abcc2 cDNA, while β-actin forward (5'gttaccaactgggacgac3') and β-actin reverse (5'gatcttgatcttcatggtgc3') amplified a product of 640 bp from actin cDNA. Actin amplification was used as an internal control for normalization of Abcc2 signal. The following PCR parameters were used: 95°C, 30 sec; 56°C, 45 sec; and 72°C, 1 min.

Single-Pass Liver Perfusion Studies

Following the ligature of the common bile duct between the gallbladder and the duodenum, the gallbladder of anesthetized mice was cannulated with PE-10 tubing (Becton Dickinson, Parsippany, NJ). The portal vein was cannulated with a 20G catheter (B. Braun Medical Inc., Bethlehem, PA) and pre-perfusion of the liver was initiated at a flow rate of 5 mL/min (CDFDA-free Krebs-Henseleit buffer containing 11 mM glucose and 5 μM taurocholate continually oxygenated with 95% O_2/5% CO_2). The abdominal vena cava below the liver was severed immediately by incision, and the inferior vena cava above the liver was cannulated with a 20G catheter. Subsequently, the abdominal inferior vena cava was ligated to direct all perfusate outflow to the cannula connected to the catheter. Following an ~15-min pre-perfusion period for equilibration of liver temperature and bile flow, the liver was perfused with buffer containing 1 μM CDFDA for 30 min followed by perfusion with CDFDA-free buffer during the subsequent 30-min CDF washout phase of the experiment. Bile was collected in 10-min intervals; outflow perfusate was collected in 5-min intervals (0-30 min) and 2-min intervals during the CDF-washout period (30-60 min). CDF concentrations in bile and perfusate samples were quantified by fluorescence spectroscopy (λ_{ex} 505 nm, λ_{em} 523 nm) using a FL600 micro plate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) in accordance with previous techniques (Zamek-Gliszczynski et al., 2003). CDFDA has been reported to be hydrolyzed in phosphate-buffered saline at physiological pH and temperature with a half-life of 7.6 ± 0.1 hr (Zamek-Gliszczynski et al., 2003).

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Even though CDFDA hydrolysis was minor over this short perfusion period of time, CDF concentrations in outflow perfusate were corrected for CDFDA hydrolysis at each time point.

Pharmacokinetic Modeling

Various models were evaluated for their description of CDF basolateral and biliary excretion rate-time data. Model selection criteria included coefficients of variation on estimated parameters, correlation between parameters, rank and condition numbers, visual examination of the distribution of residuals, and Akaike's Information Criterion (Akaike, 1976). Based on these goodness-of-fit criteria, the pharmacokinetic model presented in Fig. 5 best described the data; differential equations based on this model scheme were as follows:

$$\frac{dX_{\text{sin}}}{dt} = (Q \cdot C_{in} - Cl_{up} \cdot C_{in}) + k_{perfusate} \cdot X_{liver} - Q \cdot C_{out}, \qquad t \le 30\,\text{min}, \qquad X^{0}_{\text{sin}} = 0$$

$$\frac{dX_{\text{sin}}}{dt} = k_{perfusate} \cdot X_{liver} - Q \cdot C_{out}, \qquad t > 30\,\text{min}$$

$$\frac{dX_{liver}}{dt} = (Cl_{up} \cdot C_{in}) - k_{perfusate} \cdot X_{liver} - k_{bile} \cdot X_{liver}, \qquad t \le 30\,\text{min}, \qquad X^{0}_{liver} = 0$$

$$\frac{dX_{liver}}{dt} = -k_{perfusate} \cdot X_{liver} - k_{bile} \cdot X_{liver}, \qquad t > 30\,\text{min}$$

where Q is perfusate flow, C_{in} is the concentration of unhydrolyzed CDFDA in inflow perfusate, Cl_{up} is the CDFDA hepatic uptake clearance, C_{out} is the concentration of CDF in outflow perfusate, X_{sin} is the amount of CDF(DA) in the sinusoidal space, X_{liver} is the amount of CDF in the liver, $k_{\text{perfusate}}$ is the first-order rate constant for CDF basolateral excretion, and k_{bile} is the first-order rate constant for CDF biliary excretion. These differential equations were resolved simultaneously by nonlinear least-squares regression analysis to fit CDF basolateral and biliary excretion rate-time data using a $1/\hat{y}$ weighting scheme and a Gauss-Newton (Levenberg and Hartley) minimization process (WinNonlin 4.1, Pharsight Corportion, Mountain View, CA). The model assumed

instantaneous hydrolysis of CDFDA to CDF in the liver.

Statistical Analysis

Statistical significance was assessed using one-way analysis of variance with Dunnett's *post hoc* test. Variance equality between compared groups and normality of the data were confirmed prior to parametric testing. In all cases, the criterion for statistical significance was $p < 0.05$. All data are reported as the mean ± S.D., n=3-6/group.

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Results

Transporter Expression in Mouse Livers

Protein expression of Abcg2, Abcc3, Abcb1a/1b, and Abcb11 in livers from wild-type and knockout mice was determined by Western blot analysis (Fig. 1A). The absence of Abcg2 protein in *Abcg2*-/- mice was confirmed, whereas there was no difference in protein levels of Abcg2 between wild-type and *Abcc2*-/- mice. In contrast, Abcc3 expression measured by normalized densitometry in livers of *Abcc2*-/- mice was significantly higher (57 ± 36%) relative to wild-type mice, but was unchanged in *Abcg2*-/- mice. Abcb1a/1b and Abcb11 hepatic protein levels were comparable between wild-type and knockout mice. Since we were unable to determine Abcc2 protein levels due to the lack of a proper anti-mouse Abcc2 antibody, the mRNA level of Abcc2 was examined with RT-PCR (Fig. 1B). The mRNA levels of Abcc2 were similar in livers from wild-type and *Abcg2*-/- mice, but no Abcc2 mRNA was detected in *Abcc2*-/- mice.

Single-Pass Liver Perfusion Study

Baseline bile flow before CDFDA perfusion in wild-type, *Abcg2*-/- and *Abcc2*-/- mice was 1.02 ± 0.26, 1.07 ± 0.27 and 0.50 ± 0.11 μL/min/g liver, n=3-6/group, respectively. The bile flow in each group was stable during the perfusion period (Fig. 2). Interestingly, following the pre-perfusion period, bile flow in *Abcg2*-/- mice was higher than that in wild-type mice throughout the perfusion, resulting in a significant increase in the average 0-60 min bile flow (Table 1). In contrast, bile flow in *Abcc2*-/- mice was significantly lower (~40%) than in wild-type mice.

The cumulative biliary excretion of CDF is plotted in Fig. 3. The cumulative CDF biliary excretion at each time point in *Abcg2*-/- mice was significantly higher relative to wild-type mice, reaching 65 ± 6% of the dose in *Abcg2*-/- mice and 47 ± 15% of the dose in wild-type mice at 60 min (Table 1). Biliary excretion of CDF in *Abcc2*-/- mice was negligible.

In the present liver perfusion studies, CDFDA hydrolysis in perfusate over the course of the

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30-min liver perfusion with CDFDA was less than 5% of total CDFDA at 30 min. CDF concentrations in outflow perfusate during and after liver perfusion with CDFDA are shown in Fig. 4. CDF concentrations in outflow perfusate in each group reached steady-state by the end of the 30-min liver perfusion with CDFDA, and were comparable between *Abcg2*-/- and wild-type mice; the cumulative recovery of CDF in perfusate at 60 min was similar (32 ± 3% and 35 ± 11% of dose, *Abcg2*-/- and wild-type mice, respectively). In *Abcc2*-/- mice, however, CDF concentrations in outflow perfusate were much higher than in the other mice, and the cumulative 60-min recovery of CDF in perfusate was significantly higher (90 ± 8% of dose) than in wild-type mice (Table 1). During the 30-min washout CDFDA-free phase of perfusion, CDF concentrations in outflow perfusate decreased steeply in *Abcc2*-/- mice relative to wild-type and *Abcg2*-/- mice, approaching similar concentrations at 60 min.

Pharmacokinetic Modeling

Pharmacokinetic parameter estimates recovered from the nonlinear regression analysis of basolateral and biliary CDF excretion rate-time data are summarized in Table 2. The CDFDA Cl_{up} values were almost identical between mouse groups (4.0 ± 0.7 to 4.4 ± 0.2 mL/min). The rate constant for CDF biliary excretion (k_{bile}) was increased significantly in *Abcg2*-/- mice (0.061 ± 0.005 min^{-1}) in comparison to wild-type mice (0.039 ± 0.011 min^{-1}). In contrast, the rate constant governing CDF basolateral excretion ($k_{perfusate}$) was significantly higher in *Abcc2*-/- (0.12 ± 0.02 min^{-1}) relative to wild-type mice (0.030 ± 0.011 min^{-1}), but was not altered in *Abcg2*-/- mice (0.031 ± 0.004 min^{-1}). Utilizing the model scheme in Fig. 5 and mean parameter estimates reported in Table 2, basolateral and biliary CDF excretion rate-time profiles were simulated in wild-type and knockout mice (Fig. 6).

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Discussion

In order to characterize phenotypic changes in hepatobiliary transport activity in *Abcg2*-/- and *Abcc2*-/- mice, the disposition of CDF, a substrate of Abcc2 (canalicular transporter) and Abcc3 (basolateral transporter), was investigated using single-pass liver perfusions. In rat hepatocytes, CDF is taken up via organic anion transporting polypeptides (Zamek-Gliszczynski et al., 2003) and subsequently efficiently excreted into bile (>80% of dose) in single-pass rat liver perfusion studies employing CDF directly (Chandra et al., 2005). However, in mice, the hepatic extraction of CDF and subsequent biliary excretion (< 0.1% of dose, data not shown) were extremely low, suggesting species differences in substrate specificity of mouse and rat organic anion transporting polypeptides. Therefore, mouse livers were perfused with CDFDA to enhance hepatic delivery of CDF.

Zamek-Gliszczynski et al. (2003) reported that hepatic uptake of CDFDA was not saturable, temperature independent, and insensitive to classic inhibitors of hepatic organic anion uptake (e.g., bromosulfophthalein, digoxin, *p*-aminohippurate, and probenecid), thus demonstrating that hepatic uptake of CDFDA occurred by passive diffusion. In the current mouse liver perfusion studies with perfusate flow rate of 5 mL/min, CDFDA Cl_{up} values were estimated to be 4.0-4.4 mL/min in all groups (Table 2), indicating a high (>80%) hepatic extraction ratio for CDFDA. Therefore, hepatic uptake of CDFDA appears to be a flow-rate-limited process in mice.

Recently, *Abcg2*-/- mice have been used to investigate the role of Abcg2 in the disposition of various substrates (Jonker et al., 2002; van Herwaarden et al., 2003; Mizuno et al., 2004; Merino et al., 2005). Although baseline bile flow in the current liver perfusion study before the initiation of CDFDA perfusion in wild-type and *Abcg2*-/- mice was almost the same (1.02 ± 0.26 and 1.07 ± 0.27 μL/min/g liver, respectively), the average bile flow throughout the study in *Abcg2*-/- mice was significantly higher than in wild-type mice (Table 1). These findings are consistent with

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reports of elevated bile flow in other canalicular transporter knockout mice. *Abcb11*-/- mice exhibited significantly higher bile flow than wild-type mice on a cholic acid-rich diet (Wang et al., 2003). Moreover, Huang and Vore (2001) reported that bile flow in *Abcb4 (Mdr2)*-/- mice was higher than in wild-type mice in liver perfusion studies using the Abcc2 substrate, estradiol-17β-D-glucuronide. Bile flow in rats may be increased by solvent drag associated with concentrative excretion of drugs into bile (Platzer et al., 2001). In the current study, therefore, increased bile flow in *Abcg2*-/- mice may be attributed to elevated solvent drag caused by greater CDF biliary excretion. Further investigations using Abcg2-specific substrates are necessary to determine the mechanism of increased bile flow in *Abcg2*-/- mice.

In addition to bile flow, biliary excretion of CDF in *Abcg2*-/- mice was significantly elevated (Table 1). Likewise, the rate constant governing CDF biliary excretion (k_{bile}) was significantly increased in *Abcg2*-/- mice (Table 2). The exact mechanism responsible for the increased amount of CDF in bile of *Abcg2*-/- mice is unclear, but some possible explanations include down-regulation of Abcc3, up-regulation of Abcc2, stimulation of Abcc2 activity, and/or increased trafficking of Abcc2 to the canalicular membrane. Protein expression of basolateral Abcc3 was not altered in *Abcg2*-/- mice, and there was no difference between *Abcg2*-/- and wild-type mice in the rate constant governing the basolateral excretion of CDF ($k_{perfusate}$). These findings suggest that the mechanism responsible for basolateral excretion of CDF was maintained. Additionally, the protein levels of canalicular transporters and Abcc2 mRNA were not different between *Abcg2*-/- and wild-type mice, except for the absence of the Abcg2 protein. Johnson et al. (2002) have reported that phenobarbital and pregnenolone-16α-carbonitrile increased biliary excretion of sulfhydryls in rats, in agreement with increased Abcc2 protein levels, whereas mRNA levels were not altered. In rat livers, Abcc2 mRNA levels are a poor predictor of Abcc2 protein expression (Patel et al., 2003), suggesting the possibility that Abcc2 protein expression in *Abcg2*-/-

mice may be up-regulated without an increase in mRNA. In addition, the observed increase in biliary excretion without up-regulation of the expression of other canalicular transporters has been reported previously: biliary excretion of estradiol-17β-D-glucuronide was increased in *Abcb4(Mdr2)*-/- relative to wild-type mice, even though the expression of Abcc2 was actually decreased by 35% in *Abcb4*-/- mice (Huang and Vore, 2001). Thus, alterations in the biliary excretion of probe substrates may not correlate directly with changes in the expression of canalicular transporters that are postulated to be primarily responsible for their translocation across the hepatic canalicular membrane. Furthermore, it has been reported that activity of Abcc2 can be stimulated by organic anions (Bakos et al., 2000; Evers et al., 2000), and this stimulation could be enhanced in the livers of *Abcg2*-/- mice, presumably due to elevated intrahepatic levels of the stimulant in the absence of Abcg2. In addition, it is also possible that Abcc2 trafficking from intracellular membranes to the canalicular membrane may be enhanced in *Abcg2*-/- mice. Proper trafficking of Abcc2 to the canalicular membrane is required for CDF biliary excretion (Zhang et al., 2005). Increased trafficking of Abcc2 protein to the canalicular membrane in *Abcg2*-/- mice may contribute to the higher CDF recovery in bile and perfusate (Table 1). Taking these findings into consideration, Abcc2 transport activity in *Abcg2*-/- mice may be elevated without an increase in Abcc2 mRNA. The exact mechanism of enhanced biliary excretion of CDF in *Abcg2*-/- mice requires further investigation. Microarray analysis of gene expression in several organs and tissues currently is underway to obtain further information about *Abcg2*-/- mice.

Although numerous reports regarding various knockout mice exist in the literature, there are no accounts of the *Abcc2*-/- mouse phenotype. CDF disposition was studied by Zamek-Gliszczynski et al. (2003) using isolated perfused Abcc2-deficient TR^- rat livers. In the present study, CDF disposition in *Abcc2*-/- mice was investigated using a single-pass liver perfusion method. Consistent with TR^- rats (Xiong et al., 2000; Patel et al., 2003), bile flow in

Abcc2-/- mice was lower than in wild-type mice. These findings suggest that Abcc2 plays a role in bile flow in mice, in agreement with previous reports in rats. In contrast to wild-type mice, biliary excretion of CDF in perfused livers from *Abcc2*-/- mice was negligible, demonstrating that CDF was excreted into bile solely by Abcc2 in mice, an observation consistent with the previous report using TR^- rats (Zamek-Gliszczynski et al., 2003). Perfusate concentrations of CDF were higher in isolated perfused livers of TR^- rats (Zamek-Gliszczynski et al., 2003). In the present study, CDF concentrations in outflow perfusate obtained from *Abcc2*-/- mice were much higher than those in wild-type mice; 90 ± 8% of the dose was recovered in perfusate. Additionally, the rate constant for basolateral excretion ($k_{perfusate}$) of CDF was ~4-fold higher in *Abcc2*-/- mice. Western blot analysis revealed a significant increase (57 ± 36%) in Abcc3 protein expression in *Abcc2*-/- mice (Fig. 1), consistent with Abcc3 up-regulation in TR^- rats (Xiong et al., 2002). These findings strongly support the role of Abcc3 as the hepatic basolateral efflux transporter for CDF; the expression and function of Abcc3 is up-regulated in the absence of Abcc2 to compensate for the inability to excrete organic anions into bile.

In humans, both ABCG2 and ABCC2 are widely distributed in the apical membrane of various tissues, including brain, gut, placenta, and liver (Leslie et al., 2005). In addition, ABCC2, but not ABCG2, is observed on the apical membrane of kidney proximal tubules (Schaub et al., 1999; Jonker et al., 2000; Maliepaard et al., 2001). In contrast to humans, Abcg2 is highly expressed in both rat and mouse kidney (Jonker et al., 2000; Shimano et al., 2003). In addition to the up-regulation of Abcc3 in livers of rats deficient in Abcc2, it also has been reported that Abcc3 expression was increased in kidneys of both EHBR and TR^- rats (Kuroda et al., 2004; Chen et al., 2005). In studies using *Abcg2*-/- mice and EHBR, it was reported recently that Abcg2 played an important role in intestinal secretion of glucuronide and sulfate conjugates, whereas Abcc2 was only involved in the efflux of some glucuronide conjugates (Adachi et al., 2005). Therefore,

genetically deficient animals are likely to exhibit changes in the expression of compensatory systems, suggesting that a more comprehensive approach will be necessary to fully elucidate the phenotypes of these animals.

In conclusion, loss of Abcg2 expression resulted in increased CDF biliary excretion, while the loss of Abcc2 protein resulted in the absence of CDF biliary excretion and increased CDF basolateral excretion caused by increased Abcc3 expression and function. Knockout animal models are useful tools for the characterization of the role of specific proteins. However, these results indicate that in addition to expected changes in CDF hepatobiliary disposition in the absence of Abcc2 or Abcg2, unexpected alterations in basolateral and/or canalicular transport activities might result from the loss-of-function of a single canalicular transport protein. These alterations could result in significant changes in biliary or systemic drug exposure that may impact efficacy and/or toxicity, suggesting that a better understanding of these knockout mice is necessary for appropriate use of these models. In addition, complete characterization of these alterations is required prior to accurate interpretation and utilization of these models in pre-clinical drug development.

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Footnotes

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Legends for Figures

Fig. 1 Expression levels of Abcg2, Abcc3, Abcb1a/1b, Abcb11, and Abcc2 in livers from wild-type, *Abcg2*-/-, and *Abcc2*-/- mice. A. Western blot of Abcg2, Abcc3, Abcb1a/1b, Abcb11, and actin in livers from wild-type, *Abcg2*-/-, and *Abcc2*-/- mice. B. RT-PCR amplification product of Abcc2 and actin mRNA extracted from livers of wild-type, *Abcg2*-/-, and *Abcc2*-/- mice.

Fig. 2 Bile flow in single-pass perfused livers from wild-type (open circle), *Abcg2*-/- (closed square), and *Abcc2*-/- (closed triangle) mice. Each symbol represents the mean ± S.D. of 3-6 experiments.

Fig. 3 Cumulative biliary excretion of CDF in single-pass perfused livers from wild-type (open circle), *Abcg2*-/- (closed square), and *Abcc2*-/- (closed triangle) mice. Each symbol represents the mean ± S.D. of 3-6 experiments. The S.D. of *Abcc2*-/- mouse data is contained within the symbols. The difference in cumulative biliary excretion of CDF between wild-type, and *Abcg2*-/- or *Abcc2*-/- mice was significant at every time point ($p < 0.05$).

Fig. 4 CDF concentrations in outflow perfusate in single-pass perfused livers from wild-type (open circle), *Abcg2*-/- (closed square), and *Abcc2*-/- (closed triangle) mice. Each symbol represents the mean ± S.D. of 3-6 experiments.

Fig. 5 Scheme depicting the compartmental pharmacokinetic model used to describe the hepatobiliary disposition of CDF in single-pass perfused livers. *Q*, flow rate of perfusate;

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C_{in}, concentration of CDFDA in inflow perfusate; C_{out}, concentration of CDF in outflow perfusate; Cl_{up}, hepatic uptake clearance of CDFDA; k_{bile}, first-order rate constant governing CDF biliary excretion; $k_{perfusate}$, first-order rate constant governing CDF basolateral excretion; X_{sin}, amount of CDF(DA) in sinusoidal space; X_{liver}, amount of CDF in liver; X_{bile}, amount of CDF in bile; $X_{perfusate}$, amount of CDF in outflow perfusate.

Fig. 6 Simulation of the excretion rate-time profiles of CDF into perfusate and bile using the compartmental model presented in Fig. 5 and mean parameter estimates summarized in Table 2. Actual excretion rates of CDF into perfusate (open symbols) and bile (closed symbols) from wild-type (A), *Abcg2*-/- (B), and *Abcc2*-/- (C) mice are plotted together with the simulated rates. Each symbol represents the mean ± S.D. of 3-6 experiments.

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Table 1 Bile flow and CDF recovery at 60 min in bile and perfusate of single-pass perfused livers from wild-type, *Abcg2*-/-, and *Abcc2*-/- mice.

	Wild-type	*Abcg2* -/-	*Abcc2* -/-
Average of bile flow (μL/min/g liver)	0.82 ± 0.07	0.98 ± 0.04*	0.48 ± 0.20*
CDF excretion (% of dose)			
Bile	47 ± 15	65 ± 6*	0.027 ± 0.023*
Perfusate	35 ± 11	32 ± 3	90 ± 8*
Total	82 ± 9	97 ± 5*	90 ± 8

Data represent the mean ± S.D. of 3-6 experiments.

*$p < 0.05$, compared with wild-type mice.

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Table 2 Pharmacokinetic parameters governing CDF disposition in single-pass perfused livers from wild-type, *Abcg2*-/-, and *Abcc2*-/- mice were estimated using nonlinear regression analysis with the compartmental model shown in Fig. 5.

	Wild-type	*Abcg2* -/-	*Abcc2* -/-
Cl_{up} (mL/min)	4.0 ± 0.7	4.4 ± 0.2	4.2 ± 0.5
k_{bile} (min^{-1})	0.039 ± 0.011	0.061 ± 0.005*	N.C.
$k_{perfusate}$ (min^{-1})	0.030 ± 0.011	0.031 ± 0.004	0.12 ± 0.02*

Data represent the mean ± S.D. of 3-6 experiments.

*$p < 0.05$, compared with wild-type mice.

N.C. Not calculated

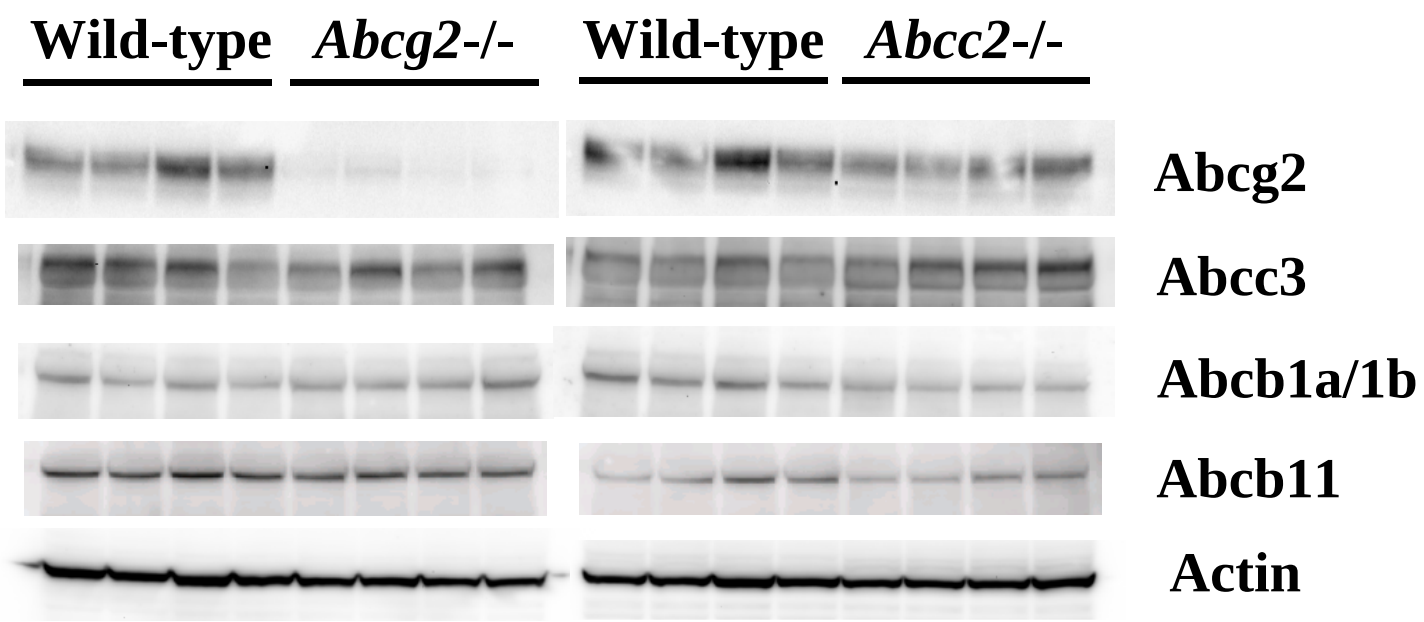

Figure 1A

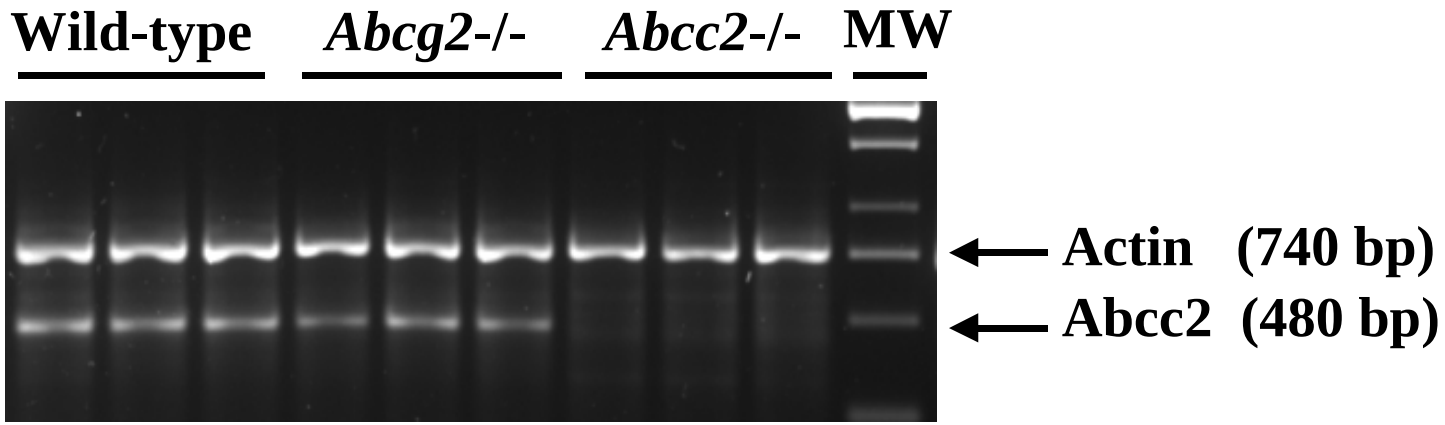

Figure 1B

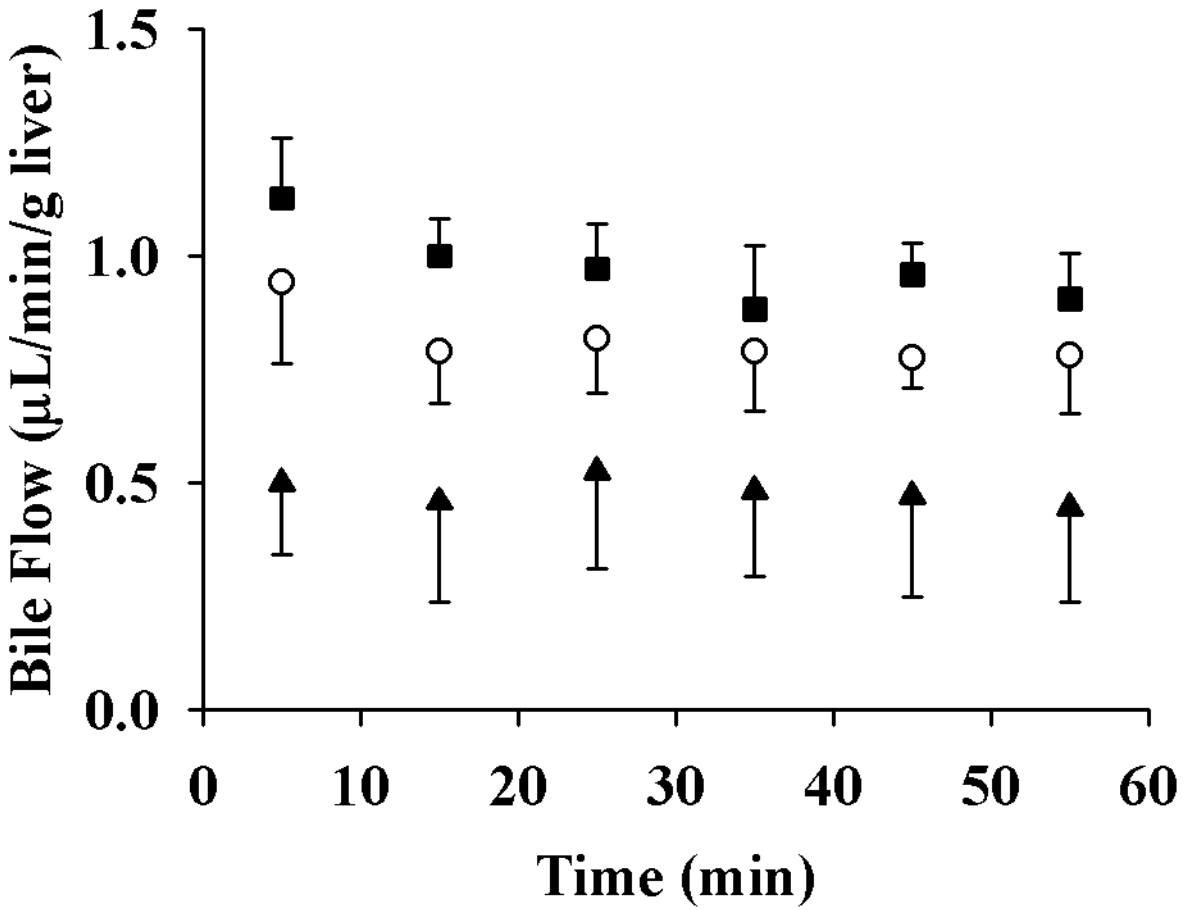

Figure 2

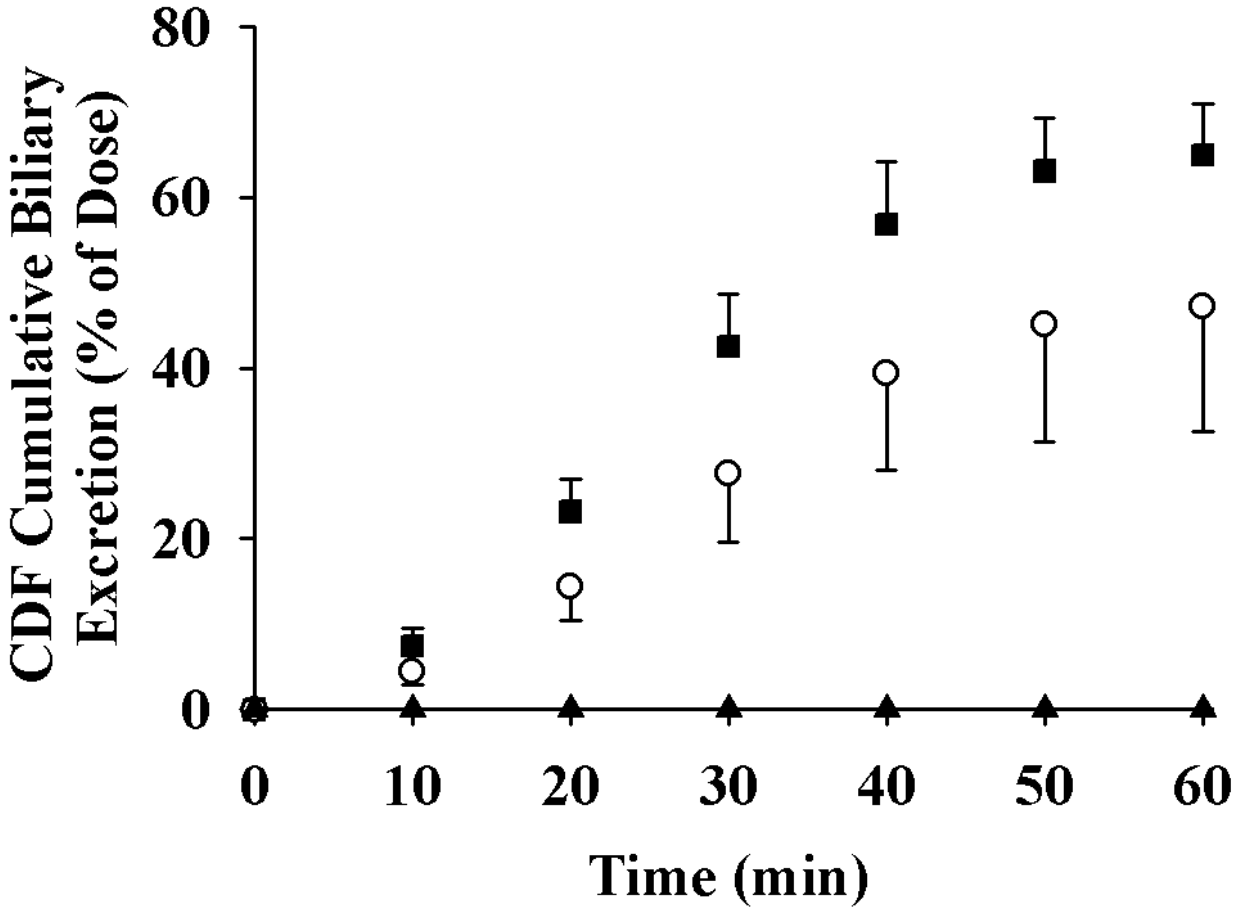

Figure 3

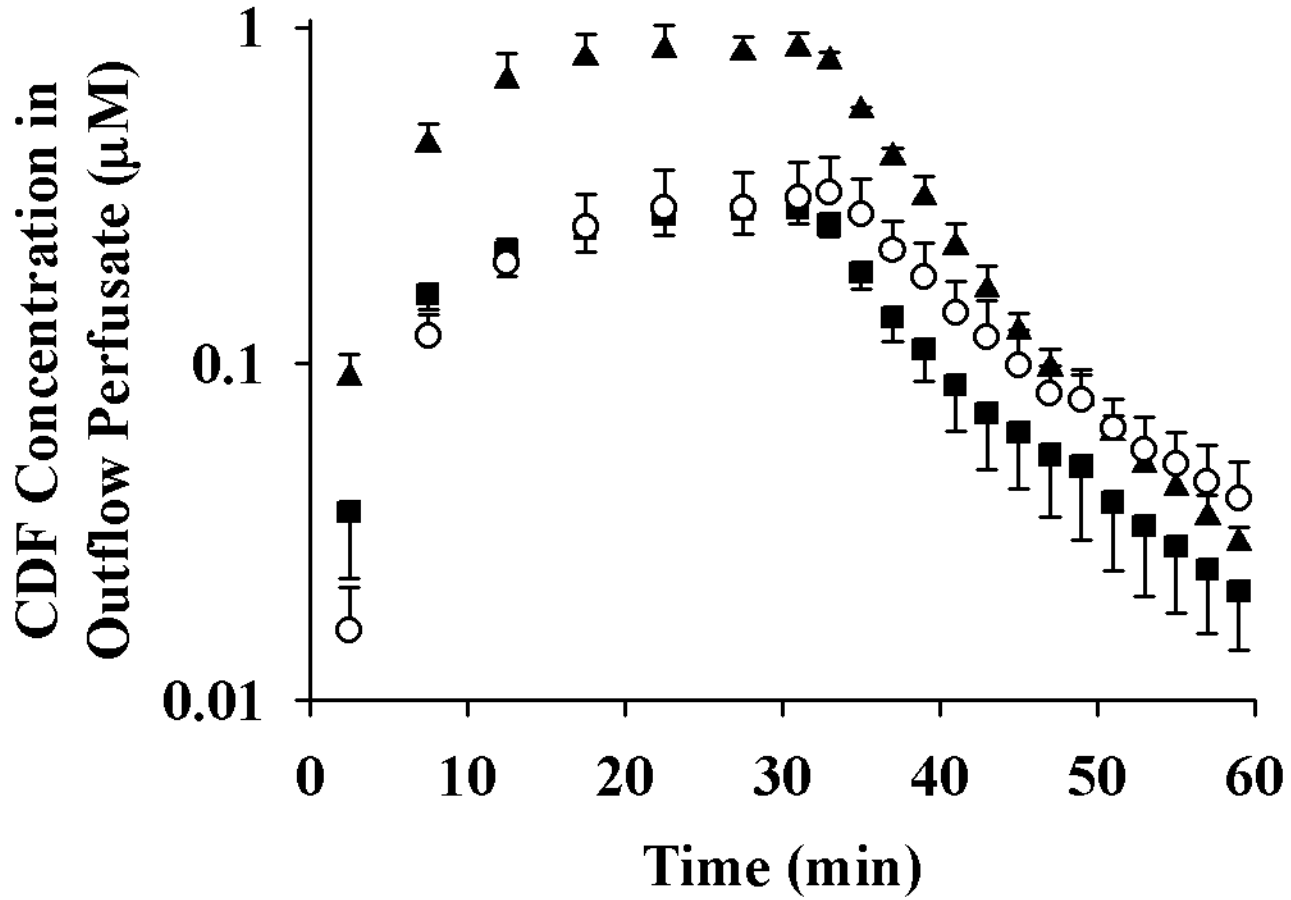

Figure 4

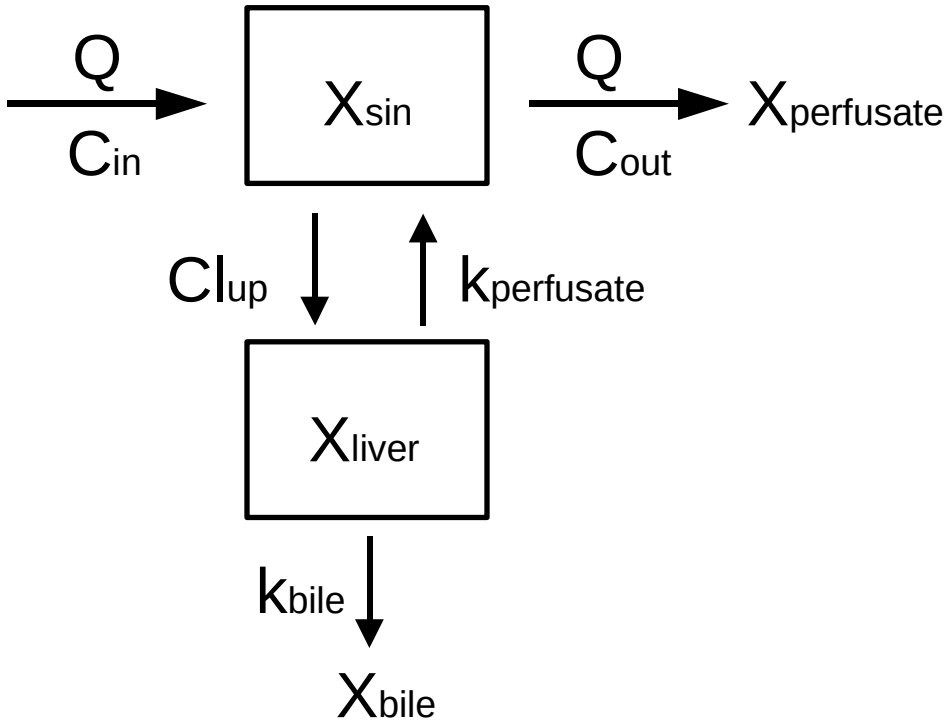

Figure 5

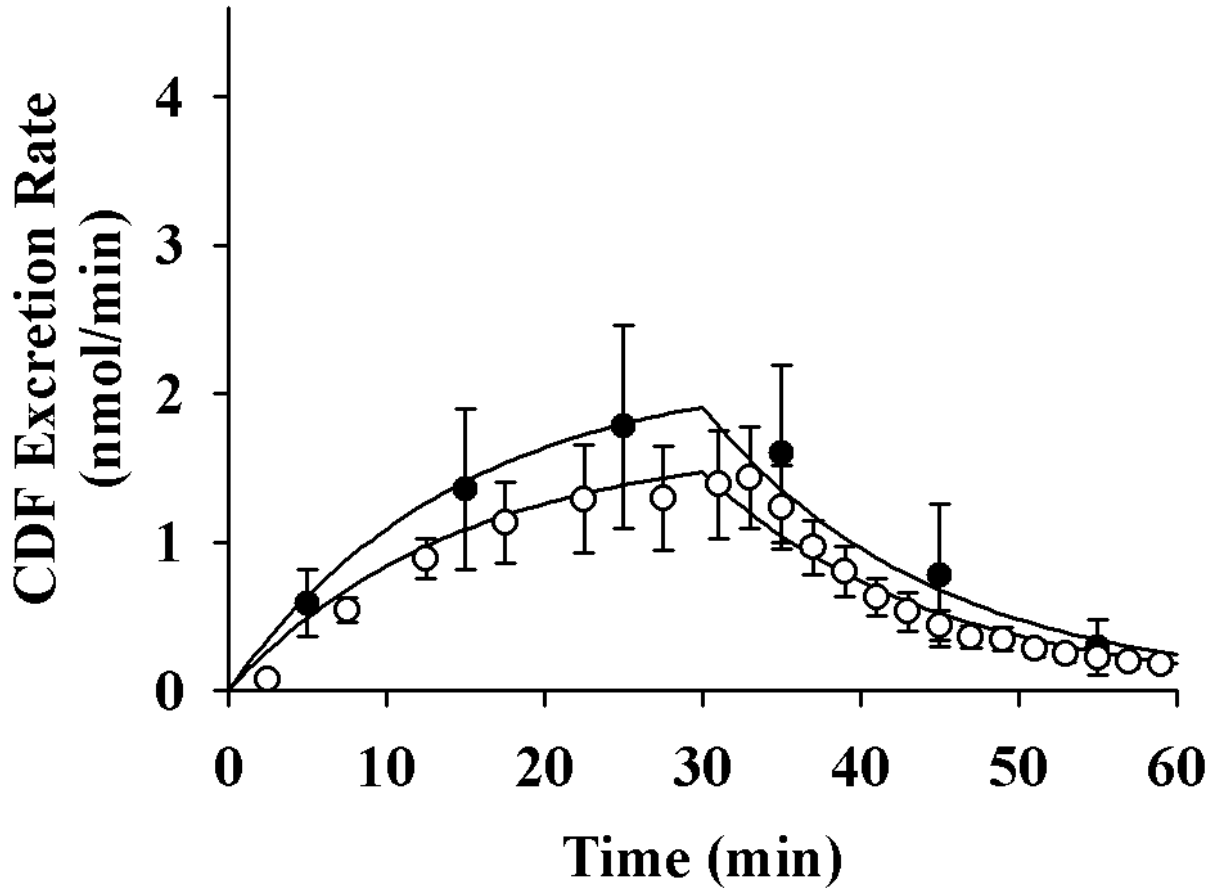

Figure 6A

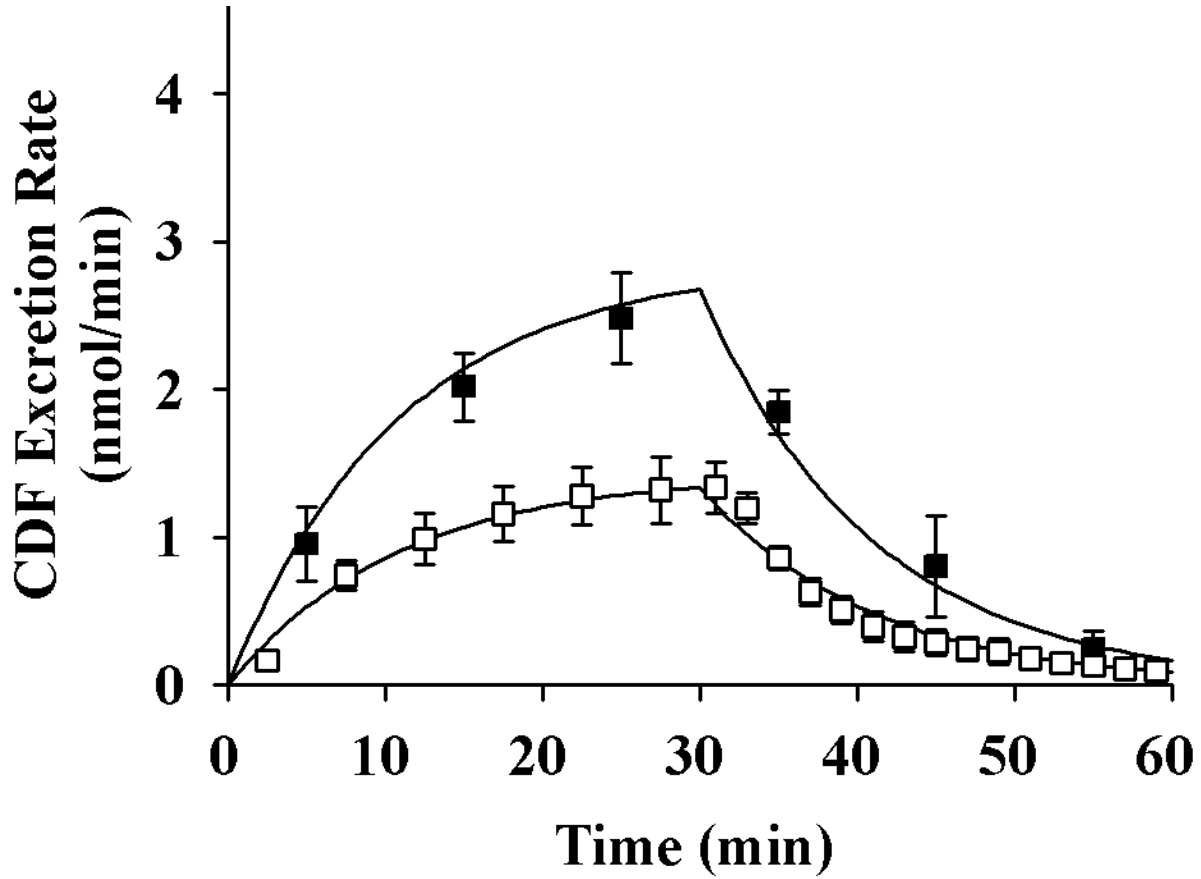

Figure 6B

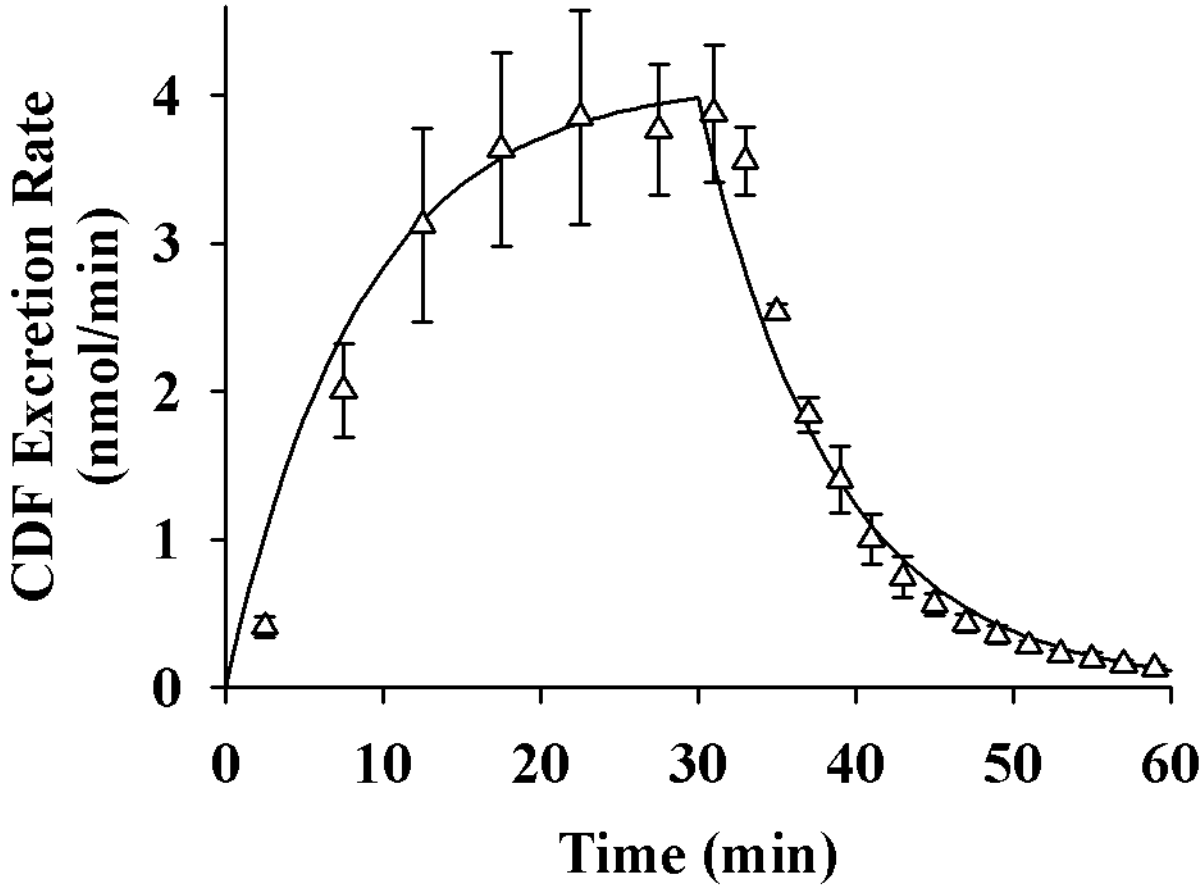

Figure 6C