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Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters

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Running title: ABC transporters determine diclofenac disposition

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Abbreviations:

ABCC, ATP-binding cassette transporter family C; ABCG, ATP-binding cassette transporter family G; DF, diclofenac; DF-AG, diclofenac acyl glucuronide; 4OH-DF, 4'-hydroxydiclofenac; 5OH-DF, 5-hydroxydiclofenac; MRP, multidrug-resistance protein

ABSTRACT

Diclofenac is an important analgesic and anti-inflammatory drug, widely used for treatment of post-operative pain, rheumatoid arthritis, and chronic pain associated with cancer. Diclofenac is extensively metabolized in the liver, and the main metabolites are hydroxylated and/or glucuronidated conjugates. We show here that loss of Multidrug Resistance Protein 2 (MRP2/ABCC2) and Breast Cancer Resistance Protein (BCRP/ABCG2) in mice results in highly increased plasma levels of diclofenac acyl glucuronide, both after oral and intravenous administration. Absence of Mrp2 and Bcrp1, localized at the canalicular membrane of hepatocytes, leads to impaired biliary excretion of acyl glucuronides, and consequently, to elevated liver and plasma levels. Mrp2 also mediates the biliary excretion of two hydroxylated diclofenac metabolites, 4'-hydroxydiclofenac and 5-hydroxydiclofenac. We further show that the sinusoidal efflux of diclofenac acyl glucuronide, from liver to blood, is largely dependent on Multidrug Resistance Protein 3 (MRP3/ABCC3). Diclofenac acyl glucuronides are chemically instable and reactive, and in patients these metabolites are associated with rare but serious idiosyncratic liver toxicity. This might explain why *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice, which have markedly elevated levels of diclofenac acyl glucuronides in their liver, display acute, albeit very mild, hepatotoxicity. We believe that the handling of diclofenac acyl glucuronides by ABC transporters may be representative for the handling of acyl glucuronide metabolites of many other clinically relevant drugs.

INTRODUCTION

Diclofenac (DF, Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID) that exhibits potent analgesic and anti-inflammatory properties and it is widely used to treat post-operative pain, rheumatoid arthritis, osteoarthritis and acute gouty arthritis (Davies and Anderson, 1997). When given orally, absorption is rapid and complete (Stierlin, et al., 1979; Peris-Ribera, et al., 1991; Davies and Anderson, 1997). The metabolism of DF partitions between acyl glucuronidation and aryl hydroxylation (Tang, 2003), and the major metabolites are DF acyl glucuronide (DF-AG), 4'hydroxy DF (4OH-DF) and 5-hydroxy DF (5OH-DF; Fig 1). In humans, UDP-glucuronosyltransferase 2B7 (UGT2B7) catalyzes the glucuronidation of DF, whereas in rats this is Ugt2b1 (King, et al., 2001). The hydroxylation of DF to 4OH-DF and 5OH-DF is catalyzed by CYP2C9 and 3A4, respectively (Tang, 2003). After glucuronidation, DF-AG can undergo further hydroxylation (Kumar, et al., 2002), and hydroxylated DF-AG conjugates are major urinary metabolites (Stierlin, et al., 1979).

Extensive first-pass metabolism combined with low enterohepatic circulation reduces the oral bioavailability of DF in humans to 50-60% of the administered dose, and the metabolites of DF are predominantly eliminated in the urine (Willis, et al., 1979; John, 1979; Willis, et al., 1980). In contrast, in rats the biliary excretion of DF glucuronides plays an important role in the elimination of DF (Peris-Ribera, et al., 1991). Once excreted into the intestines, DF glucuronides can be hydrolyzed by bacterial β-glucuronidases, and reabsorption of DF results in significant enterohepatic circulation (Peris-Ribera, et al., 1991; Seitz and Boelsterli, 1998). However, acyl glucuronides are chemically unstable and can undergo epimerization by acyl migration to the 2-, 3-, or 4-O-glucuronide, especially in the alkaline environment of bile, and these isomers are believed to be resistant to cleavage by bacterial β-glucuronidases (Dickinson and King, 1991; Ding, et al., 1993; Sallustio, et al., 2000; Seitz and Boelsterli, 1998).

Multidrug Resistance Proteins 2 and 3 (MRP2/*ABCC2* and MRP3/*ABCC3*) and Breast Cancer Resistance Protein (BCRP/ABCG2) are ATP binding cassette (ABC) multidrug transporters that have broad and substantially overlapping substrate specificities (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003). MRP2 and BCRP are situated at apical membranes of important epithelial barriers, such as intestine and kidney and at the canalicular membrane of hepatocytes. Consequently, they

extrude their substrates into bile, urine and feces, and restrict the (re)uptake of transported compounds from the gut (Schinkel and Jonker, 2003). In contrast, MRP3 is located at the basolateral membrane of epithelial cells of kidney and intestine and at the sinusoidal membrane of hepatocytes, and pumps its substrates towards the blood circulation (Borst, et al., 2006).

DF can cause rare but serious idiosyncratic hepatotoxicity and the formation of protein adducts with reactive DF glucuronides is believed to play a role herein [reviewed in (Boelsterli, 2003; Tang, 2003)]. Using TR^- rats, that naturally lack Mrp2, Seitz and colleagues (1998) showed that hepatic Mrp2 mediates the efflux of DF glucuronides from the liver into the bile. Moreover, the formation of hepatic protein adducts by the reactive acyl glucuronides of DF was critically dependent on Mrp2, i.e. TR^- rats displayed no hepatic adducts, whereas control rats did (Seitz, et al., 1998), suggesting that Mrp2 deficiency might protect the liver from diclofenac-induced toxicity. This might explain why intra-hepatic protein adduct formation most frequently occurs in the biliary tree and not within the hepatocytes, because Mrp2 efficiently concentrates the reactive DF glucuronides in the biliary tree (Sallustio, et al., 2000).

Furthermore, DF glucuronides, excreted into the bile by Mrp2, were shown to be involved in the formation of ulcers in the small intestine (Seitz and Boelsterli, 1998). Although DF treatment in humans also leads to the formation of gastrointestinal ulcers, DF glucuronides are predominantly exported from the human liver to the blood circulation and subsequently excreted in the urine (John, 1979; Willis, et al., 1979; Stierlin, et al., 1979). The hepatic efflux pump(s) that is responsible for the sinusoidal transport of DF glucuronides remains to be identified. MRP3, localized in the sinusoidal membrane of hepatocytes, might be a candidate, as this transporter accepts organic anions with a preference for glucuronidated substrates (Borst, et al., 2007).

In this study we investigated the impact of MRP2, MRP3 and BCRP on the pharmacokinetics of DF, using *Mrp2*$^{-/-}$, *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$ mice and all combination knockout strains. We included BCRP in this study, because we recently identified DF as a Bcrp1 substrate *in vitro* (Lagas, et al., 2009).

MATERIALS AND METHODS

Chemicals. DF (Voltaren; 25 mg/ml) was obtained from Novartis (Arnhem, The Netherlands). DF-AG originated from United States Biological (Swampscott, MA, USA). 5OH-DF was from Toronto Research Chemicals (North York, Canada). 4OH-DF was a kind gift from Becton Dickinson Bioscience (Breda, The Netherlands). [^{14}C]DF, specific activity 55 Ci/mol, was from Campro Scientific (Veenendaal, The Netherlands). Heparin (5000 IE/ml) originated from Leo Pharma BV (Breda, The Netherlands). Methoxyflurane (Metofane) was from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). Bovine serum albumin (BSA), fraction V, was from Roche (Mannheim, Germany). Ketamine (Ketanest-S®) was from Pfizer (Cappelle a/d IJssel, the Netherlands). Xylazine was from Sigma Chemical Co (St. Louis, MO, USA). L(+)-ascorbic acid and sodium acetate were of analytical grade and originated from Merck (Darmstadt, Germany). Acetic acid of analytical quality originated from Riedel-de Haën (Sigma Aldrich, Seelze, Germany).

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male *Bcrp1*$^{-/-}$ (Jonker, et al., 2002), *Mrp2*$^{-/-}$ (Vlaming, et al., 2006), *Mrp3*$^{-/-}$ (Zelcer, et al., 2005), *Mrp2/Bcrp1*$^{-/-}$ (Vlaming et al., 2009a), *Mrp2/Mrp3*$^{-/-}$ (van de Wetering, et al., 2007), *Mrp3/Bcrp1*$^{-/-}$ (Vlaming et al., 2009b), *Mrp2/Mrp3/Bcrp1*$^{-/-}$ (Vlaming et al., 2009b) and wild-type (WT) mice of a >99% FVB genetic background, between 9 and 15 weeks of age. See cited references for physiological characterization of single and combination knockout mice. Animals were kept in a temperature-controlled environment with a 12-hour light / 12-hour dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Plasma Pharmacokinetic Experiments and Tissue Distribution. For oral studies, DF (Voltaren; 25 mg/ml) was 50-fold diluted with a 5% glucose solution in water and a total volume of 10 ml/kg (5 mg/kg) body weight was administered by gavage into the stomach, using a blunt ended needle (n = 5). To minimize variation in absorption, mice were fasted 3 hours before drug administration. Blood samples (~30 µl) were collected in heparinized capillary tubes (Oxford Labware, St. Louis, USA) from the tail vein at 15 and 30 min and at 1, 2, 4, and 6 h after administration of the drug. For i.v. studies, DF (Voltaren; 25 mg/ml) was 25-fold diluted with a saline solution (0.9% NaCl) and a total

volume of 5 ml/kg (5 mg/kg) body weight was injected into a tail vein (n = 5). Blood samples were collected by cardiac puncture under methoxyflurane anesthesia 60 minutes after administration of the drug. Blood samples were kept on melting ice. After centrifugation at 2,100 x *g* for 6 min at 4°C, plasma was supplemented with 4% (v/v) of 2 M acetic acid in water and 1% (v/v) of 0.5 M ascorbic acid in water and stored at -80°C until LC-MS/MS analysis. Acetic acid and ascorbic acid were used to improve the stability of DF-AG and 5OH-DF, respectively [see (Sparidans, et al., 2008) for details about the stabilization of these metabolites]. In the i.v. experiments, livers were collected immediately after cardiac puncture and stored at -80°C until homogenization. Livers were homogenized in ice-cold 0.3 M sodium acetate, 20 mM ascorbic acid and 4% BSA (m/v) solution (pH 4.5) and homogenates were stored at -80°C until LC-MS/MS analysis.

Biliary Excretion. In gall bladder cannulation experiments mice were anesthetized by intraperitoneal injection of a solution of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), in a volume of 4.33 µl per gram body weight. After opening the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex limited, Hythe, UK) with an inner diameter of 0.28 mm, was inserted into the incised gall bladder and fixed with an additional ligation. Bile was collected for 60 min after i.v. injection of 5 mg/kg DF in a tube placed on ice, containing 10 µl 2M acetic acid and 1µl 0.5 M ascorbic acid. At the end of the experiment, blood was collected under methoxyflurane anesthesia by cardiac puncture and processed as described above. Mice were sacrificed by cervical dislocation and livers were collected en processed as described above. To determine the total biliary output of DF and metabolites, biliary excretion after dosing of 5 mg/kg DF, supplemented with [^{14}C]DF (~0.5 µCi per animal), was assessed in WT mice (n = 4). The levels of radioactivity were determined by liquid scintillation counting.

LC-MS/MS Analysis. For the quantitative analysis of DF and its three principal metabolites we set up and validated a fast and sensitive LC-MS/MS method (Sparidans, et al., 2008).

Toxicity studies. WT and *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice were fasted overnight and i.p. injected with 5 ml/kg (25 mg/kg) body weight DF (Voltaren, 5-fold diluted with 0.9% NaCl solution to 5 mg/ml). Blood was collected from the tail vein 3 hr after administration, using heparinized capillary tubes (Oxford Labware, St. Louis, USA). Twenty-four hr after administration, blood was collected under

methoxyflurane anesthesia by cardiac puncture, mice were sacrificed by cervical dislocation and livers were isolated and fixed in acidified formalin [ethanol/acetic acid/formaldehyde/saline at 40:5:10:45 (v/v)] and embedded in paraffin. Blood was centrifuged at 2,100 x *g* for 6 min at 4°C, plasma was collected and stored at -20 °C until analysis. Alanine aminotransferase (ALAT) and alkaline phosphatase levels in plasma were determined as markers for hepatotoxicity, using a Roche Hitachi 917 analyzer (Roche Diagnostics, Basel, Switzerland). Sections of livers were cut at 2 µm from the paraffin blocks and stained with H&E according to standard procedures. Images were captured with a Zeiss AxioCam HRc digital camera and processed with AxioVision 4 software (both from Carl Zeiss Vision).

Pharmacokinetic Calculations and Statistical Analysis. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule, without extrapolating to infinity. To assess the statistical significance, we performed one-way ANOVA followed by Dunnett's multiple comparison test. Differences were considered statistically significant when $P < 0.05$. Data are presented as means ± SD.

RESULTS

DF plasma pharmacokinetics in *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. To assess the roles of Mrp2 and Bcrp1 in the plasma pharmacokinetics of DF, we orally administered 5 mg/kg DF to WT, *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice and collected blood at multiple time points (Fig. 2). Mrp2 and Bcrp1 did not affect the oral uptake of DF, i.e. no differences in plasma concentrations were observed among all genotypes (Fig. 2A). In contrast, plasma concentrations of DF-AG, the main glucuronide metabolite of DF were highly increased in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice (Fig. 2B). Consequently, the area under the plasma-concentration time curve (AUC), which is a measure for the exposure to DF-AG, was 8-fold higher in *Mrp2*$^{-/-}$ mice (2.18 ± 0.60 mg.hr/l) and 14-fold elevated in *Mrp2/Bcrp1*$^{-/-}$ mice (3.79 ± 1.53 mg.hr/l), when compared to WT mice (0.27 ± 0.10 mg.hr/l; Fig. 1B, insert). Furthermore, *Mrp2/Bcrp1*$^{-/-}$ mice had significantly higher DF-AG plasma concentrations than *Mrp2*$^{-/-}$ mice at 2 and 4 hr after oral administration, although the AUC_{0-6} was not significantly different between these genotypes ($P = 0.06$; Fig. 2B). Deficiency of Mrp2 thus seems the main cause of the highly increased DF-AG plasma concentrations. The maximal plasma concentrations of DF as well as DF-AG were probably reached before the first sampling time point, i.e.15 min after oral administration (Fig. 2A and 2B), testifying to the rapid kinetics of these compounds.

Biliary excretion of DF and DF-AG in *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. As the metabolic conversion of DF predominantly occurs in the liver (Vargas and Franklin, 1997), the highly increased plasma concentrations of DF-AG in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice might be the result of disrupted biliary elimination of DF-AG via Mrp2 and/or Bcrp1. We therefore measured the biliary excretion of DF-AG in *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice (Fig. 3; Table 1). DF was i.v. administered at 5 mg/kg to mice with a cannulated gall bladder and bile was collected for 60 minutes, immediately followed by isolation of plasma and liver. The amounts of DF recovered in bile, liver and plasma were not significantly affected by Mrp2 and/or Bcrp1 deficiency (Fig. 3A, C and E). The amount of DF occurring in bile was also very small (< 0.05% of the dose), in spite of considerable accumulation in the liver (~4% of the dose). In contrast, the much higher biliary output of DF-AG (~4% of the dose) was ~2-fold (i.e., by 50%) reduced in both single *Mrp2*$^{-/-}$ and *Bcrp1*$^{-/-}$ mice compared to WT mice, and

approximately 4-fold (i.e., by 75%) in compound *Mrp2/Bcrp1*$^{-/-}$ mice (Fig. 3B). Interestingly, the ~2-fold lower biliary excretion of DF-AG in *Mrp2*$^{-/-}$ and *Bcrp1*$^{-/-}$ mice was associated with elevated DF-AG plasma but not liver concentrations in *Mrp2*$^{-/-}$ mice, whereas the inverse was true for *Bcrp1*$^{-/-}$ mice, i.e., elevated liver but not plasma levels of DF-AG (Fig. 3D and 3F). We will return to this difference in the discussion. Furthermore, the fact that biliary output of DF-AG was not completely abrogated in *Mrp2/Bcrp1*$^{-/-}$ mice points towards additional efflux mechanism(s) for DF-AG in the canalicular membrane, other than Mrp2 and Bcrp1. Nonetheless, the lower biliary excretion of DF-AG in *Mrp2/Bcrp1*$^{-/-}$ mice was associated with both markedly increased liver concentrations (Fig. 3D) and higher plasma concentrations (Fig. 3F).

Biliary excretion of 4OH-DF and 5OH-DF in *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. The levels of the two principal hydroxylated metabolites of DF, 4OH-DF and 5OH-DF (Fig. 1), were also measured in plasma, bile and liver (Table 1). Interestingly, although liver and plasma levels of these metabolites were not significantly different, the biliary output of 4OH-DF was 17.3- and 19.2-fold (i.e., by 94% and 95%) decreased in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice, respectively ($P < 0.01$; Table 1). Furthermore, the excretion of 5OH-DF in the bile was 2.4- and 3.2-fold (i.e., 58% and 69%) lower in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice, respectively ($P < 0.05$; Table 1). In single *Bcrp1*$^{-/-}$ mice, the biliary excretion of these metabolites was not different from WT mice. The biliary output of these hydroxylated DF metabolites, particularly of 4OH-DF, thus seems to depend largely on Mrp2. However, the impact of this process on liver and plasma levels of these metabolites was negligible, in line with the modest amounts excreted into bile.

Biliary excretion of DF glucuronide metabolites other than DF-AG in *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. To determine the total biliary output of DF and its metabolites in mice, we i.v. administered 5 mg/kg DF, supplemented with a tracer amount of [^{14}C]DF, to WT mice and we measured the biliary excretion. Over a period of 60 minutes, 42.6 ± 4.4% of the dose was excreted into the bile as ^{14}C-label. This is consistent with a study in rats with a cannulated bile duct that received 3 mg/kg [^{14}C]DF i.v. and excreted 57% of the dose as ^{14}C-label into the bile within 90 min (Seitz, et al., 1998). As depicted in Table 1, the biliary excretion of DF and DF-AG in WT mice was 0.04 and 4.2% of the dose, respectively. In addition, the excretion of 4OH-DF and 5OH-DF into the

bile accounted for 0.14 and 0.053% of the dose, respectively (Table 1). Together, DF and its primary metabolites therefore represent only ~10% of the ^{14}C-label excreted into bile. The majority of the radioactivity in the bile must thus originate from DF metabolites other than DF-AG, 4OH-DF or 5OH-DF. When analyzed with LC-MS/MS, we indeed observed additional peaks in the chromatograms of the bile samples. Based on mass, retention time, and fragmentation patterns, 4OH-DF-AG and 5OH-DF-AG could be identified. Another peak represented isomer(s) of DF-AG, which originated from epimerization of the 1-*O*-glucuronide by acyl migration to 2-, 3- or 4-*O*-glucuronide (Seitz and Boelsterli, 1998; Sallustio, et al., 2000). Acyl migration especially occurs at alkaline pH, and since mouse bile has a pH of ~9, it is likely that a significant amount of the DF-AG was converted to isomeric isoforms before we could stabilize the compound with acetic acid. As we do not have reference standards for these compounds, these metabolites could not be quantified and therefore their biliary output is given in arbitrary units, normalized to the output in WT bile (Fig. 4). By and large, the excretion patterns of 4OH-DF-AG, 5OH-DF-AG and the isomer(s) of DF-AG were qualitatively similar to that of DF-AG, indicating that biliary excretion of these glucuronide metabolites was predominantly mediated by Mrp2 and Bcrp1.

Basolateral efflux of DF-AG in the liver is mediated by Mrp3. In the liver, Mrp3 is expressed at the sinusoidal (basolateral) membranes of hepatocytes and it transports its substrates towards the blood circulation. Because Mrp3 is a typical organic anion transporter with a preference for glucuronidated substrates [reviewed in (Borst, et al., 2007)], we tested whether Mrp3 is involved in the basolateral efflux of DF-AG from the liver. DF was i.v. administered at 5 mg/kg to conscious, freely moving WT, *Mrp2*$^{-/-}$, *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$ mice and all possible combinations of these single knockout strains, and plasma and livers were collected 60 minutes after injection. As shown in figures 5A and 5C, plasma and liver concentrations of DF were not affected by single and combined transporter deficiencies. In contrast, plasma concentrations of DF-AG were 6.2-fold increased in *Mrp2*$^{-/-}$ mice and 24-fold in *Mrp2/Bcrp1*$^{-/-}$ mice (Fig. 5B), consistent with the results obtained after oral administration (Fig 2B). Strikingly, the highly increased plasma concentrations in *Mrp2/Bcrp1*$^{-/-}$ mice were restored to near WT levels in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice (Fig. 5B), and a similar shift was seen between *Mrp2*$^{-/-}$ and *Mrp2/Mrp3*$^{-/-}$ mice. This suggests that Mrp3 is the main transporter

responsible for the efflux of DF-AG across the basolateral membrane. The observation that plasma DF-AG levels in single *Mrp3*$^{-/-}$ mice and in combination *Mrp2/Mrp3*$^{-/-}$ and *Mrp3/Bcrp1*$^{-/-}$ mice were even significantly lower than in WT mice further supports an important role of Mrp3 in the efflux of DF-AG from the liver towards the blood (Fig. 5B). Accordingly, the DF-AG concentrations in the liver, determined at 60 minutes after administration, were ~1.8-fold elevated in *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice, and 3.3-fold in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice (Fig. 5D). Interestingly, in the livers of *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice, but not in WT livers, also substantial accumulation of 4OH-DF-AG, 5OH-DF-AG and of DF-AG isomers was observed (not shown). As mentioned above, the lack of reference compounds made it impossible to quantify these metabolites. However, as DF acyl glucuronide metabolites are associated with idiosyncratic hepatotoxicity [reviewed in (Tang, 2003; Boelsterli, 2003)] we hypothesized that *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice might be more prone to DF-induced liver toxicity than WT mice.

***Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice develop mild DF-induced acute hepatotoxicity.** To test whether *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice were more sensitive to DF-induced liver toxicity, DF was i.p. administered at 25 mg/kg to WT and *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice (n = 4) and ALAT and alkaline phosphatase levels in plasma were determined 3 and 24 hr after administration. In addition, livers were isolated 24 hr after administration and examined for histological signs of toxicity. Both WT and *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice that were treated with DF did not display changes in plasma alkaline phosphatase or histological signs of hepatotoxicity (data not shown). Furthermore, ALAT levels in WT mice were not altered after treatment with DF. However, treatment of *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice with DF resulted in significantly higher (~2-fold, $P < 0.05$) ALAT levels at both time points (Fig. 6), suggesting that these mice displayed acute, albeit very mild, liver toxicity.

DISCUSSION

In this study we identify Mrp2, Mrp3 and Bcrp1 in the mouse as important determinants for the pharmacokinetics of reactive glucuronide metabolites of DF. Mrp2 and Bcrp1 are involved in the biliary excretion of DF glucuronides, whereas Mrp3 is a major hepatic transporter for the extrusion of DF-AG across the sinusoidal membrane towards the blood circulation. Simultaneous loss of Mrp2, Mrp3 and Bcrp1 results in substantial accumulation of reactive glucuronide metabolites in the liver, with acute but mild hepatotoxicity as a consequence.

We note that, although not yet identified, other efflux and/or uptake transporters may be involved in the hepatic transport of DF and/or its metabolites as well. Differential expression of these transporters in single or combination knockout mice might thus affect our data. Thus far, expression of Mdr1, Mrp4, Oatp1b2 and Oatp1a4 has been analyzed in livers of single (*Bcrp1*$^{-/-}$ and *Mrp2*$^{-/-}$) and combination (*Mrp2/Mrp3*$^{-/-}$, *Mrp2/Bcrp1*$^{-/-}$, *Mrp3/Bcrp1*$^{-/-}$ and *Mrp2/Mrp3/Bcrp1*$^{-/-}$) knockout mice, but differences > 2-fold were not observed (Vlaming, et al., 2006; Vlaming, et al., 2009a; Vlaming, et al., 2009b). This makes it unlikely that alterations in expression of these transporters could substantially affect the interpretation of our data.

We recently demonstrated that mouse Bcrp1 can transport DF *in vitro* (Lagas, et al., 2009). In the present study, however, loss of Bcrp1 in mice did not affect the oral uptake or biliary excretion of DF. On the other hand, deficiencies in Bcrp1 and Mrp2 resulted in impaired biliary output of DF glucuronides. In addition to glucuronides, we also found that Mrp2, but not Bcrp1, mediates the biliary excretion of the hydroxylated DF metabolites, 4OH-DF and 5OH-DF.

From studies in rats, Mrp2 was already known to mediate the biliary excretion of DF glucuronides (Seitz, et al., 1998; Seitz and Boelsterli, 1998). Moreover, hepatobiliary excretion of DF glucuronides in TR^{-} rats, a spontaneous mutant lacking Mrp2, is almost completely abrogated (Seitz, et al., 1998). In contrast, in mice we show that the biliary excretion of DF glucuronides is mainly dependent on both Mrp2 and Bcrp1. This species difference is in line with previous findings showing that the biliary excretion of 4-methylumbelliferyl glucuronide in rats is primarily mediated by Mrp2, whereas in mice both Mrp2 and Bcrp1 play a role in this process (Zamek-Gliszczynski, et al., 2006a; Zamek-Gliszczynski, et al., 2006b). Our observation that the biliary output of DF-AG was not

completely abrogated in *Mrp2/Bcrp1*$^{-/-}$ mice may point towards the existence of yet other efflux mechanism(s) in the canalicular membrane, in addition to MRP2 and BCRP. Nonetheless, simultaneous loss of Mrp2 and Bcrp1 resulted in increased liver concentrations (Figs 3D and 5D) and, as a consequence, highly increased plasma concentrations of DF-AG (Figs 2B, 3F and 5B).

Notably, after oral DF administration, maximal plasma concentrations of DF-AG are reached within 15 minutes after administration (Fig 2B). This might suggest that the glucuronidation of DF already occurs in the gastrointestinal tract, as the gut, in addition to the liver, is an important organ for glucuronidation (Tukey and Strassburg, 2000). However, studies in rats demonstrate that DF is predominantly glucuronidated in the liver and not in the gut (Ware, et al., 1998; Vargas and Franklin, 1997; Seitz, et al., 1998). The glucuronidation of DF in rats is catalyzed by Ugt2b1 (King, et al., 2001) and since rat and mouse Ugt2b1 proteins share >85% homology (www.ensembl.org), murine Ugt2b1 may also be primarily responsible for the glucuronidation of DF. In rats and mice, the liver is the predominant tissue for the expression of Ugt2b1, whereas expression in the intestine is low (Shelby, et al., 2003; Buckley and Klaassen, 2007). We find that approximately 40% of an i.v. dose of DF is excreted as glucuronide metabolites in the bile of WT mice within 60 minutes, indicating that the liver indeed is the major tissue for DF glucuronidation in mice. We therefore believe that the early C_{max} of DF-AG (Fig. 2B) may be explained by very fast oral absorption of DF (Fig. 2A), enabling rapid hepatic uptake and subsequent conversion to DF-AG. The fact that DF was given as an aqueous solution in combination with temporary food deprivation before administration may explain the rapid absorption of the drug. Indeed, in humans the T_{max} of an aqueous DF solution was reached in 10-30 min, whereas this was ~2 hr for a tablet with the same DF dose (John, 1979).

Mrp3 has a preference for glucuronidated compounds and plays a dominant role in the transport of glucuronides across the sinusoidal membrane of hepatocytes (reviewed in: Borst, et al., 2007). Our results suggest that Mrp3 also predominantly mediates the transport of DF-AG from the liver toward the blood circulation. Interestingly, in humans DF acyl glucuronides are predominantly excreted from the liver to the blood circulation and subsequently excreted in the urine (John, 1979; Willis, et al., 1979; Stierlin, et al., 1979). This could mean that MRP3 in the sinusoidal membrane of human hepatocytes has a higher affinity for DF acyl glucuronides than canalicular MRP2, whereas in mice

this appears to be the other way around. We have recently shown that Mrp3 is upregulated in livers of *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice (Vlaming, et al., 2006; Vlaming, et al., 2009a) . This likely explains why, although the biliary DF-AG excretion is impaired in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice, substantial hepatic accumulation of DF-AG is not observed (Fig. 5D) and that plasma concentrations of DF-AG are highly elevated in *Mrp2*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice (Figs 2B, 3F and 5B). In contrast, in *Bcrp1*$^{-/-}$ mice, which do not have altered Mrp3 expression in their liver (Vlaming, et al., 2009a), impaired biliary excretion of DF-AG results in liver accumulation without elevated plasma concentration (Figs 3D and 3F).

The 24-fold increased plasma level of DF-AG in *Mrp2/Bcrp1*$^{-/-}$ mice (Fig. 5B) was restored to WT levels in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice. We therefore had expected that DF-AG would be highly accumulated in the livers of in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice, but hepatic levels of DF-AG in this strain were only 3.3-fold higher than in WT mice (Fig. 5D). The relatively low DF-AG accumulation in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice might partially be explained by the fact that biliary excretion of DF-AG in *Mrp2/Bcrp1*$^{-/-}$ mice is not completely abrogated, but reduced by ~75% (Fig. 3B). There must thus be other canalicular efflux system(s) for DF-AG, possibly with low affinity and high capacity. However, subsequent hydroxylation of DF-AG and/or acyl migration of DF-AG in the liver may also contribute to the relatively low DF-AG concentration in the liver of *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice. Indeed, DF-AG can undergo further hydroxylation in the liver (Kumar, et al., 2002). In fact, in rats the biliary excretion of metabolites that were hydroxylated and glucuronidated was approximately equal to that of DF-AG (Seitz and Boelsterli, 1998). Furthermore, substantial acyl migration has been observed *in vivo* as well (Sallustio, et al., 2000; Boelsterli, 2003). Our results suggest that the majority of radioactivity in the bile of WT mice can be attributed to 4OH-DF-AG, 5OH-DF-AG and DF-AG isomer(s). Unfortunately, these metabolites could not be quantified, but their biliary efflux was largely dependent on Mrp2 and Bcrp1 (Fig. 4). Furthermore, these metabolites are putative substrates for Mrp3, which is supported by their presence in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ livers, whereas they could not be detected in WT livers (data not shown). Overall, circumstantial evidence thus suggests that *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice accumulate substantial amounts of DF acyl glucuronides in their livers. Acyl glucuronides are electrophilic, chemically reactive compounds that can form protein adducts via

non-enzymatic reactions (Sallustio, et al., 2000). Hepatic protein adducts of DF acyl glucuronides, including hydroxylated DF-AG metabolites and DF-AG isomers, are believed to play an important role in DF-induced idiosyncratic hepatotoxicity [reviewed in (Boelsterli, 2003; Tang, 2003)]. We therefore tested if *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice were more prone to DF-induced acute hepatotoxicity and challenged these mice with a high dose of DF (25 mg/kg). Indeed, *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice displayed 2-fold elevated plasma ALAT levels, whereas WT did not, indicating that *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice experienced acute, albeit very mild liver toxicity.

In humans, the rare idiosyncratic hepatotoxicity that can be induced by DF is characterized by a delayed onset of symptoms and usually occurs between 1 and 6 months after starting the treatment (Boelsterli, 2003; Tang, 2003). There are many indications that, in addition to hepatic adduct formation by reactive metabolites, other factors, including immune-mediated responses, contribute to the liver toxicity (Boelsterli, 2003; Tang, 2003). Our results suggest some acute but mild liver toxicity in *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice, and although it was beyond the scope of this study, it might be interesting to investigate this toxicity in more detail or in a much longer time frame in these transporter-deficient mouse models.

In conclusion, our results show that Mrp2, Mrp3 and Bcrp1 play an important role in the distribution and elimination of DF acyl glucuronides in mice, both after oral and intravenous administration. We expect that acyl glucuronide metabolites of many more drugs will be similarly affected by these ABC transporters.

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Legends for figures:

Fig. 1. Structures and biotransformation routes of DF and its three most predominant primary metabolites with the putative metabolic enzymes involved in the mouse. Cyp = Cytochrome P450; Ugt = UDP-glucuronosyltransferase.

Fig. 2. Plasma concentration-time curves of DF (*A*) and DF-AG (*B*) in male FVB WT (■), *Mrp2*$^{-/-}$ (□), *Bcrp1*$^{-/-}$ (Δ), and *Mrp2/Bcrp1*$^{-/-}$ (▲) mice, after oral administration of 5 mg/kg DF. Data are means ± SD, n = 5. Inserts in the panels show the area under the curve from 0-6 hr (AUC_{0-6}) for DF (*A*) and DF-AG (*B*) in the different strains. *** $P < 0.001$, compared to WT mice.

Fig. 3. Levels of DF and DF-AG in bile (*A*, *B*), liver (*C*, *D*) and plasma (*E*, *F*) of male gall bladder cannulated WT, *Mrp2*$^{-/-}$, *Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. After ligation of the common bile duct and cannulation of the gall bladder DF (5 mg/kg) was given i.v. followed by bile collection for 60 min and tissue isolation and plasma collection at 60 min. Data represent means ± SD (n = 5 per strain). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared to WT mice. # $P < 0.05$ and ## $P < 0.01$, compared to *Mrp2*$^{-/-}$ mice. † $P < 0.05$ and †† $P < 0.01$, compared to *Bcrp1*$^{-/-}$ mice.

Fig. 4. Levels of DF-AG (*A*), isomeric isoform(s) of DF-AG (*B*), 4OH-DF-AG (*C*) and 5OH-DF-AG (*D*) in bile of male gall bladder cannulated WT, *Mrp2*$^{-/-}$,*Bcrp1*$^{-/-}$ and *Mrp2/Bcrp1*$^{-/-}$ mice. After ligation of the common bile duct and cannulation of the gall bladder DF (5 mg/kg) was given i.v. followed by bile collection for 60 min. Levels are given in arbitrary units. Data represent means ± SD (n = 5 per strain). * $P < 0.05$ and ** $P < 0.01$, compared to WT mice. # $P < 0.05$, compared to *Mrp2*$^{-/-}$ mice. † $P < 0.01$, compared to *Bcrp1*$^{-/-}$ mice.

Fig. 5. Levels of DF and DF-AG in plasma (*A*, *B*) and liver (*C*, *D*) of male WT, *Mrp2*$^{-/-}$, *Mrp3*$^{-/-}$, *Bcrp1*$^{-/-}$, *Mrp2/Mrp3*$^{-/-}$, *Mrp2/Bcrp1*$^{-/-}$, *Mrp3/Bcrp1*$^{-/-}$ and *Mrp2/Mrp3/Bcrp1*$^{-/-}$ mice. DF (5 mg/kg) was given i.v. followed by plasma collection and tissue isolation at 60 min. Data represent means ± SD (n = 5 per strain). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared to WT mice. ## $P < 0.01$, compared to *Mrp2*$^{-/-}$ mice.

Fig. 6. Plasma levels of alanine aminotransferase (ALAT) in WT and KO (*Mrp2/Mrp3/Bcrp1*$^{-/-}$) mice. Mice were i.p. injected with vehicle (V) or DF (25 mg/kg) and blood was collected 3 hr and 24 hr after administration. Data represent means ± SD (n = 4 per strain). * $P < 0.05$ and ** $P < 0.01$, compared to WT mice receiving vehicle.

Table 1. Diclofenac and three primary metabolites as determined in bile, liver and plasma of mice with cannulated gall bladder, 60 minutes after i.v. administration of diclofenac at 5 mg/kg.

		Genotype			
Biological Matrix	Compound	WT	*Mrp2*$^{-/-}$	*Bcrp1*$^{-/-}$	*Mrp2/Bcrp1*$^{-/-}$
Bile	Diclofenac	0.036 ± 0.033	0.033 ± 0.007	0.042 ± 0.014	0.044 ± 0.010
(% of dose)	Diclofenac AG	4.22 ± 1.39	2.12 ± 0.33*	1.96 ± 0.72*	1.11 ± 0.22**/#/†
	4'-hydroxy diclofenac	0.140 ± 0.055	0.008 ± 0.004**	0.145 ± 0.034	0.007 ± 0.002***/††
	5-hydroxy diclofenac	0.053 ± 0.024	0.022 ± 0.008*	0.039 ± 0.010	0.017 ± 0.003**/†
Liver	Diclofenac	4.57 ± 2.00	2.88 ± 1.23	3.92 ± 2.09	4.32 ± 1.44
(% of dose)	Diclofenac AG	0.47 ± 0.23	0.36 ± 0.31	0.84 ± 0.28*/#	1.76 ± 0.40***/##/†
	4'-hydroxy diclofenac	0.46 ± 0.17	0.42 ± 0.18	0.44 ± 0.20	0.42 ± 0.15
	5-hydroxy diclofenac	1.31 ± 0.59	0.77 ± 0.38	1.28 ± 0.45	0.94 ± 0.28
Plasma	Diclofenac	6.74 ± 3.06	3.75 ± 1.71	5.06 ± 3.39	6.54 ± 1.74
(mg/l)	Diclofenac AG	0.23 ± 0.10	0.95 ± 0.16***	0.30 ± 0.11	1.64 ± 0.49***/#/††
	4'-hydroxy diclofenac	0.23 ± 0.11	0.19 ± 0.08	0.22 ± 0.16	0.21 ± 0.07
	5-hydroxy diclofenac	0.90 ± 0.30	0.58 ± 0.15	0.82 ± 0.32	0.63 ± 0.15

Plasma concentrations are expressed as mg/l and liver and bile concentrations are given as percentage of the dose. Data are given as means ± SD, n = 4-6. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared to WT mice. # $P < 0.05$ and ## P < 0.01, compared to *Mrp2*$^{-/-}$ mice. † $P < 0.05$ and †† P < 0.01, compared to *Bcrp1*$^{-/-}$ mice.

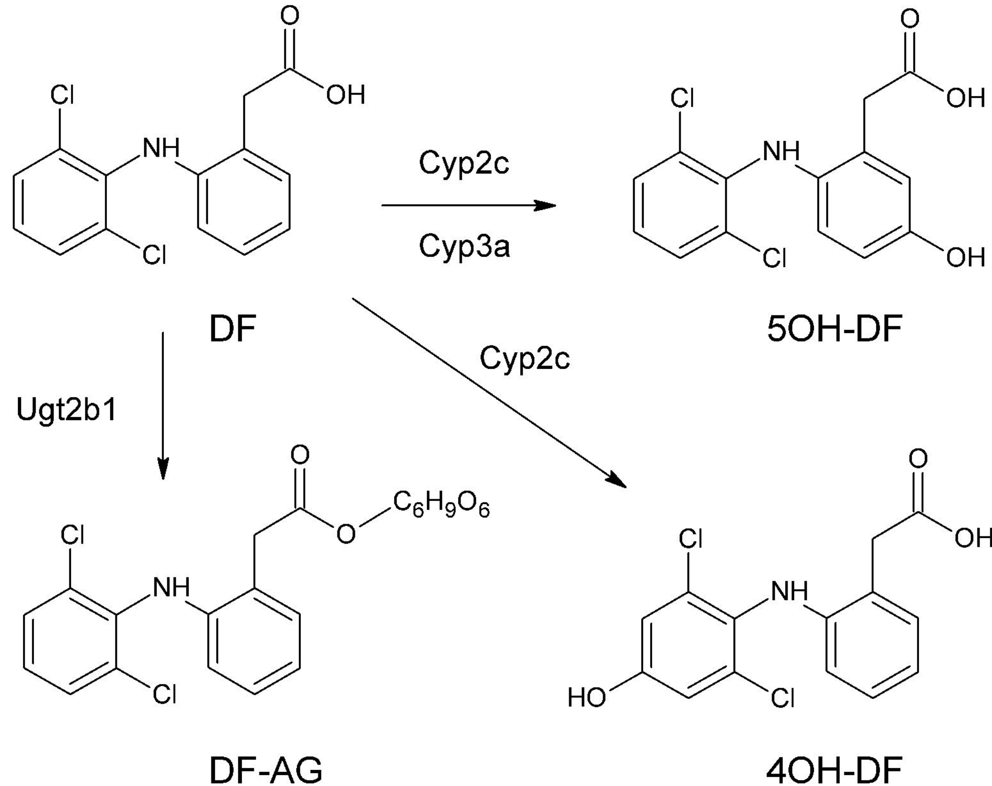
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Figure 2

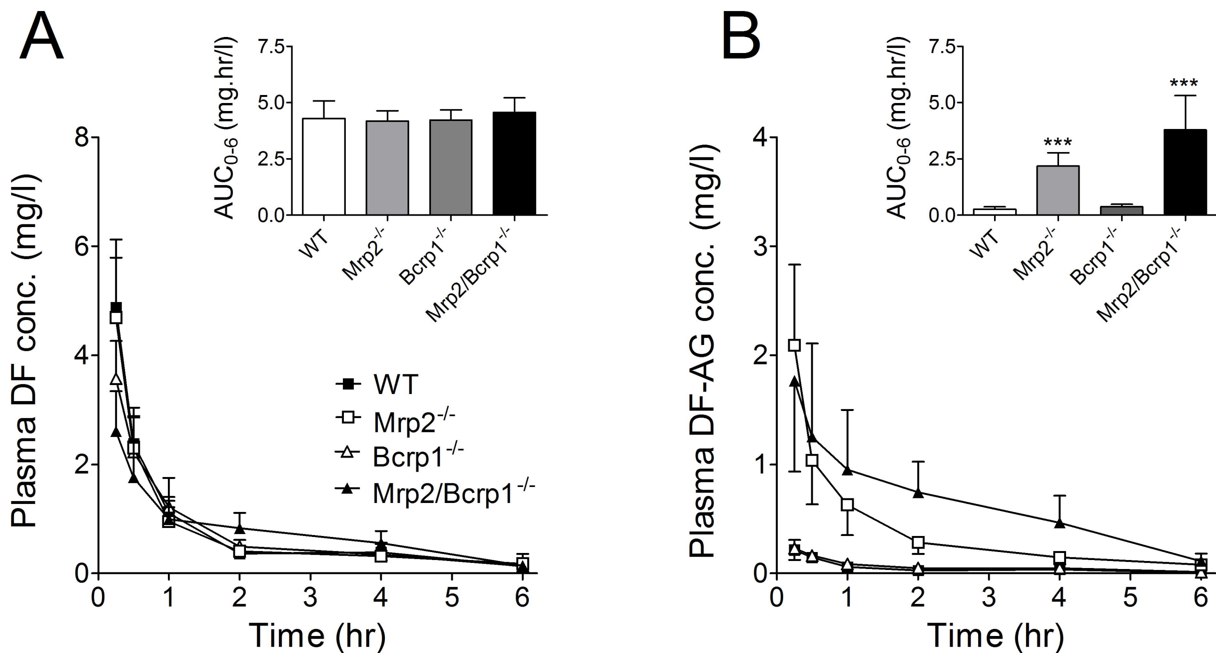


Figure 3

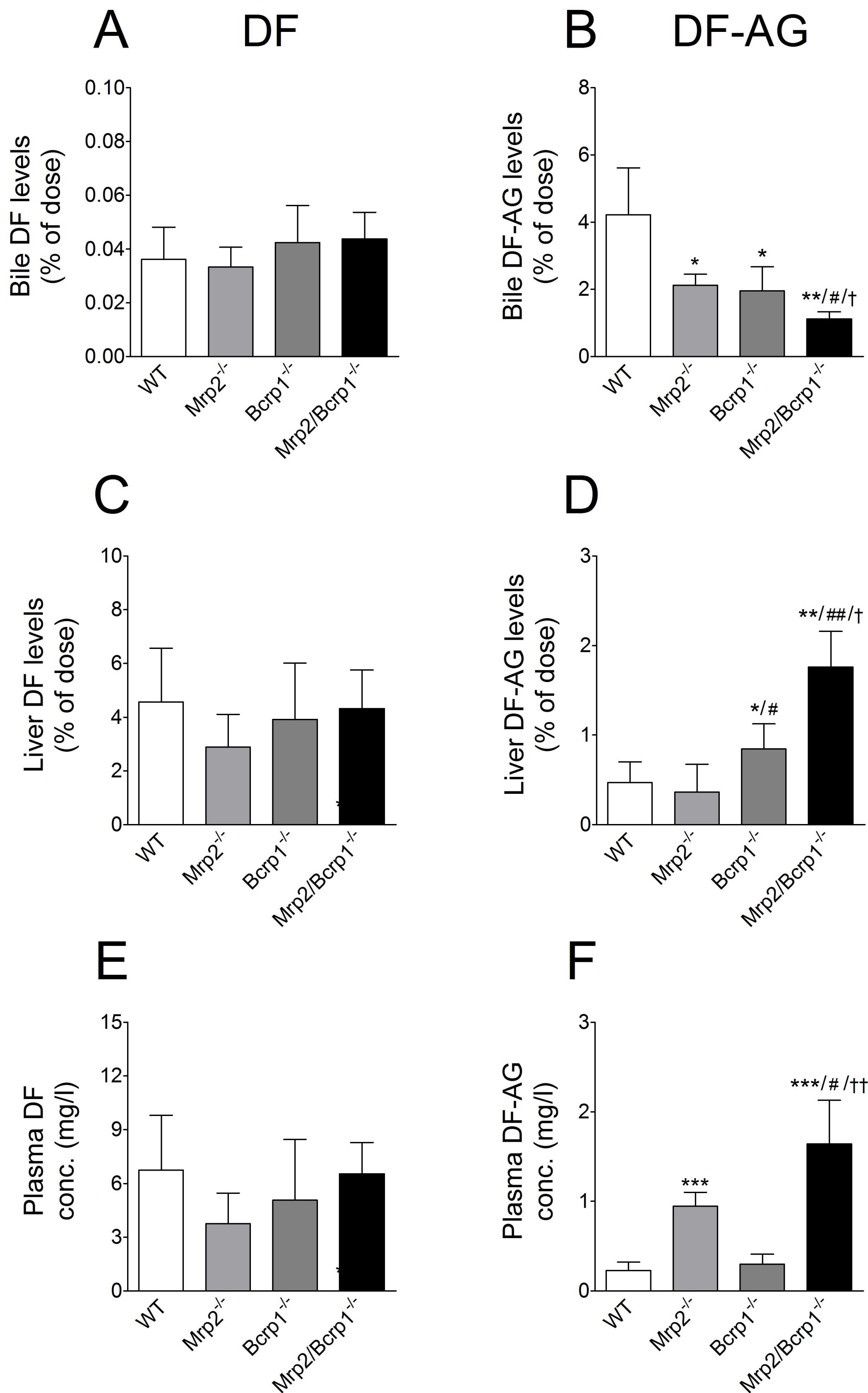


Figure 4

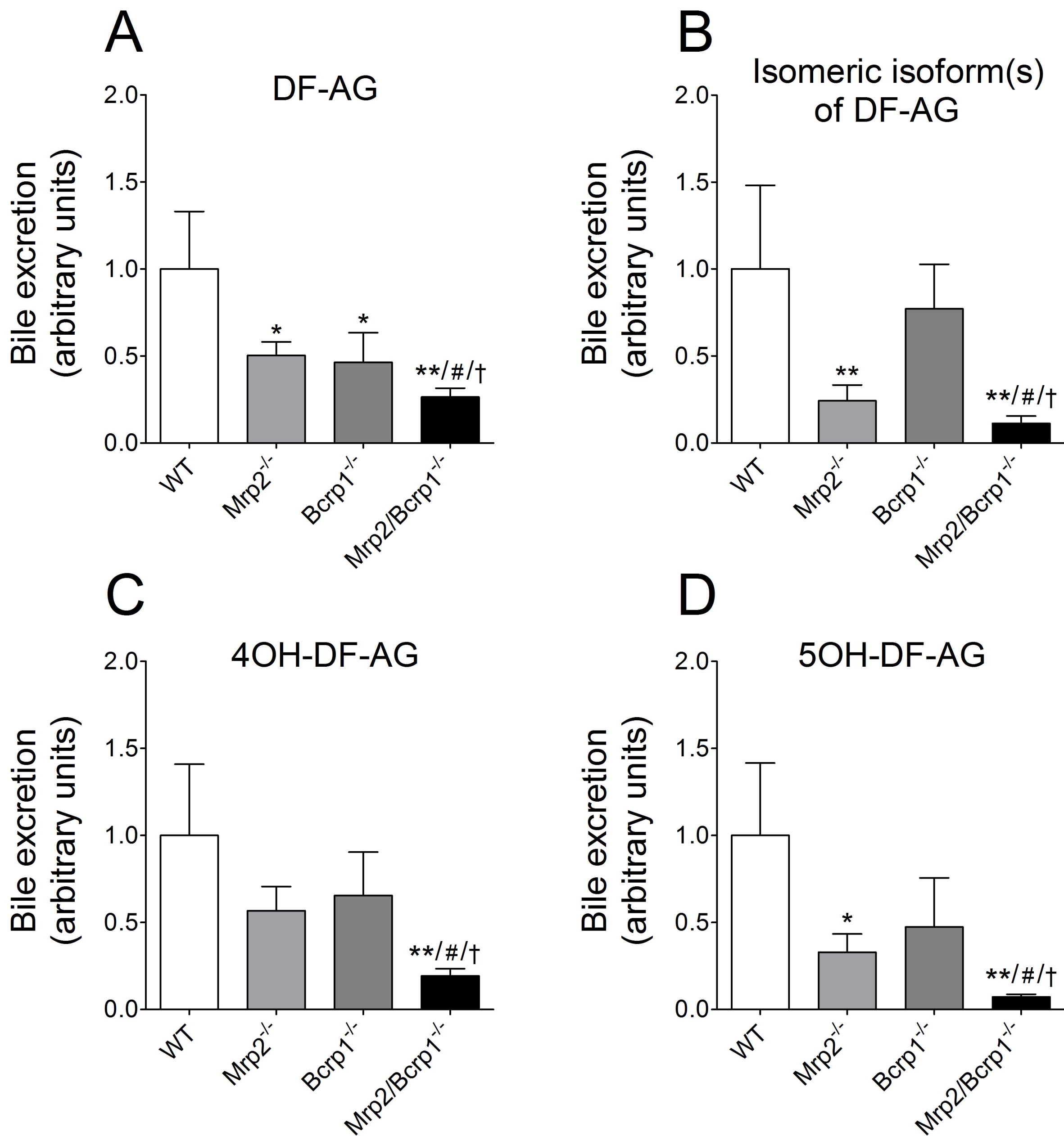
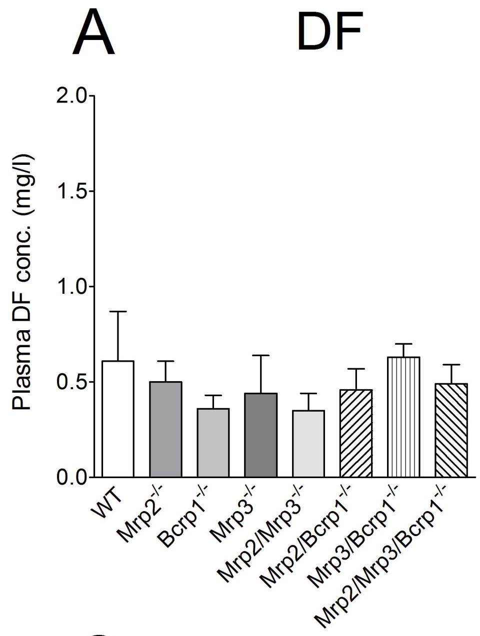
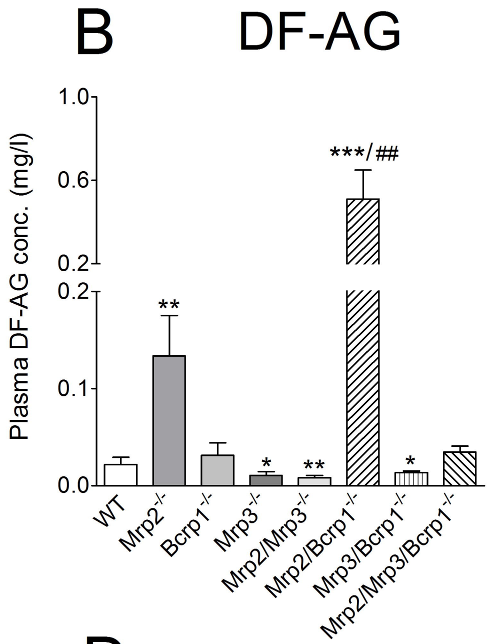
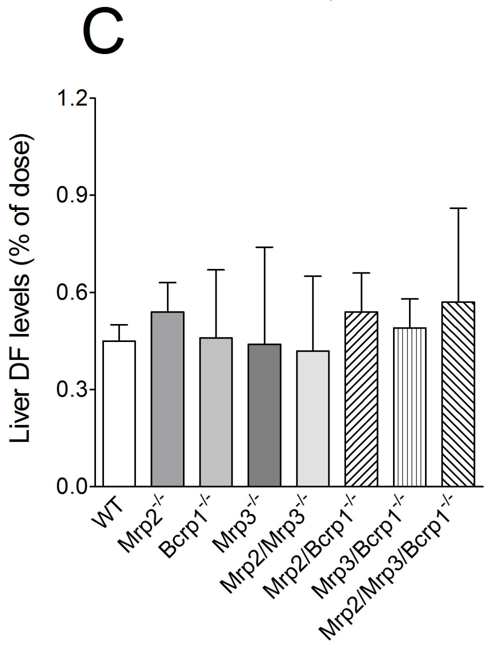


Figure 5







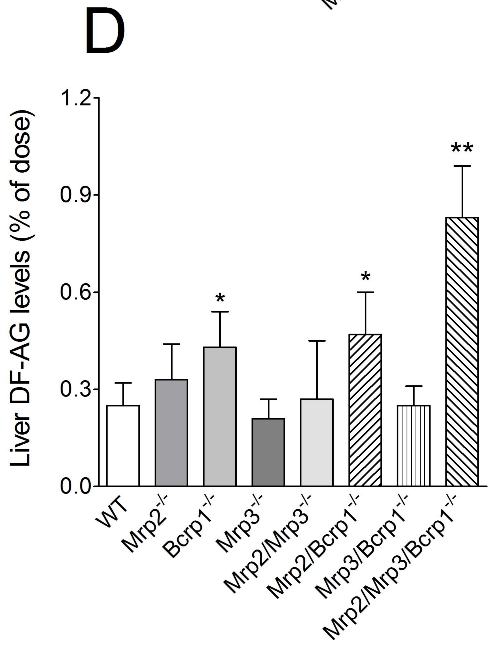


Figure 6