

# Hepatic Clearance of Reactive Glucuronide Metabolites of Diclofenac in the Mouse Is Dependent on Multiple ATP-Binding Cassette Efflux Transporters

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## ABSTRACT

Diclofenac is an important analgesic and anti-inflammatory drug that is widely used for the treatment of postoperative 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, after both oral and intravenous administration. The 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 unstable 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 ATP binding cassette transporters may be representative for the handling of acyl glucuronide metabolites of many other clinically relevant drugs.

Diclofenac (DF, Fig. 1) is a nonsteroidal anti-inflammatory drug that exhibits potent analgesic and anti-inflammatory properties, and it is widely used to treat postoperative 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 CYP3A4, 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 to 60% of the administered dose, and the metabolites of DF are predominantly eliminated in the urine (John, 1979; Willis et al., 1979, 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  $\beta$ -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

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**ABBREVIATIONS:** DF, diclofenac; DF-AG, diclofenac acyl glucuronide; 4OH-DF, 4'-hydroxydiclofenac; 5OH-DF, 5-hydroxydiclofenac; MRP, multidrug-resistance protein; BCRP, breast cancer resistance protein; ABC, ATP binding cassette; WT, wild type; AUC, area under the plasma concentration-time curve; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ALAT, alanine aminotransferase; UGT, UDP-glucuronosyltransferase.

to be resistant to cleavage by bacterial  $\beta$ -glucuronidases (Dickinson and King, 1991; Ding et al., 1993; Seitz and Boelsterli, 1998; Sallustio et al., 2000).

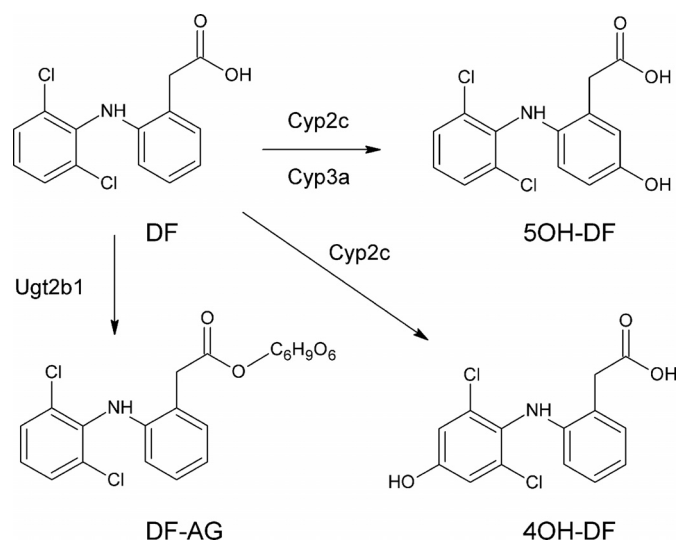
Multidrug resistance proteins 2 and 3 (MRP2/ABCC2 and MRP3/ABCC3) and breast cancer resistance protein (BCRP/ABCG2) are ATP binding cassette (ABC) multidrug transporters that have broad and substantially overlapping substrate specificities (Borst and Elferink, 2002; Schinkel and Jonker, 2003). MRP2 and BCRP are situated at apical membranes of important epithelial barriers, such as intestine and kidney and at the canalicular membrane of hepatocytes. As a consequence, 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 toward 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 (Boelsterli, 2003; Tang, 2003). Using TR<sup>-</sup> rats, which naturally lack Mrp2, Seitz and colleagues (1998) showed that hepatic Mrp2 medi-

ates 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<sup>-</sup> 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 intrahepatic 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; Stierlin et al., 1979; Willis 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, because this transporter accepts organic anions with a preference for glucuronidated substrates (Borst et al., 2007).

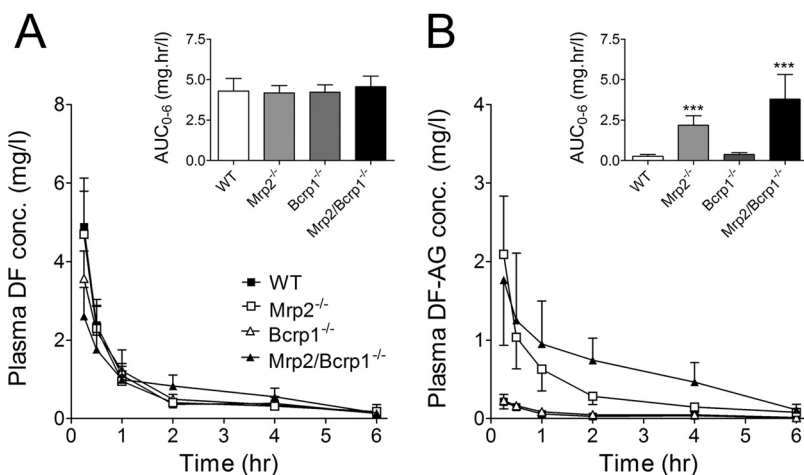
In this study, we investigated the impact of MRP2, MRP3, and BCRP on the pharmacokinetics of DF, using *Mrp2*(-/-), *Mrp3*(-/-), and *Bcrp1*(-/-) mice and all combination knockout strains. We included BCRP in this study, because we recently identified DF as a Bcrp1 substrate in vitro (Lagas et al., 2009).



**Fig. 1.** Structures and biotransformation routes of DF and its three most predominant primary metabolites with the putative metabolic enzymes involved in the mouse.

## Materials and Methods

**Chemicals.** DF (25 mg/ml; Voltaren) was obtained from Novartis (Arnhem, The Netherlands). DF-AG originated from United States Biological (Swampscott, MA). 5OH-DF was from Toronto Research Chemicals (North York, ON, Canada). 4OH-DF was a kind gift from BD Bioscience (Breda, the Netherlands). [<sup>14</sup>C]DF, with specific activity of 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, VIC, Australia). Bovine serum albumin, fraction V, was from Roche Diagnostics (Mannheim, Germany). Ketamine (Ketanest-S) was from Pfizer (Cappelle a/d IJssel, the Netherlands). Xylazine was from Sigma-Aldrich (St. Louis, MO). L(+)-Ascorbic acid and sodium acetate were



**Fig. 2.** Plasma concentration-time curves of DF (A) and DF-AG (B) in male FVB WT (■), *Mrp2*(-/-) (□), *Bcrp1*(-/-) (△), and *Mrp2/Bcrp1*(-/-) (▲) mice after oral administration of 5 mg/kg DF. Data are means ± S.D., *n* = 5. Insets, area under the curve from 0 to 6 h (AUC<sub>0-6</sub>) for DF (A) and DF-AG (B) in the different strains. \*\*\*, *P* < 0.001 compared with WT mice.

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-h light/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 (25 mg/ml Voltaren) 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 h before drug administration. Blood samples ( $\sim 30 \mu\text{l}$ ) were collected in heparinized capillary tubes (Oxford Labware, St. Louis, MO) from the tail vein at 15 and 30 min and at 1, 2, 4, and 6 h after administration of the drug. For intravenous studies, DF (25 mg/ml Voltaren) 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 min after administration of the drug. Blood samples were kept on melting ice. After centrifugation at 2100g 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  $-80^\circ\text{C}$  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 intravenous experiments, livers were collected immediately after cardiac puncture and stored at  $-80^\circ\text{C}$  until homogenization. Livers were homogenized in ice-cold 0.3 M sodium acetate, 20 mM ascorbic acid, and 4% bovine serum albumin (m/v) solution, pH 4.5, and homogenates were stored at  $-80^\circ\text{C}$  until LC-MS/MS analysis.

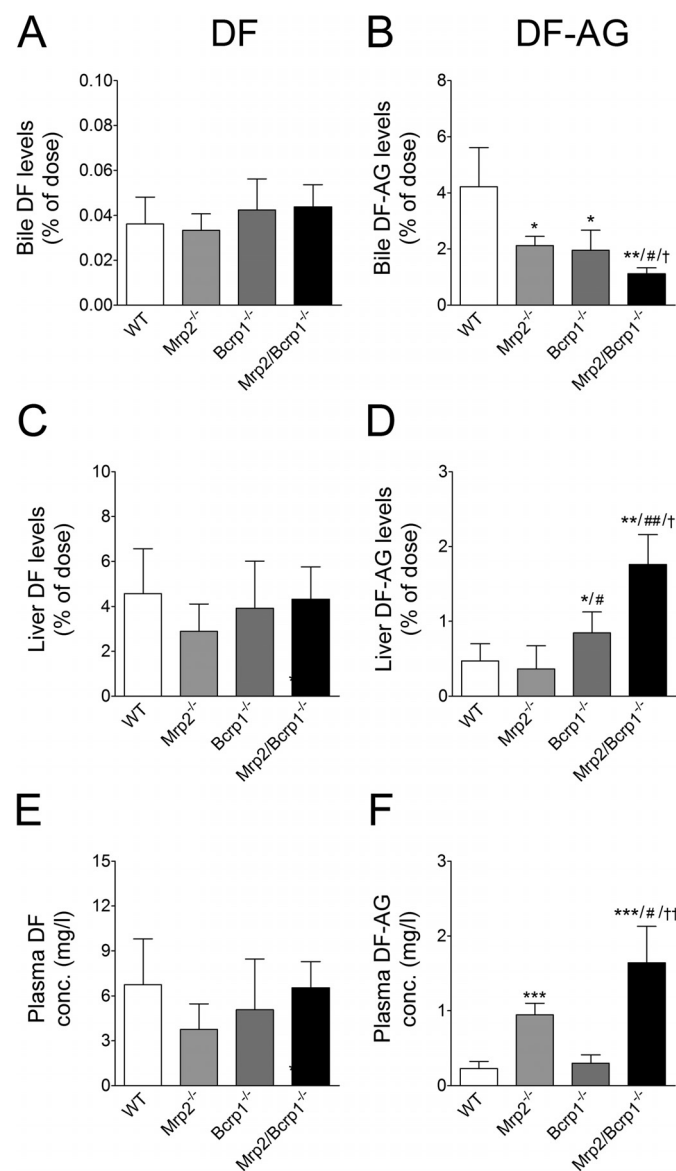
**Biliary Excretion.** In gallbladder cannulation experiments, mice were anesthetized by intraperitoneal injection of a solution of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), in a volume of 4.33  $\mu\text{l}$ /g body weight. After opening the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex Limited, Hythe, Kent, UK) with an inner diameter of 0.28 mm was inserted into the incised gallbladder and fixed with an additional ligation. Bile was collected for 60 min after intravenous injection of 5 mg/kg DF in a tube placed on ice containing 10  $\mu\text{l}$  of 2 M acetic acid and 1  $\mu\text{l}$  of 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 and 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 [ $^{14}\text{C}$ ]DF ( $\sim 0.5 \mu\text{Ci}$  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 intraperitoneally 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 h after administration using heparinized capillary tubes (Oxford Labware, St. Louis, MO). Twenty-four hours 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 2100g for 6 min at 4°C, and plasma was collected and stored at  $-20^\circ\text{C}$  until analysis. Alanine aminotransferase (ALT) 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  $\mu\text{m}$  from the paraffin blocks and stained with hematoxylin and eosin according to standard procedures. Images were captured with a Zeiss AxioCam HRc digital camera and processed with AxioVision 4 software (both from Carl Zeiss GmbH, Jena, Germany).

**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



**Fig. 3.** Levels of DF and DF-AG in bile (A and B), liver (C and D), and plasma (E and F) of male gallbladder cannulated WT, *Mrp2*( $-/-$ ), *Bcrp1*( $-/-$ ), and *Mrp2/Bcrp1*( $-/-$ ) mice. After ligation of the common bile duct and cannulation of the gallbladder, DF (5 mg/kg) was given intravenously followed by bile collection for 60 min and tissue isolation and plasma collection at 60 min. Data represent means  $\pm$  S.D. ( $n = 5$  per strain). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$  compared with WT mice. #,  $P < 0.05$ ; ##,  $P < 0.01$  compared with *Mrp2*( $-/-$ ) mice. †,  $P < 0.05$ ; ††,  $P < 0.01$  compared with *Bcrp1*( $-/-$ ) mice.

assess the statistical significance, we performed one-way analysis of variance followed by Dunnett's multiple comparison test. Differences were considered statistically significant when  $P < 0.05$ . Data are presented as means  $\pm$  S.D.

## 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). As a consequence, the AUCs, which is a measure for the exposure to DF-AG, was 8-fold higher in *Mrp2*( $-/-$ ) mice ( $2.18 \pm 0.60$  mg  $\cdot$  h/l) and 14-fold elevated in *Mrp2/Bcrp1*( $-/-$ ) mice ( $3.79 \pm 1.53$  mg  $\cdot$  h/l) compared with WT mice ( $0.27 \pm 0.10$  mg  $\cdot$  h/l; Fig. 1B, inset). Furthermore, *Mrp2/Bcrp1*( $-/-$ ) mice had significantly higher DF-AG plasma concentrations than *Mrp2*( $-/-$ ) mice at 2 and 4 h after oral administration, although the AUC<sub>0-6</sub> was not significantly different between these genotypes ( $P = 0.06$ ; Fig. 2B). Deficiency of *Mrp2* thus seems to be the main cause of the highly increased DF-AG plasma concentrations. The maximal plasma concentrations of DF and DF-AG were probably reached before the first sampling time point (i.e., 15 min after oral administration) (Fig. 2, A and B), testifying to the rapid kinetics of these compounds.

**Biliary Excretion of DF and DF-AG in *Mrp2*( $-/-$ ), *Bcrp1*( $-/-$ ), and *Mrp2/Bcrp1*( $-/-$ ) Mice.** Because 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 and Table 1). DF was intravenously administered at 5 mg/kg to mice with a cannulated gallbladder, and bile was collected for 60 min, immediately followed by the 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. 3, A, C, and E). The amount of DF occurring in bile was also very small ( $<0.05\%$  of the dose), despite considerable accumulation in the liver ( $\sim 4\%$  of the dose). In contrast, the much higher biliary output of DF-AG ( $\sim 4\%$  of the dose) was  $\sim 2$ -fold (i.e., by 50%) reduced in both single *Mrp2*( $-/-$ ) and *Bcrp1*( $-/-$ ) mice compared with WT mice and by approximately 4-fold (i.e., by 75%) in compound *Mrp2/Bcrp1*( $-/-$ ) mice (Fig. 3B). It is interesting that the  $\sim 2$ -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. 3, D and F). We will return to this difference under *Discussion*. Furthermore, the fact that biliary output of DF-AG was not completely abrogated in *Mrp2/Bcrp1*( $-/-$ ) mice points toward 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). It is interesting that although liver and plasma levels of these metabolites were not significantly different,

TABLE 1

Diclofenac and three primary metabolites as determined in bile, liver, and plasma of mice with cannulated gallbladder 60 min after intravenous administration of diclofenac at 5 mg/kg

Plasma concentrations are expressed as milligrams per liter, and liver and bile concentrations are given as percentage of the dose. Data are given as means  $\pm$  S.D.,  $n = 4$  to 6.

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

\*  $P < 0.05$  compared with WT mice.

\*\*  $P < 0.01$  compared with WT mice.

\*\*\*  $P < 0.001$  compared with WT mice.

$^\dagger$   $P < 0.05$  compared with *Mrp2*( $-/-$ ) mice.

$^\ddagger$   $P < 0.01$  compared with *Mrp2*( $-/-$ ) mice.

$^\S$   $P < 0.05$  compared with *Bcrp1*( $-/-$ ) mice.

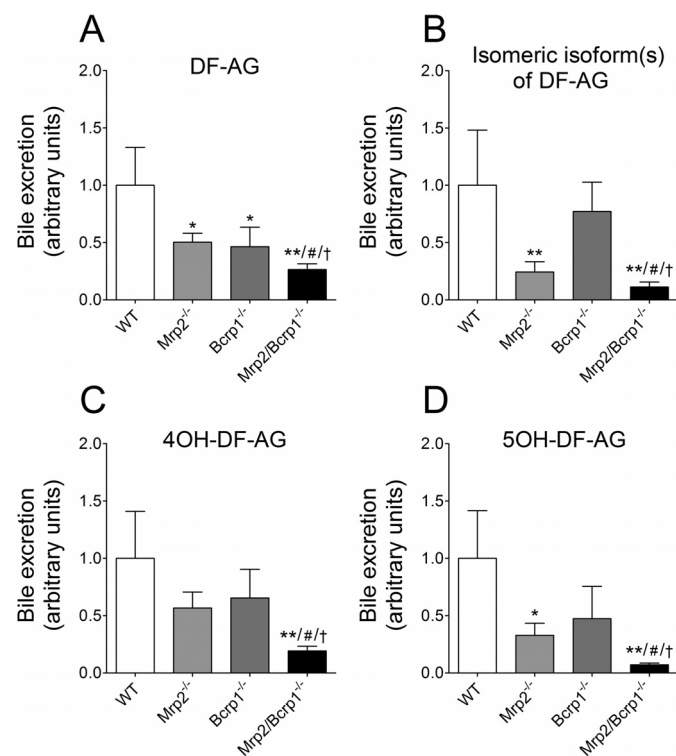
$^{**\dagger}$   $P < 0.01$  compared with *Bcrp1*( $-/-$ ) mice.

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 intravenously 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 min,  $42.6 \pm 4.4\%$  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  $\sim 10\%$  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 because mouse bile has a pH of  $\sim 9$ , 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. Because 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 toward 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 min after injection. As shown in Fig. 5, A and C, 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). It is striking that 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 toward the blood (Fig. 5B). Accordingly, the DF-AG concentrations in the liver, determined at 60 min after administration, were  $\sim 1.8$ -fold elevated in *Bcrp1*( $-/-$ ) and *Mrp2/Bcrp1*( $-/-$ ) mice and 3.3-fold in *Mrp2/Mrp3/Bcrp1*( $-/-$ ) mice (Fig. 5D). It is interesting that, in the livers of *Mrp2/Mrp3/Bcrp1*( $-/-$ ) mice, but not in WT livers, substantial accumulation of 4OH-DF-AG, 5OH-DF-AG, and DF-AG isomers was observed (data not shown). As mentioned above, the lack of reference compounds made it impossible to quantify these metabolites. However, because DF acyl glucuronide metabolites are associated with idiosyncratic hepatotoxicity (Boelsterli, 2003; Tang, 2003), we hypothesized that *Mrp2/Mrp3/Bcrp1*( $-/-$ ) mice might be more prone to DF-induced liver toxicity than WT mice.



**Fig. 4.** Levels of DF-AG (A) and 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 gallbladder, DF (5 mg/kg) was given intravenously followed by bile collection for 60 min. Levels are given in arbitrary units. Data represent means  $\pm$  S.D. ( $n = 5$  per strain). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with WT mice. #,  $P < 0.05$  compared with *Mrp2*( $-/-$ ) mice. †,  $P < 0.01$  compared with *Bcrp1*( $-/-$ ) 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 intraperitoneally 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 h after administration. In addition, livers were isolated 24 h 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 ( $\sim 2$ -fold,  $P < 0.05$ ) ALAT levels at both time points (Fig. 6), suggesting that these mice displayed acute, albeit very mild, liver toxicity.

## Discussion

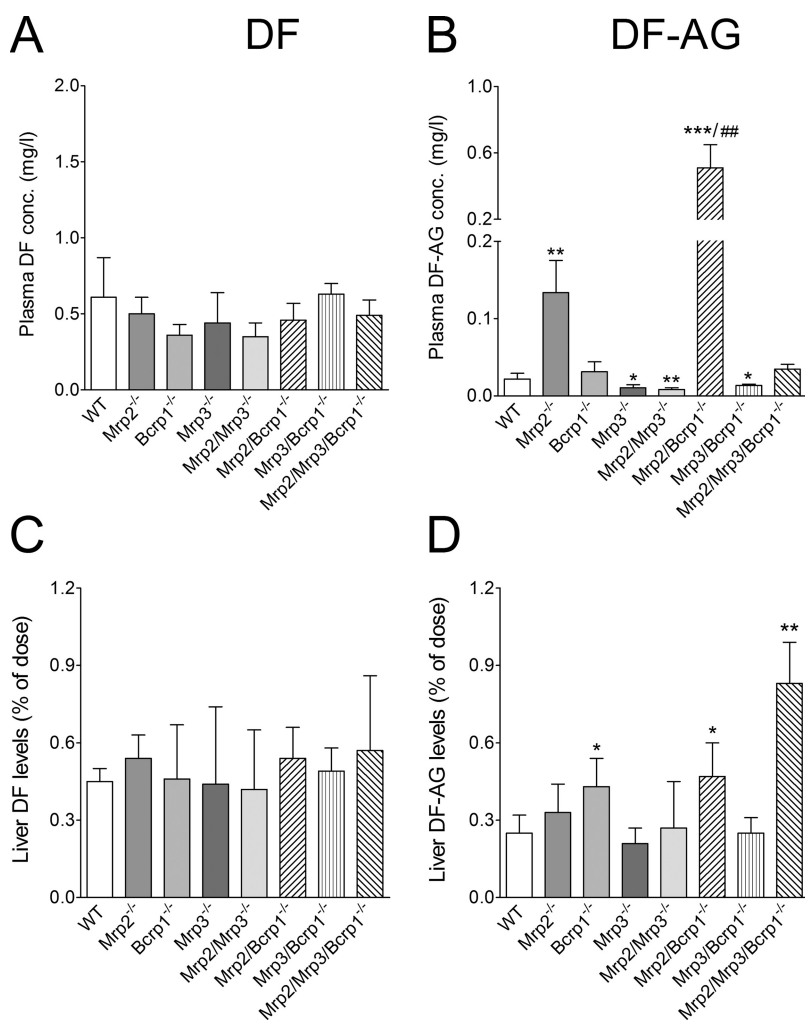
In this study, we identify Mrp2, Mrp3, and Bcrp1 in the mouse as important determinants for the pharmacokinetics of reactive glucuronide metabolites of DF. Mrp2 and Bcrp1 are involved in the biliary excretion of DF glucuronides, whereas Mrp3 is a major hepatic transporter for the extrusion of DF-AG across the sinusoidal membrane toward 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, 2009a,b). This makes it unlikely that alterations in expression of these transporters could substantially affect the interpretation of our data.

We demonstrated recently 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 and Boelsterli,



**Fig. 5.** Levels of DF and DF-AG in plasma (A and B) and liver (C and D) of male WT, *Mrp2*(-/-), *Mrp3*(-/-), *Bcrp1*(-/-), *Mrp2/Mrp3*(-/-), *Mrp2/Bcrp1*(-/-), *Mrp3/Bcrp1*(-/-), and *Mrp2/Mrp3/Bcrp1*(-/-) mice. DF (5 mg/kg) was given intravenously followed by plasma collection and tissue isolation at 60 min. Data represent means  $\pm$  S.D. ( $n = 5$  per strain). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with WT mice. #,  $P < 0.01$  compared with *Mrp2*(-/-) mice.

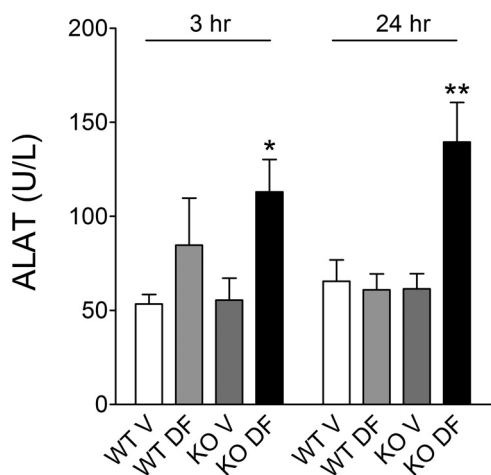
1998; Seitz et al., 1998). Moreover, hepatobiliary excretion of DF glucuronides in TR<sup>-</sup> rats, a spontaneous mutant lacking Mrp2, is almost completely abrogated (Seitz et al., 1998). In contrast, in mice we show that the biliary excretion of DF glucuronides is dependent mainly on both Mrp2 and 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,b). Our observation that the biliary output of DF-AG was not completely abrogated in *Mrp2/Bcrp1*( $-/-$ ) mice may point toward 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).

It is noteworthy that after oral DF administration, maximal plasma concentrations of DF-AG are reached within 15 min after administration (Fig. 2B). This might suggest that the glucuronidation of DF already occurs in the gastrointestinal tract, because 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 (Vargas and Franklin, 1997; Seitz et al., 1998; Ware et al., 1998). The glucuronidation of DF in rats is catalyzed by Ugt2b1 (King et al., 2001), and because rat and mouse Ugt2b1 proteins share >85% homology (available at <http://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 intravenous dose of DF is excreted as glucuronide metabolites in the bile of WT mice within 60 min, 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 to 30 min, whereas this was ~2 h 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 (Borst et al., 2007). Our results suggest that Mrp3 also predominantly mediates the transport of DF-AG from the liver toward the blood circulation. It is interesting that in humans, DF acyl glucuronides are predominantly excreted from the liver to the blood circulation and subsequently excreted in the urine (John, 1979; Stierlin et al., 1979; Willis 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 seems to be the other way around. We have shown recently that Mrp3 is up-regulated in livers of *Mrp2*( $-/-$ ) and *Mrp2/Bcrp1*( $-/-$ ) mice (Vlaming et al., 2006, 2009a). This probably 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 (Fig. 3, D and F).

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 *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 is 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



**Fig. 6.** Plasma levels of ALAT in WT and knockout (KO) *Mrp2/Mrp3/Bcrp1*( $-/-$ ) mice. Mice were intraperitoneally injected with vehicle (V) or DF (25 mg/kg) and blood was collected 3 and 24 h after administration. Data represent means  $\pm$  S.D. ( $n = 4$  per strain). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with WT mice receiving vehicle.

electrophilic, chemically reactive compounds that can form protein adducts via nonenzymatic 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 (Boelsterli, 2003; Tang, 2003). We therefore tested whether *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, after both 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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