

Bcrp1;Mdr1a/b;Mrp2 combination knockout mice : altered disposition of the dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and its genotoxic metabolites

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Bcrp1;Mdr1a/b;Mrp2 Combination Knockout Mice: Altered Disposition of the Dietary Carcinogen PhIP (2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine) and Its Genotoxic Metabolites[§]

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ABSTRACT

The multidrug transporters breast cancer resistance protein (BCRP), multidrug-resistance protein 1 (MDR1), and multidrug-resistance-associated protein (MRP) 2 and 3 eliminate toxic compounds from tissues and the body and affect the pharmacokinetics of many drugs and other potentially toxic compounds. The food-derived carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) is transported by BCRP, MDR1, and MRP2. To investigate the overlapping functions of *Bcrp1*, *Mdr1a/b*, and *Mrp2* in vivo, we generated *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice, which are viable and fertile. These mice, together with *Bcrp1;Mrp2;Mrp3*^{-/-} mice, were used to study the effects of the multidrug transporters on the pharmacokinetics of PhIP and its metabolites. Thirty minutes after oral or intravenous administration of PhIP (1 mg/kg), the PhIP levels in the small intestine were reduced 4- to 6-fold in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice compared with wild-type mice. Fecal excretion of PhIP was reduced 8- to 20-fold in knockouts. Biliary PhIP excretion was

reduced 41-fold in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice. Biliary and small intestine levels of PhIP metabolites were reduced in *Bcrp1;Mrp2*-deficient mice. Furthermore, in both knockout strains, kidney levels and urinary excretion of genotoxic PhIP-metabolites were significantly increased, suggesting that reduced biliary excretion of PhIP and PhIP metabolites leads to increased urinary excretion of these metabolites and increased systemic exposure. *Bcrp1* and *Mdr1a* limited PhIP brain accumulation. In *Bcrp1;Mrp2;Mrp3*^{-/-}, but not *Bcrp1;Mdr1a/b;Mrp3*^{-/-} mice, the carcinogenic metabolites N2-OH-PhIP (2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) and PhIP-5-sulfate (a genotoxicity marker) accumulated in liver tissue, indicating that *Mrp3* is involved in the sinusoidal secretion of these compounds. We conclude that *Bcrp1*, *Mdr1a/b*, *Mrp2*, and *Mrp3* significantly affect tissue disposition and biliary and fecal elimination of PhIP and its carcinogenic metabolites and may affect PhIP-induced carcinogenesis as a result.

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Introduction

The ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp or multidrug-resistance protein [MDR] 1 or ABCB1), breast cancer resistance protein (BCRP or ABCG2), multidrug-resistance protein (MRP) 2 (ABCC2), and MRP3 (ABCC3) have very broad and substantially overlapping substrate specificities and can actively extrude potentially toxic compounds from cells. They are expressed in organs with excretory functions, such as liver, kidney, and small intestine, and are involved in the elimination of endogenous and exogenous compounds from the body. P-gp, BCRP, and, to a lesser extent, MRP2 are

ABBREVIATIONS: 5-OH-PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-5-hydroxypyridine; ABC transporters, ATP-binding cassette transporters; BCRP, breast cancer resistance protein; D3-PhIP, 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine; DMSO, dimethyl sulfoxide; FVB, Friend Virus B type; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLQ, lower limit of quantification; MDR1, multidrug-resistance protein 1; MRP2, multidrug-resistance-associated protein 2; MRP3, multidrug-resistance-associated protein 3; N2-OH-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; P-gp, P-glycoprotein; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

additionally present in tissue sanctuaries, such as brain, testis, and placenta, where they protect these important organs from entry of potentially toxic compounds. Whereas P-gp, BCRP, and MRP2 are located at the apical membrane of epithelial cells, transporting their substrates into bile, feces, and urine, MRP3 is located basolaterally in the intestine and liver, where it transports its substrates into the blood circulation (Borst and Elferink, 2002; Schinkel and Jonker, 2003; Leslie et al., 2005).

Besides endogenous compounds and a wide range of drugs, the dietary heterocyclic amine carcinogens PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) and IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) are also substrates for ABC transporters *in vitro* and *in vivo* (Dietrich et al., 2001a,b; van Herwaarden et al., 2003, 2006; Leslie et al., 2005; Vlaming et al., 2006; Enokizono et al., 2008). PhIP is the most abundant heterocyclic amine in fried or cooked meat, chicken, and fish. In mice, PhIP primarily causes lymphomas and tumors of the small intestine, whereas in rats it causes colon, prostate, and mammary gland tumors. PhIP is rapidly taken up after ingestion and heavily metabolized, leading to detoxification products but also to activated, potentially carcinogenic metabolites (Supplemental Fig. 1; Gooderham et al., 2001, 2002; Lauber et al., 2004; Nakagama et al., 2005). Here, we focused on the precarcinogen N2-OH-PhIP (2-hydroxy-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), and the derivatives of 5-OH-PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-5-hydroxypyridine) and PhIP-5-sulfate, which represent breakdown products of the ultimate genotoxic nitrenium radical cation that also forms mutagenic PhIP-DNA adducts. Therefore, 5-OH-PhIP and PhIP-5-sulfate are considered good markers for genotoxic exposure upon PhIP exposure. PhIP-*N*-sulfate and *N*-acetyl-PhIP, the primary precursors of the genotoxic nitrenium radical, are too short-lived to be detectable in pharmacokinetic studies. Furthermore, part of the carcinogenic potential of PhIP may also be explained by the fact that PhIP possesses estrogenic activity and could therefore stimulate cell proliferation (Schut and Snyderwine, 1999; Frandsen and Alexander, 2000; Gooderham et al., 2002; Lauber et al., 2004; Chen et al., 2007; Lauber and Gooderham, 2007, 2011).

Using *Bcrp1*- or *Mrp2*-deficient mice and rats, it was previously shown that *Bcrp1* and *Mrp2* significantly influence the pharmacokinetics of [¹⁴C]PhIP *in vivo* (Dietrich et al., 2001b; van Herwaarden et al., 2003, 2006; Vlaming et al., 2006). However, since in several studies only radioactivity was measured, no differentiation between parent PhIP and its mutagenic metabolites could be made. In *Mrp2*-deficient TR⁻ rats, it was found that *Mrp2* influences the biliary excretion of PhIP, 4'-OH-PhIP and some glucuronide conjugates (Dietrich et al., 2001a). Furthermore, in *Bcrp1*^{-/-} mice, it was shown that *Bcrp1* restricts the penetration of PhIP, N2-OH-PhIP, and 4'-OH-PhIP into the brain or testis (Enokizono et al., 2008). Murine *Mdr1a* transports [¹⁴C]PhIP *in vitro*, but no effect of murine *Mdr1a/b* on [¹⁴C]PhIP plasma elimination was found (van Herwaarden et al., 2003), perhaps as a result of overlapping activity of *Bcrp1* or *Mrp2*. The effect of other ABC transporters on the pharmacokinetics of PhIP and its metabolites has not been investigated yet.

To investigate the overlapping or complementary roles of *Bcrp1*, *Mdr1a/b*, *Mrp2*, and *Mrp3* *in vivo*, we generated a set of compound knockout mice, deficient in up to three of these

ABC transporters (Schinkel et al., 1997; Jonker et al., 2002; Vlaming et al., 2006, 2008, 2009a,b). These strains proved to be useful tools for pharmacokinetic studies. To be able to determine the combined effects of the apically located transporters *Bcrp1*, *Mdr1a/b*, and *Mrp2* on physiology and pharmacology, we have now generated *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice. Furthermore, we recently developed and validated a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for the quantitative determination of PhIP and its metabolite N2-OH-PhIP in various matrices from mice and expanded it to include multiple phase 1 and phase 2 metabolites (Teunissen et al., 2010, 2011). We here used the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice to investigate the combined effect of *Bcrp1*, *Mdr1a/b*, and *Mrp2* on the elimination of PhIP and its metabolites *in vivo*. Furthermore, as the basolateral transporter *Mrp3* is often upregulated when *Mrp2* is absent, and thus may compensate for the decreased apical (biliary) efflux from the liver (Donner and Keppler, 2001; Zelcer et al., 2005; Vlaming et al., 2006), we additionally investigated the pharmacokinetics of PhIP and its metabolites in *Bcrp1;Mrp2;Mrp3*^{-/-} mice (Vlaming et al., 2009b).

Materials and Methods

Animals. In compliance with Dutch legislation, mice were housed and handled according to institutional guidelines, and approval of the local animal care and use committee was obtained before the start of experiments. *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice were generated by cross-breeding *Bcrp1;Mdr1a/b*^{-/-} (Jonker et al., 2002) and *Bcrp1;Mrp2*^{-/-} (Vlaming et al., 2009a) mice. The generation of *Bcrp1;Mrp2;Mrp3*^{-/-} mice was described before (Vlaming et al., 2009b). All animals were of >99% Friend Virus B-type (FVB) background and 9–14 weeks old. Animals were kept in a temperature-controlled environment with a 12-hour light/dark cycle. They received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Chemicals. PhIP and its deuterated internal standard D3-PhIP [2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine] were purchased from Toronto Research Chemicals (North York, ON, Canada). N2-OH-PhIP was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas City, MO). 5-OH-PhIP was a kind gift from Henrik Frandsen of the National Food Institute, Technical University of Denmark. Bovine serum albumin, dimethyl sulfoxide (DMSO), and formic acid were purchased from Merck (Darmstadt, Germany) and were used as received. Methanol was obtained from Biosolve Ltd. (Amsterdam, The Netherlands). Distilled water was from B. Braun (Melsungen, Germany). Ketamine was from Parke-Davis (Hoofddorp, The Netherlands), xylazine from Sigma-Aldrich (St. Louis, MO), and methoxyflurane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, VIC, Australia). All other chemicals and reagents were from Sigma-Aldrich (Steinheim, Germany).

Histologic, Clinical-Chemical, and Hematologic Analysis of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} Mice. Histologic analysis of male and female mouse tissues (*n* = 6), clinical chemistry analyses (including total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase) on the serum of male and female mice (*n* = 6), as well as standard hematologic analysis of male and female mice (*n* = 6, twice within a time span of 1.5 years), were performed as described (Vlaming et al., 2006).

Real-Time Polymerase Chain Reaction Analysis. RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction analysis on liver, kidney, and small intestine of male mice (*n* = 3) were done as described (van Waterschoot et al., 2008).

Pharmacokinetic Experiments. For oral administration, 10 μg of body weight of a 0.1 mg/ml PhIP solution in 20% (v/v) DMSO, and 5% (w/v) D-glucose was dosed by gavage in the stomach of male mice. For intravenous administration, 5 μg of body weight of a 0.2 mg/ml PhIP solution in 20% (v/v) DMSO and 0.9% (w/v) NaCl solution was injected into the tail vein of male mice. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia, and organs were removed. Intestinal contents and tissue were separated.

Biliary Excretion of PhIP and Its Metabolites. Gallbladder cannulations in male mice were performed as described (van Herwaarden et al., 2003). After cannulation, PhIP was administered intravenously at 1 mg/kg as described above. Bile was collected in 15-minute fractions for 60 minutes after administration of PhIP. Subsequently, mice were killed by cardiac puncture and plasma and organs were collected.

Fecal and Urinary Excretion of PhIP and Its Metabolites. Male mice were individually housed in Tecniplast metabolic cages (Milan, Italy) and allowed 24 hours to adapt before PhIP (1 mg/kg) was injected into the tail vein, as described already herein. Feces and urine were collected over 24 hours. Subsequently, mice were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia. Organs were removed, and intestinal contents (feces) and tissue were separated.

Sample Preparation. After sampling, urine, bile, and plasma samples were snap-frozen. Tissue and feces samples were weighed before snap-freezing. The complete organ or the total volume of sampled feces was homogenized in a 4% (w/v) bovine serum albumin solution using a Polytron blender. Feces homogenates were centrifuged for 10 minutes at 11,300g. A 10- μl volume of bile was diluted in 400 μl of 4% (w/v) bovine serum albumin solution.

Sample Processing. A 100- μl aliquot of plasma, diluted bile, tissue, or clear supernatant of centrifuged feces homogenate was processed immediately after thawing at ambient temperature by the addition of 300 μl of internal standard (D3-PhIP) solution in acetonitrile. The mixture was vortex-mixed for 10 seconds, followed by centrifugation for 10 minutes at 11,300g; 100 μl of the clear supernatant was diluted in a 1:1 ratio with 100 μl of 3.5 mM ammonium formate buffer, pH 3.5. Urine was 10 \times diluted by addition of a 180 μl of internal standard solution in methanol-ammonium formate buffer, pH 3.5 (30:70, v/v) to a 20- μl urine sample aliquot.

LC-MS/MS Analysis of PhIP and Its Metabolites. LC-MS/MS was used for the separation and detection of PhIP and its metabolites. Mobile phase A was prepared by adjusting a 5 mM ammonium formate solution to pH 3.5 with a 98% formic acid solution. Mobile phase B consisted of methanol. Mobile phase A and B were pumped through a Synergi Hydro 110 \AA column (150 \times 2.0 mm I.D., 4 μm ; Phenomenex, Torrance, CA) at a flow rate of 0.2 ml/min.

The LC eluate was directed to an API 3000 triple quadrupole MS (AB Sciex, Foster City, CA) equipped with an electrospray ion source operating in the positive ion mode. For quantification, multiple-reaction monitoring chromatograms were acquired and processed. Calibration curves of analyte/internal standard peak area ratio versus, respectively, PhIP and N2-OH-PhIP concentrations were constructed, and a weighted $1/x^2$ (the reciprocal of the squared concentration) linear regression was applied to the data. Quantification of metabolites was performed based on the calibration curve of PhIP. PhIP-glucuronides and OH-PhIP-glucuronide metabolites (Supplemental Fig. 1) could not be unequivocally distinguished, as reference standards were not available in all cases. Therefore, in this study, *PhIP-glucuronides* stands for the sum of PhIP-N2-glucuronide and PhIP-N3-glucuronide, and *OH-PhIP-glucuronides* is defined as the sum of all OH-PhIP-glucuronides detected (Supplemental Fig. 1). After purification from mouse urine, the identity of both PhIP-4'-sulfate and PhIP-5-sulfate was confirmed by NMR analysis (Teunissen et al., 2011). The lower limit of quantification (LLQ) of PhIP and its metabolites was 1 ng/ml.

Statistical Analysis. Unless otherwise indicated, the two-sided unpaired Student's *t* test was used to assess statistical significance of differences between two sets of data. For analysis of statistical difference when compared with data below the detection limit, calculations were made based on the $\text{LLQ} \pm \text{LLQ}$. Results are presented as the means \pm S.D. Differences were considered statistically significant when $P < 0.05$.

Results

Macroscopic and Microscopic Analysis of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} Mice. *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice were, like *Bcrp1;Mrp2;Mrp3*^{-/-} mice (Vlaming et al., 2009b), viable and fertile, and they had normal life spans, body weights, and anatomy. Adult male *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice had a 46% increased liver weight compared with wild-type mice (7.0% \pm 0.5% versus 4.8% \pm 0.2% of body weight, respectively, $n = 5$; $P = 2.8 \cdot 10^{-5}$). As previously described, the liver weight of male *Bcrp1;Mrp2;Mrp3*^{-/-} mice was \sim 70% higher than that of wild-type mice (Vlaming et al., 2009b). Despite the increased liver size, detailed microscopic analysis of liver sections did not reveal obvious pathologic changes in either strain. An increase (somewhat smaller) in liver weight was previously seen in other *Mrp2*-deficient mouse strains (Vlaming et al., 2006, 2008, 2009a; Tian et al., 2008).

Plasma Clinical Chemistry and Hematologic Analysis of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} Mice. Plasma clinical chemistry parameters in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice did not show consistent significant differences from wild-type, except for increased plasma bilirubin levels (conjugated and unconjugated), as previously shown for *Bcrp1;Mrp2*^{-/-} mice (Vlaming et al., 2009a). Additional deletion of *Mdr1a/b* did not further affect the plasma bilirubin levels of the mice (data not shown). Hematologic analysis of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice showed that hemoglobin levels were mildly but significantly reduced compared with wild-type mice (males: 6.7 \pm 0.4 mM versus 7.4 \pm 0.1 mM; females: 6.8 \pm 0.1 mM versus 7.6 \pm 0.1 mM, $n = 3$ –5; $P < 0.05$). This was previously also found in other *Mrp2*-deficient mouse strains (Vlaming et al., 2006, 2009a). Other measured parameters did not show consistent differences with wild-type mice (not shown). Overall, despite the complete absence of the three main apical detoxifying ABC transporters, the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice displayed only a few modest phenotypic alterations that were previously seen in the single knockout strains. They appear, therefore, fully amenable to pharmacological and toxicological studies.

Expression Levels of Metabolizing Enzymes and ABC Transporters in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} Mice. We determined the RNA expression levels of a set of genes involved in xenobiotic metabolism and transport in general (*Cyp3a11*, *Cyp3a13*, *Cyp3a25*, *Cyp3a41*, and *Mrp4*), as well as a number of genes that have been associated with transport or metabolism of PhIP and/or its main metabolites (*Cyp1a1*, *Cyp1a2*, *Ugt1a1*, *Sult1a1*, *Nat1*, *Nat2*, *Mdr1a*, *Mdr1b*, and *Mrp3*) in liver, small intestine, and kidney of male mice.

In liver, expression was significantly increased for *Cyp3a11*, *Cyp3a13*, *Ugt1a1*, *Mrp3*, and *Mrp4*. *Cyp3a11* and *Cyp3a25* were mildly (2.5- and 1.5-fold, respectively) increased in livers of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice (Supplemental Table 1). *Cyp3a13* was increased 3.1-fold in *Bcrp1;Mrp2;Mrp3*^{-/-} mice ($P = 0.043$), but in all strains, *Cyp3a13* RNA levels in liver were extremely low. *Mrp4* protein was

previously undetectable by Western blot in livers of wild-type FVB and *Bcrp1;Mrp2;Mrp3*^{-/-} mice (Vlaming et al., 2009b). Accordingly, we found only extremely low levels of *Mrp4* RNA in livers of all strains, even though they were increased by 28- and 43-fold in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice compared with wild-type mice, respectively ($P < 0.001$ for both strains). Of the enzymes potentially involved in PhIP metabolism, *Ugt1a1* mRNA was mildly increased (1.5- and 1.6-fold, respectively; $P < 0.05$ for both strains) in livers of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice. Furthermore, *Mrp3* mRNA levels in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice were increased 4.5-fold compared with wild-type ($P = 0.012$). Moderately increased *Mrp3* protein levels in liver were previously also found for other *Mrp2*-deficient strains (Vlaming et al., 2006, 2009a).

In small intestine, only *Cyp1a1* RNA was significantly reduced in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice, to 15 or 21% of wild-type values, respectively. None of the other tested genes displayed significant or meaningful alterations in expression in small intestine (Supplemental Table 1).

In kidney, *Cyp1a2* RNA was upregulated in *Bcrp1;Mdr1a/b;Mrp2*^{-/-}, but not *Bcrp1;Mrp2;Mrp3*^{-/-} mice, from an undetectable expression level in wild-type mice (>40 polymerase chain reaction cycles). *Cyp3a11* was also virtually undetectable in wild-type and *Bcrp1;Mrp2;Mrp3*^{-/-} kidneys and increased 64-fold in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} kidneys, but it was still very low compared with expression levels in liver tissue (Supplemental Table 1). None of the other tested genes was significantly changed, with the exception of a 12-fold increase in *Mrp4* RNA ($P < 0.05$) in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} kidneys, in line with what was found before for *Mrp4* protein levels in kidney of other *Mrp2*-deficient strains (Vlaming et al., 2006, 2008, 2009a). Overall, changes in gene expression in these strains were remarkably limited given their genetic deficiencies in major detoxifying transporters.

Effect of ABC Transporters on Plasma and Tissue Distribution of PhIP and Its Metabolites. As PhIP is heavily metabolized in the body (Supplemental Fig. 1), and, as only some of the known PhIP metabolites are potentially carcinogenic (Schut and Snyderwine, 1999; Frandsen and Alexander, 2000; Gooderham et al., 2001, 2002; Lauber et al., 2004; Nakagama et al., 2005; Chen et al., 2007; Lauber and Gooderham, 2007, 2011), we developed an LC-MS/MS assay for the quantification of PhIP and N2-OH-PhIP in mouse matrices (Teunissen et al., 2010) and expanded this assay for detection of various phase 1 and phase 2 metabolites of PhIP (Teunissen et al., 2011). We used this method to determine the plasma and tissue concentrations of PhIP and its carcinogenic metabolites 30 minutes after oral or intravenous administration of PhIP (1 mg/kg) to wild-type, *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice. Overviews of all metabolites detected in the tissues of the three strains are presented in Supplemental Table 2 (intravenous administration) and Supplemental Table 3 (oral administration). Most metabolites, for example all glucuronide conjugates, are considered detoxifying metabolites and do not have carcinogenic potential. Also, the formation of 4'-OH-PhIP and subsequently PhIP-4'-sulfate (Supplemental Fig. 1) is considered a detoxification pathway. On the other hand, besides PhIP itself, N2-OH-PhIP and the short-lived species PhIP-*N*-sulfate and *N*-acetyl-PhIP are (pre)carcinogenic (Schut and Snyderwine,

1999; Frandsen and Alexander, 2000; Gooderham et al., 2001, 2002; Lauber et al., 2004; Nakagama et al., 2005; Chen et al., 2007; Lauber and Gooderham, 2007, 2011). 5-OH-PhIP and PhIP-5-sulfate are surrogate markers for the levels of the ultimate genotoxic nitrenium radical cation that forms DNA adducts (Supplemental Fig. 1) (Alexander et al., 2002).

PhIP and its main metabolite, N2-OH-PhIP, were detected in the plasma of all mouse strains 30 minutes after intravenous or oral administration of PhIP. No significant differences between the strains were found (Fig. 1A; Supplemental Tables 2 and 3). After intravenous (but not oral) administration, PhIP-5-sulfate was detected in plasma of both knockout strains but not in wild-type plasma, suggesting that *Bcrp1* and/or *Mrp2* influence the plasma levels of this marker for genotoxic exposure (Supplemental Table 2). After oral, but not after intravenous administration, the detoxification product 4'-OH-PhIP was quite abundant in plasma of all strains, but no differences between strains were found (Supplemental Table 3). The absence of 4'-OH-PhIP in plasma after intravenous administration suggests that this compound is formed primarily by intestinal enzymes shortly after oral administration. Indeed, *Cyp1a1* and *1a2*, which can form this metabolite, are abundantly expressed in murine small intestine (Ito et al., 2007). Although *Cyp1a1* expression was reduced in small intestine of both knockout strains (Supplemental Table 1), apparently this did not significantly affect the formation of 4'-OH-PhIP in these strains.

Thirty minutes after intravenous administration, in livers of all strains, 1.8–2.5% of the given dose was found as unchanged PhIP (Fig. 1B). Similar amounts of PhIP (2.7–4.5%) were found in liver after oral administration (Fig. 1B). No significant differences between the three strains were found. Interestingly, in *Bcrp1;Mrp2;Mrp3*^{-/-} mice, after both intravenous and oral administration, the precarcinogen N2-OH-PhIP accumulated significantly in the liver (Fig. 1, C and D). This was not the case in the wild-type and *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice, suggesting that *Mrp3* protein limits accumulation of this metabolite in the liver. After intravenous administration, the same was found for the mutagenesis marker PhIP-5-sulfate (Fig. 1C). PhIP-5-sulfate was not detected in the liver (and plasma; see above) after oral administration.

The levels of the parent compound PhIP in the intestinal tract (contents and tissue combined) were determined after intravenous (Fig. 2A) or oral (Fig. 2B) application of PhIP (1 mg/kg). For both administration routes, the small intestinal levels of PhIP in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice were reduced 4- to 6-fold compared with wild-type mice. This finding suggests an important role for *Bcrp1* and/or *Mrp2* in the hepatobiliary and/or intestinal excretion of PhIP or, for oral administration, in reducing net intestinal uptake. As there was no difference between the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice, *Mdr1a/b* and *Mrp3* are probably not involved here. Also, in cecum and colon (the latter for intravenous application only) of both combination knockout strains, PhIP concentrations were reduced (Fig. 2, A and B). Small intestine levels of the carcinogenic PhIP metabolite N2-OH-PhIP and the genotoxic marker PhIP-5-sulfate were significantly reduced compared with levels in wild-type in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice, both after intravenous and oral administration of PhIP (Fig. 2, C and D). This result suggests

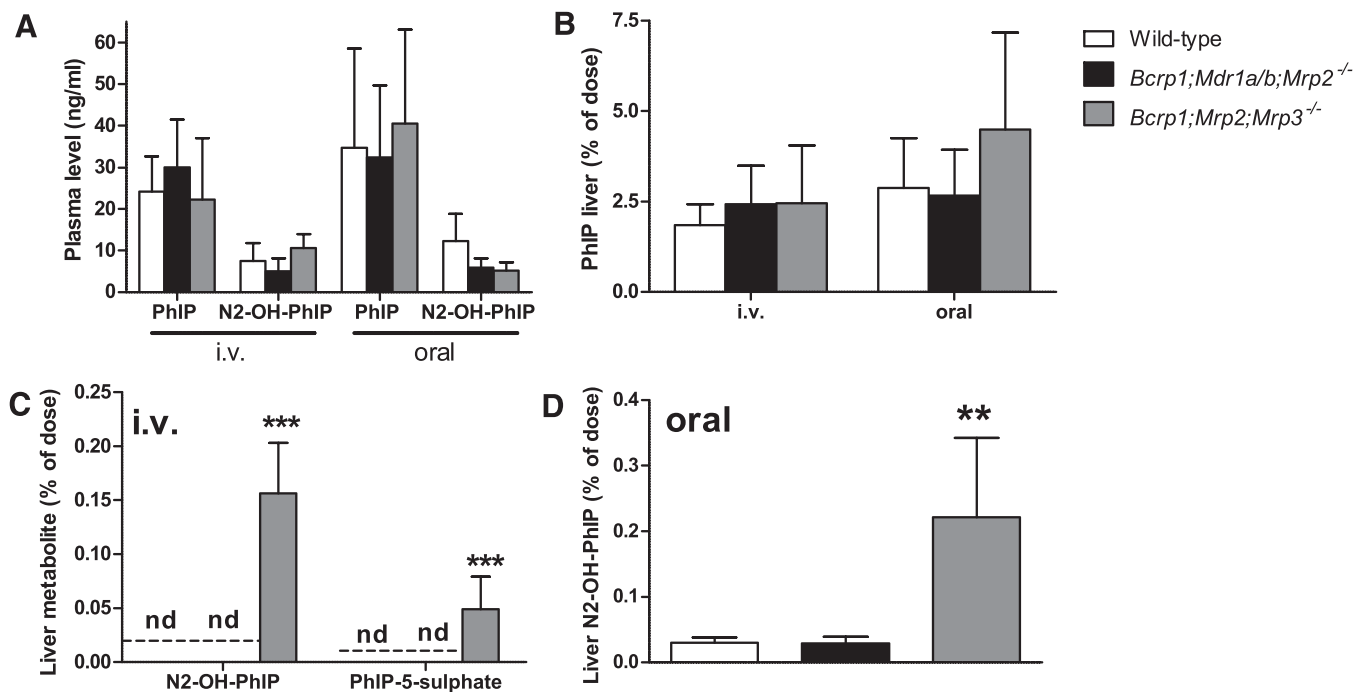


Fig. 1. Levels of PhIP and its primary carcinogenic metabolites in plasma and liver of male wild-type, *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice 30 minutes after intravenous ($n = 4-11$) or oral ($n = 5$) administration of PhIP (1 mg/kg). (A) PhIP and N2-OH-PhIP levels in plasma of the strains after intravenous or oral administration. (B) PhIP levels in liver of the strains after intravenous or oral administration. (C) Levels of N2-OH-PhIP and PhIP-5-sulfate in liver of the strains after intravenous administration. nd, not detected. Detection limits for N2-OH-PhIP and PhIP-5-sulfate were 0.02% and 0.01% of the dose, respectively (indicated by dashed lines). (D) N2-OH-PhIP levels in liver of the strains after oral administration. Data are means \pm S.D. ($n = 4-11$; $**P < 0.01$; $***P < 0.001$, compared with wild-type). In case tissue levels in wild-type mice were below the detection limit, the LLQ \pm LLQ was used to calculate statistical significance.

that *Bcrp1* and/or *Mrp2* are involved in biliary and/or direct intestinal excretion of these metabolites. In addition, the reduced levels of *Cyp1a1* in small intestine tissue of the knockout strains (Supplemental Table 1) may also lead to reduced formation of N2-OH-PhIP in small intestine. However, after intravenous and oral administration, N2-OH-PhIP

levels in small intestine of *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice were even lower than levels in the *Bcrp1;Mrp2;Mrp3^{-/-}* mice, despite an insignificant difference in *Cyp1a1* expression between these strains. Besides PhIP, N2-OH-PhIP, and PhIP-5-sulfate, various other PhIP metabolites (glucuronide and sulfate conjugates) were detected in wild-type small

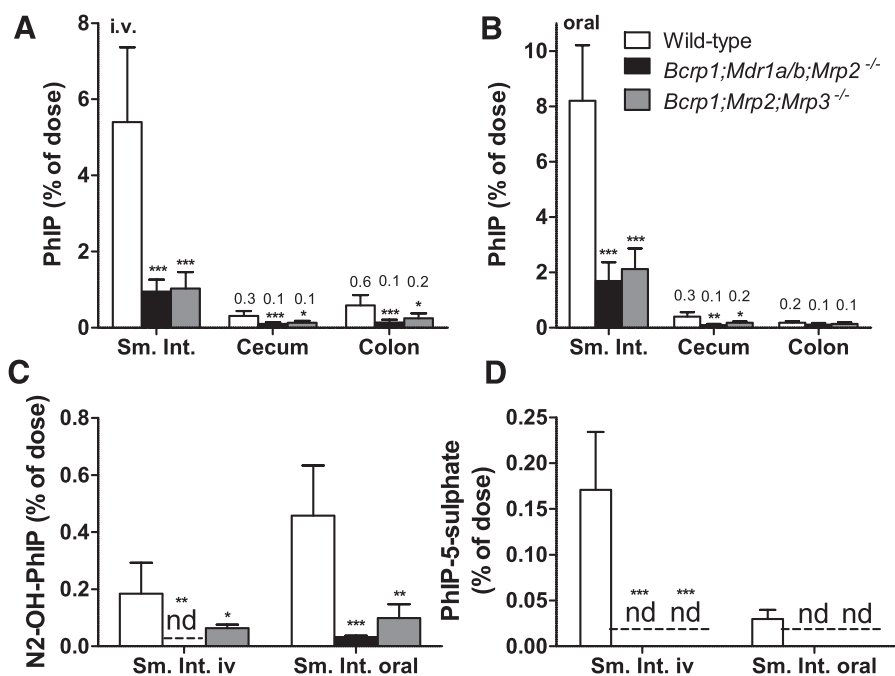


Fig. 2. PhIP, N2-OH-PhIP, and PhIP-5-sulfate levels in the intestinal tract (tissue and contents) of male wild-type, *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice 30 minutes after intravenous ($n = 4-11$) or oral ($n = 5$) administration of PhIP (1 mg/kg). (A) Parent PhIP levels in the intestinal tract of the strains after intravenous administration. (B) Parent PhIP levels in the intestinal tract of the strains after oral administration. (C) N2-OH-PhIP levels in the small intestine of the strains after intravenous or oral administration. N2-OH-PhIP detection limit was 0.02% of the dose, as indicated by the dashed line. (D) PhIP-5-sulfate levels in the small intestine of the strains after intravenous or oral PhIP administration. PhIP-5-sulfate detection limit was 0.02% of the dose (dashed line). Data are means \pm S.D. ($n = 4-11$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$); nd, not detected; Sm. Int., small intestine. In cases where values were below detection levels (nd), the statistical significance was calculated assuming values of LLQ \pm LLQ (mean \pm S.D.).

intestine, after both intravenous and oral administration (Supplemental Tables 2 and 3), but these were not detectable in small intestines of the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice, suggesting roles for Bcrp1 and/or Mrp2 in the hepatobiliary and/or intestinal elimination of these metabolites as well.

PhIP levels in kidney tissue 30 minutes after intravenous or oral administration were not significantly different between the analyzed strains (Fig. 3A). However, in the kidneys of the combination knockout mice, a significantly increased accumulation was seen of various PhIP-metabolites, including the (pre)carcinogen N2-OH-PhIP and genotoxicity marker PhIP-5-sulfate (Fig. 3, B and C). For N2-OH-PhIP after intravenous (but not oral) administration of PhIP, the increased kidney levels were more pronounced in the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice than in the *Bcrp1;Mrp2;Mrp3*^{-/-} mice. This may perhaps be caused in part by the increased expression of *Cyp1a2* in the kidney of *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice, compared with wild-type and *Bcrp1;Mrp2;Mrp3*^{-/-} mice (Supplemental Table 1). Note that N2-OH-PhIP plasma levels were not significantly different between these strains (Fig. 1A). Small amounts of PhIP-4'-sulfate and PhIP-glucuronides were also detected in the kidney after intravenous but not

after oral administration, and they were likewise increased in both knockout strains (Fig. 3B). Thus, the combined absence of Bcrp1 and Mrp2 leads to the accumulation of carcinogenic PhIP metabolites in the kidney. These increases may be due partly to reduced elimination of these compounds from the kidney, but for PhIP-5-sulfate, they may also reflect higher plasma exposure levels (Supplemental Table 2).

As previously shown in *Bcrp1*^{-/-} mice (Enokizono et al., 2008), the brain and testis penetration of PhIP after intravenous administration was significantly increased in Bcrp1-deficient mice (Fig. 3D; Supplemental Table 2). After oral administration, similar effects of Bcrp1 absence were observed, although not statistically significant in all cases, as a result of high interindividual variation (Supplemental Table 3). Interestingly, after intravenous administration, the brain levels of PhIP were higher in the *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice compared with *Bcrp1;Mrp2;Mrp3*^{-/-} mice (0.13% ± 0.03% of the dose in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice versus 0.06% ± 0.03% of the dose in *Bcrp1;Mrp2;Mrp3*^{-/-} mice, n = 4–7; P = 0.007; Fig. 3D), even though PhIP plasma levels were similar between the strains (Fig. 1A). This finding suggests that besides Bcrp1, Mdr1a/b may also be involved in restricting PhIP penetration into the brain. A similar effect

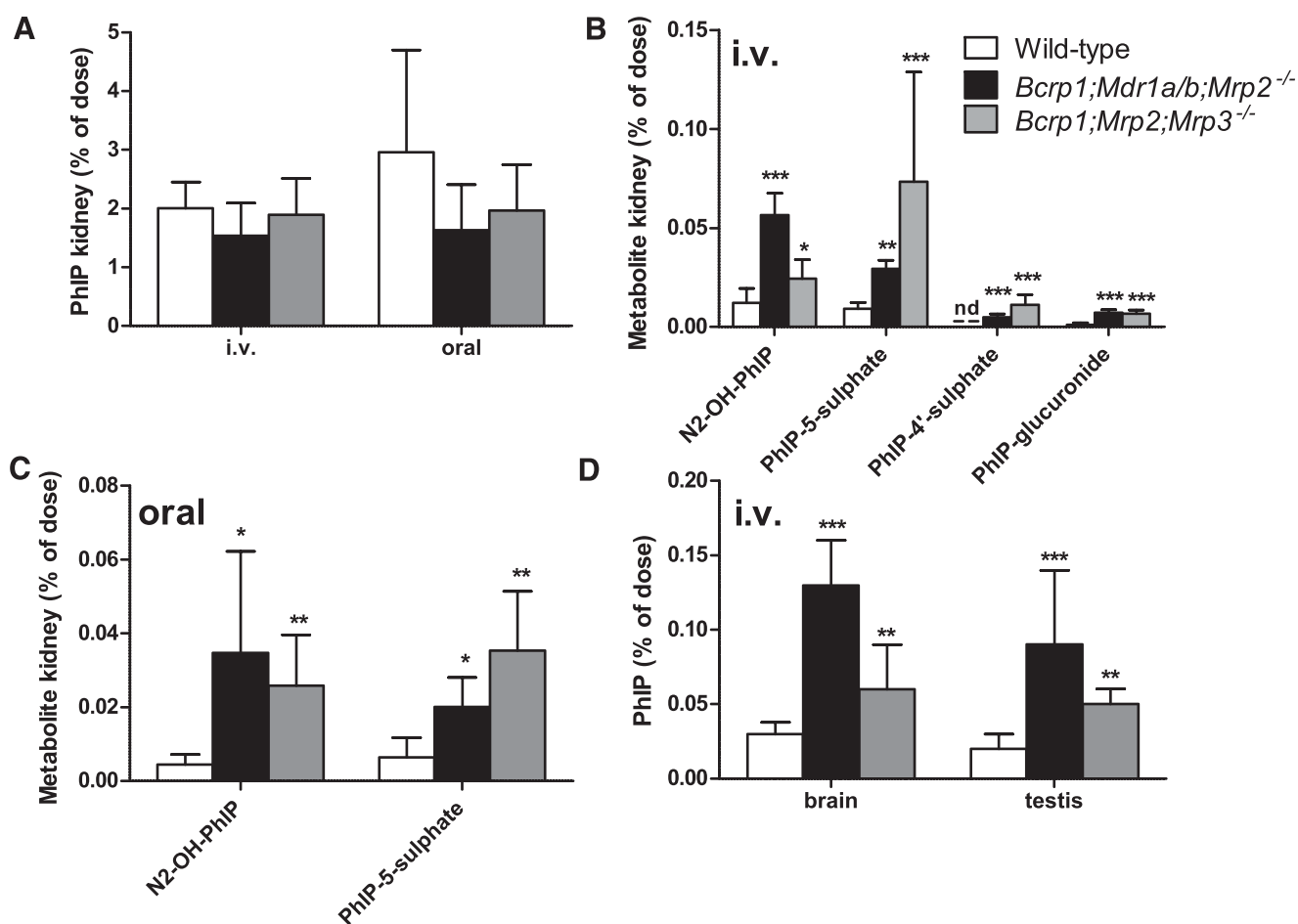


Fig. 3. PhIP and PhIP metabolite levels in kidney, brain, and testis of male wild-type, *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice 30 minutes after intravenous (n = 4–11) or oral (n = 5) administration of PhIP (1 mg/kg). (A) Parent PhIP levels in kidney of the strains after intravenous or oral administration. (B) PhIP metabolite levels in kidney of the strains after intravenous administration. nd, not detected (below 0.003% of the dose, indicated by the dashed line). (C) PhIP metabolite levels in kidney of the strains after oral administration. (D) PhIP levels in brain and testis of the strains after intravenous administration. Data are means ± S.D. (*P < 0.05; **P < 0.01; ***P < 0.001).

was observed for the testis, although the difference between *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice was (just) not statistically significant (Fig. 3D).

Effect of Apical ABC Transporters on Biliary Excretion of PhIP and Its Metabolites. Because the concentrations of PhIP and many of its metabolites in the intestinal tract after intravenous and oral administration were significantly reduced in mice deficient in *Bcrp1* and *Mrp2*, we hypothesized that this could be caused by reduced biliary excretion of these compounds. To investigate this, we performed gallbladder cannulations in male wild-type and *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice and analyzed the biliary excretion of PhIP and its metabolites in the first 60 minutes after intravenous administration of PhIP (1 mg/kg). The cumulative biliary excretion of PhIP was 41-fold lower in combination knockout compared with wild-type mice (Fig. 4A), showing that *Bcrp1*, *Mrp2*, and (possibly, to a minor extent) *Mdr1a/b* are the main transporters for biliary excretion of PhIP. The biliary excretion of PhIP metabolites (sulfate and glucuronide conjugates) was also dramatically reduced in the *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice (Fig. 4B). Surprisingly, whereas the metabolite N2-OH-PhIP was clearly detected in plasma and many tissues after intravenous and oral administration (Figs. 1–3), in bile of the mice this compound could not be detected. This finding suggests that, in contrast to PhIP, for this metabolite, which is formed both intrahepatically and extrahepatically (Frandsen and Alexander, 2000; Ma et al., 2007), biliary excretion is not a significant route of elimination.

The plasma levels of PhIP and its metabolites at the end of the gallbladder cannulation experiment are shown in Fig. 4C. PhIP concentration was not significantly altered, but significant increases were seen in the concentrations of the genotoxicity marker PhIP-5-sulfate (5.5-fold) and of OH-PhIP-glucuronide (3.3-fold) in plasma of *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice, possibly as a consequence of the reduced biliary excretion of these compounds. N2-OH-PhIP levels in plasma were relatively low and not significantly different between the strains (Fig. 4C). Interestingly, in the livers of *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice, despite highly decreased biliary excretion (Fig. 4B) and mildly increased liver RNA levels of *Ugt1a1*, OH-PhIP-glucuronide levels were significantly reduced ($0.06\% \pm 0.02\%$ of the dose in knockout versus $0.14\% \pm 0.05\%$ of the dose in wild-type, $P = 5.2 \cdot 10^{-3}$). This result suggests increased liver elimination of OH-PhIP-glucuronides over the sinusoidal membrane in the *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice. PhIP levels in the liver of these mice, like in plasma (Fig. 4C), tended to be somewhat higher in the knockout strain, but this difference was not significant ($4.4\% \pm 2.1\%$ of the dose in knockout versus $2.9\% \pm 1.0\%$ of the dose in wild-type, $P = 0.18$). Other PhIP metabolites were not detected in livers of the mice after the gallbladder cannulation experiment. To investigate the effect of *Bcrp1*, *Mrp2* and/or *Mdr1a/b* on the direct intestinal secretion of PhIP and its metabolites, we also analyzed the small intestinal contents of the mice after gall bladder cannulations (Fig. 4D). The amount of PhIP in the small intestinal contents was not significantly different between wild-type and *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice, suggesting that these transporters do not affect direct intestinal excretion of unchanged PhIP. However, the direct intestinal excretion of PhIP-5-sulfate was, like the biliary excretion (Fig. 4B), dramatically lower in

the *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice than in wild-type mice ($0.67 \cdot 10^{-3} \pm 1.5 \cdot 10^{-3\%}$ of the dose in knockout versus $14 \cdot 10^{-3} \pm 5 \cdot 10^{-3\%}$ of the dose in wild-type, $P = 6.3 \cdot 10^{-3}$), even while the plasma concentration was markedly higher (Fig. 4, C and D). For the other metabolites that were detected in the intestinal contents (N2-OH-PhIP and OH-PhIP-glucuronides), no differences between wild-type and knockout mice were found.

Effect of ABC Transporters on Urinary and Fecal Excretion of PhIP and Its Metabolites. As *Bcrp1* and *Mrp2* apparently are the main transporters for the biliary excretion of PhIP and some of its metabolites, we investigated the urinary and fecal excretion of PhIP and its metabolites in the first 24 hours after intravenous administration of PhIP at 1 mg/kg to wild-type, *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice. As shown in Fig. 5A, the urinary excretion of PhIP was not altered in the combination knockout mice, consistent with the absence of differences in plasma concentration of PhIP observed 30 minutes after intravenous (and oral) administration (Supplemental Tables 2 and 3). This suggests that these three transporters are not involved in urinary PhIP excretion (Fig. 5A). However, in line with the reduced biliary excretion of PhIP in *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice (Fig. 4A), the fecal excretion of PhIP was dramatically reduced in *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice, to 5% and 12% of wild-type excretion levels, respectively (Fig. 5A).

Although urinary excretion of PhIP was not altered in the combination knockout mice, the urinary excretion of some important metabolites was altered (Fig. 5B). The urinary excretion of the genotoxic exposure marker PhIP-5-sulfate and the (pre)carcinogen N2-OH-PhIP, as well as the detoxification products PhIP-4'-sulfate and OH-PhIP-glucuronide, was substantially (3- to 10-fold) increased in both knockout strains (Fig. 5B). This is in line with increased kidney accumulation of these compounds (Fig. 3, B and C) and may well reflect overall higher systemic exposure levels. No significant differences were seen in urinary excretion of PhIP and its metabolites between *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice, suggesting a primary role for *Bcrp1* and *Mrp2*. Because of the abundant presence of many interfering compounds, PhIP metabolite concentrations in the feces of the mice could not be reliably quantified.

Discussion

We show here that *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice, which lack all the major apical multidrug efflux transporters, are viable and fertile and show no obvious phenotypic aberrations other than an increased liver weight and increased plasma bilirubin levels, as previously found in *Bcrp1;Mrp2^{-/-}* mice (Vlaming et al., 2009a). In addition, only a few minor or modest changes in RNA expression of some drug transporters and metabolizing enzymes were observed. These mice should therefore be valuable tools for studies on the relative and combined effects of *Bcrp1*, *Mdr1a/b* and *Mrp2* on the pharmacokinetics, toxicity, and carcinogenicity of shared substrates in vivo, especially in combination with the previously generated single and double knockout mice for these transporters (Jonker et al., 2002; Vlaming et al., 2006, 2009a,b).

The exact mechanism behind the increase in liver weight of the two mouse strains studied here, and of other knockout

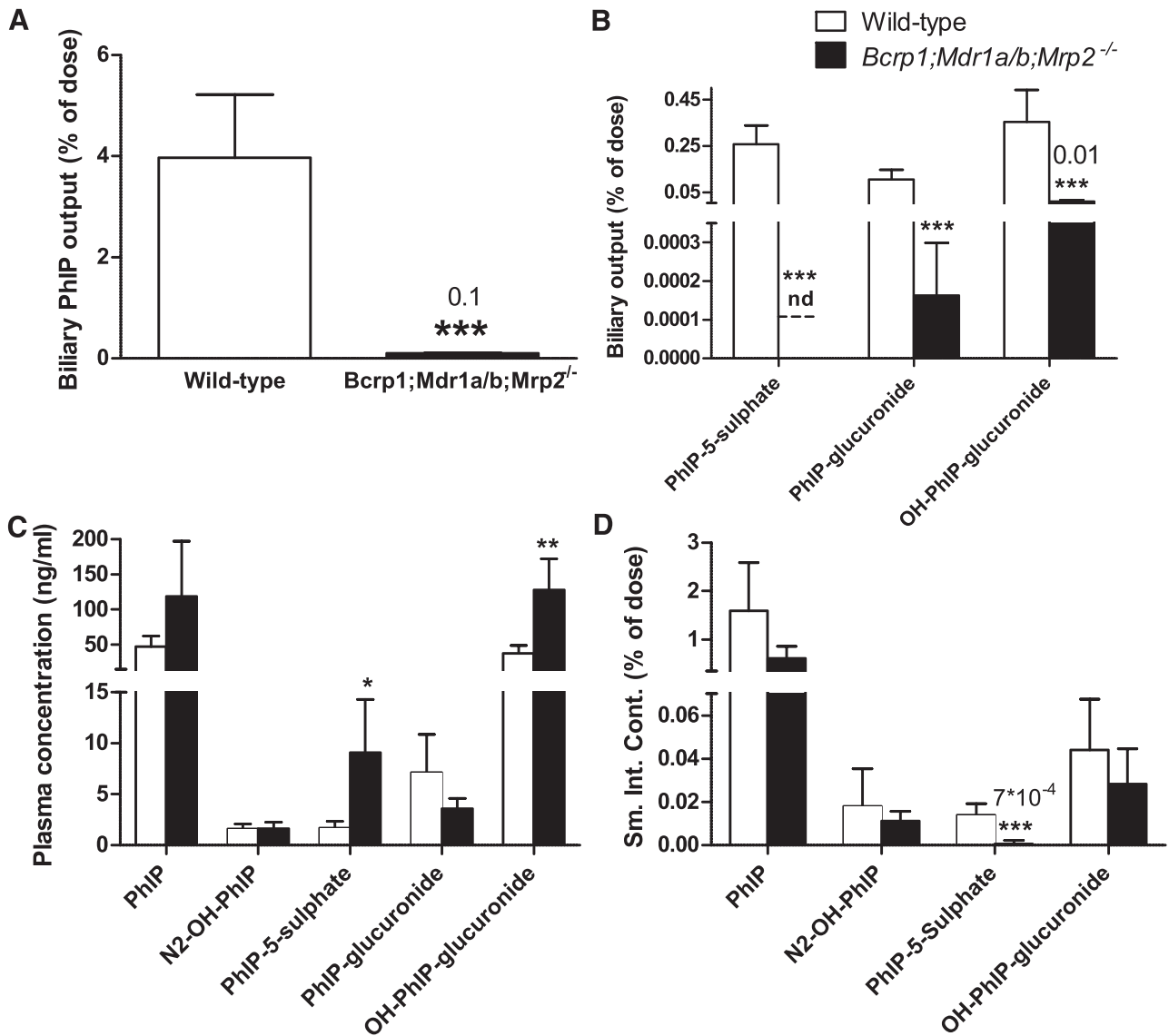


Fig. 4. Biliary excretion and plasma and small intestinal content levels of PhIP and its metabolites in the first 60 minutes after intravenous administration of PhIP (1 mg/kg) to male gallbladder-cannulated wild-type and *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice. (A) Cumulative biliary excretion of parent PhIP. (B) Cumulative biliary excretion of PhIP metabolites. PhIP-5-sulfate was not detected (nd) in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} bile (LLQ was $1 \cdot 10^{-4}$ % of the dose, indicated by the dashed line). N2-OH-PhIP and PhIP-4'-sulfate were not detectable in wild-type or knockout bile. (C) Plasma concentrations of PhIP and its metabolites at $t = 60$ minutes. (D) Small intestinal contents (Sm. Int. Cont.) levels of PhIP and PhIP metabolites at $t = 60$ minutes. Data are means \pm S.D. ($n = 5$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). In cases where values were below detection levels (nd), the statistical significance was calculated assuming values of $LLQ \pm LLQ$ (mean \pm S.D.).

strains lacking Mrp2 (Vlaming et al., 2006, 2009a,b), is not known. Likely, reduced detoxification of some compound(s) that can affect liver size causes this effect, but the modest changes we observed in expression of several other functional detoxifying systems do not suggest a drastic alteration in the overall functioning of the liver in these mice.

In this first study with *Bcrp1;Mdr1a/b;Mrp2*^{-/-} mice, combined with the *Bcrp1;Mrp2;Mrp3*^{-/-} mice (Vlaming et al., 2009b), we show that Bcrp1 and Mrp2 are the main transporters responsible for the biliary, intestinal and fecal excretion of the dietary carcinogen PhIP, as well as for the biliary and/or direct intestinal excretion of the genotoxic exposure marker PhIP-5-sulfate and several other PhIP metabolites. The urinary excretion of PhIP was not altered in the knockout mice, but the total PhIP excretion over 24

hours was markedly reduced. Furthermore, the combined absence of Bcrp1 and Mrp2 leads to increased exposure of plasma, liver, and kidney to potentially carcinogenic PhIP metabolites like N2-OH-PhIP, as well as PhIP-5-sulfate, and increased urinary excretion of all of these compounds. As most PhIP-metabolizing enzymes, except for the mildly increased *Ugt1a1* in liver, were not differently expressed in both strains compared with wild-type (Supplemental Table 1), the effects observed are likely mostly caused by absence of Bcrp1 and/or Mrp2. It appears that, when Bcrp1 and/or Mrp2 are absent, PhIP is less readily removed from the body and probably more extensively converted to metabolites, including carcinogenic ones. Since they also affect tissue distribution and elimination of these metabolites (Figs. 2–5), Bcrp1 and Mrp2 may well be involved in protecting the body from PhIP-induced carcinogenesis.

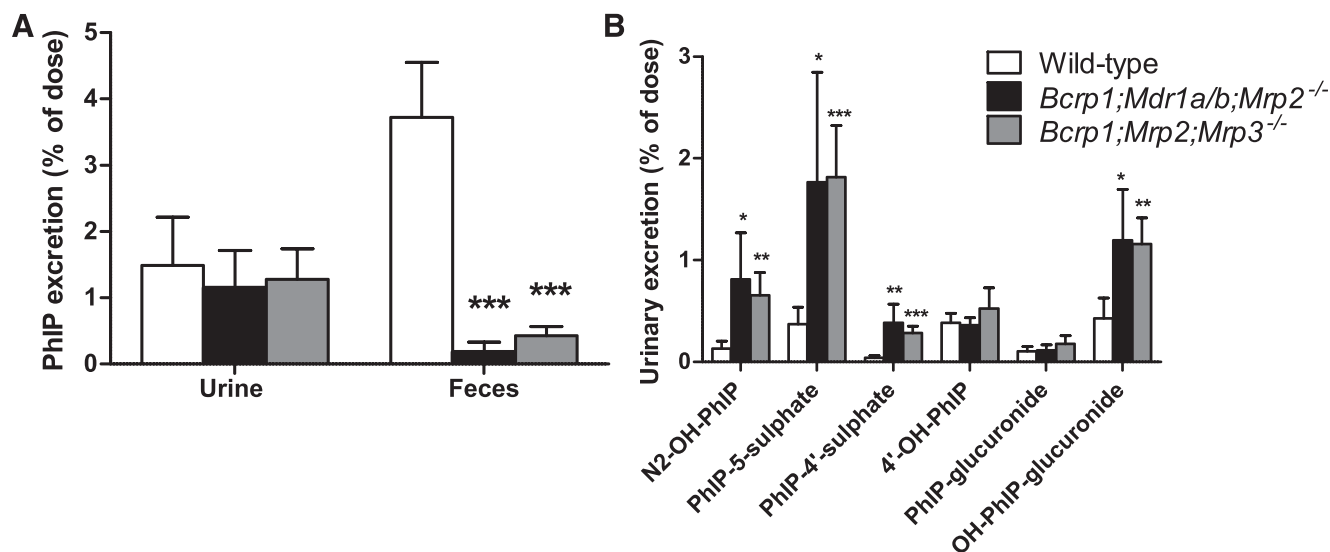


Fig. 5. Urinary and fecal excretion of PhIP and its metabolites in the first 24 hours after intravenous administration of PhIP (1 mg/kg) to male wild-type, *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice. (A) Urinary and fecal excretion of parent PhIP in the different strains. (B) Urinary excretion of PhIP metabolites in the different strains. Data are means \pm S.D. ($n = 5$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). PhIP metabolite concentrations in the feces could not be reliably quantified because of the presence of interfering compounds.

We additionally found that Mrp3 limits exposure of the liver to N2-OH-PhIP and PhIP-5-sulfate, presumably by mediating elimination of these compounds, after their formation in the liver, across the sinusoidal membrane into the blood. This process could thus have consequences for the carcinogenic potential of N2-OH-PhIP elsewhere in the body. However, we found substantially increased kidney levels and urinary excretion of N2-OH-PhIP and PhIP-5-sulfate in both knockout strains (Figs. 3, B and C, and 5B). Also the overall urinary excretion of these compounds was not different between *Bcrp1;Mdr1a/b;Mrp2^{-/-}* and *Bcrp1;Mrp2;Mrp3^{-/-}* mice (Fig. 5B). We therefore conclude that although Mrp3 seems important for short-term sinusoidal liver elimination of N2-OH-PhIP and PhIP-5-sulfate, this does not seem to markedly affect their systemic exposure levels. Possibly other basolateral ABC transporters, such as Mrp4, can also gradually transport these compounds from the liver (albeit more slowly than Mrp3), or other tissues may contribute to N2-OH-PhIP and PhIP-5-sulfate formation as well.

Similar to studies on rat Mrp2 by Dietrich et al. (2001a), we found that mouse *Bcrp1*, *Mrp2*, and *Mdr1a/b* are not essential for the urinary excretion of PhIP and its metabolites. Urinary excretion of PhIP in each of the *Bcrp1;Mrp2*-deficient strains was comparable to that in wild-type mice, and for many PhIP metabolites, urinary excretion was even increased in the knockouts, most likely as a consequence of increased plasma concentrations of these compounds (Fig. 5). The latter may result from reduced elimination by ABC transporters and occasionally from mild increases in the formation of metabolites, for instance, from upregulation of *Ugt1a1* in the liver of both knockout strains and of *Cyp1a2* in kidney tissue of *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice (Supplemental Table 1).

It is interesting to note that the brain accumulation of PhIP was significantly, if modestly, increased in the *Bcrp1;Mdr1a/b;Mrp2^{-/-}* mice compared with *Bcrp1;Mrp2;Mrp3^{-/-}* mice (Fig. 3D). Since Mrp2 and Mrp3 are unlikely to play a role in the blood-brain barrier of FVB mice, where they are not detectably expressed (Soontornmalai et al., 2006; Agarwal

et al., 2012), this indicates that the combined deficiency of *Bcrp1* and *Mdr1a/b* results in higher brain accumulation of PhIP than the single deficiency of *Bcrp1*. Thus, not only *Bcrp1* (Enokizono et al., 2008), but also *Mdr1a/b* contributes to protection of the brain from PhIP, consistent with the observed modest in vitro transport of PhIP by *Mdr1a* (van Herwaarden et al., 2003). The same may apply for the testis penetration of PhIP in the combination knockout strains (Fig. 3D).

This study shows that *Bcrp1*, *Mrp2*, and to some extent *Mdr1a/b* have a major impact on the exposure of the body to PhIP, both its primary carcinogenic metabolites and a genotoxicity exposure marker. On the one hand, *Bcrp1* and *Mrp2* deficiency resulted in markedly reduced biliary and direct intestinal excretion of PhIP and PhIP-5-sulfate and markedly reduced short-term intestinal exposure to PhIP, N2-OH-PhIP, and PhIP-5-sulfate as well as strongly decreased fecal excretion of PhIP. On the other hand, the same deficiency resulted in higher kidney and urinary levels of N2-OH-PhIP, and markedly higher plasma, kidney, and urinary levels of PhIP-5-sulfate. These data further indicate that the overall systemic exposure to carcinogenic PhIP metabolites is increased, whereas the intestinal exposure is decreased by *Bcrp1/Mrp2* deficiency. It is therefore possible that *Bcrp1/Mrp2* deficiency would have a pronounced effect on the number and tissue distribution of PhIP-induced tumors, but the direction of such effects in various tissues (increased or decreased tumor formation) may not be so easy to predict. Ultimately, only in vivo carcinogenesis studies can address these questions.

It is interesting to note that *Mrp2* (apical) and *Mrp3* (basolateral) are generally expressed on opposite poles of polarized cells in tissues analyzed in this study, such as hepatocytes, enterocytes, and kidney tubular epithelial cells. This might result in strongly increased tissue accumulation of shared substrates of both of these transporters, when both are deficient (i.e., in *Bcrp1;Mrp2;Mrp3^{-/-}* mice). Survey of our data, however, shows only a few metabolites that are consistently

and significantly accumulating more strongly in *Bcrp1;Mrp2;Mrp3*^{-/-} than in *Bcrp1;Mdr1a/b;Mrp2*^{-/-} tissues. These include N2-OH-PhIP and PhIP-5-sulfate in liver, N2-OH-PhIP in small intestine and PhIP-4-sulfate in kidney. Given the substantial other alterations in general PhIP and PhIP metabolite disposition in these mice, we think such shifts may be difficult to interpret in a straightforward manner.

Many polymorphisms and mutations in BCRP, MRP2, MRP3, and P-gp are known, and these often lead to reduced function (Huang, 2007; Maeda and Sugiyama, 2008). There are even substantial numbers of individuals with partial or complete genetic deficiencies in BCRP [e.g., Jr(a-) individuals] (Saison et al., 2012) or in MRP2 (Dubin-Johnson syndrome). Since PhIP is an abundant carcinogen, it will be of interest to assess the effect of such ABC transporter deficiencies on the carcinogenic potential of PhIP in epidemiologic studies. Moreover, one can reasonably predict that the disposition of many other dietary and environmental carcinogens, and their hydroxylated, and sulfate- and glucuronide-conjugated activated derivatives will be markedly affected by these ABC transporters as well. Our newly generated *Bcrp1;Mdr1a/b;Mrp2*^{-/-} and *Bcrp1;Mrp2;Mrp3*^{-/-} mice should therefore not only be valuable tools for pharmacokinetic studies of drugs, but also for studies on the effect of the different ABC transporters on limiting (or possibly increasing) xenobiotic-induced carcinogenesis in vivo.

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Authorship Contributions

Participated in research design: Vlaming, Teunissen, Rosing, Schellens, Beijnen, Schinkel.

Conducted experiments: Vlaming, Teunissen, van de Steeg, van Esch, Wagenaar, de Greef.

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Wrote or contributed to the writing of the manuscript: Vlaming, Teunissen, Schinkel.

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