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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Running title: PhlP PK in Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice

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List of nonstandard abbreviations:

ABC transporters   ATP-binding cassette transporters
BCRP           Breast cancer resistance protein
MDR1           Multidrug-resistance protein 1
MRP2           Multidrug-resistance-associated protein 2
MRP3           Multidrug-resistance-associated protein 3
P-gp            P-glycoprotein
PK             pharmacokinetics
ABSTRACT

The multidrug transporters BCRP, MDR1, MRP2 and MRP3 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. 30 minutes after oral or i.v. administration of PhIP (1 mg/kg), PhIP levels in small intestine were 4-6-fold reduced in Bcrp1;Mdr1a/b; Mrp2-/- and Bcrp1;Mrp2;Mrp3-/- mice compared to wild-type mice. Fecal excretion of PhIP was 8-20-fold reduced in knockouts. Biliary PhIP excretion was 41-fold reduced in Bcrp1;Mdr1a/b; Mrp2-/- mice. Biliary and small intestinal 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; Mrp2-/- mice, the carcinogenic metabolites N2-OH-PhIP and PhIP-5-sulphate (a genotoxicity marker) accumulated in liver, 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.
INTRODUCTION

The ATP-binding cassette (ABC) transporters P-glycoprotein (MDR1, ABCB1), BCRP (ABCG2),
MRP2 (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
involved in the elimination of endogenous and exogenous compounds from the body. P-
glycoprotein, BCRP and, to a lesser extent, MRP2 are 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 intestine and liver, where it transports its substrates into the
blood circulation (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003; Leslie et al.,
2005).

Besides endogenous compounds and a wide range of drugs, the dietary heterocyclic
amine (HA) carcinogens 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-
amino-3-methylimidazo[4,5-f]quinoline (IQ) are also substrates for ABC transporters in vitro
and in vivo (Dietrich et al., 2001a, 2001b; van Herwaarden et al., 2003, 2009; Leslie et al.,
2005; Vlaming et al., 2006; Enokizono et al., 2008). PhIP is the most abundant HA in fried or
cooked meat, chicken and fish. In mice, PhIP primarily causes lymphomas and small
intestinal tumors 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 (Supplementary Figure 1,
Gooderham et al., 2001, 2002; Lauber et al., 2004; Nakagama et al., 2005). We here focused
on the precarcinogen N2-OH-PhIP, and the 5-OH-PhIP and PhIP-5-sulphate derivatives, which
represent breakdown products of the ultimate genotoxic nitrenium radical cation that also forms mutagenic PhIP-DNA adducts. 5-OH-PhIP and PhIP-5-sulphate are therefore considered good markers for genotoxic exposure upon PhIP exposure. PhIP-N-sulphate 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 oestrogenic 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 brain and/or testis penetration of PhIP, N2-OH-PhIP and 4′-OH-PhIP (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 due to 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, 2009b). These strains proved to be very 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 an 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 I and phase II 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 prior to 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% FVB background and between 9-14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hour light/12-hour 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 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-b]pyridine (i.e. D3-PhIP) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N2-OH-PhIP) was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas City, USA). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]-5-hydroxypyridine (5-OH-PhIP) was a kind gift from Henrik Frandsen of the National Food Institute, Technical University of Denmark. Bovine serum albumin, dimethylsulfoxide (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 Chemical Co. (St. Louis, MO) and methoxyflurane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). All other chemicals and
reagents were from Sigma-Aldrich (Steinheim, Germany).

**Histological, clinical-chemical and hematological analysis of Bcrp1;Mdr1a/b;Mrp2–/– mice.**

Histological 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 serum of male and female mice (n = 6), as well as standard hematological 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 PCR analysis.** RNA isolation, cDNA synthesis, and real-time quantitative PCR 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 μl/g 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 i.v. administration, 5 μl/g 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.** Gall bladder cannulations in male mice were performed as described (van Herwaarden et al., 2003). After cannulation, PhIP was administered i.v. at 1 mg/kg as described above. Bile was collected in 15 min fractions for 60
min 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 above. 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) BSA solution using a Polytron blender. Feces homogenates were centrifuged for 10 min at 11,300 x g. A volume of 10 µL bile was diluted in 400 µl 4% (w/v) BSA 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 addition of 300 µl internal standard (PhIP-D3) solution in acetonitrile. The mixture was vortex mixed for 10 s followed by centrifugation for 10 min at 11,300 x g. 100 µl of the clear supernatant was diluted in a 1:1 ratio with 100 µl 3.5 mM ammonium formate buffer pH 3.5. Urine was 10 times diluted by addition of a 180 µl 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. Liquid chromatography (LC) tandem mass spectrometry (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 Å column (150 x 2.0 mm I.D., 4 µm; Phenomenex, Torrance, CA, USA) 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, USA) 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/x² (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 (Supplementary Figure 1) could not be unequivocally distinguished, as reference standards were not in all cases available. 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 (Supplementary Figure 1). After purification from mouse urine, the identity of both PhIP-4’-sulphate and PhIP-5-sulphate 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 to data below the detection limit, calculations were
made based on the LLQ +/- LLQ. Results are presented as the means ± standard deviations (SD). 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 to wild-type mice (7.0 ± 0.5% vs. 4.8 ± 0.2% of body weight, respectively, n = 5, P = 2.8*10^{-5}). As previously described, the liver weight of male $Bcrp1;Mrp2;Mrp3^{-/-}$ mice was ~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 pathological changes in either strain. A (somewhat smaller) increase 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 hematological 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 was 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). Hematological analysis of $Bcrp1;Mdr1a/b;Mrp2^{-/-}$ mice showed that hemoglobin levels were mildly but significantly reduced compared to wild-type mice (males: 6.7 ± 0.4 mM vs. 7.4 ± 0.1 mM; females: 6.8 ± 0.1 mM vs. 7.6 ± 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<sup>-/-</sup> and Bcrp1;Mrp2;Mrp3<sup>-/-</sup> 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-fold and 1.5-fold, respectively) increased in livers of *Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup>* mice (Supplementary Table 1). *Cyp3a13* was 3.1-fold increased in *Bcrp1;Mrp2;Mrp3<sup>-/-</sup>* mice (*P* = 0.043), but in all strains *Cyp3a13* RNA levels in liver were extremely low. Mrp4 protein was previously found to be undetectable by Western blot in livers of wild-type FVB and *Bcrp1;Mrp2;Mrp3<sup>-/-</sup>* mice (Vlaming et al., 2009b). Accordingly, we found only extremely low levels of *Mrp4* RNA in livers of all strains, even though they were 28- and 43-fold increased in *Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup>* and *Bcrp1;Mrp2;Mrp3<sup>-/-</sup>* mice compared to 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-fold and 1.6-fold, respectively, *P* < 0.05 for both strains) in livers of *Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup>* and *Bcrp1;Mrp2;Mrp3<sup>-/-</sup>* mice. Furthermore, *Mrp3* mRNA levels in *Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup>* mice were 4.5-fold increased compared to 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<sup>−/−</sup> and Bcrp1;Mdr1a/b;Mrp3<sup>−/−</sup> 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 (Supplementary Table 1).

In kidney, Cyp1a2 RNA was upregulated in Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup>, but not Bcrp1;Mdr1a/b;Mrp3<sup>−/−</sup> mice, from an undetectable expression level in wild-type mice (>40 PCR cycles). Cyp3a11 was also virtually undetectable in wild-type and Bcrp1;Mdr1a/b;Mrp3<sup>−/−</sup> kidney, and 64-fold increased in Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> kidney, but still very low compared to liver expression levels (Supplementary 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<sup>−/−</sup> kidney, 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 (Supplementary Figure 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; Gooderham et al., 2002; Lauber et al., 2004; Nakagama et al., 2005; Chen et al., 2007; Lauber and Gooderham, 2007; Lauber and Gooderham, 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 I and phase II 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 min after oral or i.v. administration of PhIP (1 mg/kg) to wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice. Overviews of all metabolites detected in the tissues of the three strains are presented in Supplementary Tables 2 (i.v. administration) and 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′-sulphate (Supplementary Figure 1) is considered a detoxification pathway. On the other hand, besides PhIP itself, N2-OH-PhIP and the short-lived species PhIP-N-sulphate 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-sulphate are surrogate markers for the levels of the ultimate genotoxic nitrenium radical cation that forms DNA adducts (Supplementary Figure 1; Alexander et al., 2002).

PhIP and its main metabolite N2-OH-PhIP were detected in plasma of all mouse strains 30 min after i.v. or oral administration of PhIP. No significant differences between the strains were found (Figure 1A, Supplementary Tables 2 and 3). After i.v. (but not oral) administration PhIP-5-sulphate 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 (Supplementary Table 2). After oral, but not after i.v. administration, the detoxification product 4′-OH-PhIP was quite abundant in plasma of all strains, but no differences between strains were found (Supplementary Table 3). The absence of 4′-OH-PhIP in plasma after i.v. administration suggests that this compound is primarily formed 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 (Supplementary Table 1), apparently this did not significantly affect the formation of 4′-OH-PhIP in these strains.

30 min after i.v. administration, in livers of all strains 1.8-2.5% of the given dose was found as unchanged PhIP (Figure 1B). Similar amounts of PhIP (2.7-4.5%) were found in liver after oral administration (Figure 1B). There were no significant differences between the three strains. Interestingly, in Bcrp1;Mrp2;Mrp3−/− mice after both i.v. and oral administration the precarcinogen N2-OH-PhIP accumulated significantly in the liver (Figures 1C 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 i.v. administration, the same was found for the mutagenesis marker PhIP-5-sulphate (Figure 1C). PhIP-5-sulphate 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 i.v. (Figure 2A) or oral (Figure 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 4-6-fold reduced compared to wild-type mice. This 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 i.v. application only) of both combination knockout strains PhIP concentrations were reduced (Figure 2A and B). Small intestinal levels of the carcinogenic PhIP metabolite N2-OH-PhIP and the genotoxic marker PhIP-5-sulphate were significantly reduced compared to wild-type in Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice,
both after i.v. and oral administration of PhIP (Figure 2C and D). This suggests 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 intestinal tissue of the knockout strains
(Supplementary Table 1) may also lead to reduced formation of N2-OH-PhIP in small
intestine. However, after i.v. and oral administration, N2-OH-PhIP levels in small intestine of
Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice were even lower than in the Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice, despite
an insignificant difference in Cyp1a1 expression between these strains. Besides PhIP, N2-OH-
PhIP and PhIP-5-sulphate, various other PhIP metabolites (glucuronide and sulphate
conjugates) were detected in wild-type small intestine, both after i.v. and oral administration
(Supplementary Tables 2 and 3). These were not detectable in small intestines of the
Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice, suggesting roles for Bcrp1 and/or
Mrp2 in the hepatobiliary and/or intestinal elimination of these metabolites as well.

PhIP levels in kidney 30 min after i.v. or oral administration were not significantly
different between the analyzed strains (Figure 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-
sulphate (Figures 3B and C). For N2-OH-PhIP after i.v. (but not oral) administration of PhIP,
the increased kidney levels were more pronounced in the Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice than
in the Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice. This may perhaps be caused in part by the increased
expression of Cyp1a2 in the kidney of Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice, compared to wild-type
and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice (Supplementary Table 1). Note that N2-OH-PhIP plasma levels
were not significantly different between these strains (Figure 1A). Small amounts of PhIP-4'-
sulphate and PhIP-glucuronides were also detected in the kidney after i.v. but not after oral
administration, and they were likewise increased in both knockout strains (Figure 3B). Thus,
the combined absence of Bcrp1 and Mrp2 leads to the accumulation of carcinogenic PhIP metabolites in the kidney. These increases may be partly due to reduced elimination of these compounds from the kidney, but for PhIP-5-sulphate they may also reflect higher plasma exposure levels (Supplementary Table 2).

As was previously shown in Bc r p1^{-/-} mice (Enokizono et al., 2008), the brain and testis penetration of PhIP after i.v. administration was significantly increased in Bcrp1-deficient mice (Figure 3D, Supplementary Table 2). After oral administration, similar effects of Bcrp1 absence were observed, although not in all cases statistically significant, due to a high inter-individual variation (Supplementary Table 3). Interestingly, after i.v. administration, the brain levels of PhIP were higher in the Bc r p1;M dr1a/b;Mr p2^{-/-} mice compared to Bc r p1;M r p2;Mr p3^{-/-} mice (0.13 ± 0.03 % of the dose in Bc r p1;M dr1a/b;Mr p2^{-/-} mice vs. 0.06 ± 0.03% of the dose in Bc r p1;M r p2;Mr p3^{-/-} mice, n = 4-7, P = 0.007; Fig. 3D), even while PhIP plasma levels were similar between the strains (Figure 1A). This suggests that besides Bcrp1, Mdr1a/b may also be involved in restricting PhIP penetration into the brain. A similar effect was observed for the testis, although the difference between Bc r p1;M dr1a/b;Mr p2^{-/-} and Bc r p1;M r p2;Mr p3^{-/-} mice was (just) not statistically significant (Figure 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 i.v. 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 gall bladder cannulations in male wild-type and Bc r p1;M dr1a/b;Mr p2^{-/-} mice and analyzed the biliary excretion of PhIP and its metabolites in the first 60 min after i.v. administration of PhIP (1 mg/kg). The cumulative biliary excretion of
PhIP was 41-fold lower in combination knockout compared to wild-type mice (Figure 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 (sulphate and glucuronide conjugates) was also dramatically reduced in the Bcrp1;Mdr1a/b;Mrp2\(^{-/-}\) mice (Figure 4B). Surprisingly, whereas the metabolite N2-OH-PhIP was clearly detected in plasma and many tissues after i.v. and oral administration (Figures 1-3), in bile of the mice this compound could not be detected. This 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 gall bladder cannulation experiment are shown in Figure 4C. PhIP concentration was not significantly altered, but there were significant increases in the concentrations of the genotoxicity marker PhIP-5-sulphate (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 (Figure 4C). Interestingly, in the livers of Bcrp1;Mdr1a/b;Mrp2\(^{-/-}\) mice, despite highly decreased biliary excretion (Figure 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, \(P = 5.2 \times 10^{-3}\)). This 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 (Figure 4C), tended to be somewhat higher in the knockout strain, but this 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

19
metabolites were not detected in livers of the mice after the gall bladder 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 analysed the small intestinal contents of the mice after gall bladder cannulations (Figure 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-sulphate was, like the biliary excretion (Figure 4B), dramatically lower in the Bcrp1;Mdr1a/b;Mrp2−/− mice than in wild-type mice (0.67±1.5·10^−3 % of the dose in knockout versus 14±5·10^−3 % of the dose in wild-type, P = 6.3·10^−3), even while the plasma concentration was markedly higher (Figure 4C 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 hr after i.v. administration of PhIP at 1 mg/kg to wild-type, Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice. As shown in Figure 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 min after i.v. (and oral) administration (Supplementary Tables 2 and 3). This suggests that these three transporters are not involved in urinary PhIP excretion (Figure 5A). However, in line with the reduced biliary excretion of PhIP in Bcrp1;Mdr1a/b;Mrp2−/− mice (Figure 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 (Figure 5A).

Although urinary excretion of PhIP was not altered in the combination knockout mice, the urinary excretion of some important metabolites was (Figure 5B). The urinary excretion of the genotoxic exposure marker PhIP-5-sulphate and the (pre)carcinogen N2-OH-PhIP, as well as the detoxification products PhIP-4′-sulphate and OH-PhIP-glucuronide, was substantially (3-10-fold) increased in both knockout strains (Figure 5B). This is in line with increased kidney accumulation of these compounds (Figure 3B and C), and may well reflect overall higher systemic exposure levels. There were no significant differences 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. Due to 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;Mdr1α/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, Mdr1α/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, 2009b).

The exact mechanism behind the increase in liver weight of the two mouse strains studied here, and of other knockout strains lacking Mrp2 (Vlaming et al., 2006, 2009a, 2009b), 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;Mdr1α/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-sulphate and several other PhIP metabolites. The urinary excretion of PhIP was not altered in the knockout mice, but the total PhIP excretion over 24 hrs was markedly reduced. Furthermore, 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-sulphate, 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 to wild-type (Supplementary 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 (Figures 2-5), Bcrp1 and Mrp2 may well be involved in protecting the body from PhIP-induced carcinogenesis.

We additionally found that Mrp3 limits the exposure of the liver to N2-OH-PhIP and PhIP-5-sulphate, 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-sulphate in both knockout strains (Figures 3B, C and 5B). Also the overall urinary excretion of these compounds was not different between Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice (Figure 5B). We therefore conclude that although Mrp3 seems important for short-term sinusoidal liver elimination of N2-OH-PhIP and PhIP-5-sulphate, 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-sulphate 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 (Figure 5). The latter may result from reduced elimination by ABC transporters and occasionally from mild increases in the formation of metabolites, for instance due to upregulation of Ugt1a1 in the liver of both knockout strains and of Cyp1a2 in kidney of Bcrp1;Mdr1a/b;Mrp2\(^{-/-}\) mice (Supplementary 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 to Bcrp1;Mrp2;Mrp3\(^{-/-}\) mice (Figure 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., 2005; Agarwal et al., 2012), this indicates that the combined deficiency of Bcrp1 and Mdr1a/1b results in higher brain accumulation of PhIP than the single deficiency of Bcrp1. Thus, not only Bcrp1 (Enokizono et al., 2008) but also Mdr1a/1b 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 (Figure 3D).

This study shows that Bcrp1, Mrp2 and to some extent Mdr1a/1b 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-sulphate, and markedly reduced short-term intestinal exposure to PhIP, N2-OH-PhIP and PhIP-5-sulphate.
as well as strongly decreased fecal excretion of PhilP. On the other hand, the same deficiency resulted in higher kidney and urinary levels of N2-OH-PhilP, and markedly higher plasma, kidney and urinary levels of PhilP-5-sulphate. These data further indicate that the overall systemic exposure to carcinogenic PhilP 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 PhilP-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-PhilP and PhilP-5-sulphate in liver, N2-OH-PhilP in small intestine, and PhilP-4'-sulphate in kidney. Given the substantial other alterations in general PhilP and PhilP 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 et al., 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 PhilP 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 epidemiological studies. Moreover, one can reasonably predict that the disposition of many other dietary and environmental carcinogens, and their hydroxylated, and sulphate- and glucuronide-conjugated activated derivatives will be markedly affected by these ABC transporters as well. Our newly generated Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> and Bcrp1;Mrp2;Mrp3<sup>-/-</sup> 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 <i>in vivo</i>. 
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- “Bioanalysis and metabolism of tamoxifen and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine – Applications in pharmacology” by Bas Teunissen (University of Utrecht, The Netherlands, 2011)

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FIGURE LEGENDS

Figure 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 min after i.v. (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 i.v. or oral administration. B, PhIP levels in liver of the strains after i.v. or oral administration. C, Levels of N2-OH-PhIP and PhIP-5-sulphate in liver of the strains after i.v. administration. nd, not detected, detection limits for N2-OH-PhIP and PhIP-5-sulphate 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 ± SD (n = 4-11, **, P < 0.01, ***, P < 0.001, compared to wild-type). In case tissue levels in wild-type mice were below the detection limit, the LLQ +/- LLQ was used to calculate statistical significance.

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

**Figure 3**

PhIP and PhIP metabolite levels in kidney, brain and testis of male wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice 30 min after i.v. (n = 4-11) or oral (n = 5) administration of PhIP (1 mg/kg). A, parent PhIP levels in kidney of the strains after i.v. or oral administration. B, PhIP metabolite levels in kidney of the strains after i.v. 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 i.v. administration. Data are means ± SD (*, P < 0.05, **, P < 0.01, ***, P < 0.001).

**Figure 4**

Biliary excretion and plasma and small intestinal content levels of PhIP and its metabolites in the first 60 min after i.v. administration of PhIP (1 mg/kg) to male gall bladder-cannulated wild-type and Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice. A, cumulative biliary excretion of parent PhIP. B, cumulative biliary excretion of PhIP metabolites. PhIP-5-sulphate was not detected (nd) in Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> bile (LLQ was 1*10<sup>−4</sup> % of the dose, indicated by the dashed line). N2-OH-PhIP and PhIP-4′-sulphate were not detectable in wild-type or knockout bile. C, plasma concentrations of PhIP and its metabolites at t = 60 min. D, small intestinal contents (Sm. Int. Cont.) levels of PhIP and PhIP metabolites at t = 60 min. Data are means ± SD (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 ± LLQ (mean ± SD).
Figure 5

Urinary and fecal excretion of PhIP and its metabolites in the first 24 hr after i.v. 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 ± SD (n = 5, *, P < 0.05, **, P < 0.01, ***, P < 0.001). PhIP metabolite concentrations in the feces could not be reliably quantified due to the presence of interfering compounds.
Figure 1

(A) Plasma level (ng/ml) of PhIP, N2-OH-PhIP, i.v. and oral administration.

(B) PhIP liver (% of dose) for Wild-type, Bcrp1;Mdr1a/b;Mrp2−/−, Bcrp1;Mrp2;Mrp3−/−.

(C) Liver metabolite (% of dose) for i.v. administration.

(D) Liver N2-OH-PhIP (% of dose) for oral administration.

Legend:
- Wild-type
- Bcrp1;Mdr1a/b;Mrp2−/−
- Bcrp1;Mrp2;Mrp3−/−

Not detected (nd)
Figure 3

(A) N2-OH-PhIP kidney (% of dose)

(B) PhIP-5-sulphate kidney (% of dose)

(C) PhIP-4'-sulphate kidney (% of dose)

(D) PhIP-glucuronide kidney (% of dose)

Wild-type

Bcrp1;Mdr1a/b;Mrp2−/−

Bcrp1;Mrp2;Mrp3−/−

Metabolite kidney (% of dose)

**

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**

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i.v.

oral

Brain

Testis

PhIP (% of dose)

0.00

0.05

0.10

0.15

0.20
Figure 5

A

PhIP excretion (% of dose)

Urine

Feces

B

Urinary excretion (% of dose)

N2-OH-PhIP

PhIP-5-sulphate

PhIP-4'-sulphate

4'-OH-PhIP

PhIP-glucuronide

OH-PhIP-glucuronide

Wild-type

Bcrp1;Mdr1a/b;Mrp2−/−

Bcrp1;Mrp2;Mrp3−/−

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