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GENOTOXICITY AND CARCINOGENICITY

ABC transporters Mdr1a/1b, Bcrp1, Mrp2 and Mrp3 determine the sensitivity to PhIP/DSS-induced colon carcinogenesis and inflammation

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is an abundant dietary carcinogen, formed during high-temperature cooking of meat. In this study, we investigated whether clinically relevant ATP-binding cassette (ABC) efflux transporters can modulate PhIP-induced colorectal carcinogenesis in vivo using wild-type (WT), *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice. We used a physiological mouse model of colorectal cancer; a combination of a single high-dose oral PhIP administration (200 mg/kg), followed by administering a colonic inflammatory agent, dextran sodium sulfate (DSS), in drinking water for 7 days. Pilot experiments showed that both knockout strains were more sensitive to DSS-induced colitis compared to WT mice. Lack of these transporters in mice also led to clearly altered disposition of activated PhIP metabolites after a high-dose oral PhIP administration. The results suggest that Mdr1a/1b, Bcrp1 and Mrp2 contributed to biliary excretion and Mrp3 to sinusoidal secretion of the pre-carcinogenic metabolite N2-OH-PhIP. The levels of a genotoxicity marker, PhIP-5-sulphate, were at least 4- and 17-fold reduced in the intestinal tissue and intestinal content of both knockout strains compared to WT mice. In line with these findings, the level of colon carcinogenesis was reduced by two- to four-fold in both knockout strains compared to WT mice when PhIP and DSS treatments were combined. Thus, perhaps counterintuitively, reduced activity of these ABC transporters may in part protect from PhIP-induced colon carcinogenesis. Collectively, these data suggest that ABC transporters are important in protecting the body from inflammatory agents such as DSS, in the disposition of carcinogenic metabolites, and in determining the sensitivity to dietary PhIP-induced carcinogenesis.

Keywords ABC transporters · PhIP · DSS · Colon cancer · Carcinogenesis · Inflammation

Introduction

Even in modern life, diet is still one of the significant risk factors for developing various cancers. Several studies have shown strong associations between westernized dietary habits, especially intake of fried, grilled or processed meat, and increased risk of colorectal cancer (Bernstein et al. 2015; Chan et al. 2011; Cross et al. 2010; Cross and Sinha 2004; Sinha 2002; Sinha et al. 1999). Heterocyclic amines (HCAs) are one class of mutagenic compounds that are prevalent in the human environment. One of the most abundant HCAs found in food is 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP), which is produced during high-temperature grilling, charring or frying of meat (Layton et al. 1995). PhIP is mutagenic and carcinogenic in rodents, and increased exposure to it is associated with a higher risk of colorectal, stomach, prostate and breast cancers in humans (Cross et al. 2005; Sinha 2002; Sinha et al. 1999; Ward et al. 1997).

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Carcinogenicity of PhIP arises from its bioactivated metabolites that have DNA-binding capacity. PhIP is mainly metabolized in liver, but also in the intestinal tract. The first pre-carcinogenic metabolite formed during PhIP metabolism is N2-OH-PhIP (2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine). This N-hydroxylation of PhIP occurs primarily by hepatic CYP1A2, and to a lesser extent by extra-hepatic CYP1A1 and 1B1 (Boobis et al. 1994; Crofts et al. 1997, 1998; Edwards et al. 1994). CYP1A2-dependent metabolism differs between rodents and humans, with comparatively more activation (leading to more carcinogenic metabolites) and less detoxification in the metabolism of PhIP in humans (Turteltaub et al. 1999). Highly mutagenic activation products may be formed from PhIP or N2-OH-PhIP by sulphation (PhIP-*N*-sulphate) or acetylation (*N*-acetyl-PhIP), with heterolytic cleavage of those sulfate or acetate groups resulting in a reactive radical cation (Buonarati et al. 1990; Glatt 2000; Malfatti et al. 1994; Minchin et al. 1992; Suzuki et al. 2008) (Supplementary Fig. 1). This radical reacts covalently with DNA, leading to mutagenic DNA adducts, and with proteins and other cellular constituents resulting in unstable products (Chou et al. 1995; Turesky et al. 1991). The DNA adducts lead to errors during DNA replication and mutations with cancer-initiation capacity. The various adducts are further degraded into 5-OH-PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-5-hydroxypyridine) in proportional amount, and hence 5-OH-PhIP abundance is considered to be a surrogate marker of the mutagenic capacity (i.e., carcinogenic capacity) (Alexander et al. 1997; Frandsen and Alexander 2000; Reistad et al. 1994; Schut and Snyderwine 1999). Therefore, in PhIP pharmacokinetic and carcinogenesis studies, N2-OH-PhIP is used as a precarcinogenic marker, whereas 5-OH-PhIP and its further conjugation product PhIP-5-sulfate (Supplementary Fig. 1) are usually considered as good genotoxicity markers.

In rodents, PhIP induces epithelial tissue malignancies such as prostate, mammary gland and intestinal cancers, and hematological malignancies such as lymphomas and leukemias (Esumi et al. 1989; Ito et al. 1991; Kristiansen et al. 1998; Ochiai et al. 2002; Shirai et al. 1997, 2002). The type of malignancy and onset time seem to be highly dependent on the experimental model; in all models, spontaneous tumor induction by PhIP alone requires long periods of time, ranging from 52 to 82 weeks (Cheung et al. 2011; Nakanishi et al. 2007; Sugimura et al. 2004). However, when PhIP is combined with a colonic inflammatory agent such as dextran sodium sulphate (DSS), mice develop colon adenomas and adenocarcinomas, and the time of tumor onset is reduced to 6–24 weeks depending on the mouse strain (Cheung et al. 2011; Nakanishi et al. 2007; Nishikawa et al. 2005; Tanaka et al. 2005). DSS is a synthetic sulfated polysaccharide inducing colitis in experimental animals after oral administration and DSS-induced colitis in mice has been used as a model for studying human ulcerative colitis (Chassaing et al. 2014; Eichele and Kharbanda 2017; Okayasu et al. 1990).

Tissue distribution characteristics of precarcinogenic and mutagenic metabolites of PhIP will obviously impact the target tissue and incidence of carcinogenicity. Many ATP-binding cassette (ABC) efflux transporters have wide and overlapping substrate specificities, making them important in the net absorption, distribution, excretion and toxicity of numerous xenobiotics. Transporters such as MDR1 (P-gp/ABCB1), BCRP (ABCG2), MRP2 (ABCC2) and MRP3 (ABCC3) are highly expressed in excretory organs such as liver, kidney and intestine, where they are involved in the elimination or disposition of endogenous and exogenous compounds from the body. MDR1, BCRP and MRP2 are expressed at the apical membrane of epithelial cells and they mediate excretion of their substrates into bile, feces and urine. At the intestinal epithelium, these proteins are particularly important in limiting the intestinal absorption of their substrates, thus affecting oral availability. MRP3 is expressed at the basolateral membrane of the intestinal and liver cells, where it transports its substrates into the blood circulation (Borst and Elferink 2002; Leslie et al. 2005; Schinkel and Jonker 2003). PhIP and some of its metabolites are substrates of various ABC transporters (Dietrich et al. 2001a, b; Enokizono et al. 2008; Leslie et al. 2005; Pavek et al. 2005; van Herwaarden et al. 2003; Vlaming et al. 2006). Our previous work indicated that in vivo disposition of PhIP and, therefore, by implication, also its short-lived (and hence practically undetectable) carcinogenic metabolite, PhIP-N-sulphate, are substantially altered in compound knockout mice for Mdr1a/b, Bcrp1, Mrp2 or Bcrp1, Mrp2, and Mrp3 (Teunissen et al. 2010; Vlaming et al. 2014). Interestingly, some studies also reported a negative correlation between the susceptibility to DSS-induced colitis and P-gp expression (Iizasa et al. 2003; Staley et al. 2009), suggesting that P-gp expression somehow protects from DSS effects in colon.

As PhIP and DSS both seem to be interacting in vivo with ABC transporters, either directly or indirectly, we here investigated the overlapping or complementary roles of Bcrp1, Mdr1a/b, Mrp2 and Mrp3 in combined PhIP-plus-DSS-induced carcinogenesis in vivo. Our model consisted of administering mice one high oral dose (200 mg/kg) of PhIP, followed a week later by exposure to the intestinal inflammatory agent DSS in drinking water for 7 days ad libitum (Fig. 3A). The high-PhIP dose allows for a more rapid and frequent development of tumors, allowing qualitative carcinogenesis studies using manageable numbers of mice within a reasonable time frame. We used *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice to investigate the combined effects of the apically located transporters Bcrp1, Mdr1a/b

and Mrp2 on this carcinogenesis process. We further investigated the contribution of (basolateral) Mrp3 on top of the Bcrp1 and Mrp2 deficiency using *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice, also because Mrp3 is often upregulated when Mrp2 is absent (Vlaming et al. 2006). This may function as a compensatory mechanism for decreased apical efflux of PhIP or its metabolites from the liver into the intestinal lumen (via biliary excretion) and could thus present a possible mechanism for lowering colon carcinogenesis.

Materials and methods

Animals

Mice were housed and handled according to institutional guidelines complying with Dutch and EU legislation. Male wild-type (WT), *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$, and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, all of a > 99% FVB genetic background, were used between 8 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h-light/12-h-dark cycle, and received a standard diet (Hope Farms AM-II, Woerden, the Netherlands) and acidified water ad libitum.

Chemicals and solutions

PhIP hydrochloride was purchased from Wako Chemicals Gmbh (Neuss, Germany) and dextran sulfate sodium (DSS; molecular weight ~ 39,900) was obtained from TdB Consultancy (Uppsala, Sweden).

PhIP hydrochloride (20 mg/ml) was dissolved in sterile water by stirring for ~ 20 min at room temperature. Mice received 10 µl/g of body weight of the 20 mg/ml PhIP solution (200 mg/kg) or water as vehicle via oral gavage on day 0.

DSS (2.25–3%, w/v) was dissolved in acidified drinking water (as routinely used for our mice) by stirring for ~ 1–2 min at room temperature. 7 days after PhIP administration, mice received DSS-containing or standard acidified drinking water ad libitum for another 7 days. PhIP-plus-DSS solutions were each prepared fresh on the day of the start of their experimental use and DSS solution was refreshed after 4 days.

DSS dose optimization experiments

Animals were randomly divided into DSS dose groups ($n = 5–6$). Experimental colitis was induced by DSS treatments in drinking water ad libitum for 7 days and was followed by normal acidified drinking water throughout the experiments in all genotypes. The first cohorts of mice treated with 3% DSS were killed on day 7 to assess the DSS-associated histological changes in the intestinal tract and rectum of each genotype. After initial assessment of genotype-driven DSS sensitivity, a dose escalation study was performed to decide the optimal DSS doses for each genotype that would ideally yield similar levels of inflammation. This was done to standardize the PhIP-plus-DSS carcinogenesis model in all the genotypes as much as possible to be able to assess the effect of each genotype.

In DSS dose escalation studies, a minimum of three different DSS doses, ranging from 1.5 to 3% were administered to each genotype group (Table 1). After a week of treatment, mice were kept without treatment and were killed between days 14 and 21 depending on the general welfare of mice and presence of DSS-related symptoms. During this time, mice were closely monitored for body weight loss, diarrhea, rectal bleeding and general behavior, daily until day 14, and at least five times a week until the end of the experiment. Diarrhea-related symptoms such as diarrhea, rectal bleeding and histological inflammation were graded as explained in Supplementary Table I. Due to limited availability of mice and the large size of each group, experiments had to be executed in part at different times. This was also applicable for the PhIP-plus-DSS treatment experiments as each group consisted of 16–28 mice. To correct for any strain-dependent interference, we aimed to include all the groups each time with manageable numbers from each strain. However, we have observed that the same strain could show different sensitivity to DSS alone or PhIP-plus-DSS treatments, sometimes resulting in high loss and sometimes very mild effects.

PhIP pharmacokinetic experiments

200 mg/kg PhIP was orally dosed to the animals by administering 10 µl/g of body weight of 20 mg/ml PhIP solution prepared in water. Mice were killed 30 min after PhIP administration by cervical dislocation after blood collection via cardiac puncture under isoflurane anesthesia and organs were removed. Organs were homogenized on ice in water and plasma was isolated from blood samples right after collection by centrifugation at 2100*g* for 6 min at 4 °C. All samples were immediately stored at − 20 °C after preparation until analysis.

Concentrations of PhIP and its metabolites in samples were determined using a sensitive and specific LC–MS/MS assay as described previously (Teunissen et al. 2010). When % of dose calculations were made, molecular mass changes in PhIP metabolites were considered to be negligible.

PhIP-plus-DSS treatments to induce colon carcinogenesis

Mice were randomly divided into four experimental groups consisting of control, PhIP only, DSS only, and

Table 1 DSS dose optimization experiments in WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice

DSS dose	WT			*Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$			*Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$		
	3%	2.75%	2%	3%	2.25%	2%	3%	2.5%	1.5%
Body weight loss	Severe	Moderate	Slight	Severe	Severe	None	Severe	Slight	None
Diarrhea	Moderate	Slight–moderate	Slight	Severe	Slight–moderate	Slight	Moderate	Slight–moderate	None
Visible rectal blood	Moderate	Slight	None	Moderate	Slight	Slight	Slight	Slight	None
Inflammation[a]	Moderate[a]	Severe	Moderate-severe	Very severe	Severe	Moderate-severe	Severe	Severe	Moderate
Total number of animals (*n*)	17	12	12	6	12	12	5	12	3
Number of loss (*n*)	9	3	0	4	7	2	1	3	0
% loss	53	25	0	67	58	17	20	25	0

Body weight loss, diarrhea, rectal bleeding, inflammation levels and % loss of animals after DSS dose escalation experiments were evaluated to find the optimal DSS dose. Animals received DSS in drinking water between days 0 and 7, and were killed for further evaluation of organ damage between days 14 and 28. Inflammation was scored by H&E staining of whole colons. The DSS dose and corresponding pathology levels selected for subsequent carcinogenesis studies were 2.75% for WT mice, 2% for *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice and 2.5% for *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice

[a]Inflammation levels in surviving mice only could be scored. Hence the "moderate" inflammation score for surviving WT mice receiving 3% DSS

PhIP-plus-DSS receiving mice for all genotypes. PhIP-receiving groups were administered 200 mg/kg PhIP (10 µl/g of body weight) by oral gavage. Control and DSS groups received water as vehicle. Starting 1 week after oral PhIP administration, DSS-receiving groups were given DSS in the drinking water for 7 days. For carcinogenesis experiments, the administered DSS concentrations were 2.75% (w/v) for WT, 2% for *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and 2.5% for *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice. All groups were followed without any further treatment for 22 more weeks. Mice were evaluated for body weight changes, diarrhea, rectal bleeding and general behavior for the first month after the start of the experiment at least five times a week and minimally two times per week for the rest of the periods. Mice were checked more frequently when necessary such as in the presence of abnormal behavior, trending body weight loss, diarrhea, rectal prolapse or rectal bleeding, i.e., the symptoms that are usually colorectal tumor related. During the experiment, mice were killed when they became moribund or showed more than moderate levels of tumor-related symptoms or >%15 body weight loss within a week, and all experiments were terminated at day 180 by CO_2 overdose. After killing, various organs including small intestine, colorectal tissues and tumors were isolated and fixed for further histopathological analyses. Histopathological examination was also carried out on other organs in a small set of mice ($n = 5$–6) from each group for any neoplasms occurring outside of the colorectal tract.

Histological analysis

Tissue samples were fixed in EAF fixative (absolute ethanol/acetic acid/37% formaldehyde/saline at 45:5:10:40 v/v) and embedded in paraffin. Sections were prepared from the paraffin blocks at 2 µm for hematoxylin and eosin staining or at 4 µm for ß-catenin (Abcam; ab32572) staining according to standard procedures. The sections were blindly examined by a mouse pathologist.

Statistical analysis

For PhIP pharmacokinetic experimental results, one-way analysis of variance (ANOVA) followed by post hoc tests with Tukey correction was used to determine the significant differences between groups.

For carcinogenesis experiments, three possible outcomes were statistically evaluated: tumor incidence, tumor multiplicity and normalized total tumor number. Tumor incidence indicated the fraction of mice in a cohort with one or more tumors. Tumor multiplicity was calculated by dividing the total number of tumors by the number of mice with one or more tumors. Normalized total tumor number was calculated by dividing the total number of tumors by the total number of mice in the cohort.

Fisher's exact test was used to compare differences in tumor incidence between genotypes. For tumor multiplicity and normalized total tumor number, the Wilcoxon rank-sum

test was used to assess differences in pairwise comparison between two genotypes (e.g. WT and one of the knockout strains) and the Kruskal–Wallis test to compare that between the three genotypes.

All results are presented as the means ± SD, when applicable. Differences were considered to be statistically significant when $P < 0.05$.

Results

DSS-induced colitis model optimization in WT and ABC-transporter compound knockout mice

We chose our protocol based on the extensive literature on DSS-induced colitis in mice (Chassaing et al. 2014; Melgar et al. 2005; Nakanishi et al. 2007; Whittem et al. 2010). Considering our aim of inducing colorectal tumors by combining DSS with pre-treatment of PhIP, we first started with just a 3% DSS dose and a 7-day treatment protocol to reach an appropriate level of colitis in these mice. The effect of DSS treatment causing colitis was assessed closely in terms of loss in body weight, diarrhea and presence of rectal blood during the experimental period, followed by histopathologic examination of the entire gastrointestinal tract at the end of the experiment. All these measures except body weight loss were graded based on the scoring system defined in Supplementary Table I.

While receiving 3% DSS in drinking water for 7 days, WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice responded differently, showing various degrees of DSS-related symptoms. The level of body weight loss after 3% DSS treatment was highest in *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and lowest in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice. Although the levels of diarrhea and rectal blood were acceptable, the levels of body weight loss were unacceptably high in all the strains, leading to exclusion of 20% or more of the mice from all the experimental groups (Table 1, Supplementary Fig. 2). Histologically, degeneration of the mucosal epithelia, various levels of edema and high levels of inflammatory infiltrations in the mucosal and submucosal area of the colon were observed in all the analyzed mice in the 3% DSS-treated mouse strains (Fig. 1). Lesions were generally more severe in distal colon and rectum than in the rest of the intestinal tract. In accordance with the differences in physiological effects observed between the strains, the impact of 3% DSS on the *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ colons was much more severe compared to that in

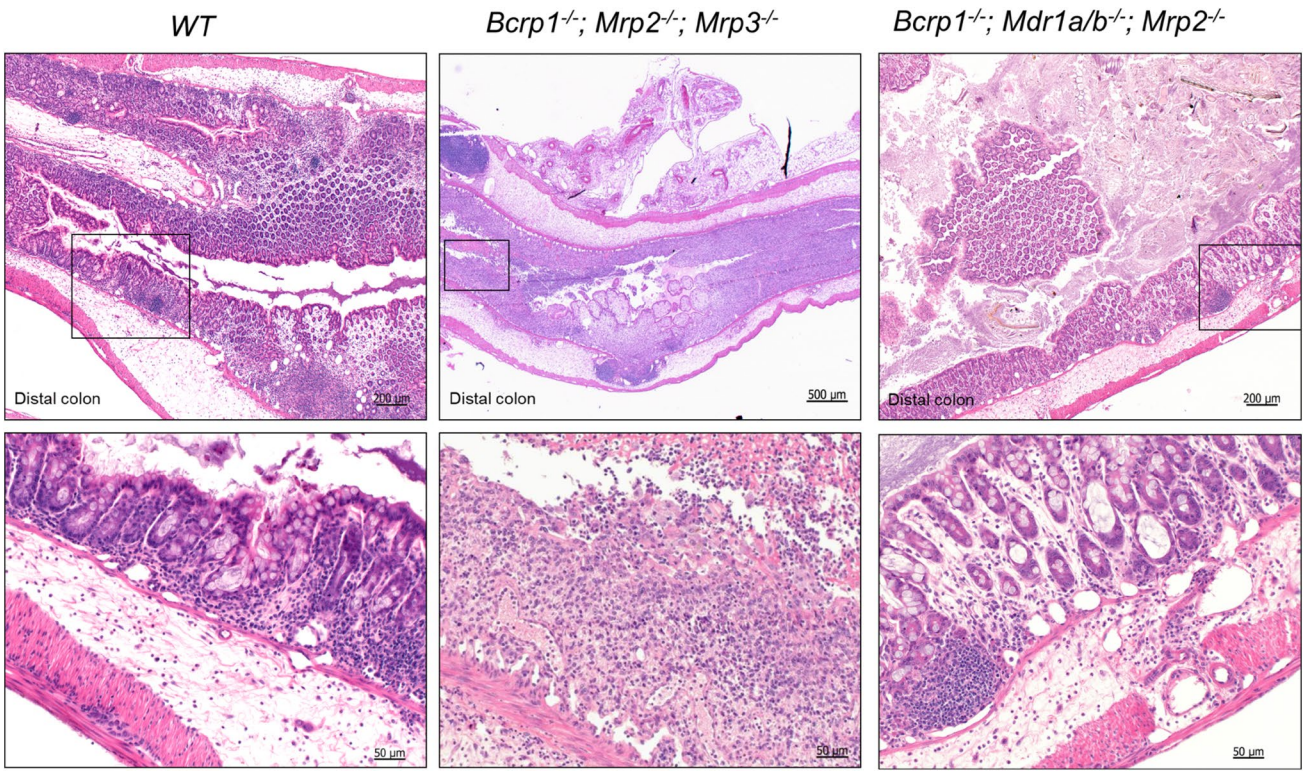

Fig. 1 Differential susceptibility of WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice to DSS exposure (3% in drinking water between days 0 and 7). H&E stainings of distal colons on day 8 are presented; the lower pictures are enlarged representations of the boxed area of the images above

the other strains, with severe necrosis of the covering epithelia of distal colon and rectum and severe degeneration of the corresponding mucosa (Fig. 1). These findings suggest clear mouse strain differences in inflammatory responses of colonic mucosa induced by DSS.

As this high inter-strain variation could easily cause differential susceptibility to PhIP-induced large bowel carcinogenesis, we aimed to normalize DSS doses for each strain to reach similar colonic inflammation levels in the strains. Thus, each strain was divided into four groups (including vehicle controls), receiving different dose ranges of DSS (1.5–3%) for 7 days and then closely checked for DSS effects during the next few weeks to assess the longer-term susceptibility. Findings of the DSS effects are reported in Supplementary Figs. 2, 3 and 4. In general, the combination of body weight loss, diarrhea and rectal blood levels appeared to correlate well with the colonic inflammation level in mice. Thus, in subsequent long-term carcinogenesis experiments, these first three parameters were closely monitored for all individual mice during and soon after DSS treatment. As can be seen with the high standard deviations in 3% DSS responses (Supplementary Figs. 2, 3, and 4), we also found a significant level of intra-strain variation in response to DSS started at different times. However, the relatively high number of mice per experimental group allowed assessment of the general response of the whole group. Interestingly, there was a narrow DSS dose range especially in WT mice (in which all ABC transporters are present), with a 0.25% difference in DSS dose resulting in clearly different effects on body weight loss and inflammation level. Strikingly, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice were much more sensitive to DSS compared to the other two strains. These mice could not handle a DSS dose that led to a high level of inflammation and diarrhea. Therefore, the maximum acceptable DSS dose (2%) in this strain yielded an inflammation level that was lower than that in the other two strains. Overall, we, therefore, decided to use strain-dependent optimal DSS doses to reach a moderate-severe, but physiologically tolerable level of inflammation in each strain (i.e., $\leq$25% loss in each mouse cohort using all criteria including excessive weight loss), yielding 2.75% DSS for WT, 2% DSS for *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and 2.5% DSS for *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice (Table 1).

Impact of ABC transporters on plasma and tissue distribution of PhIP and its metabolites

PhIP is mainly metabolized in liver and formation of its potentially carcinogenic metabolites is summarized in Supplementary Fig. 1 (Chen et al. 2007; Frandsen and Alexander 2000; Gooderham et al. 2001, 2002; Lauber et al. 2004; Lauber and Gooderham 2007, 2011; Nakagama et al. 2005; Schut and Snyderwine 1999). The LC–MS/MS-based assay we developed for quantification of PhIP and its metabolites (Teunissen et al. 2010, 2011) previously allowed us to assess the changes in distribution kinetics of these compounds in the absence of ABC transporters after a low dose of PhIP (1 mg/kg) (Vlaming et al. 2014). As we planned to use high-dose PhIP in our carcinogenicity study, we analyzed PhIP pharmacokinetics also at a dose of 200 mg/kg in the WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ strains. Low-dose (Vlaming et al. 2014) and high-dose disposition (this study) showed more or less similar profiles with roughly linear concentration increases in plasma PhIP levels and similar % of the total dose disposition in various tissues (Fig. 2 and Supplementary Table II).

Besides on PhIP itself, we also focused on the disposition of the (pre)carcinogenic metabolite N2-OH-PhIP and the surrogate markers for DNA adduct formation, 5-OH-PhIP and PhIP-5-sulphate. PhIP and its metabolites N2-OH-PhIP and PhIP-5-sulphate were detected in the plasma of all mouse strains 30 min after oral administration of PhIP. Although all their levels showed an increasing trend in the plasma of the two knockout strains, neither PhIP nor metabolite levels were significantly different from those in WT mice or between the knockout strains (Fig. 2I).

Thirty minutes after oral administration, 1.5–3.0% of the administered dose was found as unchanged PhIP in the livers of all strains (Fig. 2A), but no significant difference was observed between the strains (Fig. 2A, B). Interestingly, the precarcinogen N2-OH-PhIP levels in the *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ livers were 13.5-fold higher than in WT livers ($P < 0.001$; Fig. 2C), whereas this was only 2.2-fold increased ($P < 0.05$) in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ livers. Liver-to-plasma ratios of N2-OH-PhIP were also increased by 6.2-fold in *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ ($P < 0.001$), but not in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice (Fig. 2D). In line with this, liver levels of the mutagenesis marker 5-OH-PhIP were also increased in *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice by 6.4-fold ($P < 0.01$) and in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice by 2.2-fold ($P < 0.05$) (Fig. 2G), with the first strain showing a markedly more elevated level. When liver-to-plasma ratios were plotted, the increase in 5-OH-PhIP in the *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice was down to 2.0-fold, but still significant ($P < 0.05$, Fig. 2H). These data suggest that Mrp3 is a major transporter for N2-OH-PhIP and 5-OH-PhIP, likely mediating their sinusoidal transport from liver to blood efficiently. Despite the more limited effect in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, our data indicate that Bcrp1, Mdr1a/1b and/or Mrp2 can also transport N2-OH-PhIP and 5-OH-PhIP. Increased *Mrp3* expression in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ livers (Vlaming et al. 2014) may lead to increased sinusoidal secretion of these metabolites, therefore, lowering the actual liver levels and masking the effect of Bcrp1, Mdr1a/1b and/or Mrp2 absence. PhIP levels in kidney at

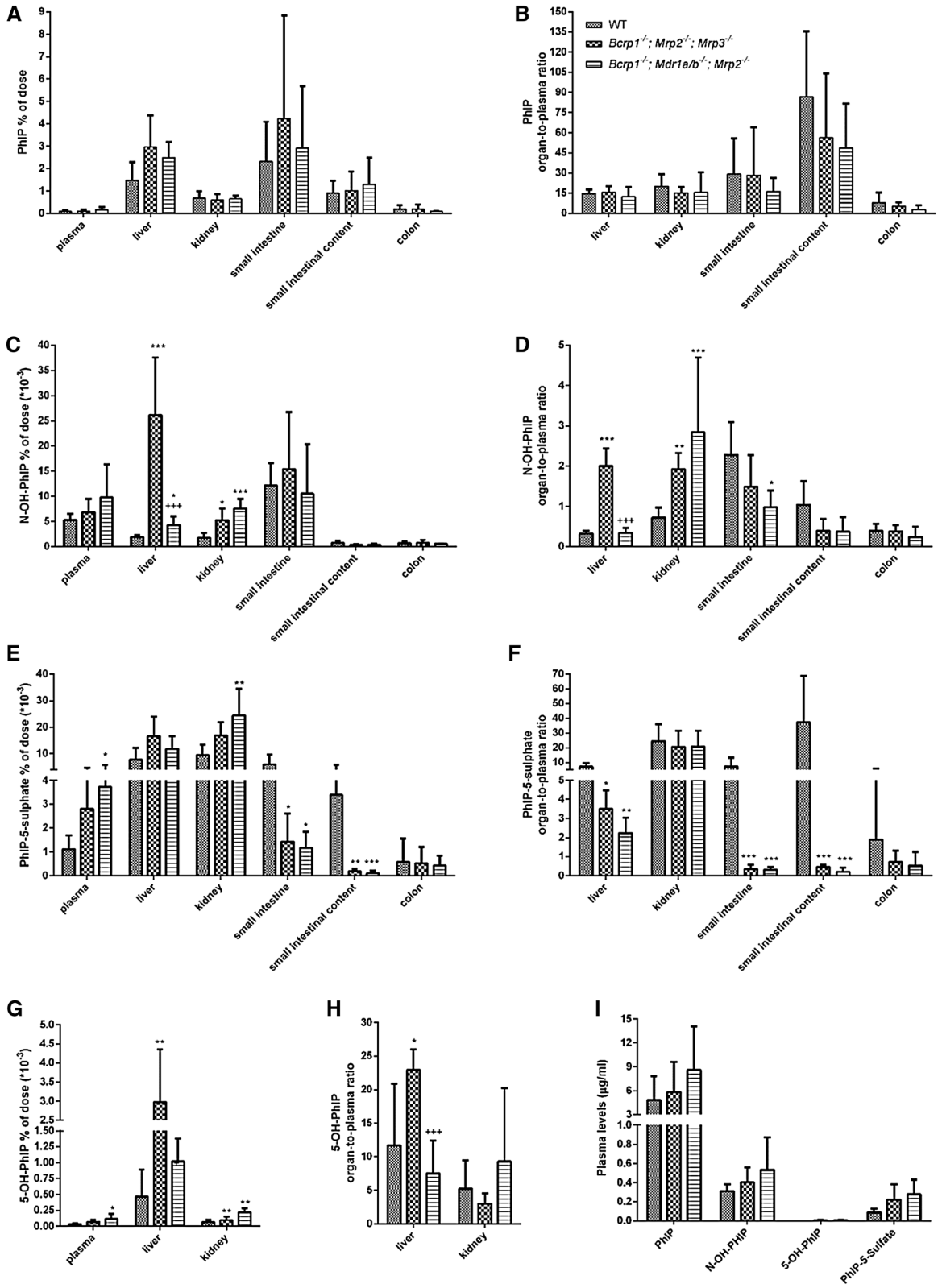

Fig. 2 PhIP and PhIP metabolite levels in plasma, liver, kidney, small intestine and colon of WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice 30 min after oral administration of 200 mg/kg PhIP. Mass % of the total administered PhIP dose (**A, C, E, G**), organ-to-plasma ratios (**B, D, F, H**) and plasma levels (**I**) of PhIP, N-OH-PhIP, PhIP-5-sulphate and 5-OH-PhIP are shown. When the variances were not homogeneous, data were log-transformed before statistical tests were applied. Data are shown as mean ± S.D. $n = 5$–6, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to WT, $^{+++}P < 0.001$ compared to *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice)

30 min were not significantly different between the three strains (Fig. 2A, B). However, PhIP metabolites including the (pre)carcinogen N2-OH-PhIP and genotoxicity markers PhIP-5-sulfate and 5-OH-PhIP were significantly increased in kidneys of both combination knockout strains (Fig. 2C, E, G). For all these metabolites, the increased kidney levels were more pronounced (mostly significantly) in the *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice than in the *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice. For N2-OH-PhIP, both knockout strains showed increased kidney-to-plasma ratios (Fig. 2D). This may reflect reduced kidney elimination of this compound in the knockout strains. Moreover, increased kidney *Cyp1a2* expression in *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice (Vlaming et al. 2014) may also contribute to the higher accumulation of N2-OH-PhIP in this strain due to more efficient conversion of PhIP to N2-OH-PhIP.

Like in other tissues, small intestinal levels of PhIP (both in tissue and in contents) were similar between the three strains. (Pre)carcinogen N2-OH-PhIP levels were also not different in the small intestinal tissue or content (Fig. 2C, D). Although livers of *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice had much higher N2-OH-PhIP levels, these mice could apparently not efficiently excrete this metabolite into the small intestine (Fig. 2C, D), suggesting an impaired biliary excretion route in this strain. 5-OH-PhIP levels were lower than the detection limit in both small intestinal tissue and contents in all strains and, therefore, not plotted in Fig. 2G. Interestingly, compared to WT mice, levels of the genotoxicity marker PhIP-5-sulfate were significantly reduced, by 4.2- to 5.2-fold ($P < 0.05$) in the small intestinal tissue and by 17.2- ($P < 0.01$) and 33.9-folds ($P < 0.001$) in the intestinal contents of *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, respectively (Fig. 2E). These reduced levels were even more pronounced when organ-to-plasma ratios were considered: compared to WT mice, PhIP-5-sulfate levels were 20- and 23-fold decreased in small intestinal tissue and 83- and 188-fold decreased in intestinal contents of *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, respectively (Fig. 2F). In colon, in addition to generally very low levels, there were no significant differences in PhIP, N2-OH-PhIP and PhIP-5-sulphate levels between the strains.

PhIP- and DSS-induced colon tumorigenesis model in WT and compound ABC-transporter knockout mice

The carcinogenicity of PhIP was assessed using the DSS model in WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice. Colon tumors were induced by a one-time oral dose of PhIP (200 mg/kg) at day 0, followed by DSS in drinking water between days 7 and 14 (Table 2; see Fig. 3A for the administration schedule). As described above, the DSS dosages used were adapted between the strains to induce similar levels of intestinal inflammation. Tumorigenicity of PhIP and of DSS alone was also assessed in each strain to evaluate the possible contribution of each factor by itself. As an experimental control, a group of mice from each strain also received only the PhIP vehicle solution. All groups were sacrificed at day 180, except for a few mice that needed to be sacrificed earlier due to tumor-related symptoms in the PhIP-plus-DSS-receiving groups. These mice were also taken up in the analysis if the reason of sacrifices turned out to be tumor related at the histopathological level.

Table 2 Tumor incidence in WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice according to treatment groups

Strain	Treatment group	# Mice in total (*n*)	# Mice with tumor (*n*)	Tumor incidence (%)
WT	Vehicle	26	0	**0**
	PhIP	28	0	**0**
	DSS	16	0	**0**
	PhIP + DSS	24	11	**46**
Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	Vehicle	27	0	**0**
	PhIP	28	1	**4**
	DSS	27	0	**0**
	PhIP + DSS	27	3	**11**
Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	Vehicle	23	0	**0**
	PhIP	25	0	**0**
	DSS	16	0	**0**
	PhIP + DSS	26	9	**35**

Treatment groups include vehicle control, PhIP only, DSS only or PhIP and DSS. Animals received 200 mg/kg of PhIP orally once on day 0, and 2–2.75% DSS in drinking water between days 0 and 7. They were sacrificed on day 180 (unless obvious tumor development required earlier termination) and organs were isolated for further histopathological examination. All mice were closely observed for body weight loss, diarrhea, rectal bleeding and rectal prolapse during the experimental period

Despite the substantial number of mice tested in each group, the tumorigenicity of vehicle, PhIP, or DSS alone appeared to be virtually absent, except for one case. One out of 28 *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice developed a (single) dysplastic adenoma in rectum after PhIP treatment. Obviously, a single high dose of PhIP alone was not a strong carcinogenic inducer, even in the ABC transporter-deficient mice. Although ABC transporters clearly affected the sensitivity of mice to DSS-induced colitis (Table 1), this did not translate into detectable carcinogenicity when DSS was administered alone (Table 2). However, when the PhIP-plus-DSS treatments were combined, all strains developed colon tumors, and the incidence varied depending on the presence of ABC transporters. The 46% tumor incidence observed in WT mice was reduced to 11% in the absence of Bcrp1,

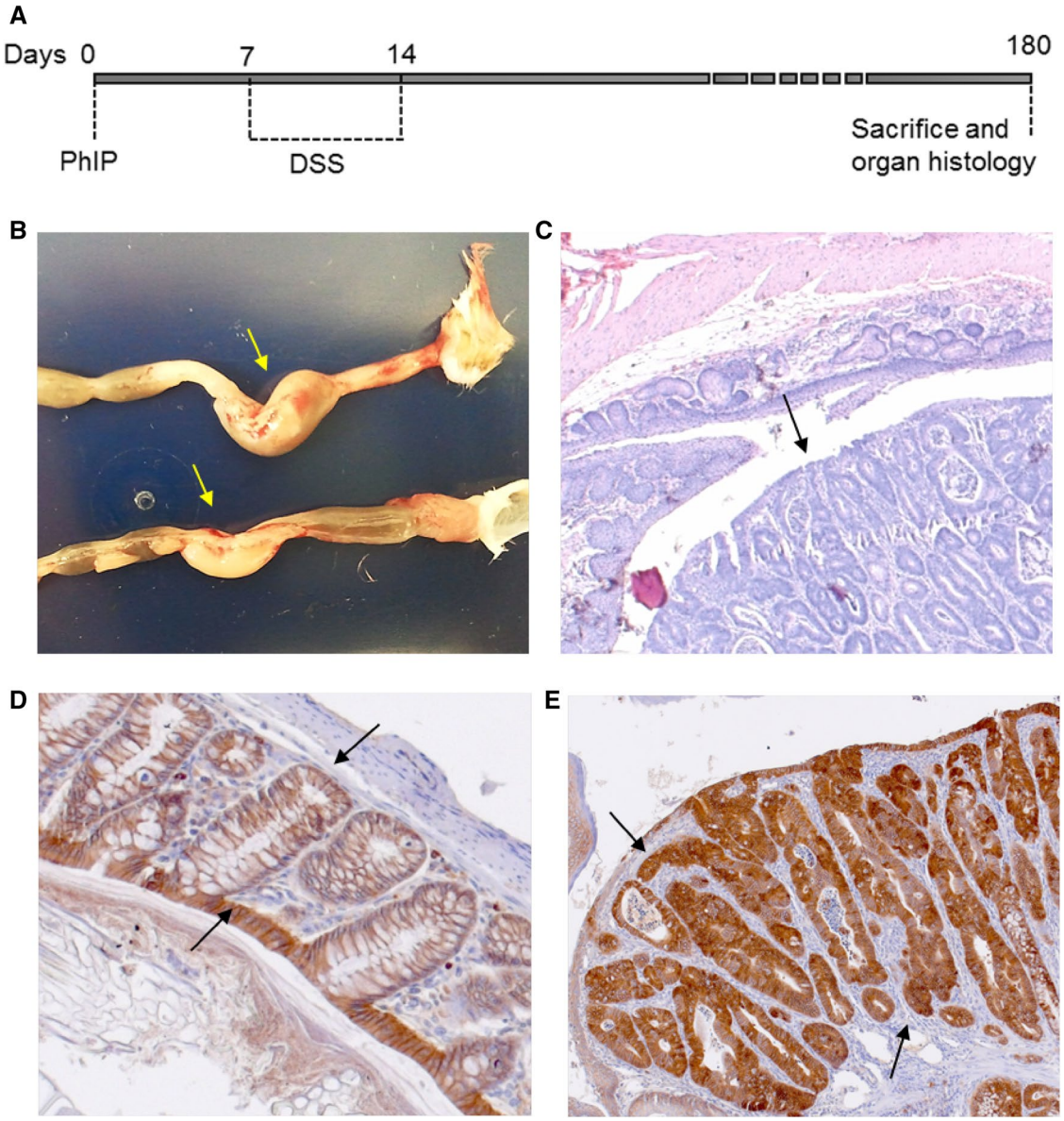

Fig. 3 PhIP and DSS induced colorectal tumors. **A** Experimental schedule of PhIP and DSS carcinogenesis experiment and **B** macroscopic image of induced tumors in colon indicated by arrows. **C**, H&E Staining showing adenocarcinoma of colon indicated with the arrow. **D** Immunohistochemical β-catenin stainings of normal colon. Arrows show the staining for normal crypt structure and epithelial lining of the colon mucosa. **E** Immunohistochemical β-catenin stainings of adenocarcinoma tumor induced by the PhIP and DSS carcinogenesis. Note that tumors show stronger staining with β-catenin compared to **D**. Images were taken at ×10 magnification

Mrp2 and Mrp3 and to 35% when Bcrp1, Mdr1a/1b and Mrp2 were lacking (Table 2). Extensive histopathological analyses done on vehicle controls ($n = 5$–6) and PhIP-plus-DSS-receiving mice ($n = 6$) in each genotype revealed no detectable tumor induction outside of the colorectal tissues. These data suggest that this tumor induction model is specific for colorectal carcinogenicity and highly dependent on combined PhIP induction and DSS-induced inflammation. Moreover, ABC transporters can have a substantial impact on the potential of tumorigenicity in this model.

The size of the induced tumors was generally large, i.e., macroscopically visible (Fig. 3B). Most of the induced colorectal tumors were adenomas as shown by H&E staining in Fig. 3B, with various subtypes such as multinodular or polypous adenomas and adenocarcinomas (Fig. 4). Immunohistochemistry of colon tissues showed a pronounced β-catenin staining in tumors compared to normal mucosa, and this was mostly due to cytoplasmic but also to some extent to nuclear staining (Fig. 3C, D). The number of tumors per mouse varied between 1 and 5, being higher in frequency in

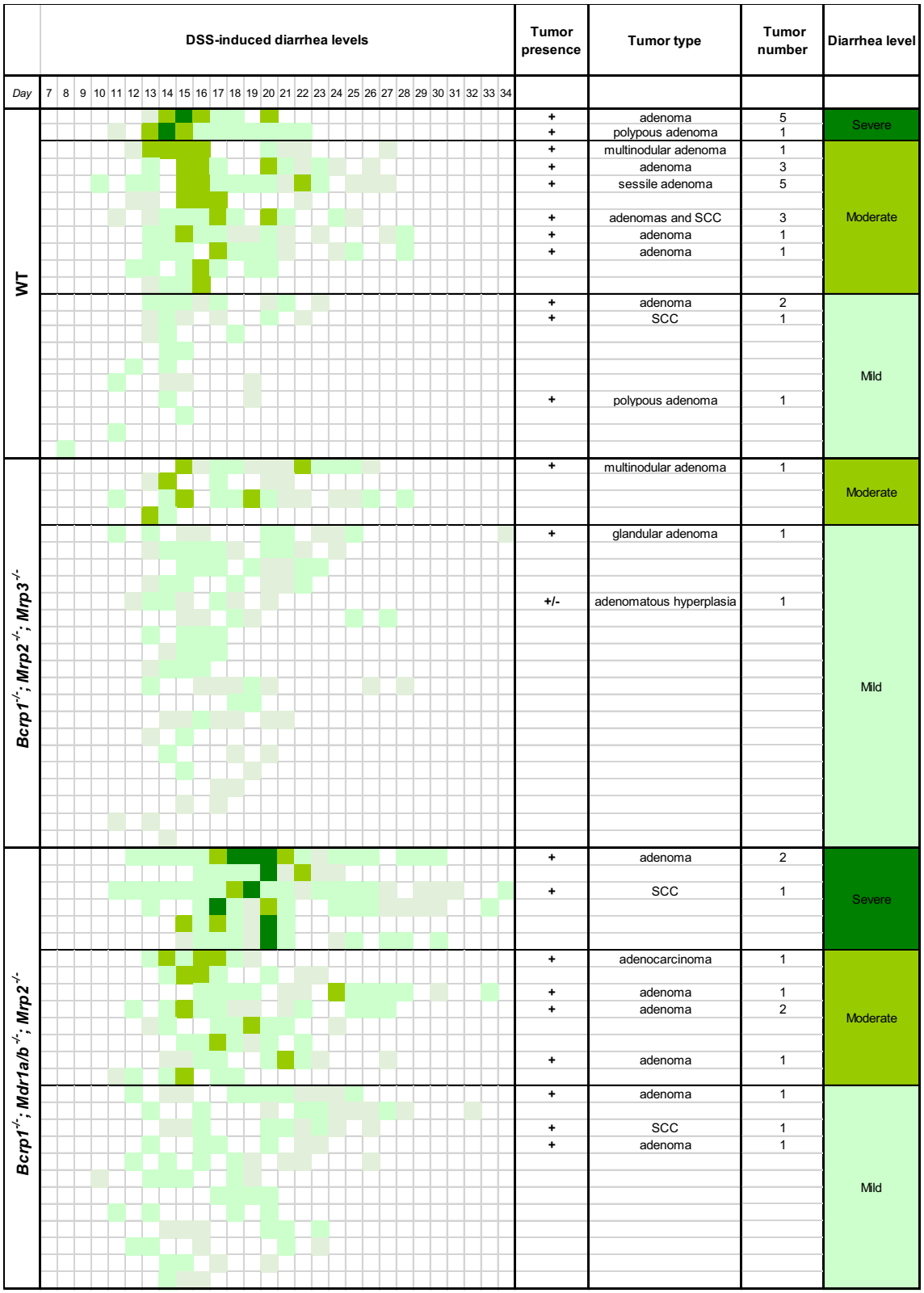

Fig. 4 Details of PhIP- and DSS-induced carcinogenesis in relation to levels of inflammation (as assessed by severity of observed diarrhea) in individual WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice. The days are shown from the beginning of DSS treatment on day 7 after PhIP treatment. Color intensity denotes the level of severity of diarrhea. *SCC* squamous cell carcinoma

WT mice compared to both the ABC-transporter knockout strains (Fig. 4).

Role of ABC transporters in PhIP-plus-DSS-induced colorectal carcinogenesis

To assess the impact of ABC transporters on PhIP-plus-DSS-induced colorectal carcinogenesis independent of the different levels of inflammation, we stratified all individual mice in the PhIP-plus-DSS-receiving strains according to their diarrhea level (Fig. 4; Table 3). As the diarrhea levels generally correlated well with the histological inflammation levels in DSS dose optimization experiments, we use this subgrouping as a surrogate marker for the inflammation levels. Level of diarrhea and rectal bleeding were graded as mild, moderate and severe based on a close assessment of the symptoms given in Supplementary Table I. Mice were checked for these criteria daily during, and

Table 3 Tumor incidence, multiplicity, and normalized total tumor number in PhIP-plus DSS-treated WT, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice according to inflammation level as inferred from the diarrhea level

Diarrhea levels	Strain	# Mice in total	# Mice with tumor	Tumor incidence (%)	Tumor multiplicity[a]	Normalized total tumor number[b]	Diarrhea score[c]
None	*WT*	3	0	–	–	–	–
	Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	4	0	–	–	–	–
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	0	0	–	–	–	–
Mild	*WT*	10	3	30	1.3 ± 0.6	0.40	0.39
	Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	19	2	11	1.0 ± 0	0.11	0.63
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	12	3	25	1.5 ± 0.7	0.25	0.75
Moderate	*WT*	9	6	67	2.3 ± 1.6	1.56	1.46
	Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	4	1	25	1.3 ± 0.6	0.25	1.08
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	8	4	50	1.3 ± 0.5	0.63	1.29
Severe	*WT*	2	2	100	3.0 ± 2.8	3.00	2.10
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	6	2	33	1.0 ± 0	0.50	1.88
Mild + moderate	*WT*	19	9	47	2.2 ± 1.7	0.95	0.89
	Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	23	3	13*	1	0.13*	0.70
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	20	7	35	1.1 ± 0.3	0.40	0.97
Moderate + severe	*WT*	11	8	73	2.7 ± 1.8	1.82	1.58
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	14	6	43	1.3 ± 0.5	0.57	1.54
Mild + moderate + severe	*WT*	21	11	52	2.3 ± 1.6	1.14	1.01
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	26	9	35	1.2 ± 0.3	0.42	1.18
All	*WT*	24	11	46	2.3 ± 1.6	1.00	0.88
	Bcrp1$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$	27	3	11**	1	0.11**	0.67
	Bcrp1$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$	26	9	35	1.2 ± 0.3	0.42	1.18

Tumor incidence was statistically analyzed by *P* value Fisher's exact test; tumor multiplicity and normalized total tumor numbers were statistically analyzed by *P* value Kruskal–Wallis test (*$P < 0.05$; **$P < 0.01$ comparing to WT mice)

[a]Tumor multiplicity is calculated by dividing the total number of tumors by the number of mice with tumor

[b]Normalized total tumor number is calculated by dividing the total number of tumors by the total number of mice in the cohort

[c]Diarrhea score is calculated by taking the average of the numbers allocated to the diarrhea level of each mouse within a cohort daily between days 13 and 22 as in Fig. 4

for a few weeks after DSS treatment until the symptoms were completely resolved. In addition to the qualitative grading (as mild, moderate, and severe), a semi-quantitative diarrhea score was also calculated by averaging annotated numbers given to the diarrhea grade between days 13 and 22 after the PhIP administration, when the diarrhea was most apparent across all strains (Fig. 4; Table 3). As can be seen in Fig. 4, there was also considerable intra-strain variation in response to DSS treatment. In addition to the baseline variation between individuals, there might also be a contribution of the mice batches treated at different times.

The results indicate that, for all genotypes, within each mouse strain the tumor incidence (fraction of mice in a cohort with one or more tumors) generally correlated well with the diarrhea level (Table 3). The increase of tumor incidence with the diarrhea grading was most pronounced in WT mice, from 30% tumor incidence at the mild diarrhea level increasing towards 67% at the moderate and 100% at the severe diarrhea level. In *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice, 11% tumor incidence in the mild diarrhea group was increased to 25% in the moderate diarrhea group. This strain could not tolerate a high level of diarrhea after the PhIP-plus-DSS administration; therefore, there was no severe diarrhea group. Loss of *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice after only DSS treatment was also the highest at all doses (Table 1). This suggests that this strain is very sensitive to DSS-induced inflammation, presumably due to the lack of this specific combination of ABC transporters. *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice could tolerate DSS-induced inflammation leading to all diarrhea levels; however, the correlation of tumor incidence with the diarrhea level was not as clearly linear as with the other two genotypes. 25% tumor incidence in the mild diarrhea group was increased to 50% in the moderate but 33% in the severe diarrhea group. When the mild and moderate diarrhea groups were analyzed together and compared between the strains, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice showed a 3.6-fold ($P < 0.05$) and 2.7-fold lower tumor incidence compared to WT and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, respectively (Table 3). Overall, the tumor incidence was decreased when ABC transporters were lacking, and especially the combination of Bcrp1, Mrp2 and Mrp3 deficiency seemed to be more critical than that of Bcrp1, Mdr1a/b and Mrp2 in modulating the carcinogenicity in the PhIP-plus-DSS model.

The tumor multiplicity parameter (average number of tumors in mice with one or more tumors) also showed a similar trend as the tumor incidence in all diarrhea groups; the numbers of tumors per genotype consistently decreased when ABC transporters were lacking (Table 3). Moreover, with increases in the diarrhea level, WT mice showed somewhat higher tumor multiplicity suggesting that diarrhea, and thus indirectly colonic inflammation not only contributes to tumor progression but may also influence the number of tumors initiated in a wild-type background.

To take also the changes in tumor multiplicity into account, we further evaluated the level of carcinogenesis by the "normalized total tumor number", defined as the total number of tumors detected in a cohort divided by the total number of mice in that cohort. This is arguably the most comprehensive tumorigenesis measure as it encompasses both the number of mice affected, and the number of tumors formed. Also as judged by this parameter, *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice were significantly less susceptible to PhIP/DSS-induced carcinogenesis than WT mice (Table 3). This parameter will be discussed in more depth in the "Discussion" section.

Discussion

Exposure to PhIP is associated with the risk of colorectal carcinogenesis in humans. PhIP is believed to cause the tumor initiation by causing mutations in the DNA. Factors that induce cellular proliferation such as DSS-mediated colonic inflammation in the scope of this study stimulate the ready progression of initiated tumors and lead to a comparatively rapid colon carcinogenesis in mice. Using these models, we show in this study that the clinically relevant ABC transporters, BCRP, MDR1, MRP2 and MRP3, influence the PhIP-plus-DSS-mediated colon carcinogenesis. The absence of these transporters seems to increase the intestinal damage by DSS and reduce the intestinal exposure of activated PhIP metabolites due to their reduced biliary excretion. Altogether, lack of these ABC transporters led to reduced numbers of colonic tumors in our PhIP-plus-DSS-induced colon tumorigenesis model.

The increased sensitivity to DSS in the ABC-transporter compound knockout mice compared to WT mice suggests that these transporters in the intestinal tissue have a protective role for DSS toxicity. There are studies suggesting that Mdr1a expression is inversely related to the susceptibility to DSS-induced colitis and deletion of Mdr1a can even lead to spontaneous colitis in mice, depending on the intestinal microflora (Iizasa et al. 2003; Panwala et al. 1998; Staley et al. 2009). In humans, genetic polymorphisms in the MDR1 gene or reduced expression of MDR1 or BCRP in the intestine are associated with ulcerative colitis (UC), and inflammatory bowel disease (IBD) (Brinar et al. 2013; Englund et al. 2007; Ho et al. 2006; Mijac et al. 2018; Onnie et al. 2006). Thus, our results are in line with these reports and suggest that in addition to Mdr1a, also Bcrp1, Mrp2 and Mrp3 influence the DSS-sensitivity and inflammatory diseases in the gut.

DSS itself, given its high molecular mass (~40.000 Da), is unlikely to be substantially transported by these ABC

transporters, which typically export molecules below 1000–1500 Da in size. It, therefore, seems unlikely that the ABC transporter deficiency directly resulted in higher intestinal wall exposure to DSS, and thus higher inflammation. One intriguing possibility is that the ABC-transporter-deficient intestine more readily develops DSS-induced inflammation because it is intrinsically more prone to inflammation. This might be the result of higher exposure of the intestinal wall to pre-inflammatory bacterial or fungal toxins produced by the intestinal microflora, that are normally kept out by one or more of the intestinal ABC transporters. This idea is consistent with the previous findings that Mdr1a-deficient mice can spontaneously develop severe colitis, but only when exposed to certain bacterial strains in the intestinal microflora (Maggio-Price et al. 2002; Panwala et al. 1998). Possibly the colitis-inducing bacterial strain produces one or more inflammation-inducing toxins that are normally kept out of the intestinal wall by Mdr1a P-glycoprotein. Alternatively, one or more of the studied ABC transporters might be involved in clearance of inflammatory mediators from the intestinal wall (Pazos et al. 2008). However, these various possibilities must be considered hypothetical for now.

We also found that Mrp3 appears to have a prominent role in the sinusoidal secretion of the (pre)carcinogenic metabolite N2-OH-PhIP and mutagenesis marker 5-OH-PhIP in liver 30 min after oral PhIP administration to mice. This function of Mrp3 may indirectly lower the intestinal exposure to precarcinogenic and genotoxic PhIP metabolites by reducing their hepatobiliary excretion. Moreover, in both the *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ and *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice especially levels of PhIP-5-sulphate were markedly reduced in the intestine and intestinal content. As the small intestinal content generally passes through the colon as well, reduced exposure to all precarcinogenic and genotoxic metabolites of PhIP over time is, therefore, expected to reduce the risk of PhIP carcinogenicity. This is indeed what we observed in the ABC transporter compound knockout mice; lowered PhIP-mediated colonic tumor onset in the absence of ABC transporters.

Interestingly, the association of colon carcinogenesis with ABC transporters and inflammation was further pronounced when the semi-quantitative diarrhea score was assessed. The normalized total tumor number (total number of tumors detected in a cohort divided by the total number of mice in that cohort) arguably offers the most integrated assessment of tumor susceptibility of the cohort. Within each genotype (but not when comparing between different genotypes), there was a good correlation between the diarrhea score and the normalized total tumor number (Table 3). This indicates that, for the PhIP-plus-DSS model, the diarrhea score is a good predictive marker for the ultimate development of tumors within a specific mouse strain (genotype). For instance, in WT mice, the normalized total tumor numbers were numerically quite similar to the diarrhea scores across all the diarrhea levels (the diarrhea number generally being slightly lower). In contrast, for the *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice, the normalized total tumor number was generally at least two times lower than the diarrhea score across all the diarrhea levels (Table 3, last two columns). This strongly suggests the *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ mice are less susceptible to developing tumors than the WT mice (at similar diarrhea/inflammation levels). For *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice, the normalized total tumor number was always at least four times lower than the diarrhea score, suggesting that this genotype is far less sensitive than WT (and *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$) mice with respect to tumor formation. Thus, also when assessing the normalized total tumor number *vs*. diarrhea score, the *Bcrp1*$^{-/-}$; *Mdr1a/b*$^{-/-}$; *Mrp2*$^{-/-}$ and especially the *Bcrp1*$^{-/-}$; *Mrp2*$^{-/-}$; *Mrp3*$^{-/-}$ mice demonstrated clearly reduced sensitivity to PhIP/DSS-induced carcinogenesis compared to the WT mice. This is well in line with the reduced intestinal exposure to PhIP-5-sulphate we observed in the knockout strains (Fig. 2E, F).

One limitation of our experiments was the substantial intra-strain variation we observed both in DSS-sensitivity and PhIP-plus-DSS-induced tumorigenesis experiments. As each treatment group included a large number of mice, the whole experiment could only be completed in several parts. This resulted in a degree of intrastrain differences in response to DSS or PhIP-plus-DSS treatments that we could not fully explain. A very recent study suggests that the structure of gut microbiota can affect the sensitivity of mice to DSS-induced colitis in different batches of C57BL/6 mice (Li et al. 2018). The high intrastrain variation might, therefore, also be related in part to a putative variability in the microbiota composition of the gut between the different mouse batches we utilized in our experiments. However, as our mouse cohorts were roughly equally composed of the different mouse batches we do not expect that this resulted in a systematic bias in our experiments.

The activity of BCRP and, to a more limited extent, MDR1 (P-gp) in humans can vary considerably due to genetic deficiencies, polymorphisms or mutations (Huang 2007; Maeda and Sugiyama 2008; Saison et al. 2012), or due to dietary and drug-dependent induction or inhibition (Alvarez et al. 2010; Brand et al. 2006; Fleisher et al. 2015). For example, flavonoid-mediated inhibition of intestinal ABC transporters was suggested to increase the absorption of PhIP in vitro (Schutte et al. 2006, 2008). These studies suggest a risk for increased PhIP exposure in vivo, when, for example, a well-fried meat is taken together with a flavonoid-containing drink. However, our study with knockout mice for these transporters showed that deletion (or inhibition) of these transporters does not necessarily lead to increased PhIP absorption in vivo but may instead lead to

reduced intestinal exposure to carcinogenic metabolites such as N2-OH-PhIP. Possibly this is due to altered PhIP activation in the liver and reduced biliary excretion, further highlighting the importance of liver transporters in carcinogen exposure. These findings suggest that, perhaps counterintuitively, inactive BCRP or MDR1 in humans due to mutation or pharmacological inhibition may have a protective role in PhIP-induced colorectal carcinogenesis. In general, better insight into the role of these transporters in colon carcinogenesis may be helpful in reducing the risk of diet-induced colorectal cancer or in identifying individuals at increased risk for accumulation of carcinogenic metabolites of PhIP in certain tissues such as liver and small intestine.

In conclusion, our study supports the view that the clinically relevant ABC transporters MDR1, BCRP, MRP2 and MRP3 may have important functions in DSS-sensitivity and tissue disposition of the pre-carcinogenic metabolites of PhIP, especially in liver and intestine, leading to altered PhIP carcinogenicity in the colon.

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Compliance with ethical standards

Conflict of interest This project was funded by internal funding from the Netherlands Cancer Institute. The authors declare that they have no conflict of interest.

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