MRP2 and the defence against drugs and toxins
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Chapter III

Mrp2-deficiency in the rat impairs biliary and intestinal excretion and influences metabolism and disposition of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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Mrp2-deficiency in the rat impairs biliary and intestinal excretion and influences metabolism and disposition of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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While metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the most abundant food-derived heterocyclic amine and carcinogen, has been studied extensively in several species, transport and its metabolites has not been defined yet. Therefore we studied metabolism and disposition of PhIP in Wistar and Mrp2-deficient TR- rats to determine the role of Mrp2 in the defence against this compound. In the first 2 h after intravenous dosing, total excretion of PhIP and its metabolites in bile was >4-fold reduced in TR- rats compared with Wistar rats, while excretion in the urine of the TR- rat was 1.8-fold higher. This difference was the result of an almost complete absence of secretion of glucuronidated metabolites but also a reduced level of secretion of unchanged PhIP into bile of the TR- rat. Direct intestinal excretion of unmetabolized PhIP was 3-fold higher in Wistar versus TR- rats. As a consequence, PhIP tissue levels in the liver were 1.7-fold higher in TR- rats, and tissue binding of PhIP, determined after ethanol extraction, was elevated by a similar magnitude. Mrp2-mediated transport of the parent compound PhIP is glutathione (GSH)-dependent, because GSH depletion by l-buthionine-[S,R]-sulfoximine (BSO) treatment in Wistar rats reduced intestinal secretion to the same level as that in TR- rats. TR- rats produced less glucuronides and 4'-OH-PhIP in the 2 h following PhIP administration. We conclude that Mrp2 protects against the carcinogen PhIP by biliary excretion of the parent compound and all major phase-II metabolites, but, more importantly, also by direct extrusion of the parent compound from the gut mucosa.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant heterocyclic amine formed during the cooking, frying and grilling of meat (1). It was first described in 1986 (2) and was shown to induce colon and breast carcinomas in rats (3). Data from several animal models led to the conclusion that PhIP may be involved in human carcinogenesis (4).

The metabolism of this compound has been studied extensively in different species (5–14). In general, after intake, PhIP is metabolized in the liver to its active form N-OH-PhIP mainly by CYP1A1 and CYPA2 (15), N-OH-PhIP is further metabolized to N-acetoxy-PhIP (by N-acetyltransferase) and N-sulfoy-PhIP (by sulfotransferases). The latter two compounds can form DNA adducts (16) and are thought to be the main mutagenic metabolites of PhIP. N-OH-PhIP can also be further metabolized to N-OH-PhIP-glucuronide (depending on the species, mainly N2- or N3-glucuronide (17)), which is a detoxification metabolite (8). The main detoxification pathway though is the conversion of PhIP to 4'-OH-PhIP, which subsequently can be glucuronidated and sulfated (5). Conjugation with glutathione was found in PCB pretreated hepatocytes but was never observed in vivo (18).

Much less is known about the transport of PhIP and its metabolites. Transport of both the parent compound and/or its metabolites is necessary for absorption, disposition and for uptake in the cells of peripheral organs. In a very recent publication it was shown that there might be active secretion of PhIP from Caco-2 cells (19); inhibition studies indirectly implied MRP2 as the most probable transporter involved.

MRP2 was initially identified as the canalicular multispecific organic anion transporter (cMOAT) in the canalicular membrane of hepatocytes (20). First studies showed that it transports a wide range of conjugated compounds, including glucuronidated, sulfated molecules and molecules conjugated to glutathione (21). Uncharged compounds have not been shown to be substrates for MRP2 yet and such a finding would need clarification of the transport mechanisms involved. The homologous transporter MRP1, which is expressed in a variety of organs except liver, has been shown to transport uncharged compounds in co-transport with glutathione (GSH) (22), a mechanism which could account for MRP2-mediated PhIP transport as well. Since PhIP is, after hydroxylation, subsequently glucuronidated and sulfated, MRP2 is a candidate for biliary excretion of the parent compound and several of its metabolites.

We therefore studied metabolism, disposition and excretion of PhIP in Mrp2-deficient TR- rats and GSH-depleted Wistar rats in comparison to control animals, which were untreated Wistar rats. We show here that Mrp2 mediates biliary excretion of PhIP and all its major phase-II metabolites and excretion of PhIP into the lumen of the small intestine. Furthermore we show that deficiency of Mrp2 in the rat, resulting in impaired biliary secretion, influences metabolism and leads to higher tissue binding of PhIP and its metabolites in the liver.

Materials and methods

Chemicals

\(^{14} \text{C}\)PhIP (specific activity 10 mCi/mmol) and unlabeled Nitro-PhIP were obtained from Toronto Research Chemicals (North York, Ontario, Canada).
Unlabeled PhIP was bought from ICN (Costa Mesa, CA, USA). All batches were found to be >98% pure by HPLC.

Sulfatase H-5, saccharo-1,4-lactone, l-thiobenzoin-[5,6]-sulfoximine (BSO) and glucuronidase (Escherichia coli Type VII-A and bovine liver Type B3) were from Sigma (St Louis, MO, USA). All solvents used for HPLC separation (HPLC or analysis grade) were from Merck (Darmstadt, Germany).

Animals
Female Wistar rats were obtained from Harlan-CPB (Zeist, The Netherlands). Age- and weight-matched female TR− rats were from our own breeding colony which has been characterized previously (23). Rats were housed in cages with a 12 h light/dark cycle and given access to food and water ad libitum. Rats were used for experiments at age 12–16 weeks (200–250 g weight). All animal experiments in this work have been carried out in accordance with the Declaration of Helsinki.

Metabolism and excretion studies (four rats each group)
After anesthetization (50 mg pentobarbital sodium (Nembutal)/kg i.p.), the jugular vein, the common bile duct and the urinary bladder were cannulated. After cannulation a continuous infusion of taurocholatecholate (100 mmol/min/100 g rat) in the jugular vein was started to maintain bile flow. [14C]PhIP (2 μCi/200 g rat) was injected in the jugular vein and bile and urine samples were obtained over a period of 2 h after injection.

BSO pretreatment
To inhibit glutathione (GSH) synthesis, we treated Wistar rats with 4 mmol BSO/kg 3.5 and 1.5 h i.p. prior to beginning of the experiments. Efficiency of BSO pretreatment was determined by controlling GSH levels (according to the method of Tietze (24)) from liver and gut tissue pieces immediately homogenized after killing.

Organs
After sample collection in each experiment, animals were killed by bleeding from the aorta and a 4 ml blood sample was collected. Liver, whole intestine (with feces), kidney, pancreas, heart and lungs were excised. Feces were separated from the intestine before storage. All material was stored at −80°C.

Sample treatment
Directly after sampling aliquots of urine and bile samples were injected on HPLC for separation of metabolites and used for counting. Weighed portions of the organs from at least two different sites were completely solubilized in Soluene 350 (Packard, Meriden, CT, USA) (1 ml/100 mg tissue) to determine tissue levels of radioactivity. Two additional portions were homogenized in ethanol (1:1, v/v), centrifuged and, after another step of washing with ethanol and centrifuging, the pellet was dissolved in Soluene as described above to determine tissue binding of radioactivity. The supernatant and wash-out from these steps were counted separately. All organ samples were decolorized with 10% (v/v) H2O2 and mixed with scintillation fluid (to a total volume of 10 ml) before counting. For determination of metabolites in liver tissue, homogenates, 2–3 g tissue was homogenized in ethanol, centrifuged and, after another step of washing with ethanol and centrifuging, the supernatant was dried under nitrogen. The sample was reconstituted in 2 ml ethanol again, centrifuged and again dried. Then the sample was dissolved in 20 μl water and placed directly on HPLC.

Bile and urine samples treated with enzyme for metabolite identification (see below) were passed over a C18-OASIS 30 mg column (Waters, Milford, MA, USA). Metabolites were eluted with methanol, dried and then reconstituted in water and injected. Total radioactivity recovery with this method was >99%.

Feces were homogenized in 50% methanol in water (1:5, w/v), the homogenate was counted and after centrifugation the supernatant was dried, reconstituted in water and analyzed by HPLC. Radioactivity recovery with this method was around 95%.

For detection of metabolites in blood we used the method of Kadirkamanathan et al. (25).

Enzyme treatment
β-Glucuronidase (3000 U/ml, bovine as well as E.coli) and sulfatase (20 U/ml, in presence of 1 mM saccharo-1,4-lactone to inhibit glucuronidase activity) were dissolved in 15 mM acetate buffer pH 5.0 (E.coli glucuronidase in potassium phosphate buffer pH 6.8) and 1 ml was added to 20 μl sample. Incubation lasted 3 h at 37°C.

HPLC
Analysis was done on a system with Gynkotek-Pumps (Gynkotek, Germany), connected to a Rheodyne 7125 injection valve (Cotati, CA, USA) and an Inertsil 5 ODS 3 column (Chrompack, Bergen on Zoom, The Netherlands). Injection volume was 20 μl. We used an UV-Detector Spectroflow 757 (Kratos, Ramsey, NJ, USA) at 315 nm, a fluorescence detector (Jasco FP-920, Tokyo, Japan) and an on-line Scintillation Analyzer (SLOTR, Packard, Meriden, CT, USA). The mobile phase (0.4 ml/min) consisted of (A) 10% methanol in 0.1% diethylamine pH 5.0, 6.0 and 7.0 and (B) 90% methanol in 0.1% diethylamine with the same pH. After 5 min isocratic flow with 10% (B) the gradient was linearly adjusted to 25% (B) at 15 min, then to 75% (B) at 30 min, where it was held constant until 35 min. Fractions for fluorescence spectra and mass spectroscopy (MS) identification were collected in 30 s intervals.

Metabolite identification
HPLC buffers at three different pHs (pH 5.0, 6.0 and 7.0) were used to determine the relative retention times of all metabolites under different conditions; these data were compared with previously reported metabolite characteristics at these pH values (7,14,25,26). Treatment with different enzymes as described above already led to a tentative identification of most metabolites. We used highly concentrated bile and urine samples from rats exposed intravenously to very high doses of PhIP (at the limit of solubility at pH 5.0, where PhIP is best soluble) to determine UV and fluorescence spectra of each metabolite after separation by HPLC. Our spectra were compared with those in the literature (13,17,18,25,27–29). In a last step all metabolites identified with the methods described were subjected to MS–MS to confirm this identification. For further details of the results from the metabolite identification studies see Table 1. N-OH-PhIP was identified by a standard synthesized from Nitro-PhIP as reported earlier by Lin et al. (30).

UV and fluorescence spectra
UV spectra were taken online on a diode array detector (Applied Biosystems 1000S, Cheshire, UK) from 240 to 370 nm. Fluorescence spectra were recorded on a Amino Bowman Luminescence Spectrometer (Spectronic, Cambridge, UK) with a fixed emission–excitation wavelength difference of 55 nm according to Crofts et al. (29).

MS identification of metabolites
Mass spectra were recorded using a Quattro triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an electrospray source (ESP). Nitrogen was used as the spraying and drying gas while the source temperature was kept at 80°C. Fractions from the HPLC separation, dried under nitrogen, were reconstituted in methanol/water (1:1) and injected directly. Positive ion scans were taken using a capillary voltage of 3500 V and negative ion scans with one of 3000 V. The cone voltage was optimized for each fraction, generally around 35 V. After that the collision energy, used for the daughter-ion scans, was optimized individually, generally around 25 eV. Argon was used as collision gas at a pressure of 2.5 mbar.

Results
Excretion of PhIP and its metabolites 2 h after intravenous dosing of PhIP
In the 2 h following intravenous administration of PhIP, the percentage of total radioactivity excreted into bile for Wistar and TR− rats was 41.4 ± 7.4 and 9.4 ± 2.7%, respectively (Figure 1, P < 0.001). The excretion of radioactivity in the urine was ~2-fold higher in TR− rats (24.6 ± 7.3%) than in Wistar rats (13.8 ± 4.4%; P < 0.05; Figure 1). Nevertheless, the total body elimination of radioactivity was still significantly lower in TR− rats compared with Wistar rats (34 ± 9.7 versus 55.2 ± 4.7%; P < 0.01; Figure 1).

Metabolites in bile and urine were analyzed using HPLC (Figure 2). This revealed four major and several minor metabolites. All major metabolites and one minor metabolite could be identified by relative retention times, UV and fluorescence spectra, enzyme treatment with glucuronidase and sulfatase and electrospray/tandem mass spectrometry (see Materials and methods and Table I for details). Quantification of these metabolites (Figure 3) showed that in the bile of TR− rats the glucuronides of 4′-OH-PhIP and the N2-glucuronide of N'-OH-PhIP were virtually absent (P < 0.001), while the N3-glucuronide of N'-OH-PhIP was strongly reduced (0.88 ± 0.31 versus 11.5 ± 3% in the TR− and Wistar rats, respectively; P = 0.003). The hepatobiliary secretion of the original compound was also significantly impaired in TR− rats (0.7 ± 0.33 and 3.09 ± 0.59% for TR− and Wistar rats, respectively; P < 0.001) as was the secretion of 4′-OH-PhIP.
Mrp2 deficiency and PhIP metabolism

Table I. Retention times of major metabolites on HPLC (pH 6.0) and data of enzyme treatment, UV and fluorescence spectra and MS identification (see Materials and methods)

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Retention time (min)</th>
<th>Metabolite</th>
<th>Enzyme treatment</th>
<th>UV max (nm)</th>
<th>Fluoresc. max (nm)</th>
<th>Mass (MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>6/8</td>
<td>4'-OH-PhIP-Gluc</td>
<td>Gluc.→4'-OH-PhIP</td>
<td>328</td>
<td>326</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>4'-PhIP-sulfate</td>
<td>Sulf.→4'-OH-PhIP</td>
<td>324</td>
<td>322</td>
<td>ESP-319</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>Not identified</td>
<td>Sulf.→?</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>4'-OH-PhIP</td>
<td>–</td>
<td>315</td>
<td>–</td>
<td>ESP+241</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>N-OH-PhIP-N3-sulf.</td>
<td>Gluc.→N-OH-PhIP</td>
<td>328</td>
<td>–</td>
<td>ESP+415</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>Not identified</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>31</td>
<td>PhIP</td>
<td>–</td>
<td>317</td>
<td>318</td>
<td>ESP+225</td>
</tr>
</tbody>
</table>

Peak numbers refer to Figure 3. Under the applied conditions, the indicated enzyme treatment resulted in a shift of the peak to the position of the indicated product. Metabolites at 23, 26 and 30 min were too minor to be identified by MS. N-OH-PhIP was identified by a synthesized standard (retention time ~30.6 min).

Abbreviations: gluc., glucuronidase treatment; b-gluc, bovine glucuronidase treatment; sulf., sulfatase treatment; ESP, electrospray ionization.

Fig. 1. Excretion of radioactive material in bile (open bars) and urine (closed bars) in the 2 h following intravenous administration of [14C]PhIP in Wistar, TR and BSO pretreated Wistar rats (BSO). Data represent means ± SD (n = 4 in all groups). Significance compared with untreated Wistar rats: +, P < 0.05; *, P < 0.01; **, P < 0.001.

Fig. 2. Representative HPLC chromatograms (pH 6) of bile samples 30 min after intravenous [14C]PhIP administration. Above Wistar rat, below TR rat. For identification data see respective peak number in Table I.

(P = 0.02). The transport of unmetabolized PhIP into bile was not significantly changed (P = 0.15; Figure 3A) in BSO pretreated Wistar rats, while glucuronides in bile were slightly but significantly reduced.

In urine (Figure 3B), the differences were much less striking. There was increased urinary excretion of glucuronides and

Fig. 3. Amount of identified metabolites excreted in (A) bile, (B) urine and (C) total elimination of each metabolite in 2 h after intravenously administered [14C]PhIP in Wistar (closed bars), TR (open bars) and BSO-pretreated Wistar rats (hatched bars). Significance levels (+, P < 0.05; *, P < 0.01; **, P < 0.001) of TR and BSO pretreated Wistar rats are compared with untreated Wistar rats. Data are given as means ± SD (n = 4 in all groups).
To determine the distribution of radioactivity remaining in the body at 2 h after intravenous administration, we solubilized aliquots of several organs (liver, kidney, small and large intestine, pancreas, heart and lung) and determined radioactivity. There was no significant difference in whole organ radioactivity between TR- and Wistar except for the liver, where a 1.7-fold increased radioactivity was measured in TR- rats.

**Tissue levels and tissue binding of radioactivity 2 h after injection**

To determine the distribution of radioactivity remaining in the body at 2 h after intravenous administration, we solubilized aliquots of several organs (liver, kidney, small and large intestine, pancreas, heart and lung) and determined radioactivity. There was no significant difference in whole organ radioactivity between TR- and Wistar except for the liver, where a 1.7-fold increased radioactivity was measured in TR- rats.

**Fig. 4. Amount of radioactivity in homogenized luminal contents of small intestine (closed bars) and colon (without cecum, open bars) of Wistar, TR- and BSO pretreated Wistar (BSO) rats 2 h after intravenously administered [14C]PhIP. Significance levels (*, P < 0.01) of TR- and BSO pretreated rats are compared with untreated Wistar rats. Data are given as means ± SD (n = 4 in all groups).**

sulfate of 4'-OH-PhIP in TR- rats. This was not observed in the BSO pretreated Wistar rats. Total body elimination (Figure 3C) of all glucuronides, 4'-OH-PhIP and the original compound PhIP was reduced in TR- rats. There was a tendency (P = 0.053) in TR- rats to excrete more 4'-PhIP-sulfate. The BSO pretreated Wistar rats also excreted in total less amounts of all glucuronides.

The pattern of metabolites detected in blood did not differ from that in urine (data not shown). The activated compound N-OH-PhIP was not detected in blood, bile or urine. Analysis of bile and urine was done at several time points after injection, but over time there were no significant changes in the relative amount of metabolites in either Wistar or TR- rats except for a minor metabolite at 30 min retention time which was not identified further.

In the same experiment we also collected the total luminal content of small intestine and colon (without cecum) separately. Any radioactivity measured in this material must have been secreted by the mucosa because PhIP was injected intravenously while bile was continuously diverted during the 2 h experimental period. There was much less radioactivity in the small intestinal luminal contents of the TR- rat than in that of the Wistar rats (0.28 ± 0.19% versus 0.95 ± 0.22%, respectively, P = 0.007), while the difference in the colon showed only a tendency (P = 0.074; Figure 4). In BSO pretreated Wistar rats active secretion in the intestine was reduced to a similar extent as in the TR- rat (0.28 ± 0.26%, P = 0.008).

We analyzed the supernatant of the homogenized intestinal contents with HPLC and found that 75% of the radioactivity consisted of the original compound PhIP, the rest was 4'-OH-PhIP. This was not different between Wistar, BSO pretreated Wistar and TR- rats.

**Fig. 5. Total (left) and covalently bound (i.e. ethanol insoluble portion, right) levels of radioactivity in the liver of Wistar (closed bars), TR- (open bars) and BSO pretreated Wistar rats (hatched bars) at 2 h after intravenous administration of [14C]PhIP. Significance levels (*, P < 0.01; ***, P < 0.001) of TR- and BSO pretreated rats are compared with untreated Wistar rats. Data are given as means ± SD (n = 4 in all groups).**

**Fig. 6. Amounts of metabolites (percentage of injected dose) in the ethanol soluble fraction of liver homogenate in Wistar (closed bars) and TR- rats (open bars) at 2 h after intravenous administration of [14C]PhIP. Liver material was homogenized in ethanol and the supernatant was analyzed by HPLC (see Materials and methods). Significance levels (*, P < 0.01; ***, P < 0.001) of TR- rats are compared with untreated Wistar rats. Data are given as means ± SD (n = 4 in all groups).**

Figure 5). Tissue binding of radioactivity in the TR- rat, as determined by ethanol insolubility, was more than doubled (P < 0.001; Figure 5). Tissue levels of radioactivity in liver of the BSO pre-treated Wistar rats were not significantly different from untreated Wistar rats, whereas tissue binding of radioactivity in this organ was significantly elevated (2.15 ± 0.42 versus 1.52 ± 0.22% for BSO pre-treated and untreated Wistar rats, respectively; P = 0.01; Figure 5).

To determine the pattern of PhIP metabolites in the liver, we analyzed the ethanol soluble fraction of liver homogenate by HPLC (Figure 6). The portions of the respective metabolites in Figure 6 are given as percentage of the total dose. All metabolites are significantly elevated in the liver of the TR- rat, with PhIP-sulfate >5-fold elevated. Surprisingly, PhIP itself is lowered in the TR- rat. Another metabolite eluting at ~15 min which was not detected in bile, urine or blood,
accounted for ~5% of the ethanol soluble fraction of all animals and could not be identified further (data for this metabolite not shown in Figure 6).

Discussion

Data from the present study demonstrate that rat Mrp2 mediates the excretion of all major phase-II metabolites of PhIP. We could show that directly for the glucurononides, the biliary excretion of which was dramatically reduced. Although the absolute amount of PhIP-sulfate excretion in bile is equal in Wistar and TR^- rats, we observed 5-fold elevated levels of this metabolite in the liver as well as higher levels in blood and urine. Thus, in TR^- rats the same sulfate excretion is only reached at higher intracellular concentrations. Based on the known characteristics of Mrp2, this excretory function for glucuronidated and sulfated conjugates is not surprising; in a large number of studies this transporter was characterized as having affinity for a broad spectrum of organic anions, particularly glucurononides and glutathione conjugates (21,31).

In addition, this study shows that Mrp2-mediated transport in kidney proximal tubules (32) is relatively unimportant compared with glomerular filtration; urinary excretion of glucurononides was increased rather than decreased in the TR^- rat. The higher phase-II metabolite levels in urine of the TR^- rats are a consequence of higher blood levels of these metabolites. This is most likely due to basolateral secretion of unexcreted glucurononides and sulfates out of the liver into the plasma. The latter process might be mediated by Mrp3 in the hepatocyte basolateral membrane, which also transports xenobiotics (33). Importantly, Mrp3 expression was shown to be increased in Mrp2-deficient rats (34,35), indicating that the actual accumulation of PhIP and/or its metabolites may partly be counteracted.

Though compensatory excretion of glucurononides occurred in urine of the TR^- rat, excretion of all glucurononides as well as 4'-OH-PhIP, is significantly reduced in the Mrp2-deficient rat (Figure 3). While the higher amounts of these metabolites in liver parenchym only indicate a delay in excretion, the total amounts of excreted metabolites even then are significantly lower when these amounts in liver parenchyma (and presumably gut parenchyma since glucurononidation takes place there, too) are taken into consideration. This additionally indicates a reduction or at least a delay in metabolism of PhIP in Mrp2-deficiency and confirms the recent finding (36) that the expression level of a transport protein can also influence metabolic reactions, probably through product inhibition of the accumulated metabolites.

Another important finding of our study, though not unexpec­ted, is that the gut functions as an active excretory route for PhIP. The significant difference in intestinal excretion between Wistar and TR^- rats points at a role for Mrp2 in this function. HPLC analysis of the radioactivity found in the intestinal luminal contents revealed mainly unmethylolated PhIP as the excreted compound. The role of Mrp2 in transport of unmethylolated PhIP is underscored by the data from biliary excretion. Both the liver and the small intestine excreted unmethylolated PhIP, and in the TR^- rat transport in both organs was strongly reduced. Walle and Walle (19) recently suggested from inhibition studies in Caco-2 monolayers that MRP2 may contribute to transport of the parent compound. In addition, it has been shown very recently that Mrp2 is expressed in rat intestine (37,38). Our results support these data and show functional involvement of Mrp2 in hepatic and intestinal defence against PhIP. The Mrp2-expressing gut functions as an active excretory organ. Intestinal PhIP excretion accounts for over 1% of the dose in the first 2 h in the whole gut of Wistar rats after intravenous administration. This amount is comparable with the amount of unmetabolized PhIP excreted by the liver and suggests a role for Mrp2 in reducing oral bioavailability of this compound. The colonic excretion of PhIP was not different between Wistar and TR^- rats which is in line with the almost absent expression of Mrp2 in this part of the gut (37). Levels of radioactivity in the colon in our experiment were not influenced by the changed luminal levels in the small intestine, since the experiment lasted only 2 h and the cecum and contents of the cecum were not used for determination of radioactivity.

We speculate that the transport of the parent compound PhIP is taking place together with GSH, since PhIP itself is neither conjugated nor negatively charged. We were not able to demonstrate a significant effect of BSO on PhIP transport from liver into bile, but transport in the small intestine was significantly reduced. Within the experimental period it was difficult to reduce GSH in rat liver to an extent lower than 25% of controls, a fact also observed by others (39,40). This level might be enough to support PhIP transport sufficiently. While BSO treatment reduced hepatic GSH content to 29% of control in our experiment, the intestinal content could be reduced to 14.5% of control. The lower GSH content of the intestine might explain why PhIP transport was reduced in this organ and not in the liver.

In the past it was shown that glutathione depletion caused a 5-fold increase of DNA adducts in the liver (25) or 15-fold increase in hepatocytes (8), respectively, in the latter case without changing the metabolic profile. It was also shown that a concentration of 40 mM GSH in vitro inhibited DNA binding by N-acetoxy-PhIP (8). Our data suggest that, in addition to the reduction of DNA adduct formation, GSH may play a role in direct extrusion of the carcinogen. It is therefore likely that there is addition of several effects in GSH action. Because GSH itself is also a substrate for Mrp2, TR^- rats lack biliary GSH secretion and, as a consequence, exhibit higher hepatic GSH levels (~157% of the Wistar rat (31)). These high GSH levels may protect against increased adduct formation. Consequently, one might expect even higher tissue bound radioactivity in the liver of the TR^- rat if GSH levels were the same as in Wistar rats. In BSO pretreated Wistar rats, liver levels of radioactivity are equal to those found in untreated Wistar rats, compatible with the fact that PhIP excretion into bile in these animals is not impaired at GSH concentrations reached with our method of BSO treatment. However, the ethanol insoluble fraction of tissue radioactivity, representing covalently bound reactive metabolites, is significantly higher in BSO pretreated than in untreated Wistar rats, which possibly highlights the influence of GSH on tissue binding. We speculate that the two described effects of GSH on detoxification of PhIP in the liver have different thresholds. While reduction to 29% of the normal GSH concentration already increases tissue binding of reactive metabolites, it still drives excretion of PhIP into bile. This hypothesis also may explain the reduced total excretion of the glucurononides of N-OH-PhIP in the BSO pre-treated Wistar rats. It is not expected that GSH plays a role in transport of glucurononides as these are charged compounds by themselves. On the other hand, lower GSH levels will result in higher
tissue binding of N'-OH-PhIP derivatives, leaving less free N'-OH-PhIP available to be glucuronidated.

In summary, we propose a dual function of Mrp2 in the intestine which acts before phase-I metabolism by pumping the unmetabolized xenobiotic out of the gut mucosa and the liver. It also contributes to reduction of the body level of toxics by rapid excretion of metabolites which have been formed by phase I enzymes (cytochrome P450) and phase II enzymes (glucuronosyltransferases and sulfotransferases) to inhibit further damage through systemic deconjugation. By these two functions Mrp2 reduces the number of reactive molecules in the whole body, thereby probably reducing adduct formation.

Complete Mrp2 deficiency in humans only occurs in rare Dubin–Johnson patients. Nevertheless it is expected that polymorphisms in the MRP2 gene may lead to reduced expression or activity of the protein in otherwise completely healthy subjects. Such polymorphisms have already been shown to occur in the MDR1 gene (41), which has a functionally similar task in the gut epithelium. Ten percent of the normal level of MRP2 is sufficient to prevent jaundice in the rat (42), but at the same time such a reduction will significantly reduce excretion of substrates such as PhIP. In so far, MRP2 could become a marker of human cancer susceptibility in the future.

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**Mrp2 deficiency and PhIP metabolism**