ABC transporter compound knockout mice: physiological and pharmacological characterization
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transporter compound knockout mice: physiological and pharmacological characterization

Marijn Vlaming
ABC transporter compound knockout mice: physiological and pharmacological characterization

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof.dr. D.C. van den Boom

ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit

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Chapter 1

Compound transporter knockout mice: powerful tools to unravel the pharmacological and physiological functions of ATP binding cassette drug transporters

Maria L.H. Vlaming, Jurjen S. Lagas, Alfred H. Schinkel

To be submitted
Compound transporter knockout mice: powerful tools to unravel the pharmacological and physiological functions of ATP binding cassette drug transporters

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ATP binding cassette (ABC) transporters are cellular efflux pumps with broad and often widely overlapping substrate specificities, which can have a major impact on the pharmacology and pharmacokinetics of many drugs. To study their separate roles and functional overlap, a collection of mice deficient in two or more ABC transporters, termed “compound transporter knockout mice”, has been generated and characterized. This review discusses recent findings obtained with these models, focusing on pharmacokinetic studies with a number of clinically relevant drugs. In addition, the characterization of these mice and some physiological aspects of ABC transporters are addressed.

INTRODUCTION
ATP binding cassette (ABC) transporters, including the multidrug efflux transporters P-gp (ABCB1/MDR1), ABCC1-4 (MRP1-4) and ABCG2 (BCRP), are transmembrane proteins that can actively extrude a wide variety of endogenous and exogenous compounds from cells. Most multidrug ABC transporters have broad and substantially overlapping substrate specificities, and their substrates include many drugs and drug metabolites [extensively reviewed in (1;2)].

P-gp, ABCC2 and ABCG2 are localized at the canalicular membranes of hepatocytes and at the apical membrane of epithelial cells of kidneys and the intestinal tract where they pump their substrates into bile, urine and feces. Consequently, these efflux pumps can have an important impact on the elimination of many clinically relevant drugs and they can restrict their intestinal uptake (1;2). In addition, these transporters are found at the blood-placenta, blood-testis and blood-brain barriers, where they protect the corresponding tissue sanctuaries from the penetration of potentially harmful compounds (1;2). ABCC3 is a basolateral transporter, predominantly present in liver, gut and kidney, where it transports its substrates towards the systemic circulation (1;2). In contrast, ABCC4 is expressed apically in brain capillaries and kidney proximal tubules, but in liver, prostate, urogenital tissues and choroid epithelial cells it is localized at the basolateral membrane, pumping its substrates into the circulation (1;2). The subcellular location
membrane of proximal tubular cells and extrude their substrates into the urine. ABCC1 and ABCC3 are present at the basolateral membrane, pumping their substrates towards the blood circulation. D) ABC transporters at the BBB. P-gp, ABCG2, ABCC2 and ABCC4 are located at the apical membrane of capillary endothelial cells, pumping their substrates towards the blood. ABCC1 is also expressed in the basolateral membrane of epithelial cells of the choroid plexus, preventing the entry of its (potentially harmful) substrates in the cerebrospinal fluid (CSF) (not shown, reviewed in (1)).
in various tissues of the ABC transporters discussed in this review is depicted in Fig. 1.

For most ABC transporters, transporter deficient (knockout) mouse models have been generated and these models have been and still are widely used to study the in vivo functions of these efflux pumps. However, due to the extensively overlapping substrate specificities of ABC transporters, it is often difficult to unravel their separate roles and functional overlap using single knockout mice. For example, when one transporter is absent, another one may partly or completely compensate for its loss. As a consequence, often no or only minor effects of the single deletion are seen. Interestingly, a suggestion for overlapping functions of ABC transporters in vivo was recently seen in a clinical study that investigated the protective roles of ABCB1 (P-gp) and ABCG2 (BCRP) during cancer chemotherapy in children (3). This study showed that a combination of two SNP variants of ABCB1 and ABCG2 correlated with increased encephalopathy (brain toxicity), whereas patients with either one of these variants did not suffer from increased toxicity.

To study the overlapping effects of ABC transporters in vivo, a set of mice deficient in two or more ABC transporters, also termed “compound transporter knockout mice”, has been generated and characterized (4-13). This review discusses recent findings obtained with these compound transporter knockout mice. The main focus will be on pharmacokinetic studies with a number of clinically relevant drugs, illustrating that these models are powerful tools to study the separate roles and the functional overlap of ABC transporters. An overview of the generated compound knockout mice and the pharmacokinetic studies performed with these mice so far is given in Table 1. The ABC transporter compound knockout models will be discussed one by one in chronological order. For optimal understanding, the substrate specificity and tissue distribution of the particular ABC transporters will be briefly addressed in each section. In addition, for a number of compound knockout models the characterization and some physiological aspects will be addressed.

Abcb1a/1b-/- mice, the first compound ABC transporter knockout model
P-glycoprotein (P-gp/ABCB1) is the first discovered and most extensively studied member of the mammalian ATP binding cassette (ABC) multidrug transporter family (14). P-gp was discovered in cancer cells where it functions as an efflux pump and extrudes a wide variety of anticancer drugs. In addition to cancer cells, P-gp is also widely expressed in normal tissues, including the epithelial cells of intestine and kidneys and the canalicular membrane of hepatocytes, where it pumps its substrates into feces, urine and bile [Fig. 1; reviewed in (1)]. P-gp has a very broad substrate specificity, including bulky amphiphatic anticancer drugs such as taxanes, anthracyclines and Vinca alkaloids. Consequently, (over)expression of P-gp in tumor cells can lead to multidrug resistance [MDR, (15)]. In contrast to humans, who have one ABCB1 gene coding for P-gp, mice have two, Abcb1a and Abcb1b,
and the products of these genes together appear to fulfill the same functions as the single human ABCB1 (P-gp). To study the in vivo functions of P-gp, Abcb1a<sup>−/−</sup> mice were initially established by inactivation of the Abcb1a gene via homologous recombination (16). Because Abcb1a is expressed at the blood–brain barrier (BBB) and in the intestinal epithelium, whereas Abcb1b is not, Abcb1a<sup>−/−</sup> mice proved to be a valuable tool to elucidate two important physiological functions of P-gp, namely restricting the brain penetration and limiting the oral uptake of potentially harmful compounds (16;17). Shortly after the generation of Abcb1a<sup>−/−</sup> mice, mice lacking Abcb1b were generated, but profound biological effects in this genotype were not observed (4). Therefore, a secondary targeting was performed and by disrupting both Abcb1a and Abcb1b in the same embryonic stem cell chromosome the first ABC transporter compound knockout mouse model was realized (4). Remarkably, Abcb1a/1b<sup>−/−</sup> mice had normal viability, fertility and life span and no apparent physiological abnormalities were observed, despite the complete absence of P-gp (4). Moreover, compound Abcb1a/1b<sup>−/−</sup> mice appeared to be a better model than the single knockout mice, and are still extensively used as a standard model for studies on the roles of P-gp in physiology, pharmacology and toxicology [reviewed in (18;19)]. Furthermore, these mice are also used as the basis for many other compound knockout mice (see below).

**Abcb1a/1b;Abcc1<sup>−/−</sup> mice, the first triple ABC transporter knockout model**

ABCC1 (MRP1) is a versatile efflux transporter that can extrude a wide variety of endogenous and exogenous compounds from cells. In contrast to P-gp, which mainly transports non-conjugated neutral or weakly basic lipophilic substrates, ABCC1 is primarily an organic anion transporter, capable of transporting a broad spectrum of drugs conjugated to glutathione, glucuronic acid and sulfate. In addition, ABCC1 is the major transporter for the endogenous glutathione-conjugate leukotriene C<sub>4</sub> (LTC<sub>4</sub>), an important mediator of the inflammatory response (20;21). Moreover, ABCC1 can also extrude lipophilic and amphipathic xenobiotics from cells as was shown for the anticancer drugs vincristine and etoposide (22;23). A number of experiments suggested that this transport was mediated by co-transport with glutathione. However, the presence of co-transport was later on questioned [reviewed in (24)].

ABCC1 is located basolaterally in polarized cells of many normal tissues, but it also occurs in some unpolarized cells. Expressing tissues include lung, heart, kidney, liver, muscle, colon, testes, bone marrow cells, blood erythrocytes and epithelial cells of the choroid plexus (see also Fig. 1). In addition, ABCC1 is found in tumor cells where it can contribute to multidrug resistance (1). The main function of ABCC1 seems to be the protection of individual cells from accumulation of toxic compounds (1). Two groups independently generated Abcc1<sup>−/−</sup> mice (25;26), and both mutants had normal viability, fertility and life span. However, Abcc1<sup>−/−</sup> mice
had an impaired response to inflammatory stimuli, which could be attributed to decreased secretion of LTC₄ from leukotriene secreting cells (25). Furthermore, tissue levels of glutathione were elevated in tissues that normally express substantial levels of Abcc1 (26). In addition, Abcc1⁻/⁻ mice were found to be hypersensitive to the anticancer drugs etoposide and vincristine (25;26), indicating that Abcc1 plays an important role in drug detoxification. Moreover, absence of Abcc1 resulted in increased etoposide-induced damage to the mucosa of the oropharyngeal cavity and to the seminiferous tubules of the testis (27).

Both groups that had generated Abcc1⁻/⁻ mice crossed these with the previously established Abcb1a/1b⁻/⁻ mice (4) to obtain Abcb1a/1b;Abcc1⁻/⁻ mice (5;6). These triple knockout (TKO) mice were more sensitive to toxicity induced by etoposide and vincristine than Abcb1a/1b⁻/⁻ and Abcc1⁻/⁻ mice (5;6), indicating that P-gp and ABCC1 had additive effects in the protection from toxicity. Importantly, Abcb1a/1b;Abcc1⁻/⁻ mice also allowed investigators to demonstrate a protective function of ABCC1 in the choroid plexus (5). Etoposide was used as a probe drug. In P-gp deficient animals, the brain accumulation of etoposide was substantially higher than in their wild-type counterparts, whereas in Abcc1⁻/⁻ mice brain accumulation was similar to the wild-type situation. However, mice deficient for both P-gp and Abcc1 had about 10-fold higher etoposide concentrations in their cerebrospinal fluid than mice only lacking P-gp. Apparently, absence of P-gp at the BBB allowed the drug to accumulate in the brain, and subsequently the important function of ABCC1 at the blood-cerebrospinal fluid barrier (preventing the accumulation of potentially harmful compounds in the cerebrospinal fluid) could be demonstrated. The latter finding is a nice example illustrating the power of compound transporter knockout models to elucidate novel transporter functions that otherwise would not have been found.

Abcb1a/1b;Abcg2⁻/⁻ mice, a useful tool to study the overlapping roles of P-gp and ABCG2

ABCG2 (BCRP) can transport a broad range of endogenous and exogenous substrates and shares a substantial overlap in substrate specificity with P-gp (1). Furthermore, in contrast to ABCC1, the tissue distribution of ABCG2 is roughly equal to that of P-gp, including expression at apical membranes of excretory organs (Fig.1). Consequently, ABCG2 limits the oral availability and tissue penetration of its substrates and mediates their excretion into bile, feces and urine. In addition, ABCG2 can confer multidrug resistance to tumor cells. Recent work, relying mainly on the use of Abcg2⁻/⁻ mice, has revealed important contributions of ABCG2 to the blood-brain, blood-testis and blood-fetal barriers [reviewed in (28-31)]. Compound Abcb1a/1b;Abcg2⁻/⁻ mice were generated by crossing the established Abcb1a/1b⁻/⁻ (4) and Abcg2⁻/⁻ mice (32). Despite the fact that these mice lack two important apical efflux transporters, normal viability, fertility and life span and no
apparent physiological abnormalities were observed (7). These TKO mice thus seemed amenable for physiological and pharmacological analyses. The first study employing these mice was conducted to establish the respective contributions of P-gp and ABCG2 to the side population (SP) phenotype in mammary gland and bone marrow of mice (7;33). Many tissues contain a SP (or side population) of cells with stem cell characteristics, that can be identified by the ability of these cells to export the dye Hoechst 33342. Both P-gp and ABCG2 had been implicated to be responsible for this Hoechst 33342 export (33;34). By comparing Abcb1a/1b−/−, Abcg2−/− and Abcb1a/1b;Abcg2−/− mice, it was found that Abcg2 is almost exclusively responsible for the SP phenotype in bone marrow, whereas both transporters contributed to the SP in the mammary gland (7). However, it was recently also shown that mouse mammary stem cells are Hoechst 33342 positive, and therefore likely not components of the SP (35;36).

In addition to normal tissues, it has been found that cancer cell lines and primary tumor cells also contain a SP. This has led to the hypothesis that expression of P-gp and/or ABCG2 in cancer stem cells may render these cells multidrug resistant and possibly explains their poor tractability. Although there remains controversy whether the SP is a universal stem cell marker, knowledge of the contribution of P-gp and ABCG2 to drug-resistance of specific tumor SPs may be useful to optimize cancer chemotherapy. Abcb1a/1b;Abcg2−/− mice may be useful to further address these issues.

Abcb1a/1b;Abcg2−/− mice have also been extensively used to study the overlapping functions of P-gp and ABCG2 at the BBB. Like P-gp (described above), ABCG2 is also abundant in the apical membranes of endothelial cells that form the BBB (37). However, in contrast to P-gp, for ABCG2 it was not as straightforward to establish a functional role at the BBB, despite the availability of Abcg2-deficient mice (32;38). In fact, retrospectively, most early studies used shared P-gp and ABCG2 substrates and failed to show higher brain penetration in single Abcg2−/− mice than in their wild-type counterparts [reviewed in (28)]. However, when the brain penetration in compound Abcb1a/1b;Abcg2−/− mice was compared to that in Abcb1a/1b−/− mice, a clear function of Abcg2 at the BBB could be demonstrated. This was first shown for the anticancer drug topotecan, which is a good substrate of ABCG2/Abcg2 and a weaker P-gp substrate. Compared to wild-type mice, the brain-to-plasma AUC ratios were not significantly different in Abcg2−/− mice, whereas these ratios were 2.0-fold higher in Abcb1a/1b−/− mice and 3.2-fold higher in Abcb1a/1b;Abcg2−/− mice (39). Although topotecan appears to be a better substrate for ABCG2 than for P-gp in vitro as well as in the mouse intestine (40), apparently P-gp dominates at the BBB. Nonetheless, when P-gp is absent Abcg2 can partly take over the function of P-gp at the BBB and when both transporters are absent the brain penetration is highly increased. This clearly shows that, in addition to P-gp, Abcg2
also has a functional role at the BBB in restricting the entry of topotecan to the brain.

Using these compound knockout models, qualitatively similar results regarding brain penetration were recently obtained for the tyrosine kinase inhibitors imatinib, lapatinib and dasatinib (41-43). Although these three compounds are all good \textit{in vitro} substrates for P-gp and ABCG2, a contribution of Abcg2 in restricting the brain penetration was only observed in \textit{Abcb1a/1b;Abcg2}\textsuperscript{2/-} mice, i.e. when P-gp is absent too. A possible explanation for this apparent discrepancy could be that Abcg2 expression at the BBB is lower than that of P-gp. Indeed, it was recently shown in mice of a ddy background that P-gp protein levels in brain capillaries were about 3-fold higher than protein levels of Abcg2 (44). This suggests that P-gp is the dominant player at the murine BBB and might explain why only when P-gp is absent the contribution of Abcg2 becomes visible. It must be noted, however, that the above described experiments on the brain penetration of tyrosine kinase inhibitors (41-43) were performed in mice of an FVB background and it was previously shown that the expression of ABC transporters at the murine BBB can differ dramatically between mouse strains (45). In addition, we checked the RNA expression of Mdr1a P-gp in the brain of \textit{Abcg2}\textsuperscript{2/-} mice, but found no difference compared to wild-type mice (Lagas et al, unpublished results). The relative contribution of Abcg2 at the BBB thus seems not to be underestimated by an increased expression of P-gp at the BBB of FVB \textit{Abcg2}\textsuperscript{2/-} mice.

Interestingly, examination of human brain capillaries revealed that mRNA levels of ABCG2 were about 8-fold higher than P-gp mRNA levels (46). It should be noted that the brain capillaries in that study were isolated from 7 patients who all suffered from brain disease (epilepsia or glioma), which might affect the RNA expression levels. However, there was more ABCG2 mRNA than ABCB1 mRNA in the microvessels from all patients, regardless of their pathology or treatment (46). If RNA levels correspond relatively well with protein levels of both transporters at the BBB, these studies might indicate that there are species differences in the relative expression of P-gp and ABCG2 at the BBB. In that case, the contribution of ABCG2 at the human BBB might be more important than thought thus far.

Although in mice P-gp thus seems to dominate at the BBB, we recently observed that the brain penetration of orally administered sorafenib, another tyrosine kinase inhibitor, was 4.3-fold increased in \textit{Abcg2}\textsuperscript{2/-} mice, not altered in \textit{Abcb1a/1b}\textsuperscript{2/-} mice and 9.3-fold higher in \textit{Abcb1a/1b;Abcg2}\textsuperscript{2/-} mice (Lagas et al, unpublished results). In contrast, for orally administered dasatinib we previously found that Abcg2 deficiency did not affect the brain penetration, whereas absence of P-gp resulted in a 3.6-fold increase and \textit{Abcb1a/1b;Abcg2}\textsuperscript{2/-} mice had 13.2-fold higher brain penetration (43). This discrepancy might simply be explained by the fact that sorafenib is a good Abcg2 substrate \textit{in vitro}, but a very poor P-gp substrate (Lagas et al, unpublished results).
Table 1. Impact of different ABC transporters on pharmacokinetics (PK) of drugs and endogenous substrates as analyzed using ABC transporter compound knockout mice.

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<tr>
<td>Etoposide</td>
<td>Abcb1a/1b;Abcc1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>P-gp restricts the brain penetration. Abcc1 limits the accumulation in the cerebrospinal fluid. Both transporters protect against etoposide induced toxicity.</td>
<td>5, 6</td>
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<tr>
<td>Topotecan</td>
<td></td>
<td>P-gp mediates the elimination and restricts the brain penetration. Abcg2 limits the oral uptake and mediates the elimination. Abcg2 also restricts the brain penetration when P-gp is absent.</td>
<td>39</td>
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<td>Imatinib</td>
<td>Abcb1a/1b;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>P-gp mediates the fecal excretion and restricts the brain penetration. Abcg2 has a minor impact on the fecal excretion, but partially limits the brain penetration when P-gp is absent.</td>
<td>41</td>
</tr>
<tr>
<td>Dasatinib</td>
<td></td>
<td>P-gp limits the oral availability and restricts the brain penetration. Abcg2 partially restricts the brain penetration when P-gp is absent.</td>
<td>43</td>
</tr>
<tr>
<td>Lapatinib</td>
<td></td>
<td>P-gp restricts the brain penetration. Abcg2 partially restricts the brain penetration when P-gp is absent.</td>
<td>42</td>
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<tr>
<td>Sorafenib</td>
<td></td>
<td>Abcg2 restricts the brain penetration. P-gp partially restricts the brain penetration when Abcg2 is absent.</td>
<td>Lagas et al., unpublished results</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Abcb1a/1b;Abcc2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>P-gp is the main transporter for the biliary excretion. Abcc2 has a modest impact on biliary excretion, but can partly compensate for the absence of P-gp.</td>
<td>52</td>
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<tr>
<td>Paclitaxel</td>
<td></td>
<td>P-gp limits the oral uptake and facilitates the direct intestinal excretion. Abcc2 is the main transporter for the biliary excretion, and has an impact on the oral PK when P-gp is absent. Both transporters equally affect the iv PK.</td>
<td>8</td>
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<tr>
<td>PMEA</td>
<td>Abcc4;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc4 restricts the accumulation in the spleen. Abcc4 further restricts the liver, kidney and heart accumulation when Abcg2 is absent. Abcg2 restricts the liver, kidney, ovary and brain accumulation.</td>
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<tr>
<td>Methotrexate</td>
<td>Abcc2;Abcc3&lt;sup&gt;−/−&lt;/sup&gt; and Abcc2;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc2 is the main transporter for biliary excretion. Abcg2 mediates the biliary and urinary excretion when Abcc2 is absent. Abcc3 mediates sinusoidal excretion from the liver when Abcc2 is absent.</td>
<td>12, 70, Vlaming et al., unpublished results</td>
</tr>
<tr>
<td>7-Hydroxymethotrexate</td>
<td>Abcc2;Abcc3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc2 is the main transporter for biliary excretion. Abcg2 mediates the biliary and urinary excretion when Abcc2 is absent. Abcc3 mediates the sinusoidal excretion from the liver when Abcc2 is absent.</td>
<td></td>
</tr>
<tr>
<td>Morphine-3-glucuronide</td>
<td>Abcc2;Abcc3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc2 is the main transporter for biliary excretion. Abcc3 mediates the sinusoidal excretion from the liver when Abcc2 is absent.</td>
<td>11</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>Abcc2;Abcc3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc2 mediates the biliary excretion. Abcc3 mediates the sinusoidal excretion from the liver.</td>
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This further illustrates that for each substrate the interactions with ABC transporters in the BBB are unique, making it difficult to draw general conclusions on the pharmacological impact of ABC transporters.

Taken together, in addition to \textit{Abcb1a/1b}\textsuperscript{−/−} and \textit{Abcg2}\textsuperscript{−/−} mice, \textit{Abcb1a/1b};\textit{Abcg2}\textsuperscript{−/−} mice have proven to be a valuable tool to study the separate and combined impact of P-gp and ABCG2 at the BBB.

\textit{Abcb1a/1b};\textit{Abcc2}\textsuperscript{−/−} mice, a model to study hepatic versus intestinal elimination

Like P-gp and ABCG2, ABCC2 (MRP2) is expressed at the apical membranes of epithelial cells in kidney and intestine and at the canalicular membrane of hepatocytes (Fig. 1). Consequently, ABCC2 plays an important role in the hepatobiliary and renal excretion of its substrates, but, in contrast to P-gp and ABCG2, its contribution in restricting uptake of compounds from the intestine seems limited (1). ABCC2 was long thought to mainly affect organic anionic drugs \textit{in vivo}, with a preference for substrates conjugated to glutathione, glucuronic acid and sulfate [reviewed in (47;48)]. Studies in two rat strains that naturally lack \textit{Abcc2} (EHBR and TR\textsuperscript{−}) and in humans suffering from Dubin-Johnson syndrome (a hereditary deficiency in ABCC2) revealed that ABCC2/Abcc2 plays an important role in the elimination of bilirubin glucuronides from hepatocytes into the bile (49-51). Recent work has shown that ABCC2 can also transport bulky amphipathic anticancer drugs \textit{in vivo} (8;52), and ABCC2 thus has a substantial overlap in substrate specificity with P-gp. Recently, we and others independently generated \textit{Abcc2}\textsuperscript{−/−} mice (52;53), and we additionally crossed our \textit{Abcc2}\textsuperscript{−/−} mice with \textit{Abcb1a/1b}\textsuperscript{−/−} mice (4) to obtain compound \textit{Abcb1a/1b};\textit{Abcc2}\textsuperscript{−/−} mice (8;52). Similar to single \textit{Abcc2}\textsuperscript{−/−} mice, compound \textit{Abcb1a/1b};\textit{Abcc2}\textsuperscript{−/−} mice had a ~25\% increased liver weight, and the bile flow was reduced by 40\% to 50\% due to absence of Abcc2-mediated biliary glutathione excretion (52). Furthermore, as a consequence of reduced biliary excretion of conjugated bilirubin caused by Abcc2-deficiency (52), conjugated bilirubin concentrations in plasma were ~3-fold elevated, compared to wild-type mice (8). Overall, \textit{Abcb1a/1b};\textit{Abcc2}\textsuperscript{−/−} mice appear in many respects very similar to \textit{Abcc2}\textsuperscript{−/−} mice and are likely as amenable to pharmacological analysis. We used the \textit{Abcb1a/1b};\textit{Abcc2}\textsuperscript{−/−} mice to study the separate and combined impact of P-gp and Abcc2 on the elimination of the lipophilic amphipathic anticancer drugs doxorubicin (52) and paclitaxel (8). The hepatobiliary excretion of doxorubicin was mainly dependent on P-gp, with a modest role for Abcc2 (52). In contrast, rather surprisingly, the excretion of paclitaxel into the bile was dominated by Abcc2, with a very minor contribution of P-gp (8). The abrogated biliary excretion of paclitaxel in \textit{Abcc2}\textsuperscript{−/−} mice resulted in a 1.3-fold higher area under the plasma concentration-time curve (AUC) upon intravenous paclitaxel administration. Interestingly, the \textit{AUC}_{i.v.} for paclitaxel in \textit{Abcb1a/1b}\textsuperscript{−/−} mice was also 1.3-fold higher. This could be explained by the dominant role of P-gp in the gut, where it mediates the direct
intestinal excretion of paclitaxel from the blood across the intestinal wall into the gut lumen. Very likely it also restricts the intestinal re-uptake of paclitaxel after hepatobiliary secretion of the drug in the gut. Moreover, absence of both transporters resulted in an additive 1.7-fold higher AUC\textsubscript{i,v.} in \textit{Abcb1a/1b;Abcc2\textsuperscript{-/-}} mice [reviewed in (54)]. These studies demonstrate the power of this compound transporter knockout model to elucidate the tissue specific contribution as well as the separate and combined impact of P-gp and ABCC2 on the elimination of lipophilic amphipathic drugs.

\textit{Abcc4;Abcg2\textsuperscript{-/-}} mice, a model to study the impact of ABCC4 and ABCG2 on tissue accumulation of shared substrates

ABCG2 (BCRP) and ABCC4 (MRP4) are both expressed in liver and kidney, as well as in tissue sanctuaries such as brain, testis, prostate and ovary [Fig.1; (9,28;54)]. As described above, ABCG2 is present in the apical membranes of epithelial cells, pumping its substrates into bile, urine and feces (1). ABCC4 is expressed apically in brain capillaries and kidney proximal tubules, but in liver, prostate, urogenital tissues and choroid epithelial cells it is localized at the basolateral membrane, pumping its substrates into the circulation (54). In \textit{Abcc4\textsuperscript{-/-}} mice, Abcg2 expression is increased in spleen and brain, whereas thymus and spleen from \textit{Abcg2\textsuperscript{-/-}} mice had increased \textit{Abcc4\textsuperscript{-/-}} expression (9), suggesting compensatory changes in these tissues when either one of the transporters is absent.

Abcc4 and Abcg2 have broad and substantially overlapping substrate specificities. They can both confer resistance to many (antiviral and anti-cancer) drugs such as PMEA (9-(2-(phosphonomethoxy)ethyl)-adenine) (9;55), camptothecin analogs (e.g. topotecan and irinotecan (56-58)), and methotrexate (59;60). Besides various drugs, Abcc4 and Abcg2 also transport endogenous compounds, such as cGMP (10) and steroid conjugates (61;62).

Recently, two research groups independently generated \textit{Abcc4;Abcg2\textsuperscript{-/-}} mice (in a mixed C57BL6;129SVJ and a mixed 129Ola/BL6;FVB background, respectively) (9;10). Both strains were viable and fertile. Clinical chemistry and hematologic analysis of the \textit{Abcc4;Abcg2\textsuperscript{-/-}} mice in C57BL6;129SVJ background did not reveal any specific aberrations due to absence of both transporters (9), suggesting that Abcc4 and Abcg2 do not have any vital, overlapping physiological functions. However, these mice appeared very useful to investigate the overlapping pharmacological functions of Abcc4 and Abcg2. Intravenous administration of the purine nucleoside phosphonate analogue $[^3$H]PMEA to \textit{Abcc4\textsuperscript{-/-}} mice resulted in accumulation in the spleen but not in other tissues, suggesting that Abcc4 plays a modest role in limiting the tissue accumulation of PMEA. In contrast, in \textit{Abcg2\textsuperscript{-/-}} mice $[^3$H]PMEA accumulated in liver, kidney, brain and ovary, showing a significant effect of Abcg2 on the tissue accumulation of the drug. In \textit{Abcc4;Abcg2\textsuperscript{-/-}} mice, however, $[^3$H]PMEA concentrations in liver, kidney and heart were even more
increased than in $Abcg2^{-/-}$ mice (9). This suggests that when Abcg2 is absent, Abcc4 can also reduce $[^3H]$PMEA concentrations in these tissues, although it cannot completely compensate for the absence of Abcg2. On the other hand, $[^3H]$PMEA concentrations in the spleen were similarly increased in $Abcc4^{-/-}$ and $Abcc4;Abcg2^{-/-}$ mice, indicating that Abcg2 does not affect PMEA concentrations in this organ. This first pharmacological experiment employing $Abcc4;Abcg2^{-/-}$ mice shows that these are a valuable tool to elucidate the overlapping functions of both transporters in the pharmacokinetics of shared substrates. Therefore, it will be interesting to study the pharmacokinetics of additional common substrates in these mice.

$Abcc2;Abcc3^{-/-}$ mice, a useful tool to study the complementary functions of $ABCC2$ and $ABCC3$ in the liver

The ABC transporters ABCC2 (MRP2) and ABCC3 (MRP3) are both family members of the multidrug resistance protein (MRP) family and have similar structure and substrate specificities (48). They can both transport a range of physiological substrates, such as bilirubin glucuronides, estradiol-17β-glucuronide and some bile salts (48). Furthermore, ABCC2 and ABCC3 can transport many drugs, such as anthracyclines, epipodophyllotoxins and methotrexate, as well as a range of drug conjugates, in particular drug glucuronides such as morphine glucuronide (11;48).

The tissue distribution of ABCC3 is quite similar to that of ABCC2 [Fig.1; (1)]. ABCC3 is additionally present in the adrenal glands and pancreas [Fig.1; (48)]. In contrast to the apical localization of ABCC2, ABCC3 is expressed basolaterally, pumping its substrates towards the circulation (1;48). Interestingly, in Abcc2/ABCC2-deficient mice, rats and humans, Abcc3/ABCC3 protein expression was significantly increased in the liver. This suggests a compensatory role of Abcc3/ABCC3 when Abcc2/ABCC2 is absent (52;63;64), transporting substrates that cannot be excreted into the bile back into the circulation, and hence leading to increased urinary excretion of these compounds.

Recently, $Abcc2;Abcc3^{-/-}$ mice have been generated, both in C57BL/6 and FVB background (11-13) and they have been used to study the overlapping physiological and pharmacological functions of both transporters in vivo. Physiological characterization of these mice in both backgrounds showed that $Abcc2;Abcc3^{-/-}$ had normal life spans and body weights, but that the liver weights were significantly (36-49%) increased compared to wild-type mice (12;13), although the livers appeared normal, both macroscopically and microscopically (12). It could be that accumulation of shared Abcc2 and Abcc3 substrates in the liver induces liver proliferation. Furthermore, in $Abcc2;Abcc3^{-/-}$ mice of both backgrounds, the bile flow was significantly decreased compared to wild-type (12;13), as shown previously for $Abcc2^{-/-}$ mice (52;53).
It was previously hypothesized that (increased) Abcc3 protein in liver of Abcc2-deficient rats, mice and humans was, in combination with the decreased biliary clearance, responsible for the increased plasma levels and urinary excretion of conjugated bilirubin (52;63). Analysis of bilirubin concentrations in plasma, bile and urine of the Abcc2;Abcc3−/− mice indeed showed that Abcc3 in the liver of Abcc2/− mice was necessary for the increased sinusoidal efflux of bilirubin glucuronides, and their increased plasma concentrations and urinary excretion (12). Similar observations using Abcc2;Abcc3−/− mice were made for the shared substrate drugs or drug metabolites methotrexate (12), 7-hydroxymethotrexate (12), morphine-3-glucuronide (11) and fexofenadine (13).

Administration of methotrexate to Abcc2;Abcc3−/− mice further led to significantly increased liver concentrations of methotrexate and its toxic metabolite 7-hydroxymethotrexate, which was not seen (or only to a minor extent) in the single knockout strains (12). Also treatment of the Abcc2;Abcc3−/− mice with morphine led to dramatic accumulation of its metabolite morphine-3-glucuronide (but not morphine itself) which was not found in the single knockout strains (11). This shows that Abcc2 and Abcc3 together are very important for reducing liver exposure of potentially toxic compounds, and that when one of them is absent or reduced, the other can (at least partly) compensate for this deficiency.

It was previously shown that co-administration of morphine to methotrexate-treated mice significantly reduced plasma clearance of methotrexate (65), which is of clinical interest because morphine and methotrexate are often co-administered in cancer treatment. The results obtained with the Abcc2;Abcc3−/− mice described above suggest that these effects could be caused by competition between methotrexate and morphine-3-glucuronide for elimination via Abcc2 and Abcc3. This would suggest that co-administration of these drugs to patients with reduced expression or activity of ABCC2 and/or ABCC3, or to Dubin-Johnson patients (66), should be done with caution. Overall, Abcc2;Abcc3−/− mice have already proven to be useful models for studying the overlapping and compensatory roles of Abcc2 and Abcc3 in vivo.

Abcc2;Abcg2−/− mice, a model to study the functional overlap of ABCG2 and ABCC2 in hepatobiliary excretion
As described above, the tissue distributions of ABCC2 and ABCG2 are quite similar (Fig. 1), and the substrate specificities of ABCC2 and ABCG2 are broad and substantially overlapping as well. Both proteins can transport many drugs, including anti-cancer drugs like methotrexate, doxorubicin and SN-38, as well as dietary toxins such as the carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and a range of glucuronide and sulphate conjugates of endogenous and exogenous compounds (1;68;69).
The substantial overlap in substrate specificity and tissue distribution suggests that ABCC2 and ABCG2 are able to compensate for each other when one of the two proteins is absent or non-functional. Functions of transporters assessed with single knockout mice may therefore be obscured due to activity of the other transporter, which is still present. To investigate this, we have recently generated Abcc2;Abcg2⁻/⁻ mice in 99% FVB background (70). Like the other ABC transporter compound knockout mice generated so far, these mice are viable and fertile and do not display any phenotypes other than what was seen previously in the single knockout mice, such as the hypersensitivity to the phototoxic dietary compound pheophorbide a of Abcg2⁻/⁻ mice and the conjugated hyperbilirubinemia of Abcc2⁻/⁻ mice (32;52). This suggests that the physiological functions of ABCC2 and ABCG2 are not overlapping, or may still be taken over by other systems such as enzymes or other transporters.

Because wild-type mice of an FVB background, in contrast to other genetic backgrounds, do not have Abcc2 protein in brain capillary endothelial cells, i.e., at the BBB (46), the Abcc2;Abcg2⁻/⁻ mice we have generated cannot be used to investigate overlapping functions of Abcc2 and Abcg2 in the BBB. However, we did use these mice to investigate the effect of Abcc2 and Abcg2 on the disposition of the anti-cancer drug methotrexate and its main (toxic) metabolite 7-hydroxymethotrexate in vivo (70). We found that Abcc2 and Abcg2 have additive effects on the plasma elimination of methotrexate, which was mainly caused by their impact on the biliary excretion of the drug. Whereas in both single knockout strains still substantial biliary excretion was present, in the double knockouts this was almost completely abolished, showing that Abcc2 and Abcg2 in mice are the main transporters for the excretion of methotrexate into the bile. Interestingly, in Abcg2⁻/⁻ mice, we found no differences in the plasma concentration-versus-time curves for the toxic methotrexate metabolite 7-hydroxymethotrexate. However, compared to Abcc2⁻/⁻ mice, an additional effect of Abcg2 absence on the plasma concentrations was found in Abcc2;Abcg2⁻/⁻ mice, indicating that when Abcc2 is absent, Abcg2 can partly compensate for its loss. This clearly illustrates the value of these compound transporter knockout mice to determine the relative impacts of both transporters on the elimination of shared substrates from the body. We therefore expect that Abcc2;Abcg2⁻/⁻ mice will be extensively used to determine the in vivo effects on pharmacokinetics of known, but also of newly discovered drugs which are substrates of both transporters.

CONCLUSIONS

In the past few years, a large set of ABC transporter compound knockout mice have been generated and used for the analysis of overlapping effects of these proteins in vivo. The results obtained will be helpful to determine the consequences of reduced expression or activity of ABC transporters in patients treated with potentially toxic
drugs. Furthermore, as ABC transporters can also transport endogenous compounds and food-derived toxins (e.g. carcinogens), these mice can be used to investigate the relative effects of ABC transporters on normal health. For example, ABC transporter (compound) knockout mice may be used to study whether loss of functional activity of one or more ABC transporters can influence the chance to develop cancer. Also studies on the overlapping or complementary effects of ABC transporters in multidrug resistance could be performed in vivo using these compound knockout strains.

The results obtained so far have shown that the relative effect of each ABC transporter on drug pharmacokinetics can be highly dependent on the substrate, administration route and the tissue or organ under investigation. Very likely, also the given dose determines which ABC transporter is more important for the pharmacokinetics of the drug. It is therefore very difficult to use in vitro assays to predict the in vivo effects of ABC transporters, and compound knockout mice are therefore invaluable tools for these types of studies.

So far, mainly combinations of two ABC transporters have been deleted simultaneously in mice. Because various drugs and toxins, as well as their metabolites, can be transported by more than two ABC transporters, it is obvious to extend the current set of models with triple, quadruple or even higher order compound knockout strains. Furthermore, crossing ABC transporter knockout mice with knockout models of other drug elimination mechanisms, such as drug-metabolizing enzymes, will give more insight into the interplay between these different systems in vivo.

Of course, as ABC transporters are involved in protection of the organism from endogenous and exogenous toxins, it will be interesting to see how many additional ABC transporter genes can be deleted without causing serious health problems to mice. Besides increasing fundamental knowledge on ABC transporter function, this is of interest because attempts are made to improve drug response in patients by inhibition of one or more ABC transporters (71,72). We have recently even been able to generate Abcb1a/b;Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice which are viable, fertile and have normal life spans (Vlaming et al., unpublished results). This suggests that the physiological functions of these proteins are not essential, at least not in the protective environment of the lab. In the near future, these strains can be used for pharmacological analyses. Further investigation of the generated ABC transporter compound knockout mice will likely reveal more physiological and pharmacological functions of ABC transporters, and help to improve treatment of patients with drugs of which the efficacy and toxicity are influenced by ABC transporters.
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Chapter 2

Carcinogen and anti-cancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice


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Carcinogen and anti-cancer drug transport by Mrp2

in vivo: studies using Mrp2 (Abcc2) knockout mice

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The ABC transporter MRP2 (ABCC2) forms a natural barrier and efflux system for various (conjugates of) drugs, other xenotoxins and endogenous compounds. To obtain insight in the pharmacological and physiological functions of Mrp2, we generated Mrp2 knockout mice, which were viable and fertile but suffered from mild hyperbilirubinemia due to impaired excretion of bilirubin monoglucuronides into bile. The mice also had an 80-fold decreased biliary glutathione excretion and a 63% reduced bile flow. Levels of Mrp3 (Abcc3) in liver and Mrp4 (Abcc4) in kidney of Mrp2⁻/⁻ mice were about 2-fold increased. After oral administration of the food-derived carcinogens [¹⁴C]PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and [¹⁴C]IQ (2-amino-3-methylimidazo[4,5-f]quinoline) plasma values were 1.9-fold and 1.7-fold higher in Mrp2⁻/⁻ mice vs. wild-type mice respectively, demonstrating the role of Mrp2 in restricting exposure to these compounds. At a high dose of 50 mg/kg of the drug [³H]methotrexate, the plasma AUC for i.v. administration was 1.8-fold higher in Mrp2⁻/⁻ mice (1345 ± 207 min·µg/ml vs. 734 ± 81 min·µg/ml). No clear plasma concentration difference arose at low dose (1 mg/kg). Subsequently Mdr1a/b/Mrp2 knockout mice were generated. Their biliary excretion of doxorubicin after i.v. administration (5 mg/kg) was 54-fold decreased (0.32 ± 0.13 nmol/gr liver vs. 17.30 ± 6.59 nmol/gr liver in wild-type), and a role for both Mdr1a/b and Mrp2 in this process was revealed. Our results demonstrate that the Mrp2⁻/⁻ mouse provides a valuable tool for studies of the impact of Mrp2 on behavior of drugs and other toxins, especially when combined with other ABC transporter knockout mice.

Introduction

The multidrug transporter MRP2 (ABCC2, cMOAT), member of the ATP-binding-cassette (ABC) superfamily, confers resistance to a range of anti-cancer drugs (1,2). The protein is mainly present in the apical membranes of polarized cells in liver, small intestine and kidney and mediates active transport of both endogenous and
xenobiotic compounds to bile, urine or feces (2). MRP2 is functionally deficient in patients with the Dubin-Johnson syndrome (3), in TR rats (4) and EHBRS (Eisai hyperbilirubinemic rats) (5), which all show impaired secretion of bilirubin glucuronides into the bile and as a consequence suffer from conjugated hyperbilirubinemia (3-5). The mutant rat strains also show substantially reduced biliary excretion of glutathione and glutathione conjugates (6).

Besides its role in transport of endogenous compounds, MRP2 plays an important role in the transport of various drugs and their metabolites. MRP2 is expressed in many solid human tumors originating from kidney, colon, breast, lung and ovary (7), and it was shown that the protein actively transports many types of anti-cancer and other drugs like vinca alkaloids, anthracyclines, protease inhibitors, antibiotics, etoposide, cisplatin, methotrexate and irinotecan in vitro (1,2,8,9). Mrp2 is involved in vivo in the excretion of methotrexate and irinotecan and its metabolites into the bile, as was demonstrated in Mrp2 deficient rats (10,11). Experiments with Mrp2 deficient rats indicate that the protein furthermore plays a role in reducing the oral availability and biliary and intestinal excretion of the heterocyclic amine PhIP, a food-derived carcinogen (12,13).

To obtain a more complete view of the relative role of the various ABC transporters that perform related functions in vivo, it will be of great interest to establish the precise role of Mrp2 in physiology and pharmacology of drugs and other toxic compounds. The in vivo roles of other ABC transporters, such as P-gp (Abcb1), Bcrp1 (Abcg2), Mrp1 (Abcc1) and Mrp3 (Abcc3) have been studied using previously generated knockout mice for these proteins (14-18). MRP2 shows substantial overlap in substrate specificity with these members of the ABC transporter family (1,2). Mrp2 knockout mice will therefore be very useful tools to study the in vivo role of Mrp2 and compare this with that of other ABC transporters. Importantly, the existence of Mrp2 knockout mice allows generation of compound knockout mice by crossing this strain with the other ABC transporter knockout strains, permitting systematic analysis of the relative role of each ABC transporter in physiology, drug resistance, and pharmacokinetics of drugs and xenotoxins.

In the present study we generated and subsequently characterized Mrp2−/− mice by analysis of basic physiological parameters, comparing our results with previous results from mutant rats and humans deficient in Mrp2. We used the generated Mrp2−/− mice to analyze the effect of murine Mrp2 deficiency on in vivo behavior of the anti-cancer drugs methotrexate and irinotecan and of the dietary carcinogens PhIP and IQ. The Mrp2−/− mice were subsequently crossed with Mdr1a/b−/− mice to determine the relative roles of both ABC transporters in the biliary excretion of the anti-cancer drug doxorubicin.
Materials and Methods

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The animals used were Mrp2−/− and wild-type mice of a comparable mixed genetic background (approximately 50% 129/Ola, 50% FVB), between 9-14 weeks of age. For the doxorubicin experiment Mrp2−/− mice were backcrossed to 99% FVB background and subsequently crossed with Mdr1a/b−/− mice to obtain Mdr1a/1b/Mrp2 triple knockout mice (19). 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. [3H]MTX (7.0 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). PhIP, [14C]PhIP (10 Ci/mol), IQ and [14C]IQ (10 Ci/mol) were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Doxorubicin-HCl (Doxorubicin 0.2%), irinotecan (Campto® 20 mg/ml) and MTX (Emthexate PF® 25 mg/ml) were obtained from Pharmachemie (Haarlem, The Netherlands). Ketamine was obtained 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). M2III-5 was a kind gift of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands). Sf-9 vesicles containing MRP4, C219, MRPr1, M3-18 and M3-I-10 were kind gifts of Dr. N. Zelcer and Dr. K. van de Wetering in our institute. BXP-53 was described before (15).

Generation of Mrp2−/− and Mdr1a/b/Mrp2 knockout mice. Using Mrp2 cDNA probes, a 129/Ola mouse genomic sequence containing exons 3-7 of Mrp2 was identified (Fig. 1A). A 1.7 kb fragment containing exons 4-6 was deleted and replaced by a 2.0 kb pgk-hygro cassette in reverse transcriptional orientation. Deletion of exons 4-6 results in removal of amino acids 111 to 209, encoding the transmembrane (TM) regions 3-5 and part of the large intracellular loop between TM regions 5 and 6, and in a frame shift. Electroporation of the targeting construct and selection for recombinant E14 embryonic stem cells was done as described (20). Of 262 hygromycin-resistant clones, 14 were targeted correctly as judged by Southern analysis of BclII-digested genomic DNA with 3’ and 5’ Mrp2 probes (Fig. 1A). The absence of additional pgk-hygro cassettes elsewhere in the genome was confirmed by hybridization with a hygro-specific probe. Chimeric mice were generated by microinjection of two independently targeted embryonic stem cell clones into blastocysts. Chimeric offspring were backcrossed to FVB mice. By using this approach, two independent Mrp2−/− mouse lines of a mixed genetic background (approximately 50% 129/Ola, 50% FVB) were established. One of these Mrp2−/− mouse lines was backcrossed to 99% FVB background and subsequently crossed
with Mdr1a/b<sup>−/−</sup> mice (16) to obtain Mdr1a/b/Mrp2 triple knockout mice of a 99% FVB background.

**Bile flow measurements and analysis.** Gall bladder cannulations in male wild-type and Mrp2<sup>−/−</sup> mice (n = 5 for each group) were performed as described (21). Bile of the mice was collected in 15 min fractions in pre-weighed tubes for 60 min. At the end of the experiment, blood was collected by cardiac puncture. Also, urine of the mice was collected. Bile flow was determined by weighing the collected bile samples, assuming a density of 1.0 g/ml. All samples were frozen immediately and stored at -80°C. Concentrations of bilirubin monoglucuronides, bilirubin diglucuronides and unconjugated bilirubin in bile and urine were determined as described (22). GSH in the bile samples was determined as described (23).

**Pharmacokinetic experiments.** For oral administration of [<sup>14</sup>C]PhIP and [<sup>14</sup>C]IQ, 5 µl/g body weight of a 0.2 mg/ml [<sup>14</sup>C]PhIP or [<sup>14</sup>C]IQ solution in 20% (v/v) DMSO and 5% (w/v) D-glucose was dosed by gavage into the stomach of female mice. After 30 min, blood samples were collected by cardiac puncture and levels of radioactivity were determined by scintillation counting. [<sup>3</sup>H]MTX was i.v. administered by injecting 5 µl of drug solution (appropriate concentration in saline)/g body weight into the tail vein of female mice lightly anesthetized with methoxyflurane. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and organs were removed and homogenized in 4% BSA using a Polytron homogenizer. Intestinal contents (feces) were separated from the intestinal tissue. Levels of radioactivity in homogenized organs, feces and blood were determined by liquid scintillation counting. For i.v. administration of irinotecan a 2 mg/ml drug solution in 5% (w/v) D-glucose was prepared and 5 µl/g body weight was injected into the tail vein of female mice lightly anesthetized with methoxyflurane. Blood samples were collected by cardiac puncture under methoxyflurane anesthesia and livers were isolated. Of each plasma sample (250 µl) obtained after centrifugation at 5,000 rpm for 5 min at 4°C, 50 µl was frozen in dry ice immediately after harvesting and used for determination of the total irinotecan and SN-38 concentrations in the blood. The remaining 200 µl of the plasma was used for the separate determination of lactone and carboxylate forms of irinotecan and SN-38 and was processed as described (24). Livers were homogenized in human plasma (5 ml/liver) and frozen in liquid nitrogen. Samples were stored at -80°C until HPLC analysis was performed. Measurements of total irinotecan and SN-38 and the lactone and carboxylate forms of irinotecan and SN-38 were based on the HPLC-technology as described previously (24). Doxorubicin-HCl was administered i.v. to female mice by injecting 2.5 µl/g body weight of a 2 mg/ml solution directly after gall bladder cannulation, which was performed as described (21). Bile was collected for 60 minutes and mice were subsequently killed by terminal bleeding through
cardiac puncture. Plasma samples, liver, small intestine and small intestinal contents were collected and stored at –80°C until HPLC analysis, which was performed as described (25).

**Clinical-chemical analysis of plasma.** Standard clinical chemistry analyses on plasma were performed on a Hitachi 917 analyzer to determine levels of total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine, urea, Na⁺, K⁺, Cl⁻, Ca²⁺, phosphate, total protein and albumin.

**Hematological analysis.** Hemoglobin, hematocrit, mean corpuscular volume, red blood cells, white blood cells, lymphocytes, monocytes, granulocytes and platelets were determined in EDTA blood on a Beckman Coulter Ac-T Diff analyzer. This analysis was performed 2 times within a time span of 2 months.

**Western analysis.** Crude membrane fractions from tissues were prepared as described (26). Western blotting was performed as described (15), the ECL signal was detected by film. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). In the dilution series for Mrp3 and Mrp4 expression, both Ponceau S and India ink staining of the membranes could readily reveal 2-fold protein loading differences. P-gp was detected with mAb C219 (dilution 1:1000) and Mrp1 with mAb MRPr1 (1:1000). For detection of Mrp2 the primary antibody M₂III-5 was used (dilution 1:50). Mrp3 and Mrp4 were detected with mAbs M₃-18 (dilution 1:50) and M₄I-10 (dilution 1:5) respectively. Bcrp1 was detected using the mAb BXP-53 (dilution 1:400). The primary antibodies were detected by incubating the blot either with rabbit anti-mouse IgG (for M₂III-5 and C219) or rabbit anti-rat IgG (for MRPr1, M₃-18, M₄I-10 and BXP-53) (both 1:1000, DAKO).

**Histological analysis.** Tissues were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Sections were cut at 4 µm from the paraffin blocks and stained with hematoxylin and eosin according to standard procedures. The sections were examined by a mouse pathologist.

**Pharmacokinetic calculations and statistical analysis.** The two-sided unpaired Student's t-test was used throughout the study to assess the statistical significance of differences between two sets of data. Results are presented as the means ± standard deviations (SD). Differences were considered to be statistically significant when \( P < 0.05 \). Averaged concentrations for each time-point were used to calculate the area under the plasma concentration versus time curve (AUC) from \( t = 0 \) to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated.
by the law of propagation of errors (27). Results of AUC measurements are presented as means ± standard error of the mean (SEM).

Figure 1. Generation and validation of Mrp2<sup>−/−</sup> mice. A, in the targeting construct, a 1.7 kb fragment containing exons 4-6 was deleted and replaced with an inverted 2.0 kb Pgk-hygro cassette. Only relevant restriction sites are indicated and only part of the Mrp2 gene is shown. For Southern analysis, 5' and 3' probes as indicated were used on BclI digested genomic DNA. Exons are indicated by closed boxes (exact sizes of exons are not drawn to scale) and the sizes of diagnostic restriction fragments for wild-type and targeted alleles are indicated by double-headed arrows (drawn to scale). B, detection of Mrp2 protein in crude membrane fractions of Mrp2<sup>−/−</sup> and wild-type mouse liver samples (10 µg/lane). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. Lysates of MDCKII cells expressing human MRP2 (Evers et al., 1998) (10 µg) were used as a positive control.
Results

**Generation and analysis of Mrp2−/− mice.** Mrp2 knockout mice were generated as outlined in Materials and Methods and Fig. 1A. Complete absence of Mrp2 protein in Mrp2−/− mice was confirmed with Western blot analysis using M2III-5, an antibody that binds to an epitope in amino acids 1339-1541 at the C-terminus of rat Mrp2 (Fig. 1B). Mrp2−/− mice were fertile and had normal life spans and body weights. Crosses of Mrp2−/− mice yielded progeny with all Mrp2 genotypes at the expected Mendelian ratio.

Macroscopic and microscopic histological and pathological analysis did not reveal any obvious specific aberrations in tissues of the Mrp2−/− mice, including the liver. However, adult Mrp2−/− mice did have a 20-25% increased liver weight compared to wild-type mice (males: 5.71 ± 0.29% of the total body weight in Mrp2−/− mice vs. 4.52 ± 0.34% in wild-types, n = 5, P = 0.0003; females: 5.43 ± 0.28% of the total body weight in Mrp2−/− mice vs. 4.32 ± 0.56% in wild-types, n = 7-8, P = 0.0003).

**Figure 2.** Protein levels of Mrp3 in crude membrane fractions of male liver (upper panel) and Mrp4 protein in crude membrane fractions of female liver (middle panel) and male kidney (lower panel) from two independent wild-type and Mrp2−/− mice, as detected by Western blot analysis. The wild-type band for Mrp3 is representative for independent wild-type samples tested with the anti-Mrp3 mAb on other blots. In the middle panel, the lane with the positive control (SF-9 vesicles containing MRP4 (Zelcer et al., 2003b)) is indicated with C, and the marker lane with M. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. The amount of protein loaded in each lane (in µg) is noted above the respective lanes.
Expression of other ABC multidrug transporter proteins in tissues of Mrp2\(^{-/-}\) mice. Since many multidrug transporters have overlapping substrate specificities, absence of Mrp2 could result in (compensatory) induction of expression of other ABC multidrug transporters. Therefore the levels of P-gp, Bcrp1, Mrp1, Mrp3 and Mrp4 protein were determined by Western blot analysis of tissues from male and female Mrp2\(^{-/-}\) mice. There was no up-regulation of P-gp and Bcrp1 in the liver of Mrp2\(^{-/-}\) mice (data not shown). Mrp1 was not detectable in livers of wild-type and Mrp2\(^{-/-}\) mice, but Mrp1 expression in the kidneys of the Mrp2\(^{-/-}\) mice was unchanged (data not shown). In contrast, the level of Mrp3 in the liver of Mrp2\(^{-/-}\) mice was ~2 fold increased compared to wild-type (Fig. 2, upper panel). Mrp4 in male liver was low and therefore hard to quantify in both wild-type and Mrp2\(^{-/-}\) mice. Since liver Mrp4 expression in female mice is higher than in males (28), we analyzed Mrp4 expression in liver of Mrp2\(^{-/-}\) females. Mrp4 protein levels were not substantially different between wild-type and Mrp2\(^{-/-}\) liver (Fig. 2, middle panel). Mrp4 levels in kidney however, were ~2-fold increased in Mrp2\(^{-/-}\) mice (Fig. 2, lower panel).

![Figure 3](image-url) **Figure 3.** Bilirubin levels in plasma of Mrp2\(^{-/-}\) mice. A, plasma values of total bilirubin in wild-type (open bars) and Mrp2\(^{-/-}\) mice (closed bars) (means ± SD, n = 5-6, *** P < 0.001). B, plasma values of conjugated bilirubin in Mrp2\(^{-/-}\) mice (closed bars) (means ± SD, n = 5-6). The values for wild-type mice were below the detection limit of the analyzer (<1 µM). In previous studies (Jonker et al., 2002), male wild-type mice displayed unconjugated bilirubin levels of 1.5 ± 0.55 µM.

**Analysis of plasma composition of Mrp2\(^{-/-}\) mice.** Clinical chemical analysis of the plasma of Mrp2\(^{-/-}\) mice showed a moderately increased (~2-fold in males and ~3-fold in females) level of total bilirubin compared to wild-type mice (Fig. 3A). This difference could be attributed mainly to elevated levels of conjugated bilirubin in the Mrp2\(^{-/-}\) mice (Fig. 3B). The absolute increase could not be quantified, because
conjugated bilirubin levels of wild-type plasma were below the detection limit of the analyzer (<1 µM). The other clinical-chemical parameters measured (see Materials and Methods) showed no significant differences between wild-type and Mrp2\(^{-/-}\) mice.

**Hematological analysis of Mrp2\(^{-/-}\) mice.** Extensive hematological analysis (see Materials and Methods) was performed on blood from wild-type and Mrp2\(^{-/-}\) mice (n = 12, 6 males and 6 females). Within two months two separate sets of analyses were performed. The only measured parameter that consistently showed significant differences between wild-type and Mrp2\(^{-/-}\) mice was hemoglobin concentration, which was lower in Mrp2\(^{-/-}\) mice. In the first analysis the hemoglobin levels were 8.7 ± 0.4 mM in wild-type mice and 8.1 ± 0.6 mM in Mrp2\(^{-/-}\) mice (n = 12, P = 0.007), which was qualitatively confirmed in the second analysis. Hemoglobin levels did not differ significantly between sexes in either wild-type or Mrp2\(^{-/-}\) mice.

**Analysis of bile and urine composition of Mrp2\(^{-/-}\) mice.** Mrp2 is involved in the excretion of many conjugated and non-conjugated anionic compounds into bile. TR\(^{-}\) rats and EHBRs therefore suffer from impaired biliary secretion of glutathione, glutathione conjugates, and bilirubin glucuronides and also have a reduced bile flow (4,6). We measured the bile flow and the excretion of GSH and bilirubin glucuronides into the bile of male Mrp2\(^{-/-}\) mice after gall bladder cannulation. The bile flow in the Mrp2\(^{-/-}\) mice was reduced quite constantly to approximately 37% of wild-type bile flow over a 1 hour period (Fig. 4A). Analysis of the collected bile showed a significant decrease in total bilirubin output (75 ± 29 pmol/min per g liver for Mrp2\(^{-/-}\) mice and 135 ± 22 pmol/min per g liver for wild-type mice), which was mainly due to a decrease in bilirubin monoglucuronide output (46 ± 18 pmol/min per g liver for Mrp2\(^{-/-}\) mice and 120 ± 16 pmol/min per g liver for wild-type mice) (Fig. 4B). The Mrp2\(^{-/-}\) mice also showed a dramatic decrease in glutathione output into the bile (~80 fold) to only 1.2% of wild-type output (0.5 ± 0.2 nmol/min per g liver for Mrp2\(^{-/-}\) mice and 41.2 ± 6.7 nmol/min per g liver for wild-type mice) (Fig. 4C). Analysis of the urine of the two mouse strains showed a 7-fold increase of total bilirubin concentration in the Mrp2\(^{-/-}\) mice (Fig. 4D). This was primarily due to a dramatic increase (47-fold) in the bilirubin monoglucuronide concentration in the urine of the Mrp2\(^{-/-}\) mice. This suggests that in Mrp2\(^{-/-}\) mice excretion via the urine is used as an alternative route for diminished bilirubin monoglucuronide elimination via the liver.
Figure 4. Analysis of bile and urine from male wild-type (open bars) and Mrp2\(^{-/-}\) mice (closed bars) after gall bladder cannulations. Bile was collected in 15 min fractions over 1 hr. Bile collected in the first 15 min was analyzed. A, bile flow in wild-type and Mrp2\(^{-/-}\) mice. B, output of total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) in bile of wild-type and Mrp2\(^{-/-}\) mice. The output of bilirubin diglucuronide in wild-type mice was below the detection limit of the analyzer (5 \(\mu\)M). C, glutathione output in bile of wild-type and Mrp2\(^{-/-}\) mice. D, total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) concentration in urine of wild-type and Mrp2\(^{-/-}\) mice (n.q., not quantifiable, the detection limit of the analyzer for BDG in urine was 5 \(\mu\)M). Data are means \(\pm\) SD (n = 5, ** P < 0.01, *** P < 0.001).

Mouse Mrp2 restricts plasma levels of the dietary carcinogens PhIP and IQ. Many heterocyclic amines formed in cooked meat are highly mutagenic and carcinogenic (29). MDCKII cells expressing human MRP2 have been shown to transport one of these heterocyclic amines, PhIP, thereby providing a possible mechanism to protect the body from this dietary carcinogen (13). To test whether Mrp2 affects availability of PhIP in the mouse, we administered 1 mg/kg \([^{14}\text{C}]\text{PhIP}\) orally to female wild-type and Mrp2\(^{-/-}\) mice and measured radioactivity in plasma after 30 minutes. This experiment showed a 1.9-fold increased plasma value of \([^{14}\text{C}]\text{PhIP}\) in Mrp2\(^{-/-}\) compared to wild-type mice (202 \(\pm\) 73 ng/ml versus 107 \(\pm\) 27
ng/ml, n = 8, P = 0.0037), as shown in Fig. 5. This indicates that murine Mrp2 contributes to protection of the body from the dietary carcinogen PhIP.

We further tested the role of murine Mrp2 in the oral availability of another heterocyclic amine dietary carcinogen, IQ. 1 mg/kg [14C]IQ was orally administered to wild-type and Mrp2−/− mice and plasma radioactivity was measured after 30 minutes. The plasma levels in Mrp2−/− mice were 1.7-fold higher than in wild-type mice (346 ± 72 ng/ml versus 209 ± 46 ng/ml, n = 5, P = 0.0071) (Fig. 5), indicating a significant role for Mrp2 in limiting exposure of the body to IQ as well.

**Figure 5.** Plasma concentrations of [14C]PhIP and [14C]IQ 30 minutes after oral administration of 1 mg/kg of the compounds to female wild-type (open bars) and Mrp2−/− (closed bars) mice (means ± SD, n = 5-8, **P < 0.01).**

### Dose-dependent role of Mrp2 in elimination of the anti-cancer drug MTX from blood

Mrp2 is known to play a role in transport of, and resistance to, the drug MTX in vitro (1,8). It was also shown using Mrp2 deficient rats that Mrp2 excretes MTX into bile (11). To test whether Mrp2 in mice plays a role in the elimination of MTX, [3H]MTX was administered i.v. to female wild-type and Mrp2−/− mice at dosages of 1, 10 and 50 mg/kg. The results show that the influence of Mrp2 on [3H]MTX elimination was dose-dependent (Fig. 6A). At 1 mg/kg, no difference in the plasma concentration of [3H]MTX between wild-type and Mrp2−/− mice was observed at t = 30 min (163 ± 39 ng/ml for Mrp2−/− and 152 ± 29 ng/ml for wild-type mice, n = 6, P = 0.57). At 10 mg/kg of [3H]MTX, a moderate (1.6-fold) but significant difference was found (2.30 ± 0.29 µg/ml for Mrp2−/− and 1.47 ± 0.37 µg/ml for wild-type mice, n = 3-4, P = 0.021), whereas for 50 mg/kg the difference in plasma level between Mrp2−/− and wild-type mice was 2.2-fold (14.5 ± 4.3 µg/ml versus 6.49 ± 1.92 µg/ml, n = 3-4, P = 0.019). This indicates that Mrp2 in mice becomes relatively more important in elimination of [3H]MTX when the administered dose and thereby the plasma level increases. The amount of [3H]MTX in the contents of the small intestine at 30 min after i.v. administration of 50 mg/kg [3H]MTX was significantly
lower in the Mrp2<sup>−/−</sup> mice (4.87 ± 4.51% versus 11.40 ± 1.84% in wild-type mice) (Fig. 6B), which suggests that Mrp2 is important in hepatobiliary and/or direct intestinal excretion of [<sup>3</sup>H]MTX into the small intestinal lumen. This difference is reflected by a lower amount of [<sup>3</sup>H]MTX in the small intestinal wall in Mrp2<sup>−/−</sup> compared to wild-type mice (1.50 ± 0.78% of the dose versus 3.94 ± 1.28%), as shown in Fig. 6B. There was no difference in radioactivity in the livers of both mouse strains 30 min after [<sup>3</sup>H]MTX administration (Fig. 6B).

**Figure 6.** Effect of Mrp2 on elimination of [<sup>3</sup>H]MTX in female mice. A, plasma concentrations of [<sup>3</sup>H]MTX 30 minutes after i.v. administration of different dosages of MTX (1, 10, 50 mg/kg) to wild-type (open bars) and Mrp2<sup>−/−</sup> (closed bars) mice (means ± SD, n = 3-6, * P < 0.05). After administration of 1 mg/kg [<sup>3</sup>H]MTX the plasma concentrations were 152 ± 29 ng/ml in wild-type and 163 ± 39 ng/ml in Mrp2<sup>−/−</sup> mice (n = 6, P = 0.57). B, [<sup>3</sup>H]MTX in small intestinal tissue, small intestinal contents and liver 30 minutes after i.v. administration of 50 mg/kg [<sup>3</sup>H]MTX to wild-type (open bars) and Mrp2<sup>−/−</sup> mice (closed bars) (means ± SD, n = 3-4, * P < 0.05). C, plasma concentration versus time curves after i.v. administration of 50 mg/kg [<sup>3</sup>H]MTX to wild-type and Mrp2<sup>−/−</sup> mice (means ± SEM, n = 3-6, P = 0.032 for area under the curves, * P < 0.05).
Plasma samples at different time points after i.v. administration of 50 mg/kg [³H]MTX were obtained to generate a plasma concentration versus time curve and investigate the time-dependence of [³H]MTX elimination by Mrp2 (Fig. 6C). The difference in elimination between wild-type and Mrp2⁻/⁻ mice was most pronounced during the first 15 min after administration, when plasma levels were high. Already at 7.5 min after administration there was a significantly higher plasma level of [³H]MTX in Mrp2⁻/⁻ mice. The area under the curve between 7.5 and 120 minutes was 1.8-fold increased in Mrp2⁻/⁻ mice (1345 ± 104 min·µg/ml versus 734 ± 37 min·µg/ml in wild-type mice, n = 3-6, P = 0.032, means ± SEM), indicating that Mrp2 plays a significant role in the plasma elimination (and thus exposure) of MTX in mice.

Murine Mrp2 does not affect irinotecan and SN-38 pharmacokinetics after i.v. administration of 10 mg/kg irinotecan. In Mrp2 deficient rats (EHBRs) the plasma clearance of total and especially the carboxylate forms of irinotecan and SN-38 after i.v. administration was found to be much slower than in wild-type rats (10). We therefore tested whether this was similar in Mrp2 deficient mice. After i.v. administration of irinotecan at a 10 mg/kg dose to female mice, no differences in plasma clearance were measured for total irinotecan and SN-38 in Mrp2⁻/⁻ mice (Supplementary Figure 1). Also, no differences in plasma values of the separate lactone or carboxylate forms of irinotecan and SN-38 were measured (data not shown). Apparently, in contrast to rats, Mrp2 in mice does not substantially impact on the clearance of irinotecan and its metabolites, at least not when the drug is administered at a dose of 10 mg/kg.

Mrp2 and Mdr1a/b play distinct roles in the biliary excretion of doxorubicin. Recently we have obtained Mdr1a/b/Mrp2 triple knockout mice by crossing the above-described Mrp2⁻/⁻ mice with previously generated Mdr1a/b double knockout mice (19). With this model, which is readily amenable to pharmacological analysis, we can determine the relative roles of both P-gp and Mrp2 in the elimination and excretion of shared substrates. To determine the relative roles of Mrp2 and Mdr1a/b in biliary and direct intestinal excretion of the anti-cancer drug doxorubicin, which is a substrate for both ABC transporters (1,2), we performed gall bladder cannulations and administered doxorubicin i.v. to female wild-type, Mrp2⁻/⁻, Mdr1a/b⁻/⁻ and Mdr1a/b/Mrp2⁻/⁻ mice (all in 99% FVB background) at a dose of 5 mg/kg. After 60 minutes, there were no significant differences in plasma doxorubicin levels between wild-type and (combination) knockout mice (not shown, see Supplementary Table 1). Similarly, there were no significant differences in doxorubicin levels of liver, nor in small intestine and small intestinal contents, indicating that neither Mdr1a/b, nor Mrp2 play a substantial role in direct intestinal excretion of doxorubicin.
(Supplementary Table 1), or that an alternative intestinal transporter can compensate for absence of Mrp2 and Mdr1a/b.

Figure 7. Hepatobiliary excretion of doxorubicin in wild-type, Mrp2\(^{-/-}\), Mdr1a/b\(^{-/-}\) and Mdr1a/b/Mrp2\(^{-/-}\) mice (means ± SD, n = 3-5, * P < 0.05, *** P < 0.001). Doxorubicin (5 mg/kg) was administered i.v. to mice with a cannulated gall bladder. Bile fractions were collected over a 60 min period. After 60 min, plasma, liver, small intestine and small intestinal contents were collected. All samples were analyzed by HPLC. Biliary doxorubicin output in 60 min as % of the administered dose in the different strains: wild-type 9.43 ± 3.05 %; Mrp2\(^{-/-}\) 6.18 ± 1.31 % (n = 4-5, P = 0.067); Mdr1a/b\(^{-/-}\) 0.89 ± 0.30 % (n = 3-4, P = 0.005); Mdr1a/b/Mrp2\(^{-/-}\) 0.23 ± 0.11 % (n = 4-5, P = 0.0002). (Doxorubicin levels in plasma, liver small intestine and small intestinal contents are shown in Supplementary Table 1.)
The biliary doxorubicin output in 60 minutes is shown in Figure 7. In \( \text{Mrp2}^\sim \) mice the biliary doxorubicin excretion was about 2-fold (51%) decreased (8.45 ± 1.85 nmol/g liver for \( \text{Mrp2}^\sim \) mice versus 17.30 ± 6.59 nmol/g liver for wild-type mice, \( n = 4-5, P = 0.041 \)), indicating that Mrp2 can transport doxorubicin into the bile. In \( \text{Mdr1a/b}^\sim \) mice the biliary excretion of doxorubicin is about 10-fold (90%) decreased (1.68 ± 0.64 nmol/g liver for \( \text{Mdr1a/b}^\sim \) versus 17.30 ± 6.59 nmol/g liver for wild-type mice, \( n = 3-4, P = 0.010 \)), indicating a more substantial role for Mdr1a/b than for Mrp2 in the biliary excretion of doxorubicin. It was previously shown in single \( \text{Mdr1a}^\sim \) mice that the biliary excretion was about 5-fold (80%) reduced compared to wild-type mice after 90 minutes (30). Our data suggest that additional removal of Mdr1b in mice doubles the difference compared to wild-type mice, suggesting that both Mdr1a and Mdr1b have a separate role in the biliary excretion of doxorubicin. When Mrp2 and Mdr1a/b are all absent, the biliary excretion of doxorubicin is even further reduced to less than 2% of wild-type biliary excretion (0.32 ± 0.13 nmol/g liver for \( \text{Mdr1a/b/Mrp2} \) knockout mice versus 17.30 ± 6.59 nmol/g liver for wild-type mice, \( n = 4-5, P = 0.0006 \)). The excretion of doxorubicin in the \( \text{Mdr1a/b/Mrp2} \) knockout mice is 5-fold lower than in the \( \text{Mdr1a/b}^\sim \) mice (\( n = 3-5, P = 0.0028 \)) and 26-fold lower than in the \( \text{Mrp2}^\sim \) mice (\( n = 4-5, P = 2.2 \times 10^{-5} \)), indicating that Mrp2 and Mdr1a/b can partly compensate for the absence of the other ABC transporter. From these data we conclude that both Mrp2 and Mdr1a/b can transport doxorubicin into the bile, although the roles of Mdr1a/b are more pronounced.

**Discussion**

Here we present the generation and characterization of an \( \text{Mrp2} \) knockout mouse. These mice display several modest aberrations, like mild hyperbilirubinemia due to impaired secretion of conjugated bilirubin into the bile, and a reduction in bile flow and biliary GSH excretion. \( \text{Mrp2}^\sim \) mice also display a 20-25% increased liver size. Nevertheless, they appear quite healthy overall and therefore provide an excellent model to study the role of Mrp2 in physiology and pharmacokinetics of drugs and other toxic compounds. While this study was prepared for submission, Chu et al. (2006) published a basic characterization of an independently generated \( \text{Mrp2}^\sim \) strain, against a different genetic background (C57BL/6) (31). Like us, also based on their complementary analysis of (limited) expression changes in detoxifying genes in \( \text{Mrp2}^\sim \) mice, Chu et al. conclude that these mice provide valuable tools to study the role of Mrp2 in drug disposition. Whereas many physiological changes in the two knockout strains are similar (e.g. reduced bile flow and reduced biliary output of conjugated bilirubin and GSH), there are also some differences, as discussed below.

Although the generated \( \text{Mrp2}^\sim \) mice look quite healthy overall, the liver weight of these mice is 20-25% increased. This could be caused by accumulation of Mrp2 substrates in the liver, possibly promoting liver proliferation. An increased
liver weight has also been shown in the EHBR previously (5). Whether liver weight was increased in the Mrp2\(^{-/-}\) strain analyzed by Chu et al. (2006) was not reported.

In our Mrp2\(^{-/-}\) mouse model the protein levels of Mrp3 in liver and Mrp4 in kidney are about 2-fold induced. Elevated levels of Mrp3 in the liver have also been found in Mrp2-deficient rats and Dubin-Johnson patients (32), although in rats and humans the Mrp3 induction is more pronounced. It is noteworthy that Chu et al. (2006) found no induction of hepatic Mrp3 expression (31), in contrast to Mrp2-deficient rats, humans and our Mrp2\(^{-/-}\) mice. Recently, Nezasa et al. (2006) did find elevated hepatic Mrp3 protein levels in the same Mrp2\(^{-/-}\) mouse strain as analyzed by Chu et al. (2006) (33). This indicates that the differences could be due to inter-laboratory variation (e.g. food composition differences).

Consistent with our results, 2-fold higher Mrp4 protein levels in kidney but not in liver were recently detected in TR\(^{-}\) rats (34). In contrast, Chu et al. (2006) did find clear induction of Mrp4 expression in liver of their Mrp2\(^{-/-}\) mice (31). Mrp4 regulation in liver is clearly dependent on the genetic background, as we did see substantial Mrp4 induction in liver of the Mrp2\(^{-/-}\) mice in 99% FVB background we recently obtained (not shown). Induction of Mrp3 in the liver and Mrp4 in the kidney as a result of Mrp2 deficiency suggests that compensatory expression mechanisms exist for these genes, which are activated due to the absence of Mrp2. The availability of Mrp2\(^{-/-}\) mice will allow systematic analysis of such compensatory elimination mechanisms by studying separate and compound (Mrp3, Mrp4) knockout mice.

Even though the transport of bilirubin monoglucuronides into the bile is significantly decreased in Mrp2\(^{-/-}\) mice, bilirubin monoglucuronides are still present in their bile. This suggests that another anion transporter, possibly Bcrp1 (Abcg2) or Bsep (Abcb11), in the apical membrane of hepatocytes is capable of transporting conjugated bilirubin into the bile.

Others have shown transport of the heterocyclic amine PhIP by human MRP2 in vitro (13) and rat Mrp2 has been shown to protect the body against this carcinogen by biliary excretion and reducing the bioavailability of PhIP (12,13). In this study we show that mouse Mrp2 also plays a clear role in protecting the body from this carcinogen upon oral administration. We further show that murine Mrp2 limits the plasma levels of another food-derived carcinogen, IQ, after oral administration. The results suggest that Mrp2 could be involved in protecting the body from these and many more (food-derived) carcinogens.

For the drug MTX we found that in mice Mrp2 plays a significant role in the elimination from the body, but only at a comparatively high dose (50 mg/kg) and not (or much less) at lower doses (1 or 10 mg/kg). Already 7.5 min after high dose (50 mg/kg) i.v. administration a clear difference between plasma MTX values in wild-type and Mrp2\(^{-/-}\) mice was observed (Fig. 6C), indicating a role for Mrp2 in the elimination of MTX at high plasma concentrations. This is consistent with the idea
that Mrp2 is a transporter with a low affinity, but high capacity for MTX (8,35), causing a relatively large impact of Mrp2 on the total MTX elimination only at high plasma concentrations. At lower dosages (and plasma concentrations) other MTX clearance mechanisms appear to predominate. Accordingly, beyond 20-30 min after MTX administration (Fig. 6C), when the plasma levels of MTX had decreased, MTX clearance was no longer higher in wild-type than in Mrp2−/− mice. A similar result was found earlier when the role of mouse Bcrp1 in MTX plasma clearance was investigated (36). Interestingly, also in humans a role for MRP2 in elimination of high-dose MTX has been suggested. A patient with a heterozygous loss of function mutation in ABCC2 who received high-dose MTX i.v. had a three-fold reduced methotrexate elimination rate, leading to severe over-dosing and nephrotoxicity (37).

Investigations in humans and rats suggest that MTX metabolism is quite limited (11,38). Our data presenting radioactivity plasma levels are quite comparable to data from the study in male mice by Li et al. (2004), who determined unchanged MTX plasma levels by HPLC (39). We conclude that the amount of radioactivity we measure in the plasma probably accounts mainly for unchanged [3H]MTX.

The reduced levels of MTX in the intestinal wall of the Mrp2−/− mice likely reflect the reduced intestinal content values, which indicate that Mrp2 is involved in biliary and/or direct intestinal excretion of MTX. MTX levels in the liver were not different in Mrp2−/− mice at t = 30 min. A possible explanation is the up-regulation of Mrp3 in the liver of Mrp2−/− mice. Since Mrp3 can transport MTX (2), Mrp3 in the hepatocytes of Mrp2−/− mice may compensate for reduced biliary MTX excretion by transporting accumulated MTX back into the circulation of Mrp2−/− mice. It could also be that the small amount of radioactivity in the liver (< 5%) mainly accounts for metabolites of [3H]MTX that are not Mrp2 substrates.

We investigated the role of Mrp2 in the elimination of irinotecan and/or its active metabolite SN-38 using our Mrp2−/− mice. In a previous study on the role of rat Mrp2 in elimination of irinotecan and SN-38 (10), there was a clear difference between SD rats and Mrp2 deficient EHBRS in the biliary excretion of irinotecan and SN-38. In our study, no role for murine Mrp2 in the elimination of irinotecan or SN-38 was found, showing a clear difference between Mrp2-deficient mice and rats. This could mean that murine Mrp2 is a comparatively poor irinotecan transporter compared to rat Mrp2, or possibly that other ABC transporters, like Bcrp1 and P-gp, of which the human homologues have been shown to transport irinotecan in vitro (9,40), play a more pronounced role in the elimination of this drug in mice.

Crossing the generated Mrp2−/− mice with other ABC transporter knockout mice enables us to study the relative roles of the various ABC transporters in transport of shared substrates in vivo, as we show here with Mdr1a/b/Mrp2 triple knockout mice. Our experiment with the anti-cancer drug doxorubicin shows that Mdr1a, Mdr1b and Mrp2 can all transport this shared substrate into the bile, but the
influence of Mdr1a and Mdr1b is more substantial than that of Mrp2. From our results it seems that both Mrp2 and Mdr1a/b can take over at least part of each other’s functions. Of course differences between humans and mice could exist, so translation to the human situation must be done cautiously. We also show with this experiment that neither Mrp2, nor Mdr1a/b play a detectable role in the direct intestinal excretion of doxorubicin. This suggests that another intestinal transporter is involved in this process.

We conclude that murine Mrp2 plays an important role in oral availability and elimination of drugs and carcinogens, most likely by biliary excretion and possibly by decreasing uptake from the small intestine. The $Mrp2^{-/-}$ mice allow us to generate many different combination knockout mice by crossing them with other ABC transporter knockout mice. These will provide important tools to define the relative contributions of the different ABC transporters to drug resistance, drug pharmacokinetics and protection against endogenous and exogenous toxins. This will eventually lead to a better understanding of the role of ABC-transporters in intra- or inter-individual variation in response to drugs and thus improve the possibility of optimizing drug treatment for individual patients, and minimizing side effects.

Acknowledgements

We thank our colleagues for critical reading of the manuscript, Rob Lodewijks and Hans Tensen for excellent technical assistance, Martin van der Valk for histological analysis, and George Scheffer, Koen van de Wetering and Noam Zelcer for kindly providing the antibodies and the Sf-9 vesicles. This work was supported by grant NKI 2003-2940 from the Dutch Cancer Society.

References


against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci USA* 99: 15649-15654.


Mrp2 (Abcc2) knockout mice


**Supplementary Table 1.** Doxorubicin levels 60 min after i.v. injection of 5 mg/kg to gall bladder cannulated mice. Plasma concentrations at t = 60 min are in nM, doxorubicin levels in small intestinal tissue and contents at t = 60 min are in nmol, doxorubicin liver concentrations at t = 60 min and total biliary doxorubicin output over 60 min are presented in nmol/gr liver.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Mrp2−/−</th>
<th>Mdr1a/b−/−</th>
<th>Mdr1a/b/Mrp2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma (nM)</strong></td>
<td>185.45 ± 23.73</td>
<td>217.85 ± 33.61</td>
<td>205.42 ± 12.49</td>
<td>208.96 ± 98.49</td>
</tr>
<tr>
<td><strong>Small intestinal tissue (nmol)</strong></td>
<td>4.24 ± 1.61</td>
<td>4.01 ± 0.92</td>
<td>4.63 ± 1.50</td>
<td>5.04 ± 1.32</td>
</tr>
<tr>
<td><strong>Small intestinal contents (nmol)</strong></td>
<td>5.39 ± 2.57</td>
<td>4.58 ± 1.11</td>
<td>3.45 ± 0.95</td>
<td>4.48 ± 1.86</td>
</tr>
<tr>
<td><strong>Bile (nmol/gr liver)</strong></td>
<td>17.30 ± 6.59</td>
<td>8.45 ± 1.85*</td>
<td>1.68 ± 0.64*</td>
<td>0.32 ± 0.13***</td>
</tr>
<tr>
<td><strong>Liver (nmol/gr liver)</strong></td>
<td>24.86 ± 4.96</td>
<td>16.79 ± 12.77</td>
<td>32.28 ± 17.59</td>
<td>18.51 ± 10.11</td>
</tr>
</tbody>
</table>

**Supplementary Figure 1.** Plasma concentration versus time curves for total irinotecan (A) and total SN-38 (B) after i.v. administration of 10 mg/kg irinotecan to female wild-type and Mrp2−/− mice (means ± SD, n = 3). Note the differences in X- and Y-axes for both graphs. CPT-11 plasma concentrations at t = 4 hrs and t = 8 hrs were not detectable and therefore are not added to this graph.
Chapter 3

Multidrug resistance protein 2 (Mrp2; Abcc2) is an important determinant of paclitaxel pharmacokinetics

Jurjen S. Lagas, Maria L.H. Vlaming, Olaf van Tellingen, Els Wagenaar, Robert S. Jansen, Hilde Rosing, Jos H. Beijnen, Alfred H. Schinkel

Multidrug resistance protein 2 (Mrp2; Abcc2) is an important determinant of paclitaxel pharmacokinetics

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Purpose: P-glycoprotein (ABCB1, P-gp) efficiently transports lipophilic amphipathic drugs, including the widely used anti-cancer drug paclitaxel (Taxol). We previously found that human MRP2 (ABCC2) also transports paclitaxel in vitro, and although we expected that paclitaxel pharmacokinetics would be dominated by P-gp, the impact of Mrp2 was tested in vivo.

Experimental design: We generated and characterized Mdr1a/b/Mrp2⁻/⁻ mice, allowing assessment of the distinct roles of Mrp2 and Mdr1a/1b P-gp in paclitaxel pharmacokinetics.

Results: Surprisingly, the impact of Mrp2 upon intravenous administration of paclitaxel was as great as that of P-gp. The AUCᵢ.v. in both Mrp2⁻/⁻ and Mdr1a/1b⁻/⁻ mice was 1.3-fold higher than in wild-type mice, and in Mdr1a/b/Mrp2⁻/⁻ mice a 1.7-fold increase was found. In spite of this similar impact, Mrp2 and P-gp had mostly complementary functions in paclitaxel elimination. Mrp2 dominated the hepatobiliary excretion, which was reduced by 80% in Mrp2⁻/⁻ mice. In contrast, P-gp dominated the direct intestinal excretion, with a minor role for Mrp2. The AUCᵢoral of paclitaxel was 8.5-fold increased by Mdr1a/1b deficiency, but not affected by Mrp2 deficiency. However, in the absence of Mdr1a/1b P-gp, additional Mrp2 deficiency increased the AUCᵢoral another 1.7-fold.

Conclusions: Thusfar, Mrp2 was thought to mainly affect organic anionic drugs in vivo. Our data show that Mrp2 can also be a major determinant of the pharmacokinetic behavior of highly lipophilic anti-cancer drugs, even in the presence of other efficient transporters. Variation in MRp2 activity might thus directly affect the effective exposure to paclitaxel, upon intravenous administration, but also upon oral administration, especially when P-gp activity is inhibited.

INTRODUCTION

ATP binding cassette (ABC) multidrug transporters, like P-glycoprotein (P-gp, ABCB1), BCRP (ABCG2) and MRp2 (ABCC2) can have an important impact on chemotherapy. These proteins share a strategic localization at apical membranes of important epithelial barriers and at the canalicular membrane of hepatocytes, where
they facilitate excretion of transported drugs via liver, intestine and kidneys, and limit their distribution to tissues such as brain or testis (1). In addition, (over-)expression of these transporters in tumor cells can lead to drug resistance through active efflux of cytostatic drugs. Many inhibitors of P-gp and/or BCRP have therefore been developed and applied to potentially improve chemotherapy response of such tumors (2).

Paclitaxel is an excellent P-gp substrate that is widely used in treatment of breast and ovarian cancer, non-small cell lung cancer and Kaposi’s sarcoma (3). We showed earlier that P-gp in epithelial cells of the small intestine actively effluxes its substrates, including paclitaxel, directly from the blood into the intestinal lumen. Moreover, using paclitaxel as model substrate, P-gp was shown to drastically limit intestinal absorption of orally administered substrates (4, 5). Based on these findings, numerous mouse studies and clinical trials have been performed, showing that the poor oral availability of paclitaxel could be dramatically improved by coadministration of a P-gp inhibitor (6-10). This is of importance, because oral administration of paclitaxel would be preferred over i.v. administration, as it is convenient to patients, reduces administration costs and facilitates the use of more chronic treatment regimes (11).

Despite virtually complete absorption of paclitaxel from the gastro-intestinal tract in Mdr1a/1b<sup>-/-</sup> mice, bioavailability does not approach 100% (5, 6). Similar results were found in patients, when paclitaxel was combined with the potent P-gp inhibitors Cyclosporine A (CsA) or GF120918 (Elacridar<sup>®</sup>) (10, 12). This might be explained by the fact that besides absorption, first-pass metabolism and elimination also affect the bioavailability of a drug. In addition to the P-gp-mediated excretion of paclitaxel from blood directly into the gut lumen (5), excretion into the bile is another important route of elimination, both in rodents and in humans (13, 14). Given its presence in the canalicular membrane of hepatocytes, P-gp seemed to be a good candidate for this elimination pathway. However, studies with Mdr1a<sup>−/−</sup> and Mdr1a/1b<sup>-/-</sup> mice (5, 15) failed to demonstrate a significant role for P-gp in hepatobiliary excretion of paclitaxel and its hydroxylated metabolites.

We recently identified human MRP2 as a transporter for taxanes in vitro (16), and we hypothesized that MRP2 may also play a role in vivo, affecting absorption, distribution and/or elimination of paclitaxel. As MRP2 is expressed at the apical membrane of epithelial cells of the small intestine (17), it might limit oral absorption of paclitaxel, similar to P-gp. Furthermore, MRP2 is found at the canalicular membrane of hepatocytes (18), and could thus mediate biliary excretion of paclitaxel and/or its principal hydroxylated metabolites. Thus, absence or reduced activity of MRP2 might increase absorption or decrease elimination of paclitaxel and hence increase overall paclitaxel exposure, potentially influencing therapeutic efficacy and risks of toxic side effects. Involvement of MRP2 in the pharmacokinetics of paclitaxel could be highly relevant for chemotherapy in patients, and possible
interpatient variability. Many MRP2 polymorphisms have been described in the human population that affect MRP2 transport activity, including fully deficient variants that occur in homozygous form in Dubin-Johnson patients (19). We have recently generated Mrp2<sup>-/-</sup> mice (20) and crossed them with Mdr1a<sub>1b</sub>Δ<sup>717</sup> mice (15) to obtain Mdr1a<sub>1b</sub>/Mrp2<sub>-/-</sub> mice. The availability of these strains allowed us to address the relative impact of Mrp2 and P-gp on paclitaxel pharmacokinetics.

**MATERIALS AND METHODS**

**Chemicals.** Paclitaxel, 2’-methylpaclitaxel and paclitaxel formulated as a 6 mg/ml solution (Taxol®) in Cremophor EL and dehydrated alcohol (1:1, v/v) were from Bristol-Myers Squibb (Princeton, NJ, USA). [³H]Paclitaxel (4.8 Ci/mmol) was from Moravek Biochemicals (Brea, CA, USA). Paclitaxel metabolites 3’p-hydroxy-paclitaxel and 6α-hydroxy-paclitaxel were purified from patient feces as described (21) or purchased from Gentest Corporation (Woburn, MA, USA). Ketamine (Ketanest-S®) was from Pfizer (Cappelle a/d IJssel, the Netherlands). Xylazine was from Sigma Chemical Co (St. Louis, MO, USA). Methoxyflurane (Metofane®) was from Medical Developments Australia (Springvale, Victoria, Australia). Heparin (5000 IE/ml) was from Leo Pharma BV (Breda, the Netherlands). Bovine serum albumin (BSA), fraction V, was from Roche (Mannheim, Germany). The organic solvents methanol, acetonitril (both HPLC grade) and diethyl ether were from Merck (Darmstadt, Germany). Blank human plasma was from healthy volunteers.

**Animals.** Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male Mdr1a<sub>1b</sub>Δ<sup>717</sup> (15), Mrp2<sup>-/-</sup> (20), Mdr1a<sub>1b</sub>/Mrp2<sup>-/-</sup> and wild-type mice, all of a >99% FVB genetic background, between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hour light / 12-hour dark circle and received a standard diet (AM-II, Hope Farms, Woerden, the Netherlands) and acidified water *ad libitum*.

**Plasma Pharmacokinetics.** For oral administration, paclitaxel formulated in Cremophor EL and dehydrated alcohol (1:1, v/v, 6mg/ml, Taxol®) was diluted with saline to 1 mg/ml and dosed at 10 mg/kg body weight (10 ml/kg). To minimize variation in absorption, mice were fasted for 3 hours, before paclitaxel was administered by gavage into the stomach, using a blunt-ended needle. Multiple blood samples (~30 µl) were collected from the tail vein at 15 and 30 min and 1, 2, 4, 6, and 8 h, using heparinized capillary tubes (Oxford Labware, St. Louis, USA). Blood samples were centrifuged at 2100 g for 10 min at 4°C, the plasma fraction was collected, completed to 200 µl with blank human plasma and stored at -20°C until analysis. For intravenous studies, paclitaxel was formulated in ethanol and...
polysorbate 80 (1:1, v/v, 6 mg/ml). This solution was diluted with saline to 2 mg/ml and injected as single bolus at a dose of 10 mg/kg (5 ml/kg) into the tail vein. Blood samples were collected by cardiac puncture under methoxyflurane anesthesia. Animals were sacrificed at 7.5, 15 and 30 min and 1, 2, 4 and 8 hr after paclitaxel administration, with 3-4 animals per time point. Blood samples were centrifuged at 2100 g for 10 min at 4°C, the plasma fraction was collected and stored at -20°C until analysis.

**Fecal and Urinary Excretion.** Mice were individually housed in Ruco Type M/1 stainless-steel metabolic cages (Valkenswaard, the Netherlands). They were allowed 2 days to adapt, before 10 mg/kg paclitaxel, supplemented with [³H]paclitaxel (~0.5 µCi per animal), was injected into a tail vein. Feces and urine were collected over a 24 h period; urine was diluted 5-fold with blank human plasma and feces were homogenized in 4% BSA (1 ml per 100 mg feces). Part of the sample was used to determine levels of radioactivity by liquid scintillation counting; the rest was stored at -20°C until analysis.

**Biliary Excretion.** In gall bladder cannulation experiments, mice were anesthetised by intraperitoneal injection of a combination of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), in a volume of 4.33 µl per gram body weight. After opening the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex limited, Hythe, UK) with an inner diameter of 0.28 mm, was inserted into the incised gallbladder and fixed with an additional ligation. Bile was collected for 60 min after i.v. injection of paclitaxel. For gall bladder cannulation experiments 5 mg/kg was used, as 10 mg/kg paclitaxel in combination with anesthesia and surgery can result in cardiac and respiratory insufficiency (5). At the end of the experiment, blood was collected by cardiac puncture and mice were sacrificed by cervical dislocation. Several tissues were removed and homogenized in 4% BSA; intestinal contents were separated from intestinal tissues prior to homogenization. Tissue homogenates, bile and plasma were stored at -20°C until analysis.

**Drug Analysis.** Amounts of paclitaxel and its hydroxylated metabolites 3’p-hydroxypaclitaxel and 6α-hydroxypaclitaxel in small plasma samples, obtained by sampling from the tail vein, were determined using a previously described sensitive and specific LC-MS/MS assay (22). All other samples were processed using liquid-liquid and solid-phase extraction, followed by reversed-phase HPLC with UV detection (23), with minor modifications. We adjusted the mobile phase for HPLC analysis of bile samples and tissue- and feces homogenate-extracts (acetonitrile-methanol-0.2 M ammonium acetate buffer (pH 5.0) (42:65:93, v/v/v)) to obtain successful separation of drug peaks and interfering peaks.
Clinical-Chemical Analysis of Plasma. Standard clinical chemistry analyses on plasma of wild-type, *Mdr1a/1b*/*, *Mrp2*/* and *Mdr1a/1b/Mrp2*/* mice (n = 6, males and females) were performed on a Roche Hitachi 917 analyzer (Roche diagnostics, Basel, Switzerland) to determine levels of total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine, urea, Na⁺, K⁺, Cl⁻, Ca²⁺, phosphate, total protein and albumin.

Haematological Analysis. Haemoglobin, haematocrit, mean corpuscular volume, red blood cells, white blood cells, lymphocytes, monocytes, granulocytes and platelets were determined in EDTA blood on a Beckman Coulter Ac-T Diff analyzer.

Pharmacokinetic Calculations and Statistical Analysis. Pharmacokinetic parameters were calculated by noncompartmental methods using the software package WinNonlin Professional version 5.0. The area under plasma concentration-time curves (AUC) was calculated using the trapezoidal rule, without extrapolating to infinity. Elimination half-lives (t₁/₂, el) were calculated by linear regression analysis of the log-linear part of the plasma concentration-time curves. Plasma clearance (Cl) after i.v. paclitaxel administration was calculated by the formula Cl = Dose/AUCᵢ.ᵣ and the oral bioavailability (F) was calculated by the formula $F = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{i.v.}}} \times 100\%$. The two-sided unpaired Student’s t-test was used for statistical analysis. Data obtained with single- and combination knockout mice were compared to data obtained with wild-type mice, unless stated otherwise. Differences were considered statistically significant when $P < 0.05$. Data are presented as means ± SD.

RESULTS

Generation and Characterization of *Mdr1a/1b/Mrp2*/* Mice. We generated *Mdr1a/1b/Mrp2*/* mice by cross-breeding *Mdr1a/1b*/* and *Mrp2*/* mice (15, 20). *Mdr1a/1b/Mrp2*/* mice were fertile and had normal life spans and body weights. Similar to *Mrp2*/* mice (20), they had a ∼25% increased liver weight (6.1 ± 0.4% of body weight in *Mdr1a/1b/Mrp2*/* vs. 4.8 ± 0.3% in wild-type, n = 5-6, $P = 0.0003$).

No other macroscopic or microscopic anatomic abnormalities were evident. The bile flow in *Mdr1a/1b/Mrp2*/* mice was reduced to 40-50% of wild-type levels ($P < 0.01$), and not significantly different from that in *Mrp2*/* mice (20).

*Mdr1a/1b/Mrp2*/* mice had a moderately increased (∼3-fold) plasma level of total bilirubin compared to wild-type mice, which could be attributed to elevated levels of conjugated bilirubin (3.2 ± 1.6 µM in males, 2.7 ± 0.8 µM in females, n = 6). Conjugated bilirubin levels in wild-type plasma were below the detection limit (<1 µM). Conjugated and total bilirubin levels in *Mdr1a/1b/Mrp2*/* mice were not significantly different from those in *Mrp2*/* mice. The other clinical-chemical
parameters measured in plasma (see Materials and Methods) showed no significant differences between wild-type and Mdr1a/1b/Mrp2⁻/⁻ mice.

Haemoglobin levels were moderately but significantly decreased in both male and female Mdr1a/1b/Mrp2⁻/⁻ mice (males: 7.0 ± 0.1 mM in knockout vs. 7.4 ± 0.1 mM in wild-type mice, n = 3-4, P = 0.017; females: 7.2 ± 0.5 mM in knockout vs. 7.6 ± 0.1 mM in wild-type mice, n = 5-6, P = 0.016). These results are qualitatively similar to those for Mrp2⁻/⁻ mice. None of the other haematological parameters measured revealed significant differences between wild-type and Mdr1a/1b/Mrp2⁻/⁻ mice.

Mdr1a/1b/Mrp2⁻/⁻ mice thus appear in many respects very similar to Mrp2⁻/⁻ mice (20), and they are likely as amenable to pharmacological analyses.

![Fig. 1](image)

**Fig. 1.** Plasma concentration-time curves of paclitaxel in male FVB wild-type (●), Mdr1a/1b⁻/⁻ (○), Mrp2⁻/⁻ (▼) and Mdr1a/1b/Mrp2⁻/⁻ (∇) mice, after oral (A) and i.v. (B) administration of paclitaxel at a dose of 10 mg/kg. Data represent mean concentrations ± SD, n = 5-6 for oral and n = 3-4 for i.v. administration.

**Impact of Mrp2 and P-gp on Plasma Pharmacokinetics of Paclitaxel.** To investigate the relative roles of Mrp2 and P-gp in absorption, distribution and elimination of paclitaxel, we studied oral and intravenous plasma pharmacokinetics in wild-type, Mrp2⁻/⁻, Mdr1a/1b⁻/⁻ and Mdr1a/1b/Mrp2⁻/⁻ mice. Upon oral administration of 10 mg/kg paclitaxel, plasma concentrations and area under the plasma concentration-time curve (AUCoral) were not different between Mrp2⁻/⁻ and wild-type mice (Fig. 1A and Table 1). For Mdr1a/1b⁻/⁻ mice the AUCoral was about 8.5-fold higher, in line with previous results (5, 6), but the elimination half life of
the drug was not changed (Table 1). Interestingly, however, in Mdr1a/1b/Mrp2<sup>−/−</sup> mice the AUC<sub>oral</sub> was increased another 1.7-fold compared to Mdr1a/1b<sup>−/−</sup> mice (and 14.2-fold compared to wild-type mice), the C<sub>max</sub> was 1.5-fold increased, and a 1.4-fold extended elimination half life was found (P < 0.01 for each parameter) (Fig. 1A, Table 1). These results confirm that P-gp is a major factor in limiting the paclitaxel AUC after oral administration, but that, in the absence of P-gp, Mrp2 also has a marked impact on oral paclitaxel plasma pharmacokinetics.

The relative impact of Mrp2 versus P-gp was even more pronounced after intravenous administration of paclitaxel. The AUC<sub>i.v.</sub> was 1.3-fold higher in Mrp2<sup>−/−</sup> mice than in wild-type mice (Fig. 1B, Table 1). A similar 1.3-fold increase in AUC<sub>i.v.</sub> was found for Mdr1a/1b<sup>−/−</sup> mice, consistent with our previous results (5, 6). This similarity in impact of Mrp2 and P-gp on paclitaxel plasma levels after i.v. administration is striking, since paclitaxel is an excellent P-gp substrate (24, 25). Nonetheless, even with P-gp present, Mrp2 is an important determinant for the disposition of paclitaxel in vivo. Absence of both Mrp2 and Mdr1a/1b resulted in a 1.7-fold higher AUC<sub>i.v.</sub> than in wild-type mice, and a significantly prolonged elimination half life (Fig. 1B, Table 1).

Table 1. Plasma pharmacokinetic parameters after oral or i.v. administration of paclitaxel at 10 mg/kg.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oral</th>
<th>i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Mdr1a/1b&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC(0-8), hr.ml/l</td>
<td>0.44 ± 0.19</td>
<td>3.75 ± 0.38***</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;, mg/l</td>
<td>0.13 ± 0.11</td>
<td>1.05 ± 0.19***</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2, el&lt;/sub&gt;, hr</td>
<td>1.96 ± 0.28</td>
<td>1.69 ± 0.16</td>
</tr>
<tr>
<td>AUC(0-8), hr.ml/l</td>
<td>5.57 ± 0.26</td>
<td>7.08 ± 0.31*</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;, mg/l</td>
<td>6.17 ± 0.21</td>
<td>7.09 ± 0.30</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2, el&lt;/sub&gt;, hr</td>
<td>1.65 ± 0.11</td>
<td>1.79 ± 0.10</td>
</tr>
<tr>
<td>Cl, l/hr.kg</td>
<td>1.80 ± 0.08</td>
<td>1.41 ± 0.06*</td>
</tr>
<tr>
<td>F, %</td>
<td>7.9 ± 3.4</td>
<td>53.0 ± 5.8**</td>
</tr>
</tbody>
</table>

AUC<sub>(0-8)</sub>, area under plasma concentration-time curve up to 8 hr; C<sub>max</sub>, maximum plasma levels; t<sub>1/2, el</sub>, elimination half life, calculated from 2-8 hr for both oral and i.v. administration; Cl, plasma clearance; F, oral bioavailability. Data are means ± SD, n = 5-6 for oral and n = 3-4 for i.v. administration. * P < 0.05, ** P < 0.01 and *** P < 0.001, compared to wild-type mice. † P < 0.01, compared to Mdr1a/1b<sup>−/−</sup> mice.
Role of Mrp2 and P-gp in Plasma and Liver Levels of 3’p-Hydroxypaclitaxel and 6α-Hydroxypaclitaxel. Because metabolism is an important detoxification pathway for paclitaxel, we also studied its primary metabolites: 3’p-hydroxypaclitaxel and 6α-hydroxypaclitaxel. Plasma levels of these monohydroxylated metabolites at \( t = 8 \) hr after i.v. administration of paclitaxel at 10 mg/kg were below the limits of detection in wild-type mice (Table 2). However, substantial levels were detected in plasma of \( Mdr1a/1b^{-/-} \) and \( Mrp2^{-/-} \) mice, and for \( Mdr1a/1b/Mrp2^{-/-} \) mice the levels were another 3 to 4-fold higher. Similar results were obtained for metabolite levels in liver at \( t = 8 \) hr (Table 2), suggesting an interrelatedness of plasma and liver metabolite levels. The same might apply to unchanged paclitaxel, as its accumulation in liver and plasma concentration were also markedly higher in each of the separate and especially the combined knockout strains.

Table 2. Levels of paclitaxel and monohydroxylated metabolites in plasma and liver at \( t = 8 \) hr after i.v. administration of 10 mg/kg paclitaxel.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biological Matrix</th>
<th>Compound</th>
<th>Wild-type</th>
<th>Mdr1a/1b^{-/-}</th>
<th>Mrp2^{-/-}</th>
<th>Mdr1a/1b/Mrp2^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma (ng/ml)</td>
<td>Paclitaxel</td>
<td>38.3 ± 5.8</td>
<td>80.5 ± 8.9**</td>
<td>73.9 ± 12.6*</td>
<td>189.6 ± 13.1***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’p-hydroxypaclitaxel</td>
<td>ND</td>
<td>2.3 ± 0.4</td>
<td>2.0 ± 0.5</td>
<td>6.5 ± 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6α-hydroxypaclitaxel</td>
<td>ND</td>
<td>0.6 ± 0.7</td>
<td>1.0 ± 0.7</td>
<td>4.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Liver (% of dose)</td>
<td>Paclitaxel</td>
<td>5.8 ± 0.8</td>
<td>9.3 ± 1.0**</td>
<td>9.2 ± 1.5*</td>
<td>12.3 ± 1.6**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’p-hydroxypaclitaxel</td>
<td>0.3 ± 0.04</td>
<td>0.5 ± 0.08**</td>
<td>1.8 ± 0.3***</td>
<td>4.1 ± 0.4***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6α-hydroxypaclitaxel</td>
<td>ND</td>
<td>0.1 ± 0.02</td>
<td>0.5 ± 0.3</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

Plasma levels of paclitaxel and metabolites are expressed as ng/ml (mean ± SD, \( n = 3\)-4) and liver levels of paclitaxel and metabolites are expressed as percentage of the dose (mean ± SD, \( n = 3\)-4). ND: not detectable. * \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \), compared to wild-type mice.

Impact of Mrp2 and P-gp on Fecal and Urinary Excretion of Paclitaxel. In both humans and mice, fecal excretion is the main route of elimination for paclitaxel, whereas almost no parent compound is found in the urine (14, 26-28). We collected urine and feces for 24 hours after i.v. administration of 10 mg/kg \([^{3}H] \)paclitaxel and determined cumulative excretion of total radioactivity as well as unchanged paclitaxel and its monohydroxylated metabolites (Table 3). 68.2% of the radioactivity was recovered from the feces in wild-type mice. In \( Mdr1a/1b^{-/-} \) and \( Mrp2^{-/-} \) mice this was reduced to 49.0% and 46.8%, respectively, whereas only 21.6% was found in the feces of \( Mdr1a/1b/Mrp2^{-/-} \) mice \( (P < 0.001 \) for each
parameter). For urinary excretion of radioactivity, a reverse pattern was found, ranging from 3.3% in wild-type mice to 27.1% in \( \text{Mdr1a/1b/Mrp2}^{-/-} \) mice. The combined radioactivity data revealed a shift from almost exclusively fecal excretion in wild-type mice to roughly equal fecal and urinary excretion in \( \text{Mdr1a/1b/Mrp2}^{-/-} \) mice.

HPLC-UV analyses showed that fecal excretion of unmodified paclitaxel in wild-type mice was 49% of the administered dose (Table 3). In \( \text{Mdr1a/1b}^{-/-} \) and \( \text{Mdr1a/1b/Mrp2}^{-/-} \) mice, less than 2% was excreted in the feces. For \( \text{Mrp2}^{-/-} \) mice, a less pronounced but still marked reduction in fecal excretion was found (to 30.8%, \( P = 0.002 \)), indicating that Mrp2 in liver and/or intestine also contributes substantially to the fecal excretion of paclitaxel (about 18% of the dose). Yet, in \( \text{Mdr1a/1b}^{-/-} \) mice, where Mrp2 is still present, paclitaxel was nearly absent from feces. This suggests that P-gp helps to keep paclitaxel, initially excreted by Mrp2, in the intestinal lumen, presumably by limiting reabsorption of the drug.

### Table 3. Cumulative fecal and urinary excretion (0-24 hr) of paclitaxel, \( 3'p \)-hydroxypaclitaxel and \( 6\alpha \)-hydroxypaclitaxel in intact mice after i.v. administration of \( [\text{3H}] \)paclitaxel at 10 mg/kg.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biological Matrix</th>
<th>Compound</th>
<th>( \text{Wild-type} )</th>
<th>( \text{Mdr1a/1b}^{-/-} )</th>
<th>( \text{Mrp2}^{-/-} )</th>
<th>( \text{Mdr1a/1b/Mrp2}^{-/-} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>Paclitaxel</td>
<td>49.0 ± 4.4</td>
<td>1.4 ± 0.6***</td>
<td>30.8 ± 8.1**</td>
<td>1.0 ± 0.3***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( 3'p )-hydroxypaclitaxel</td>
<td>14.8 ± 1.2</td>
<td>17.2 ± 1.3*</td>
<td>9.9 ± 1.9**</td>
<td>1.6 ± 0.5***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( 6\alpha )-hydroxypaclitaxel</td>
<td>8.4 ± 0.6</td>
<td>9.7 ± 0.8*</td>
<td>5.1 ± 1.6**</td>
<td>0.6 ± 0.2***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>([\text{3H}]) label</td>
<td>68.2 ± 1.6</td>
<td>49.0 ± 4.5***</td>
<td>46.8 ± 7.8***</td>
<td>21.6 ± 3.2***</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>Paclitaxel</td>
<td>0.66 ± 0.18</td>
<td>0.58 ± 0.21</td>
<td>0.73 ± 0.07</td>
<td>0.77 ± 0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( 3'p )-hydroxypaclitaxel</td>
<td>ND</td>
<td>ND</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( 6\alpha )-hydroxypaclitaxel</td>
<td>ND</td>
<td>ND</td>
<td>0.04 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>([\text{3H}]) label</td>
<td>3.3 ± 0.6</td>
<td>5.4 ± 0.8**</td>
<td>14.5 ± 1.7***</td>
<td>27.1 ± 3.3***</td>
<td></td>
</tr>
</tbody>
</table>

Excretion is given as percentage of the dose (mean ± SD, \( n = 5 \)). ND, not detectable.

* \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \), compared to wild-type mice.

### Role of Mrp2 and P-gp in Fecal and Urinary Excretion of Monohydroxylated Metabolites. The fecal excretion pattern of the hydroxylated paclitaxel metabolites was quite different from that of the parent compound (Table 3). Wild-type mice excreted 15% of the dose as \( 3'p \)-hydroxypaclitaxel and 8.5% as \( 6\alpha \)-hydroxypaclitaxel. In \( \text{Mdr1a/1b}^{-/-} \) mice, the fecal excretion of both metabolites was moderately but significantly increased compared to wild-type mice (\( P < 0.05 \) for
both), and accounted for more than half of the excreted radioactivity. Mrp2/– mice, however, displayed a reduced excretion of 3′-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel to 67% and 61% of wild-type levels, respectively. In Mdr1a/1b/Mrp2/– mice, fecal excretion of these metabolites was nearly abolished. The latter result suggests that, in addition to Mrp2, Mdr1a/1b P-gp is also important in the fecal excretion of the hydroxylated metabolites, in spite of their increased excretion in the Mdr1a/1b/– mice. This may result from strongly increased formation of the metabolites due to the extended residence time of paclitaxel in Mdr1a/1b/– mice, more than compensating for a partial reduction in their excretion capacity due to P-gp deficiency. Mrp2 appeared to be responsible for nearly all of the fecal excretion of the metabolites in the Mdr1a/1b/– mice.

Table 4. Paclitaxel and its monohydroxylated metabolites as determined in bile, plasma and different tissues of mice with cannulated gall bladder, 60 minutes after i.v. administration of [3H]paclitaxel at 5 mg/kg.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>Wild-type</th>
<th>Mdr1a/1b/–</th>
<th>Mrp2/–</th>
<th>Mdr1a/1b/Mrp2/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma†</td>
<td>Paclitaxel</td>
<td>546 ± 43</td>
<td>534 ± 65</td>
<td>825 ± 128**</td>
<td>837 ± 99**</td>
</tr>
<tr>
<td></td>
<td>[3H] label</td>
<td>936 ± 94</td>
<td>1068 ± 160</td>
<td>1324 ± 126**</td>
<td>1532 ± 111**</td>
</tr>
<tr>
<td>Bile</td>
<td>Paclitaxel</td>
<td>3.25 ± 0.83</td>
<td>2.21 ± 0.50</td>
<td>0.66 ± 0.17**</td>
<td>0.10 ± 0.05***</td>
</tr>
<tr>
<td></td>
<td>3′-p-hydroxypaclitaxel</td>
<td>0.95 ± 0.33</td>
<td>1.41 ± 0.33</td>
<td>0.10 ± 0.05***</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6α-hydroxypaclitaxel</td>
<td>0.40 ± 0.15</td>
<td>0.66 ± 0.17</td>
<td>0.03 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>[3H] label</td>
<td>19.0 ± 3.64</td>
<td>22.1 ± 3.02</td>
<td>4.26 ± 0.43***</td>
<td>3.91 ± 0.92***</td>
</tr>
<tr>
<td>Liver</td>
<td>Paclitaxel</td>
<td>27.5 ± 1.69</td>
<td>27.0 ± 1.15</td>
<td>37.9 ± 4.86**</td>
<td>36.8 ± 5.73*</td>
</tr>
<tr>
<td></td>
<td>3′-p-hydroxypaclitaxel</td>
<td>0.77 ± 0.32</td>
<td>1.03 ± 0.18</td>
<td>1.46 ± 0.38*</td>
<td>1.76 ± 0.53*</td>
</tr>
<tr>
<td></td>
<td>6α-hydroxypaclitaxel</td>
<td>0.27 ± 0.16</td>
<td>0.44 ± 0.08</td>
<td>0.73 ± 0.26*</td>
<td>0.74 ± 0.28*</td>
</tr>
<tr>
<td></td>
<td>[3H] label</td>
<td>24.6 ± 1.13</td>
<td>25.4 ± 1.16</td>
<td>37.2 ± 3.58***</td>
<td>39.7 ± 5.13***</td>
</tr>
<tr>
<td>S.I.C.</td>
<td>Paclitaxel</td>
<td>4.94 ± 0.93</td>
<td>2.00 ± 0.75**</td>
<td>3.59 ± 0.76*</td>
<td>1.63 ± 0.29**</td>
</tr>
<tr>
<td></td>
<td>3′-p-hydroxypaclitaxel</td>
<td>2.05 ± 0.33</td>
<td>3.90 ± 1.24*</td>
<td>0.70 ± 0.38***</td>
<td>0.86 ± 0.25***</td>
</tr>
<tr>
<td></td>
<td>6α-hydroxypaclitaxel</td>
<td>0.28 ± 0.07</td>
<td>0.55 ± 0.04**</td>
<td>0.14 ± 0.07*</td>
<td>0.23 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>[3H] label</td>
<td>7.55 ± 0.70</td>
<td>4.28 ± 0.71***</td>
<td>6.60 ± 1.05</td>
<td>2.78 ± 0.49***</td>
</tr>
</tbody>
</table>

Levels are given as percentage of the dose (means ± SD, n = 4-6). ND: not detectable; S.I.C.: small intestinal contents. †Plasma levels of paclitaxel are expressed as ng/ml and tritium plasma levels as ng-equivalent/ml. Metabolites were not detectable in plasma at t = 60 minutes. * P < 0.05, ** P < 0.01 and *** P < 0.001, compared to wild-type mice.
In the urine of *Mdr1a/1b*<sup>−/−</sup>, and especially *Mrp2*<sup>−/−</sup> and *Mdr1a/1b/Mrp2*<sup>−/−</sup> mice, a highly significant increase in excreted radioactivity was found. Paclitaxel and its primary hydroxylated metabolites only represented a minor fraction (Table 3), so other hydrophilic metabolites likely accounted for the majority of this excreted radioactivity.

**Impact of Mrp2 and P-gp on Biliary and Direct Intestinal Excretion of Paclitaxel and its Hydroxylated Metabolites.** We performed gall bladder cannulation experiments to clarify the roles of Mrp2 and Mdr1a/1b in biliary and direct intestinal excretion. Previous experiments suggest that P-gp does not primarily mediate biliary excretion of paclitaxel or its hydroxylated metabolites (5, 15). We measured the biliary excretion for 1 hour in anesthetized mice with a cannulated gall bladder and a ligated common bile duct, receiving i.v. [3H]paclitaxel at 5 mg/kg. In wild-type mice, 3.3% ± 0.8% of the dose was excreted over 1 hour as unchanged paclitaxel (Table 4). *Mdr1a/1b*<sup>−/−</sup> mice did not show a significant reduction in biliary excretion of paclitaxel, in line with previous findings (5, 15). In contrast, in *Mrp2*<sup>−/−</sup> mice biliary excretion of paclitaxel was reduced by 80% compared to wild-type mice, whereas in *Mdr1a/1b/Mrp2*<sup>−/−</sup> mice the excretion was almost totally abolished (97% reduction). A similar excretory pattern was found for the principal metabolites (Table 4). This indicates that Mrp2 is the predominant factor in the biliary excretion of paclitaxel and its hydroxylated metabolites and that Mdr1a/1b plays a minor role in this process. Furthermore, in *Mrp2*<sup>−/−</sup> and *Mdr1a/1b/Mrp2*<sup>−/−</sup> mice, very similar and significantly increased levels of paclitaxel in plasma (by 51% and 53%) and in liver (by 38% and 34%) and increased levels of metabolites in liver were found at the end of the cannulation experiment (Table 4). This probably reflects the decreased hepatobiliary elimination of paclitaxel and monohydroxylated metabolites owing to Mrp2 absence. The biliary radioactivity data indicate that the majority of other paclitaxel metabolites was also primarily transported into the bile by Mrp2, since in wild-type and *Mdr1a/1b*<sup>−/−</sup> mice about 20% of the radioactive dose was recovered in bile, whereas this was only ~4% in *Mrp2*<sup>−/−</sup> and *Mdr1a/1b/Mrp2*<sup>−/−</sup> bile.

Other than through biliary excretion, paclitaxel can reach the gut lumen by excretion directly across the intestinal wall. P-gp is known to play a major role in this process (5, 15). We analyzed the small intestinal contents at the end of the 1 hr gall bladder cannulation experiments. Since the common bile duct was ligated, paclitaxel and metabolites could only reach the intestinal lumen by excretion from the blood across the gut wall. In the small intestinal contents of wild-type mice 4.9 ± 0.9% of the administered dose was recovered as unchanged drug (Table 4). For *Mrp2*<sup>−/−</sup> mice this was 3.6 ± 0.8%, a modest but significant reduction (*P* = 0.035), also in view of the higher paclitaxel plasma concentration. Markedly less paclitaxel was detected in the intestinal lumen of *Mdr1a/1b*<sup>−/−</sup> and *Mdr1a/1b/Mrp2*<sup>−/−</sup> mice:
2.0% ± 0.8% and 1.6% ± 0.3%, respectively. These data confirm the dominant role of P-gp in the direct intestinal excretion of paclitaxel, while Mrp2 may contribute modestly to this process.

Different results were obtained for the hydroxylated metabolites. Mdr1a/1b−/− mice showed a significantly increased intestinal excretion of 3’p-hydroxy paclitaxel and 6α-hydroxy paclitaxel, presumably owing to higher plasma levels of these compounds. In contrast, clearly reduced amounts of these metabolites were found in the intestinal contents of Mrp2−/− and Mdr1a/1b/Mrp2−/− mice (Table 4). These data suggest that Mrp2 has a predominant function in the direct intestinal excretion of the hydroxylated paclitaxel metabolites.

DISCUSSION
In this study we describe the generation and characterization of Mdr1a/1b/Mrp2−/− mice, and their utilization in the analysis of the separate and combined impact of Mrp2 and P-gp on the pharmacokinetics of paclitaxel. Extensive analysis of the Mdr1a/1b/Mrp2−/− mice suggests that they are very similar to Mrp2−/− mice, displaying mild physiological abnormalities such as increased liver weight, mild conjugated hyperbilirubinemia, reduced bile flow and a modest decrease in blood haemoglobin levels. No severe deficiencies due to the combination of Mrp2 and Mdr1a/1b knockout were observed. Consequently, the Mdr1a/1b/Mrp2−/− mice appear as suitable for pharmacological analyses as the separate Mrp2−/− and Mdr1a/1b−/− mice (15, 20). These mice thus provide a powerful tool to study redundant or overlapping, but also complementary functions of Mrp2 and P-gp in pharmacology, toxicology and physiology.

Although we had previously demonstrated that paclitaxel is transported by human MRP2 (16), we were surprised to find that the impact of Mrp2 on the pharmacokinetics of paclitaxel after intravenous administration was at least as great as that of Mdr1a/1b P-gp. Paclitaxel is an excellent P-gp substrate, so we had expected that its pharmacokinetics would be dominated by P-gp, as is indeed the case upon oral administration of the drug. However, upon intravenous administration, even in the presence of P-gp, Mrp2 has a marked effect on paclitaxel plasma levels and excretion, at least equal to the P-gp effects. As paclitaxel is currently primarily administered to patients intravenously, variation in MRP2 activity might directly affect their effective paclitaxel exposure.

The pronounced impact of P-gp on (oral) paclitaxel pharmacokinetics appears to be determined primarily by the capability of P-gp to reduce net (re-)absorption of paclitaxel from the intestinal lumen, and, related to this, its capability to mediate direct intestinal excretion (5). Especially upon oral administration in P-gp-proficient mice, very little paclitaxel enters the circulation, leaving little room for a significant contribution of Mrp2. We observed earlier that Mrp2 has a more pronounced pharmacokinetic impact at relatively high plasma drug concentrations of
methotrexate, presumably because at lower plasma concentrations alternative, more high-affinity elimination systems dominate drug removal (20). The same might apply for elimination of the comparatively low paclitaxel levels after oral administration in P-gp-proficient animals (Fig. 1).

The results from Tables 3 and 4 indicate that Mrp2 and P-gp have rather complementary roles in hepatobiliary and intestinal excretion of paclitaxel after i.v. administration. Mrp2 is the dominant factor in biliary excretion of paclitaxel, and P-gp contributes modestly. In contrast, P-gp dominates the direct intestinal excretion of paclitaxel, while Mrp2 plays a minor role here. Table 3 shows that Mrp2 activity accounts for at least 18% of the dose being excreted in the feces over 24 hr, which must result mainly from hepatobiliary and perhaps some direct intestinal excretion. In spite of this, in the absence of P-gp in the Mdr1a/1b mice, very little paclitaxel is retrieved in the feces (Table 3). This must mean that the paclitaxel initially excreted by Mrp2 into the intestinal lumen of these mice is readily reabsorbed from the gut due to P-gp absence. This continued reabsorption of unchanged paclitaxel results in prolonged metabolism, explaining why very little unmetabolized paclitaxel leaves the body when P-gp is absent.

It is interesting to note that, in spite of the qualitatively different primary functions of P-gp and Mrp2 affecting paclitaxel pharmacokinetics, the quantitative effect of absence of both proteins on the AUC_i.v. was very similar (1.3-fold each). The combination of both deficiencies had rather an additive than a synergistic effect on the paclitaxel AUC_i.v. (1.3 x 1.3 = 1.69, corresponding well with the 1.7-fold increased AUC_i.v. in the combination knockout mice).

In the past, MRP2/Mrp2 has been considered primarily as an organic anion transporter, and earlier experiments in Mrp2-deficient rats and mice indicated that Mrp2 could have a marked effect on pharmacokinetics of the anionic anti-cancer drug methotrexate (20, 29). Our data show that Mrp2 can also be a major determinant of the pharmacokinetic behavior of a highly lipophilic anti-cancer drug, even in the presence of other very efficient transporters for this drug. As it is now clear that several other non-anionic and lipophilic (anti-cancer) drugs, including docetaxel, etoposide and various HIV protease inhibitors, are markedly transported by MRP2 in vitro (16, 30), it may well be that these other drugs are equally affected in their (i.v.) pharmacokinetics. This could mean that MRP2 activity has a much broader significance for pharmacokinetic behavior of anti-cancer and other drugs than previously appreciated. This is of importance, as extensive genetic polymorphisms in human MRP2 are known that affect functionality, some even resulting in full homozygous deficiency for MRP2 (19). In a recent study, six known allelic variants in genes involved in paclitaxel metabolism (CYP2C8, CYP3A4, CYP3A5) and in the gene coding for P-gp (ABCBI) were evaluated, but could not explain the substantial interindividual variability in paclitaxel pharmacokinetics.
It will be of interest to test whether polymorphisms in the $ABCC2$ gene contribute to these variations. Furthermore, factors affecting MRP2 expression, like hepatic diseases, renal failure or exposure to certain drugs, can result in inter-individual differences in disposition of drugs eliminated via MRP2 (19). Such variation in MRP2 activity might thus affect the therapeutic plasma levels and toxic side effects of a much broader range of anti-cancer drugs than previously realized and this should be taken into account during chemotherapy treatment of patients.

Our study shows that Mrp2 has a marked impact on both i.v. and oral paclitaxel AUC when P-gp activity is absent (Fig. 1). In a variety of clinical trials, highly efficacious P-gp inhibitors such as PSC-833 (Valspodar®), GF120918 (Elacridar®), and others are co-administered with paclitaxel or other MRP2 substrate drugs, to counteract multidrug resistance in tumors, or to improve the oral bioavailability of the anti-cancer drug (7, 10, 12, 32). Under these circumstances, variation in MRP2 activity due to genetic polymorphisms might have even more pronounced effects on effective availability of the drug, with implications for therapeutic efficacy and the risk of toxic side effects. It will thus be important to be well aware of the impact of MRP2 on the pharmacokinetic behavior of many anti-cancer drugs when P-gp is inhibited. The mouse models we have generated will provide useful tools to qualitatively assess this impact for a variety of drugs. This information can subsequently be used for rational translation of the insights to the (clinical) situation in humans, which may ultimately lead to more constant and reliable chemotherapy regimens.

REFERENCES


Role of Mrp2 (Abcc2) in paclitaxel pharmacokinetics


Chapter 4

Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the \textit{in vivo} elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate

Maria L.H. Vlaming, Zeliha Pala, Anita van Esch, Els Wagenaar, Olaf van Tellingen, Dirk R. de Waart, Ronald P.J. Oude Elferink, Koen van de Wetering and Alfred H. Schinkel

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Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the in vivo elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate

Maria L.H. Vlaming¹, Zeliha Pala², Anita van Esch¹, Els Wagenaar¹, Olaf van Tellingen³, Dirk R. de Waart⁴, Ronald P.J. Oude Elferink⁴, Koen van de Wetering⁵ and Alfred H. Schinkel¹

Divisions of ¹Experimental Therapy, ³Clinical Chemistry and ⁵Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; ²Faculty of Pharmacy, Istanbul University, Istanbul, Turkey; ⁴AMC Liver Center, Academic Medical Center, Amsterdam, The Netherlands.

Purpose: ABCC2 (MRP2) and ABCC3 (MRP3) mediate the elimination of toxic compounds such as drugs and carcinogens and have a large overlap in substrate specificity. We investigated the roles of Abcc2 and Abcc3 in the elimination of the anti-cancer drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) in vivo.

Experimental Design: Abcc2;Abcc3−/− mice were generated, characterized, and used to investigate possibly overlapping or complementary roles of Abcc2 and Abcc3 in elimination of MTX and 7OH-MTX after i.v. administration of 50 mg/kg MTX.

Results: Abcc2;Abcc3−/− mice were viable and fertile. In Abcc2−/− mice the plasma area under the curve (AUCi.v.) for MTX was 2.0-fold increased compared to wild-type, leading to 1.6-fold increased urinary excretion, which was not seen in Abcc2;Abcc3−/− mice. Biliary excretion of MTX was 3.7-fold reduced in Abcc2−/−, but unchanged in Abcc2;Abcc3−/− mice. The plasma AUCi.v.s of 7OH-MTX were 6.0-fold and 4.3-fold increased in Abcc2−/− and Abcc2;Abcc3−/− mice, respectively, leading to increased urinary excretion. The biliary excretion of 7OH-MTX was 5.8-fold reduced in Abcc2−/−, but unchanged in Abcc2;Abcc3−/− mice. 7OH-MTX accumulated substantially in liver of Abcc2−/− and especially Abcc2;Abcc3−/− mice.

Conclusions: Abcc2 is important for (biliary) excretion of MTX and its toxic metabolite 7OH-MTX. When Abcc2 is absent, Abcc3 transports MTX and 7OH-MTX back from the liver into the circulation, leading to increased plasma levels and urinary excretion. Variation in ABCC2 and/or ABCC3 activity may therefore have profound effects on the elimination and severity of toxicity of MTX and 7OH-MTX after MTX treatment of patients.
INTRODUCTION

The multidrug resistance proteins ABCC2 (MRP2) and ABCC3 (MRP3) are members of the ATP-binding cassette (ABC) transporter superfamily. ABCC2 is present in apical membranes of hepatocytes and epithelial cells of small intestine and kidney and is involved in the elimination of both endogenous and exogenous compounds from the body (1). ABCC3 is also found in liver, kidney and small intestine, as well as in adrenal glands and pancreas. In contrast to ABCC2, ABCC3 localizes to the basolateral membrane of polarized cells (2). Both transporters are expressed in various tumors and can transport a range of (anti-cancer) drugs (1;2). There is a large overlap in the substrate specificity of ABCC2 and ABCC3. They can for example both transport bilirubin glucuronides (3-5), and in patients with the Dubin-Johnson syndrome, who have functionally deficient ABCC2 (6;7), both plasma bilirubin glucuronide levels and ABCC3 protein levels are increased. It has been speculated that these two findings are related: ABCC3 upregulation in absence of ABCC2 would allow increased basolateral efflux of bilirubin glucuronides from the liver (7). Since Abcc2 and Abcc3 are both involved in the elimination of xenobiotics, absence of each or both of them can have a profound effect on the pharmacokinetics of drugs, or their glucuronide conjugates, as has been shown using single knockout mice for Abcc2 and Abcc3 (8), as well as with the recently generated Abcc2;Abcc3−/− mice (9).

Methotrexate (MTX), a widely used anti-cancer and anti-rheumatic drug, is a substrate for both ABCC2 and ABCC3 in vitro (2). Abcc2-deficient mice and rats are hampered in the (biliary) elimination of ([3H])MTX (10;11), whereas the effect of Abcc3 on the pharmacokinetics of MTX in vivo thus far has not been studied. In the treatment of cancer, MTX is given intravenously at relatively high doses (>15 mg/m²), which sometimes leads to severe and even lethal toxicity in patients (12). Interestingly, mutations in the ABCC2 gene have recently been associated with increased methotrexate toxicity in patients (13-15). Furthermore, crystalline deposits of the toxic metabolite 7-hydroxymethotrexate (7OH-MTX) (which has a very low aqueous solubility) in renal tubules have been implicated in kidney failure after high-dose MTX treatment (12;16). The effect of ABCC2 and ABCC3 on the disposition of 7OH-MTX has not yet been studied, although it was shown by Breedveld et al. (2007) that ABCC2 transports 7OH-MTX in vitro (17).

To investigate the hypothesized functional relationship between Abcc2 and Abcc3 in vivo, we have generated and characterized Abcc2;Abcc3−/− mice and analyzed plasma, bile and urine of these mice. We further used the mice to investigate the relative roles of Abcc2 and Abcc3 in the elimination of MTX and its toxic metabolite 7OH-MTX, which is primarily formed by aldehyde oxidase in the liver (18-20). We show here that Abcc2 has a major impact on the pharmacokinetics of both MTX and 7OH-MTX and that, when Abcc2 is absent, Abcc3 transports these toxic compounds from the liver back into the circulation, allowing an
alternative route of elimination via the urine. Our data illustrate the importance of the functionally overlapping and complementary roles of Abcc2 and Abcc3 in vivo.

METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Abcc2<sup>−/−</sup> (10) and Abcc3<sup>−/−</sup> (5) mice have been described. Abcc2;Abcc3<sup>−/−</sup> mice were generated by cross-breeding. 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. MTX (Emthexate PF<sup>®</sup> 25 mg/ml) was from Pharmachemie (Haarlem, The Netherlands) and 7OH-MTX from Toronto Research Chemicals Inc. (North York, ON, Canada). 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). MRPr1, M<sub>4</sub>I-80 and M<sub>5</sub>II-54 were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands), K12 was kindly provided by Dr. Bruno Stieger (University Hospital, University of Zürich, Zürich, Switzerland). BXP-53 was described before (21).

Western analysis. Crude membrane fractions from tissues were prepared as described (21;22). Western blotting was performed as described (23). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). Abcc1 (Mrp1), Abcc4 (Mrp4), Abcc5 (Mrp5), Abcg2 (Bcrp1) and Abcb11 (Bsep) were detected with Abs MRPr1 (dilution 1:1000), M<sub>4</sub>I-80 (dilution 1:400), M<sub>5</sub>II-54 (dilution 1:1), BXP-53 (dilution 1:400) and K12 (dilution 1:2000), respectively. Bound primary antibodies were detected by incubating the blot with HRP-labelled rabbit anti-rat IgG (1:1000, DAKO) (Abcc1, Abcc4, Abcc5 and Abcg2) or HRP-labelled goat anti-rabbit IgG (1:2000, DAKO) (Abcb11).

Histological, clinical-chemical and hematological analysis. Histological analysis of mouse tissues, standard clinical chemistry analyses on serum and standard hematological analysis (twice within a time span of 1.5 years) were performed as described (10).

Bile flow measurements and analysis. Gall bladder cannulations and collection of bile in male wild-type, Abcc2<sup>−/−</sup>, Abcc3<sup>−/−</sup> and Abcc2;Abcc3<sup>−/−</sup> mice (n = 5 for each group) were performed as described (10;24). Concentrations of bilirubin monoglucuronides (BMG), bilirubin diglucuronides (BDG) and unconjugated
bilirubin (UCB) in bile and urine were determined as described (25). Bile salts, choline-containing phospholipids, and cholesterol were determined enzymatically as described (26).

**Plasma and tissue pharmacokinetic experiments.** MTX was administered to female mice by injecting 5 \( \mu l/g \) body weight of a 10 mg/ml MTX in 0.9% NaCl solution into the tail vein. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and organs were removed. Intestinal contents (feces) and tissue were separated.

**Biliary excretion of MTX and 7OH-MTX.** Gall bladder cannulations in female mice were performed as described (24). After cannulation, 50 mg/kg MTX was administered i.v. as described above. Bile was collected in 15 min fractions for 60 min. Subsequently, mice were killed by cardiac puncture and blood and organs were collected.

**Fecal and urinary excretion of MTX and 7OH-MTX.** Female mice were individually housed in Ruco Type M/1 stainless steel metabolic cages (Valkenswaard, The Netherlands), and allowed 24 hours to adapt before 50 mg/kg MTX was injected into the tail vein, as described above. Feces and urine were collected over 0-24 and 24-48 hours. 48 hours after injection, mice were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia. Organs were removed and intestinal contents (feces) and tissue were separated.

**HPLC analysis of MTX and 7OH-MTX.** Collected organs were homogenized in an ice-cold 4% BSA solution before HPLC analysis. MTX and 7OH-MTX concentrations in plasma, urine and tissue homogenates were determined as described (27).

**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. When more than two groups were compared, one-way ANOVA followed by Tukey’s multiple comparison test was used, as indicated in text and/or figure legends. Results are presented as the means ± standard deviations (SD). Differences were considered statistically significant when \( P < 0.05 \). Averaged concentrations for each time-point were used to calculate the area under the plasma-concentration versus time curve (AUC) from \( t = 0 \) to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (28). Results of AUC measurements are presented as means ± SD.
Figure 1. Characterization of Abcc2;Abcc3−/− mice. A, liver weight (as percentage of body weight) of male (left panel) and female (right panel) wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice (means ± SD, n = 5, **, P < 0.01, ***, P < 0.001, ANOVA). B, levels of Abcc4 protein in crude membrane fractions of liver samples from two independent female Abcc2;Abcc3−/− and wild-type mice. C, levels of Abcc4 protein in crude membrane fractions of kidney samples from two independent female Abcc2;Abcc3−/− and wild-type mice. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. In B and C, the lane with the positive control (Sf-9 vesicles containing ABCC4 (40)) is indicated with “+”. Underglycosylation of ABCC4 in the Sf-9 cells causes the faster migration compared to the murine Abcc4. The amount of protein loaded is noted above the lanes.
RESULTS

Macroscopic and microscopic analysis of Abcc2;Abcc3−/− mice.
Abcc2;Abcc3−/− mice were viable, fertile and had normal life spans, body weights and anatomy. Adult Abcc2;Abcc3−/− mice had a 36-49% increased liver weight compared to wild-type mice (Figure 1A). The liver weight in male Abcc2;Abcc3−/− mice was also significantly higher than in the Abcc2−/− mice (Figure 1A), which by themselves already had a ~27% increased liver weight (Figure 1A and (10)). Similarly increased liver weight was seen in Abcc2−/− and Abcc2;Abcc3−/− mice in C57BL/6 background (29). Despite the markedly increased liver size, detailed microscopic analysis of liver sections did not reveal obvious pathological changes.

Protein levels of other ABC multidrug transporter proteins in tissues of Abcc2;Abcc3−/− mice.
We checked protein levels of various ABC transporters in liver, kidney and/or small intestine of male and female Abcc2;Abcc3−/− mice using immunoblot analysis. Abcc4 expression in livers (Figure 1B) and kidneys (Figure 1C) of female but not male (not shown) Abcc2;Abcc3−/− mice was about 2-fold increased compared to wild-type livers. Levels of Abcc4 in small intestine were very low, and no differences were seen between wild-type and Abcc2;Abcc3−/− mice (not shown). Abcg2 protein levels in male and female liver, kidney and small intestine of Abcc2;Abcc3−/− mice were not different from wild-type (not shown). Abcc1 and Abcc5 expression in liver of male and female Abcc2;Abcc3−/− mice were not different from those found in wild-type either (not shown), nor was Abcb11 expression in liver of male Abcc2−/− and Abcc2;Abcc3−/− mice (not shown).

Plasma clinical chemistry and hematological analysis of Abcc2;Abcc3−/− mice.
It has been hypothesized (7;10;30) that Abcc3 expression (most likely in liver) could be related to increased conjugated bilirubin levels observed in the circulation of Abcc2−/− mice, possibly as a compensation for the reduced biliary excretion of bilirubin glucuronides via Abcc2. Analysis of plasma from wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice showed that Abcc3 is indeed necessary for the increased plasma bilirubin glucuronide levels seen in Abcc2−/− mice: whereas in Abcc2−/− mice plasma levels of total and conjugated bilirubin were markedly increased as compared to wild-type controls, in Abcc2;Abcc3−/− mice these plasma levels returned to wild-type values. Abcc3−/− mice had plasma levels of total and conjugated bilirubin similar to those found in wild-type mice as well (Figures 2A and B).

All other clinical-chemical parameters measured showed no significant differences between wild-type and knockout strains (not shown). Also hematological analysis did not yield any consistent differences between Abcc2;Abcc3−/− and wild-type mice.
**Figure 2.** Bilirubin levels in plasma of *Abcc2;Abcc3* mice. 

A, plasma values of total bilirubin in wild-type, *Abcc2*+/−, *Abcc3*+/− and *Abcc2;Abcc3* mice (means ± SD, n = 3-9, ***, P < 0.001). B, plasma values of conjugated bilirubin in wild-type, *Abcc2*+/−, *Abcc3*+/− and *Abcc2;Abcc3* mice (means ± SD, n = 3-9). The values for wild-type, *Abcc3*+/− and *Abcc2;Abcc3* mice were below the detection limit of the analyzer (<1 µM).

**Biliary and urinary excretion and composition in *Abcc2;Abcc3* mice.** We analyzed the bile and urine composition of wild-type, *Abcc2*+/−, *Abcc3*+/− and *Abcc2;Abcc3* mice after ligation of the common bile duct and gall bladder cannulation. We previously found that *Abcc2*+/− mice have a significantly reduced bile flow (Figure 3A and (10)). Bile flow in the *Abcc2;Abcc3* mice was significantly reduced as well, to about 76% of wild-type bile flow (Figure 3A). Surprisingly, this was still significantly higher than in *Abcc2*+/− mice (P<0.01, ANOVA), although this was not the case after correction for differences in liver weight (ANOVA) (not shown). The biliary excretion of total bilirubin in *Abcc2;Abcc3* mice was significantly reduced compared to wild-type mice (Figure 3B). The reduced total biliary bilirubin excretion in *Abcc2;Abcc3* mice was caused by a reduction in bilirubin monoglucuronide output, which was also reduced in *Abcc2*+/− mice (Figure 3B). Interestingly, the (much lower) biliary bilirubin diglucuronide output in both strains lacking *Abcc2* was increased compared to wild-type and *Abcc3*+/− mice (Figure 3B). The (modest) output of unconjugated bilirubin in bile of *Abcc2*+/−, *Abcc3*+/− and *Abcc2;Abcc3* mice was not significantly different.
from wild-type mice (Figure 3B). Analysis of the urine of the cannulated mice showed that bilirubin monoglucuronide was found in urine of \(Abcc2^{-/-}\), but not of \(Abcc2;Abcc3^{-/-}\) mice, consistent with the increased bilirubin glucuronide levels in plasma of \(Abcc2^{-/-}\) mice (Figure 3C and 2B).

**Figure 3.** Analysis of bile and urine from male wild-type, \(Abcc2^{-/-}\), \(Abcc3^{-/-}\) and \(Abcc2;Abcc3^{-/-}\) mice after gall bladder cannulation and ligation of the common bile duct. Bile was collected in 15 min fractions over 1 h. Bile collected in the first 15 min was analyzed for bilirubin concentration (Panel B). A, average bile flow in the various mouse strains (0-60 min). B, output of total bilirubin, bilirubin monoglucuronides (BMG), bilirubin diglucuronides (BDG) and unconjugated bilirubin (UCB) in the bile of the mouse strains (first 15 min fractions) (BW, body weight). C, BMG concentration in urine of the mouse strains. BDG and UCB were not detected in any of the urine samples. Data are means ± SD (\(n=5\), *, \(P < 0.05\); **, \(P < 0.01\), ***, \(P < 0.001\)) (n.q., not quantifiable).

Biliary output of cholesterol and bile acids was not significantly different in any of the knockout strains (not shown). Biliary phospholipid output was significantly increased in \(Abcc2^{-/-}\) and \(Abcc2;Abcc3^{-/-}\) mice compared to wild-type mice (Supplementary Figure 1SA). However, after correction for liver weight, no significant differences were seen in any of the strains (Supplementary Figure 1SB).

**Influence of Abcc2 and Abcc3 on methotrexate pharmacokinetics in vivo.**
To investigate the relative roles of Abcc2 and Abcc3 in the pharmacokinetics of MTX, we administered MTX i.v. to female wild-type, \(Abcc2^{-/-}\), \(Abcc3^{-/-}\) and
Abcc2;Abcc3/- mice at a dose of 50 mg/kg (comparable to 154 mg/m² in man (31)) and measured MTX levels in plasma and a set of organs at different time points. The results for plasma are shown in Figure 4A and Supplementary Table 1S. Compared to wild-type mice, the MTX plasma AUC between 7.5 and 120 min was 2-fold increased in Abcc2/- mice (870 ± 103 min·µg/ml vs. 444 ± 44 min·µg/ml for wild-type, P = 6.2 * 10⁻⁴). In contrast, in the Abcc2;Abcc3/- mice the MTX plasma AUC was not significantly different from wild-type (435 ± 47 min·µg/ml, P = 0.76), and 2-fold lower than in single Abcc2/- mice (P = 2.2*10⁻³). In Abcc3/- mice the MTX plasma AUC was not significantly different from wild-type either, although there was a tendency of a reduced AUC_{plasma} in these mice (368 ± 34 min·µg/ml, P = 0.084). This suggests that in the presence of Abcc2 there is only a minor influence of Abcc3 on the pharmacokinetics of MTX. However, the role of Abcc3 becomes important when Abcc2 is absent.

**Figure 4.** Pharmacokinetics of MTX after i.v. administration of 50 mg/kg MTX to female wild-type, Abcc2/-, Abcc3/- and Abcc2;Abcc3/- mice. A, MTX plasma concentration versus time curves of the various strains (means ± SD, n = 3-11). B, MTX liver level versus time curves (means ± SD, n = 4-9). C, liver-to-plasma ratio versus time curves (means ± SD, n = 4-9). D, MTX small intestinal tissue + contents (SI tissue + contents) level versus time curves (means ± SD, n = 5-9).
We also measured liver levels of MTX between 7.5 and 120 min after administration (Figure 4B and Supplementary Table 1S). Already 7.5 min after administration, livers contained high amounts of MTX (44-55% of the dose), which decreased thereafter. Interestingly, the liver-to-plasma ratios of MTX over 120 min (Figure 4C and Supplementary Table 1S) were lower in Abcc2−/− mice than in wild-type mice, whereas Abcc3−/− mice tended to have increased MTX liver-to-plasma ratios. In Abcc2;Abcc3−/− mice the liver-to-plasma ratios were similar to those found in wild-type. These results, combined with the MTX plasma vs. time curves, suggest that Abcc2;Abcc3−/− mice had reduced sinusoidal elimination of MTX from the liver compared to Abcc2−/− mice.

We next determined the MTX levels in small intestinal tissue and contents of the different strains (Figure 4D and Supplementary Table 1S). MTX levels in all strains steadily increased up to 60 min after administration. At all time points MTX levels in small intestine of Abcc2−/− mice were significantly lower compared to wild-type mice (Supplementary Table 1S), indicating an important role for Abcc2 in the elimination of MTX, most likely via hepatobiliary excretion (see also below). At 60 min after administration, MTX small intestinal levels in Abcc2;Abcc3−/− mice were significantly reduced as well, compared to wild-type and Abcc3−/− mice (P<0.01, ANOVA). However, MTX levels in Abcc2;Abcc3−/− mice were significantly higher than in Abcc2−/− mice at this time point (P<0.01, ANOVA). Collectively, these data suggest that apical elimination mechanisms different from Abcc2 still mediate substantial hepatobiliary excretion of MTX in the Abcc2−/− and Abcc2;Abcc3−/− mice. Tendencies of increased liver MTX concentrations in the Abcc2;Abcc3−/− mice (Figure 4B), and higher MTX hepatobiliary excretion compared to Abcc2−/− mice (see below), likely explain the higher small intestinal values in the Abcc2;Abcc3−/− mice.

Kidney toxicity is a clinically relevant dose-limiting factor for (high-dose) MTX treatment (32). Compared to wild-type mice, kidney levels of MTX were significantly increased in Abcc2−/− mice 15-60 min after MTX administration, whereas levels in kidneys of Abcc2;Abcc3−/− mice were not (Supplementary Table 1S). MTX kidney levels appeared to correlate with MTX plasma levels in all strains.

Subsequent gall bladder cannulation experiments revealed that excretion of MTX into bile the first 60 min after i.v. MTX administration (50 mg/kg) was reduced in Abcc2−/− mice to about 27% of that found in wild-type mice. Surprisingly, in Abcc2;Abcc3−/− mice, the biliary excretion of MTX over the first 60 min did not differ from that found in wild-type or Abcc3−/− mice (ANOVA) (Figure 5A). MTX levels in small intestinal contents and tissue after gall bladder cannulation were very low (<1% of the dose) in all strains and did not differ between the strains (not shown), indicating that direct intestinal excretion is a negligible route for the elimination of MTX in mice.
Figure 5. Effect of Abcc2 and Abcc3 on biliary and urinary excretion of MTX in female mice. A, biliary excretion of MTX (as % of dose) after i.v. administration of 50 mg/kg MTX to common bile duct ligated and gall bladder cannulated female wild-type, Abcc2\(^{-/-}\), Abcc3\(^{-/-}\) and Abcc2;Abcc3\(^{-/-}\) mice (means ± SD, n = 3-5, **, P < 0.01, ANOVA). B, urinary and fecal excretion of MTX (as % of dose) in the first 24 hrs after i.v. administration of 50 mg/kg MTX to mice of the various strains (means ± SD, n = 3-9, **, P < 0.01, ANOVA). Urinary and fecal excretion of MTX 24-48 hrs after i.v. administration were around 1% of the dose and not significantly different from wild-type in any of the strains (not shown). The levels of MTX in liver, small intestine and kidney 48 hrs after i.v. administration were below 0.03% in all strains and plasma levels were below the LLQ (24 nM) (not shown).

Clearly, Abcc2\(^{-/-}\) mice excreted less MTX into bile, resulting in increased MTX plasma levels. The fact that in Abcc2;Abcc3\(^{-/-}\) mice MTX plasma levels were not increased illustrates that in the absence of Abcc2, Abcc3 substantially transports MTX across the sinusoidal membrane of the hepatocyte towards the circulation, thereby possibly facilitating the elimination of MTX via the urine. To investigate this we determined the urinary and fecal excretion of MTX (50 mg/kg) in the various strains after i.v. administration (Figure 5B). Indeed, Abcc2\(^{-/-}\) mice displayed increased elimination of unchanged MTX via the urine, whereas urinary excretion of MTX in Abcc2;Abcc3\(^{-/-}\) mice was reduced to rates found in wild-type and Abcc3\(^{-/-}\) mice. Despite the markedly reduced biliary excretion of MTX in Abcc2\(^{-/-}\) mice, the fecal excretion of MTX did not differ from wild-type in any of the strains (Figure 5B). Compared to the high amount of MTX excreted into bile (Figure 5A) and small intestine (Figure 4D) in the first hour after administration, the amount of MTX found in feces is relatively low. This indicates that MTX is substantially re-absorbed from the intestine in all mouse strains tested.

Influence of Abcc2 and Abcc3 on 7-hydroxymethotrexate pharmacokinetics in vivo.

In patients receiving high-dose MTX a significant amount of the toxic metabolite 7OH-MTX, which is primarily formed in the liver (18-20), is detected in the urine
The effects of Abcc2 and Abcc3 on the pharmacokinetics of 7OH-MTX after i.v. administration of 50 mg/kg MTX are shown in Figure 6 and Supplementary Table 2S. The plasma concentration-time curves for 7OH-MTX in wild-type, Abcc2\(^{-/-}\), Abcc3\(^{-/-}\) and Abcc2; Abcc3\(^{-/-}\) mice (Figure 6A) suggest a very important role for Abcc2 in the elimination of this toxic metabolite. Whereas the plasma AUCs for 7OH-MTX in wild-type and Abcc3\(^{-/-}\) mice were quite low and not significantly different from each other (wild-type: 17 ± 3 min· µg/ml, Abcc3\(^{-/-}\): 12 ± 1 min· µg/ml, \(P = 0.084\)), the plasma AUC for 7OH-MTX in Abcc2\(^{-/-}\) mice was 6-fold increased compared to wild-type mice (106 ± 11 min· µg/ml, \(P = 3.7 \times 10^{-5}\)). At early time-points (0-30 min), plasma levels of 7OH-MTX in Abcc2; Abcc3\(^{-/-}\) mice were not different from wild-type and Abcc3\(^{-/-}\) mice, and clearly lower than in Abcc2\(^{-/-}\) mice. This suggests that in the absence of Abcc2, Abcc3 efficiently transports 7OH-MTX from liver towards the circulation. Interestingly, at later time points after MTX administration (> 30 min) plasma 7OH-MTX levels were substantially increased in Abcc2; Abcc3\(^{-/-}\) mice, leading to a 4.3-fold higher plasma AUC compared to wild-type mice (73 ± 20 min· µg/ml, \(P = 7.1 \times 10^{-5}\)). The concomitant liver levels of 7OH-MTX in Abcc2\(^{-/-}\) mice were markedly higher than in wild-type mice at all time points tested, and the 7OH-MTX levels in livers of Abcc2; Abcc3\(^{-/-}\) mice were even more increased, especially between 30 and 120 min after administration of MTX (Figure 6B and Supplementary Table 2S). This suggests an important role for Abcc2 in clearing the liver from the toxic metabolite 7OH-MTX. Abcc3 only plays a role in the absence of Abcc2, as the 7OH-MTX levels in liver of Abcc3\(^{-/-}\) mice were not different from those found in wild-type mice. Kidney levels of 7OH-MTX in Abcc2\(^{-/-}\) (0-60 min) and Abcc2; Abcc3\(^{-/-}\) (15-60 min) mice were 3-11 fold and 4-7 fold increased compared to wild-type, respectively, correlating with the increased 7OH-MTX plasma levels (Supplementary Table 2S).

Gall bladder cannulation experiments furthermore showed that the cumulative biliary excretion of 7OH-MTX in Abcc2\(^{-/-}\) mice in the first 60 min after MTX administration was reduced to only 17% of the biliary 7OH-MTX output in wild-type mice (Figure 6C). In contrast, the biliary excretion of 7OH-MTX was not significantly different from wild-type in Abcc3\(^{-/-}\) and Abcc2; Abcc3\(^{-/-}\) mice (Figure 6C).

Whether Abcc2 and Abcc3 affect urinary and fecal excretion of 7OH-MTX in the first 24 hr after i.v. administration of MTX (50 mg/kg) was subsequently investigated (Figure 6D). Compared to control mice, urinary excretion of 7OH-MTX was markedly higher in both Abcc2\(^{-/-}\) and Abcc2; Abcc3\(^{-/-}\) mice, consistent with the increased 7OH-MTX plasma levels in these strains (Figure 6D). Urinary 7OH-MTX output in Abcc3\(^{-/-}\) mice was not significantly different from wild-type output (Figure 6D). The fecal output of 7OH-MTX was similar to wild-type in both Abcc2\(^{-/-}\) and Abcc3\(^{-/-}\) mice, but 2-fold increased in Abcc2; Abcc3\(^{-/-}\) mice (Figure 6D).
Figure 6. Pharmacokinetics of 7OH-MTX after i.v. administration of 50 mg/kg MTX to female wild-type, *Abcc2*/-;*Abcc3*/- and *Abcc2;*Abcc3*/- mice. A. 7OH-MTX plasma concentration versus time curves of the various strains (means ± SD, n = 3-11). B, 7OHMTX liver level (as % of MTX dose) versus time curves (means ± SD, n = 4-9). C, biliary excretion of 7OH-MTX (as % of MTX dose) after ligation of the common bile duct and gall bladder cannulation, followed by i.v. administration of 50 mg/kg MTX (means ± SD, n = 3-5, *, P < 0.05). D, urinary and fecal excretion of 7OH-MTX (as % of MTX dose) in the first 24 hrs after i.v. administration of 50 mg/kg MTX (means ± SD, n = 3-9, ***, P < 0.001). The urinary excretion of 7OH-MTX 24-48 hours after MTX administration was below 1% of the given dose in all strains, and 7OH-MTX was not detected in feces over that time period (not shown). 48 hrs after administration, the 7OH-MTX concentrations in liver and small intestinal contents were below 0.15 % and 7OH-MTX was undetected in plasma, kidneys and small intestinal tissue of all strains (not shown).

DISCUSSION
Abcc2 and Abcc3 have overlapping substrate specificities and Abcc3 protein expression is often increased when Abcc2 is absent (7;10). Using *Abcc2*/- and the here described *Abcc2;*Abcc3*/- mice we show that in the absence of Abcc2, Abcc3 transports bilirubin glucuronide across the sinusoidal membrane, resulting in increased plasma levels and urinary excretion. Similar results were obtained for the pharmacokinetics of the anti-cancer and anti-rheumatic drug MTX: Abcc2 transports MTX into bile whereas in the absence of Abcc2, Abcc3 transports MTX back into
the circulation, allowing increased urinary excretion. Using \( \text{Abcc2}^{+/+} \) and \( \text{Abcc2};\text{Abcc3}^{+/+} \) mice we additionally show that Abcc2 is important for the biliary excretion of 7OH-MTX, the main (toxic) metabolite of MTX. In the absence of Abcc2, Abcc3 markedly influences the sinusoidal elimination of 7OH-MTX from the liver, and the absence of both ABC transporters leads to significant liver accumulation of 7OH-MTX. Absence of Abcc2 leads to a profound increase in plasma and kidney levels of MTX and especially 7OH-MTX, which is partly dependent on presence of Abcc3. Analysis of \( \text{Abcc3}^{-/-} \) mice alone would have suggested little impact of Abcc3 on either MTX or 7OH-MTX pharmacokinetics. Our findings suggest that differences in ABCC2 and (when ABCC2 is absent or reduced) ABCC3 activity could profoundly influence plasma and tissue levels, as well as MTX and 7OH-MTX related toxicity in patients who are treated with MTX.

It is quite surprising that in the absence of both Abcc2 and Abcc3, the health of the mice does not seem to be affected. This may be caused by the fact that the mice live in a relatively protected environment and therefore may not be confronted with many toxic compounds during their life. Other elimination mechanisms possibly (partly) compensate for the absence of these ABC transporters, for example Abcc4, which is 2-fold increased in liver and kidney of female \( \text{Abcc2};\text{Abcc3}^{-/-} \) mice. Although no spontaneous health problems were seen in untreated \( \text{Abcc2};\text{Abcc3}^{-/-} \) mice, drug treatment can clearly lead to substantial accumulation of potentially toxic metabolites in liver and plasma, as we show here for 7OH-MTX after MTX treatment, and as was previously shown for morphine-3-glucuronide upon morphine treatment of \( \text{Abcc2};\text{Abcc3}^{-/-} \) mice (9).

Analysis of the plasma and urine of the \( \text{Abcc2};\text{Abcc3}^{-/-} \) mice (Figures 2 and 3C) showed that Abcc3 is necessary for the increased bilirubin glucuronide levels in blood and urine of \( \text{Abcc2}^{-/-} \) mice. This indicates that Abcc3 functions as a back-up pathway for Abcc2: when toxic compounds in the liver cannot be excreted into the bile, Abcc3 transports them into the blood and the compounds are alternatively excreted via the urine. Surprisingly, in \( \text{Abcc2};\text{Abcc3}^{-/-} \) mice both biliary and urinary excretion of bilirubin are relatively low, raising the question how the bilirubin formed is handled in these mice. \( \text{Abcc2};\text{Abcc3}^{+/+} \) mice do not display obvious pathologies, suggesting that alternative bilirubin elimination pathways are involved. Bilirubin accumulating in the liver might for example be degraded by CYP1A enzymes (34), but also (possibly unknown) other mechanisms could play a role.

Using \( \text{Abcc2}^{+/+} \) mice, we confirmed that absence of Abcc2 causes a reduction in bile flow (10;35). Surprisingly, the bile flow (when corrected for body weight) of \( \text{Abcc2};\text{Abcc3}^{+/+} \) mice was significantly higher than that of \( \text{Abcc2}^{+/+} \) mice (albeit still lower than wild-type bile flow). The reason for this is unknown. Possibly accumulation of Abcc2 and Abcc3 substrates in liver can induce compensatory mechanisms that increase the bile flow, perhaps via other ABC transporters such as Abcg2 or Abcb11, although protein expression of these two transporters was not
increased in the liver of male \( Abcc2;Abcc3^{+/−} \) mice.

Using gallbladder cannulation experiments we show that murine Abcc2 plays an important role in the biliary excretion of MTX, as was shown previously for rat Abcc2 (11). The results for rats and mice were similar, although in rats the effect of Abcc2 was even more pronounced than in mice. This suggests that additional transport mechanisms (for example Abcg2) are more important for biliary excretion of MTX in mice compared to rats. Another explanation for this difference could be that in Abcc2-deficient rats Abcc3 liver protein expression is much more increased than in \( Abcc2^{−/−} \) mice (~6-fold in rats vs. ~2-fold in mice) (10;36). Strongly induced Abcc3 in liver of Abcc2-deficient rats may lead to an overestimation of the effect of Abcc2 on biliary MTX excretion.

In absence of Abcc2, Abcc3 is clearly involved in elimination of MTX from the liver. Increased plasma and decreased liver levels of MTX in the \( Abcc2^{−/−} \) mice were reflected by increased urinary excretion of MTX, which was not observed in \( Abcc2;Abcc3^{−/−} \) mice. Thus, hepatic Abcc3 expression is necessary for the increased urinary MTX excretion in the \( Abcc2^{−/−} \) mice. The Abcc3-mediated (back-)transport of MTX from the liver into the circulation leads to a reduced volume of distribution of MTX in \( Abcc2^{−/−} \) mice due to upregulation of Abcc3. Interestingly, although hepatic Abcc3 transports MTX towards the circulation, MTX is still quite rapidly taken up by the liver, leading to accumulation of >44% of the dose 7.5 min after administration in all strains. At later time points also other transport mechanisms than Abcc2 and Abcc3 (possibly Abcg2 and/or Abcc4), passive diffusion and/or metabolism seem to play a role in liver elimination of MTX: in \( Abcc2;Abcc3^{−/−} \) mice 120 min after i.v. administration only 1% of the dose was left in the liver.

The effect of other (apical) transporters was also suggested by the gall bladder cannulation experiments, as in absence of Abcc2 there was still significant transport of MTX into the bile. When Abcc2 and Abcc3 were both absent, the biliary excretion of MTX was markedly higher than in the absence of Abcc2 alone. Combined, this suggests the presence of one or more alternative canaliculc transporters which become increasingly important in biliary excretion of MTX as a consequence of (mildly) increasing liver concentrations in \( Abcc2;Abcc3^{−/−} \) mice. An obvious candidate would be Abcg2, which has been shown to influence MTX pharmacokinetics in mice (37), but also other apical transport proteins could be involved.

The severities of MTX-related toxicities in humans appear to correlate with MTX serum levels (12;16) and might, based on our results, be related to the expression levels and/or activity of ABCC2 and ABCC3. High-dose MTX therapy additionally leads to persistent renal dysfunction in nearly 30% of all patients (32). Renal failure is thought to be caused by crystalline deposits of MTX and especially 7OH-MTX (12). In rats, 7OH-MTX is much more toxic than MTX (38), and after high dose MTX treatment (>50 mg/kg) a significant amount of 7OH-MTX is
detected in patient urine (33). This suggests that MTX-related toxicity may correlate with 7OH-MTX plasma and tissue levels. Insight into in vivo mechanisms that affect plasma levels of 7OH-MTX is therefore important. Recently, it was shown in vitro that ABCG2 and ABCC2 can transport 7OH-MTX (17) and we now show in mice that hepatic Abcc2 and Abcc3 are involved in the excretion of 7OH-MTX from the liver by transporting it into bile and circulation, respectively. Especially the absence of Abcc2 affects plasma and kidney levels of 7OH-MTX. Abcc3 provides an alternative (sinusoidal) route of 7OH-MTX elimination from the liver when Abcc2 is absent and combined absence leads to substantial accumulation of 7OH-MTX in liver and, probably secondary to that, also in plasma. Abcc3 is not exclusively responsible for transport of 7OH-MTX into the circulation and subsequent urinary excretion. Especially at somewhat later time points (>30 min) after administration another mechanism appears to be relevant as well, as was seen in the Abcc2;Abcc3<sup>−/−</sup> mice. This mechanism becomes apparent when liver concentrations of 7OH-MTX are high (Figure 6B). A possible candidate for this would be Abcc4, which is expressed at the basolateral membrane of hepatocytes and the apical membrane in renal cells, and about 2-fold upregulated in liver of female Abcc2<sup>−/−</sup> and Abcc2;Abcc3<sup>−/−</sup> mice.

In Abcc2<sup>−/−</sup> and especially Abcc2;Abcc3<sup>−/−</sup> mice, the total amount of excreted 7OH-MTX (via urine and feces) is higher than in the other strains (Figure 6D). It could be that MTX accumulation in the liver of these strains causes more 7OH-MTX formation. Another possible explanation is increased liver expression of the enzyme aldehyde oxidase, which is responsible for 7OH-MTX formation (18-20). Indeed, preliminary microarray analysis showed that mRNA expression of aldehyde oxidase in Abcc2<sup>−/−</sup> and Abcc2;Abcc3<sup>−/−</sup> mice is 1.4-2.4-fold increased. The 7OH-MTX formed is subsequently (slowly) eliminated from the liver and body, leading to a high 7OH-MTX exposure in Abcc2-deficient mice. It would be interesting to know whether the increased expression of aldehyde oxidase is also seen in patients with ABCC2 mutations, as this could make them more vulnerable to 7OH-MTX-related toxicity.

In this study we administered an i.v. bolus injection of 50 mg/kg MTX to mice, which is comparable to a dose of 154 mg/m<sup>2</sup> in man (31). It cannot be excluded that the effects of Abcc2 and/or Abcc3 found in this study might differ when higher doses or different routes of administration (e.g. oral or constant infusion) are used. However, as associations between ABCC2 mutations and MTX-related toxicity in patients have been found previously after high-dose (3-5 g/m<sup>2</sup>) MTX infusions as well (13;15), it is likely that at least the impact of ABCC2 is similar even at very high doses and longer infusion times.

For practical reasons this study was performed in female mice. In females the liver expression of Abcg2/ABCG2 protein is lower than in males (39), and the effect of Abcg2 on MTX and 7OH-MTX pharmacokinetics therefore may be lower
as well. The observed impact of Abcc2 (and Abcc3) on MTX and 7OH-MTX pharmacokinetics may thus be bigger in females. Interestingly, Rau et al. (2006) (15) found an association between an ABCC2 mutation and MTX-related toxicity in female, but not in male patients. The fact that Abcc4 protein in liver is increased in female Abcc2; Abcc3<sup>−/−</sup> mice may additionally lead to gender-specific differences in MTX pharmacokinetics.

Our data show that Abcc3 can often compensate for Abcc2 when this protein is absent or non-functional, transporting shared substrates like bilirubin glucuronides, MTX and 7OH-MTX from liver back into the circulation, thereby leading to increased elimination via the urine. Our results show a direct effect of Abcc2 absence (and concomitant Abcc3 upregulation) on the pharmacokinetics of MTX and 7OH-MTX, and are in line with previously reported associations between ABCC2 mutations in patients and increased toxicity after methotrexate treatment (13-15). Clearly, Abcc2; Abcc3<sup>−/−</sup> mice provide useful tools to investigate the overlapping and compensatory roles of the ABC transporters Abcc2 and Abcc3 in physiology and pharmacology of shared substrates in vivo.

ACKNOWLEDGEMENTS
We thank our colleagues for critical reading of the manuscript, Rob Lodewijks, Enver Delic and Hans Tensen for excellent technical assistance, Martin van der Valk, Ji-Ying Song and Nadine Meertens for histological analysis, and George Scheffer and Bruno Stieger for kindly providing antibodies.

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Supplementary Table 1S. Levels of MTX in tissues and plasma of female wild-type, *Abcc2*\(^{-/-}\), *Abcc3*\(^{-/-}\) and *Abcc2;Abcc3*\(^{-/-}\) mice at different time-points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th><em>Abcc2</em>(^{-/-})</th>
<th><em>Abcc3</em>(^{-/-})</th>
<th><em>Abcc2;Abcc3</em>(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (µg/ml)</td>
<td>7.5</td>
<td>23.3 ± 2.7</td>
<td>36.2 ± 4.9***</td>
<td>19.8 ± 2.6*</td>
<td>26.0 ± 3.5</td>
</tr>
<tr>
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<td>15</td>
<td>9.1 ± 2.7</td>
<td>18.9 ± 6.4</td>
<td>8.5 ± 1.1</td>
<td>7.5 ± 1.3</td>
</tr>
<tr>
<td></td>
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<td>3.3 ± 0.7***</td>
<td>3.5 ± 0.5**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.4 ± 0.4</td>
<td>2.7 ± 0.4***</td>
<td>1.0 ± 0.4</td>
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</tr>
<tr>
<td></td>
<td>120</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Liver (% of dose)</td>
<td>7.5</td>
<td>49.5 ± 4.4</td>
<td>44.4 ± 4.9*</td>
<td>51.0 ± 6.8</td>
<td>48.2 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>36.0 ± 1.3</td>
<td>28.1 ± 4.7**</td>
<td>43.9 ± 2.0***</td>
<td>33.7 ± 4.2</td>
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<td>16.4 ± 5.3</td>
<td>23.2 ± 3.7</td>
<td>19.4 ± 9.2</td>
<td>32.1 ± 5.4***</td>
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<td>4.8 ± 0.8</td>
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<td>6.2 ± 2.8</td>
<td>8.1 ± 2.6*</td>
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<td>Liver/plasma</td>
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<td>13.2 ± 1.3***</td>
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<tr>
<td>concentration ratio</td>
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<td>33.8 ± 2.9</td>
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<td>67.4 ± 35.7</td>
<td>68.6 ± 16.2**</td>
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<td>39.8 ± 15.2</td>
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<td>11.7 ± 4.0***</td>
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<td>47.7 ± 5.3</td>
<td>24.5 ± 8.3***</td>
<td>51.2 ± 6.8</td>
<td>36.3 ± 4.4***</td>
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<tr>
<td>Kidney (% of dose)</td>
<td>7.5</td>
<td>3.3 ± 1.6</td>
<td>5.3 ± 2.3</td>
<td>3.1 ± 0.5</td>
<td>4.0 ± 0.8</td>
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<tr>
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<td>0.9 ± 0.3</td>
<td>2.6 ± 0.7**</td>
<td>1.6 ± 0.1**</td>
<td>1.1 ± 0.3</td>
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<tr>
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<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2***</td>
<td>0.3 ± 0.02</td>
<td>0.4 ± 0.03</td>
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<tr>
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<td>0.2 ± 0.1</td>
<td>0.3 ± 0.03***</td>
<td>0.2 ± 0.02</td>
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</table>

Note: MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9) and MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis).
**Supplementary Table 2S.** Levels of 7OH-MTX in tissues and plasma of female wild-type, \textit{Abcc2}/\textit{−}/\textit{−}, \textit{Abcc3}/\textit{−}/\textit{−} and \textit{Abcc2;Abcc3}/\textit{−}/\textit{−} mice at different time-points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th>\textit{Abcc2}/\textit{−}/\textit{−}</th>
<th>\textit{Abcc3}/\textit{−}/\textit{−}</th>
<th>\textit{Abcc2;Abcc3}/\textit{−}/\textit{−}</th>
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</thead>
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<td>Plasma (µg/ml)</td>
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<td>0.23 ± 0.07</td>
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<td>0.22 ± 0.03</td>
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<td>0.28 ± 0.10</td>
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<td>0.16 ± 0.04</td>
<td>0.50 ± 0.17***</td>
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<td>60</td>
<td>0.10 ± 0.03</td>
<td>1.00 ± 0.11***</td>
<td>0.08 ± 0.04</td>
<td>0.82 ± 0.32***</td>
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<td>120</td>
<td>0.05 ± 0.02</td>
<td>0.46 ± 0.06***</td>
<td>0.04 ± 0.01</td>
<td>0.68 ± 0.45***</td>
</tr>
<tr>
<td>Liver (% of dose)</td>
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<td>4.4 ± 1.0</td>
<td>7.3 ± 1.9***</td>
<td>4.1 ± 1.3</td>
<td>9.1 ± 0.5***</td>
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<td></td>
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<td>4.5 ± 1.0</td>
<td>8.9 ± 0.7***</td>
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<tr>
<td></td>
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<td>6.2 ± 0.6***</td>
<td>2.2 ± 1.3</td>
<td>12.3 ± 4.1***</td>
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<td></td>
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<td>4.8 ± 1.6**</td>
<td>1.6 ± 0.9</td>
<td>13.9 ± 6.2***</td>
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<tr>
<td></td>
<td>120</td>
<td>0.4 ± 0.1</td>
<td>1.8 ± 0.7***</td>
<td>0.3 ± 0.2</td>
<td>8.0 ± 1.6***</td>
</tr>
<tr>
<td>SI tissue + content (% of dose)</td>
<td>7.5</td>
<td>0.08 ± 0.02</td>
<td>0.02 ± 0.01***</td>
<td>0.06 ± 0.02</td>
<td>0.02 ± 0.01***</td>
</tr>
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<td></td>
<td>15</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1**</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.04**</td>
</tr>
<tr>
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<td>1.6 ± 0.5</td>
<td>0.3 ± 0.1**</td>
<td>1.2 ± 0.9</td>
<td>0.8 ± 0.1**</td>
</tr>
<tr>
<td></td>
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<td>3.0 ± 0.5</td>
<td>0.8 ± 0.2***</td>
<td>2.8 ± 0.8</td>
<td>1.8 ± 0.6***</td>
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<tr>
<td>Kidney (% of dose)</td>
<td>7.5</td>
<td>0.009 ± 0.003</td>
<td>0.03 ± 0.02***</td>
<td>0.007 ± 0.003</td>
<td>0.012 ± 0.007</td>
</tr>
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<td>0.02 ± 0.004</td>
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<td>0.11 ± 0.01***</td>
<td>0.01 ± 0.003</td>
<td>0.07 ± 0.01***</td>
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Note: 7OH-MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9) and 7OH-MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis).
Supplementary Figure 1. Analysis of phospholipid output into bile of male wild-type, \( Abcc2^{-/-} \), \( Abcc3^{-/-} \) and \( Abcc2;Abcc3^{-/-} \) mice after gall bladder cannulation and ligation of the common bile duct. Bile was collected in 15 min fractions over 1 h. Bile collected in the first 15 min was analyzed for phospholipid concentration. A, biliary phospholipid output in the various mouse strains when corrected for body weight (BW). B, biliary phospholipid output in the various strains when corrected for liver weight. Data are means ± SD (\( n = 5 \), *, \( P < 0.05 \), **, \( P < 0.01 \)).
Chapter 5

Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo

Maria L.H. Vlaming, Zeliha Pala, Anita van Esch, Els Wagenaar, Dirk R. de Waart, Koen van de Wetering, Cornelia M.M. van der Kruijssen, Ronald P.J. Oude Elferink, Olaf van Tellingen and Alfred H. Schinkel

Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo

Maria L.H. Vlaming	extsuperscript{1}, Zeliha Pala	extsuperscript{2}, Anita van Esch	extsuperscript{1}, Els Wagenaar	extsuperscript{1}, Dirk R. de Waart	extsuperscript{3}, Koen van de Wetering	extsuperscript{1}, Cornelia M.M. van der Kruisssen	extsuperscript{1}, Ronald P.J. Oude Elferink	extsuperscript{3}, Olaf van Tellingen	extsuperscript{4} and Alfred H. Schinkel	extsuperscript{1}

Divisions of \textsuperscript{1}Molecular Biology and \textsuperscript{4}Clinical Chemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands; \textsuperscript{2}Department of Pharmacology, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey; \textsuperscript{3}AMC Liver Center, Academic Medical Center, Amsterdam, The Netherlands.

Purpose: ABCC2 (MRP2) and ABCG2 (BCRP) transport various endogenous and exogenous compounds, including many anti-cancer drugs, into bile, feces and urine. We investigated the possibly overlapping roles of Abcg2 and Abcc2 in the elimination of the anti-cancer drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX).

Experimental Design: We generated and characterized Abcc2; Abcg2\textsuperscript{-/-} mice, and used these to determine the overlapping roles of Abcc2 and Abcg2 in the elimination of MTX and 7OH-MTX after iv administration of 50 mg/kg MTX.

Results: Compared to wild-type, the plasma areas under the curve (AUCs) for MTX were 1.6-fold and 2.0-fold higher in Abcg2\textsuperscript{-/-} and Abcc2\textsuperscript{-/-} mice respectively, and 3.3-fold increased in Abcc2; Abcg2\textsuperscript{-/-} mice. Biliary excretion of MTX was 23-fold reduced in Abcc2; Abcg2\textsuperscript{-/-} mice, and MTX levels in the small intestine were dramatically decreased. Plasma levels of 7OH-MTX were not significantly altered in Abcg2\textsuperscript{-/-} mice, but the AUCs were 6.2-fold and even 12.4-fold increased in Abcc2; Abcg2\textsuperscript{-/-} and Abcc2; Abcg2\textsuperscript{-/-} mice, respectively. This indicates that Abcc2 compensates for Abcg2 deficiency, but that Abcg2 can only partly compensate for Abcc2 absence. Furthermore, 21-fold decreased biliary 7OH-MTX excretion in Abcc2; Abcg2\textsuperscript{-/-} mice and substantial 7OH-MTX accumulation in liver and kidney were seen. We additionally found that in absence of Abcc2, Abcg2 mediated substantial urinary excretion of MTX and 7OH-MTX.

Conclusions: Abcc2 and Abcg2 together are major determinants of MTX and 7OH-MTX pharmacokinetics. Variation in ABCC2 and/or ABCG2 activity due to polymorphisms or co-administered inhibitors may therefore substantially affect the therapeutic efficacy and toxicity in patients treated with MTX.
INTRODUCTION
ABCG2 (BCRP) and ABCC2 (MRP2), both members of the ATP-binding cassette (ABC) transporter superfamily, are present in the apical membranes of hepatocytes and epithelial cells of small intestine and kidney, where they play a role in the excretion of both endogenous and exogenous compounds into bile, feces and urine. They protect the body from a wide range of potentially toxic compounds and influence the pharmacokinetics of many drugs (1).

ABCG2 and ABCC2 have very broad and substantially overlapping substrate specificities. Examples of xenobiotics that are transported by both ABCG2 and ABCC2 are the anti-cancer drugs doxorubicin, methotrexate, SN-38, and the food-derived carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) (1-3). Furthermore, they can both transport a range of glucuronide and sulphate conjugates of drugs and endogenous compounds (1;3-5). Many polymorphisms and mutations of ABCG2 and ABCC2 have been identified, often leading to differences in expression and activity (2;6;7). These may partly explain the high inter-patient variability in drug response and occurrence of adverse drug reactions, a major concern in the treatment of cancer patients receiving high doses of potentially toxic drugs (2;6;7). Also effective ABCG2 and/or ABCC2 inhibitors (e.g. elacridar, cyclosporin A) are tested in clinical trials to improve treatment (8). It is therefore important to define the specific and possibly overlapping effects of the different ABC transporters on the pharmacokinetics of (anti-cancer) drugs.

Recently, knockout mouse models for both Abcg2 and Abcc2 have been generated (9-12), which have been used extensively for pharmacokinetic analyses of drugs (13;14). Since ABCG2/Abcg2 and ABCC2/Abcc2 show substantial overlap in substrate specificity, absence of one of them could lead to (partial) functional takeover by the other transporter. This may cause underestimations of the significance of Abcg2 or Abcc2 when the single knockout mice are used. An ABC transporter compound knockout model would allow more detailed investigation, as has recently been shown using other ABC transporter compound knockout mice (9;14-18).

To study the relative and possibly overlapping physiological and pharmacological functions of Abcg2 and Abcc2 in vivo we have now generated and characterized Abcc2;Abcg2-/- mice. We used them to analyze overlapping functions of Abcg2 and Abcc2 in determining the iv pharmacokinetics of methotrexate (MTX), a widely used anti-cancer drug and substrate of both transporters (1;3;19). Since it was recently shown that also 7-hydroxymethotrexate (7OH-MTX), the main (toxic) metabolite of MTX, is transported by both ABCG2 and ABCC2 in vitro (20), and that absence of Abcc2 in vivo influences 7OH-MTX pharmacokinetics (16), we additionally investigated the relative impact of both transporters on the pharmacokinetics of 7OH-MTX. We show here that Abcg2 and Abcc2 have additive as well as overlapping functions in determining the pharmacokinetics of MTX and 7OH-MTX. Moreover, Abcg2 and Abcc2 are the main efflux transporters for the
biliary excretion of MTX and 7OH-MTX after iv MTX administration and, when Abcc2 is absent, Abcg2 can partly compensate for its loss by mediating significant urinary excretion of MTX and 7OH-MTX.

METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation of Abcc2<sup>−/−</sup> (12) and Abcg2<sup>−/−</sup> (9) mice has been described. Abcc2;Abcg2<sup>−/−</sup> mice were generated by crossbreeding the single knockout strains. 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. MTX (Emthexate PF<sup>®</sup> 25 mg/ml) was from Pharmachemie (Haarlem, The Netherlands), 7OH-MTX from Toronto Research Chemicals Inc. (North York, ON, Canada) and [3H]MTX from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Ketamine was from Parke-Davis (Hoofddorp, The Netherlands) and methoxyfluorane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). MRPr1, M<sub>3</sub>II-18, M<sub>4</sub>I-80 and M<sub>5</sub>II-54 were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands). K12 was kindly provided by Dr. B. Stieger (University of Zürich, Zürich, Switzerland). RT-PCR primers were from QIAGEN (Venlo, The Netherlands). All other chemicals and reagents were from Sigma-Aldrich (Steinheim, Germany).

Western analysis. Preparation of crude membrane fractions and Western blotting were performed as described (21;22). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). Abcc1, Abcc4, Abcc5 and Abcb11 were detected as described (16). Abcc3 was detected with mAb M<sub>3</sub>II-18 (dilution 1:100). Bound primary antibodies were detected as described (12;16). Densitometric analysis was performed using the TINA 2.09 software program (Raytest, Staubenhardt).

RT-PCR analysis. RNA isolation, cDNA synthesis and real-time PCR (RT-PCR) analysis on livers of female mice (n = 3) were done as described (23).

Vesicular transport assays. ATP-dependent transport of [3H]MTX into inside-out vesicles prepared from Sf9 cells overproducing human or murine ABCC2/Abcc2 (24) and control Sf9 cells was determined as described (15).
**Histological, clinical-chemical and hematological analysis.** 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), and standard hematological analyses of male and female mice (n = 6, twice within a time span of 1.5 years) were performed as described (12).

**Bile and plasma collection for bilirubin analysis.** Gall bladder cannulations in male wild-type, \( Abcg2^{-/-} \), \( Abcc2^{-/-} \) and \( Abcc2;Abcg2^{-/-} \) mice (n = 5-6) and bile and urine collection were performed as described (16;25). Heparin plasma was collected from male mice (n = 4-6) by cardiac puncture under methoxyflurane anesthesia. To each plasma sample 10 µl of 100 mg/ml ascorbic acid solution in water was added. All samples were frozen immediately and stored at -80°C.

**Bilirubin and bile component analysis.** Concentrations of bilirubin monoglucuronides, bilirubin diglucuronides and unconjugated bilirubin in bile (100x diluted in water) of the first 15 min fraction, collected urine and plasma were determined as described (26).

**Pharmacokinetic experiments.** MTX was administered to female wild-type, \( Abcg2^{-/-} \), \( Abcc2^{-/-} \) and \( Abcc2;Abcg2^{-/-} \) mice (n = 3-13) by injecting 5 µl/g body weight of a 10 mg/ml MTX solution in saline into the tail vein. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia at 7.5, 15, 30, 60 or 120 min after MTX administration and organs were removed.

**Biliary MTX excretion experiment.** Gall bladder cannulations in female wild-type, \( Abcg2^{-/-} \), \( Abcc2^{-/-} \) and \( Abcc2;Abcg2^{-/-} \) mice (n = 3-5) were performed as described (25). After successful cannulation, 50 mg/kg MTX was administered iv as described above. Bile was collected in 15 min fractions for 60 min. Mice were killed by cardiac puncture and blood and organs were collected. Small intestinal tissue and contents were separated.

**Fecal and urinary MTX excretion experiment.** Female wild-type, \( Abcg2^{-/-} \), \( Abcc2^{-/-} \) and \( Abcc2;Abcg2^{-/-} \) mice (n = 5-9) were individually housed in Ruco Type M/1 stainless steel metabolic cages (Valkenswaard, The Netherlands). They were allowed 24 hours to adapt before 50 mg/kg MTX was injected into the tail vein as described above. Feces and urine were collected for 24 hours.

**HPLC analysis of MTX and 7OH-MTX.** Collected organs were homogenized in an ice-cold 4% BSA solution and plasma and bile were diluted in human plasma before HPLC analysis. Urine was diluted in water, feces were homogenized in ice-
cold 4% BSA solution. MTX and 7OH-MTX concentrations in the different matrices were determined as described (27).

**Statistical analysis.** Unless otherwise indicated, the two-sided unpaired Student's t-test was used to assess the statistical significance of differences between wild-type and knockout mice. When differences between more than 2 groups were analyzed, one-way ANOVA followed by Tukey’s multiple comparison test was performed, as indicated. Results are presented as means ± standard deviations (SD). Differences were considered statistically significant when \( P < 0.05 \). Averaged concentrations for each time point were used to calculate the area under the plasma concentration versus time curve (AUC) from \( t = 0 \) to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (28).

**RESULTS**

**Macroscopic and microscopic analysis of Abcc2;Abcg2−/− mice.**

\( Abcc2;Abcg2−/− \) mice were viable, fertile and had normal life spans and body weights. Macroscopic and microscopic histological and pathological analysis did not reveal obvious specific aberrations in tissues of the \( Abcc2;Abcg2−/− \) mice, including the liver. Similar to what was previously shown for \( Abcc2−/− \) mice (12), the liver weight of \( Abcc2;Abcg2−/− \) mice was significantly (26-36%) increased compared to wild-type (Figure 1A), but not significantly different from that of \( Abcc2−/− \) mice (ANOVA), suggesting that the increase in liver weight is primarily caused by \( Abcc2 \) absence.

**Protein expression of other ABC transporters in tissues of Abcc2;Abcg2−/− mice.**

Absence of both Abcg2 and Abcc2 in tissues of the double knockout mice may lead to compensatory increases in expression of other ABC transporters. Therefore we analyzed the expression of Abcc1 (Mrp1), Abcc3 (Mrp3), Abcc4 (Mrp4), Abcc5 (Mrp5) and Abcb11 (Bsep) in tissues of \( Abcc2;Abcg2−/− \) mice (>99% FVB background). Abcc3 protein levels in liver (but not kidney (not shown)) of male (not shown) and female (Figure 1B) \( Abcc2;Abcg2−/− \) mice were ~1.7-fold increased compared to wild-type. Abcc4 protein in female liver and kidney (Figures 1C and D) and male kidney (not shown) of \( Abcc2;Abcg2−/− \) mice was ~1.5-fold increased. Abcc4 in male liver was very low in both wild-type and \( Abcc2;Abcg2−/− \) mice (not shown). Abcc1 in male liver and Abcc5 in male and female liver were quite low and not different between wild-type and \( Abcc2;Abcg2−/− \) mice (not shown). Abcb11 expression was not increased in \( Abcc2;Abcg2−/− \) mice, and possibly even mildly decreased compared to wild-type (not shown). Combined, the expression patterns of Abcc3 and Abcc4 in liver and kidney of \( Abcc2;Abcg2−/− \) mice were similar to what was found for \( Abcc2−/− \) mice in >99% FVB background (12), suggesting that these inductions are mainly the result of absence of Abcc2.
**Figure 1.** General characterization of Abcc2;Abcg2\(^{-/-}\) mice. A, liver weight (as percentage of body weight) in male (left panel) and female (right panel) wild-type, Abcg2\(^{-/-}\), Abcc2\(^{-/-}\) and Abcc2;Abcg2\(^{-/-}\) mice (means ± SD, n = 5-6, **, P < 0.01, ***, P < 0.001 compared to wild-type, ANOVA). B, protein levels of Abcc3 in crude membrane fractions of liver samples from two independent female wild-type and Abcc2;Abcg2\(^{-/-}\) mice. Densitometric analysis of the blots showed that on average Abcc3 expression was 1.7-fold increased in Abcc2;Abcg2\(^{-/-}\) mice. C, protein levels of Abcc4 in crude membrane fractions of liver samples from two independent female wild-type and Abcc2;Abcg2\(^{-/-}\) mice. Densitometric analysis of the blots showed that on average Abcc4 expression was 1.5-fold increased in Abcc2;Abcg2\(^{-/-}\) mice. D, protein levels of Abcc4 in crude membrane fractions of kidney samples from two independent female wild-type and Abcc2;Abcg2\(^{-/-}\) mice. Densitometric analysis of the blots showed that on average Abcc4 expression was 1.6-fold increased in Abcc2;Abcg2\(^{-/-}\) mice. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. In C and D, the lane with the positive control (SF-9 vesicles containing ABCC4 (49)), is indicated with “+”. Underglycosylation of ABCC4 in the SF-9 cells causes the faster migration compared to the murine Abcc4. The amount of protein loaded in each lane is noted above the respective lanes.
Expression levels of genes involved in methotrexate disposition.
Since we wanted to analyze the pharmacokinetics of MTX in Abcc2;Abcg2−/− mice, we determined mRNA expression levels of genes that may be involved in MTX metabolism and transport in livers of female knockout mice. We found that mRNA levels of aldehyde oxidase 1 and 3, which are implicated in the conversion of MTX to 7OH-MTX (29) were 3-8-fold increased in livers of Abcc2+/− and Abcc2;Abcg2−/− mice (Figures S1A and S1B). mRNA levels of the uptake transporter Slco1a4 were not different from wild-type in the knockout strains (Figure S1C). mRNA of Slco1b2 was ~2-fold increased in Abcc2+/− and Abcc2;Abcg2−/− mice (Figure S1D).

Plasma clinical chemistry and hematological analysis of Abcc2;Abcg2−/− mice.
Except for bilirubin (see below), standard plasma clinical chemistry parameters were not significantly different between any of the strains analyzed (not shown). Hematological analysis of male and female Abcc2;Abcg2−/− mice showed a tendency of reduced hemoglobin levels as was previously seen in Abcc2−/− mice in a mixed genetic background (12) (not shown). Other measured parameters did not consistently show differences with wild-type mice (not shown).

Bilirubin in plasma, bile and urine of Abcc2;Abcg2−/− mice.
We have previously shown that Abcc2−/− mice had mildly increased plasma levels of conjugated bilirubin due to reduced biliary excretion of this compound (12). In Abcg2−/− mice on the other hand, the plasma levels of unconjugated bilirubin were increased (9). Clinical chemistry (not shown) and subsequent HPLC analysis (Figure 2A) of plasma confirmed these findings. In Abcc2;Abcg2−/− mice, bilirubin monoglucuronide, diglucuronide and unconjugated bilirubin concentrations were all increased, leading to 7-fold higher total bilirubin plasma levels in Abcc2;Abcg2−/− mice compared to wild-type.

Because Abcg2 and Abcc2 both excrete their substrates into bile, we analyzed bile flow and biliary bilirubin output in the different strains. Like in Abcc2−/− mice (12), the cumulative bile flow over 1 hour in Abcc2;Abcg2−/− mice was significantly reduced, to 54% of wild-type bile flow and not significantly different from Abcc2−/− mice (ANOVA) (Figure 2B). The total biliary bilirubin output in Abcg2−/− mice was comparable to wild-type (Figure 2C). As shown before (11;12), the total biliary bilirubin excretion of Abcc2−/− mice was significantly reduced, due to a decreased bilirubin monoglucuronide output. This was also seen in Abcc2;Abcg2−/− mice. Biliary bilirubin diglucuronide excretion in wild-type mice was very low, and in Abcg2−/− mice this was not detected at all. As shown before (16), biliary excretion of bilirubin diglucuronide was significantly increased in Abcc2−/− mice. This was similar for Abcc2;Abcg2−/− mice and is apparently related to Abcc2 absence. Biliary excretion of unconjugated bilirubin was modest in wild-type and Abcc2−/− mice, and not significantly different (Figure 2C). In Abcg2−/− and Abcc2;Abcg2−/− mice however,
the biliary unconjugated bilirubin excretion was significantly reduced.

In urine collected from the gall bladder cannulated mice, bilirubin monoglucuronide was detected only in Abcc2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice (Figure 2D), reflecting the increased bilirubin monoglucuronide plasma levels in these strains (Figure 2A). Bilirubin diglucuronides or unconjugated bilirubin were not detected in the urine of any of the strains.

**Figure 2.** Bilirubin in plasma, bile and urine of male wild-type, Abcg2<sup>−/−</sup>, Abcc2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice. A, total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) concentrations in plasma of the various mouse strains (nq, not quantifiable, detection limit 0.3 µM). B, bile flow in the various mouse strains. Bile was collected by gall bladder cannulation in 15 min fractions over 1h. C, output of total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) into the bile of the various mouse strains. Bile collected in the first 15 min after gall bladder cannulation was analyzed for bilirubin concentration (nq, not quantifiable, detection limit 0.05 µM). D, bilirubin monoglucuronide (BMG) concentration in urine of gall bladder cannulated mice (nq, not quantifiable, detection limit 0.05 µM). Data are means ± SD (n = 4-6, *, P < 0.05, **, P < 0.01, ***, P < 0.001, Student’s t-test).
Functionally overlapping roles of Abcg2 and Abcc2 in the elimination of methotrexate.

It was shown previously using Abcg2−/− and Abcc2−/− mice that both ABC transporters play a significant role in plasma elimination of ([3H])MTX after iv administration (12;16;30). However, whereas transport of MTX by murine Abcg2 in vitro has been shown before (19), direct transport by murine Abcc2 has not. We therefore investigated transport of [3H]MTX using Sf9-vesicles expressing murine Abcc2 (24). We found that, like human ABCC2, murine Abcc2 actively transports MTX (Figure 2S).

Figure 3. Pharmacokinetics of MTX after iv administration of 50 mg/kg to female wild-type, Abcg2−/−, Abcc2−/− and Abcc2;Abcg2−/− mice. A, MTX plasma concentration versus time curve of the various strains (n = 3-13). B, MTX liver level, C, MTX liver-to-plasma ratio, D, MTX small intestinal (S.I.) tissue + contents level versus time curve of the various strains (n = 3-9). Data are presented as means ± SD.
To investigate the relative roles of Abcc2 and Abcg2 in MTX pharmacokinetics, we administered MTX iv (50 mg/kg) to the different strains, and measured plasma and tissue concentrations over 120 min. The plasma AUCs for Abcg2−/− and Abcc2−/− mice were 1.6- and 2.0-fold increased compared with wild-type, respectively (Figure 3A, Table 1S). In Abcc2;Abcg2−/− mice the plasma AUC was 3.3-fold increased, suggesting additive effects of Abcg2 and Abcc2 on MTX plasma elimination.

MTX liver concentrations were, like plasma, highest (44-54% of the dose) early after administration and decreased quite rapidly thereafter (Figure 3B, Table 1S). As shown before for Abcc2−/− mice (16), and despite increased Slco1b2 expression (Figure 1SD), the liver-to-plasma ratios over 120 min (Figure 3C) were significantly reduced in Abcc2−/− and Abcc2;Abcg2−/− mice. The liver-to-plasma ratios of Abcg2−/− mice over 120 min were not significantly different from wild-type. This suggests more effective liver elimination of MTX towards plasma (over the sinusoidal membrane of hepatocytes), most likely via (upregulated) Abcc3 (16). This may contribute to the increased plasma concentrations of MTX in both Abcc2−/− and Abcc2;Abcg2−/− mice compared to wild-type. Note that Abcc3 was not upregulated in single Abcg2−/− mice (not shown), consistent with liver-to-plasma MTX ratios being similar to wild-type values.

The small intestinal tissue and contents levels of MTX between 7.5 and 60 min (Figure 3D, Table 1S) were significantly reduced compared to wild-type in Abcg2−/−, Abcc2−/− and Abcc2;Abcg2−/− mice at every measured time point. Whereas in Abcg2−/− and Abcc2−/− mice still substantial MTX excretion into the small intestine was observed, in Abcc2;Abcg2−/− mice this was virtually absent, and significantly lower than in the Abcg2−/− and Abcc2−/− mice 60 min after administration (P < 0.01, ANOVA). This shows that Abcg2 and Abcc2 together dictate the excretion of MTX into the small intestine and that they partly compensate for each other’s absence.

To investigate the functions of Abcg2 and Abcc2 in more detail, we measured their influence in biliary excretion and/or direct intestinal MTX excretion with gall bladder cannulation experiments. Figure 4A shows that Abcc2 and Abcg2 both influence the biliary excretion of MTX and that each can partly compensate for loss of the other. In the Abcc2;Abcg2−/− mice, the biliary excretion of MTX was dramatically reduced, to only 2 ± 1% of the dose versus 45 ± 20% in wild-type (P = 0.011), showing that Abcg2 and especially Abcc2 are the main determinants for biliary excretion of MTX.

As shown before (16), MTX levels in small intestinal tissue and contents of the gall bladder cannulated mice were low (<3% of the dose) compared to the biliary MTX levels, suggesting that direct intestinal excretion is not important in MTX elimination. Surprisingly, whereas the plasma concentrations after this experiment (Figure 3SA) were not significantly different from wild-type in any of the knockout strains (ANOVA), ~3-fold increased levels of MTX in the small intestinal contents of
Abcc2;Abcg2<sup>−/−</sup> mice compared to the other mouse strains were found (P < 0.05, ANOVA) (Figure 4B), suggesting the presence of an active direct intestinal MTX excretion mechanism in Abcc2;Abcg2<sup>−/−</sup> mice which is not present/active in the other strains.

Figure 4C shows the role of Abcg2 and Abcc2 in the urinary and fecal excretion of MTX the first 24 hr after iv administration (50 mg/kg). As described before (16), MTX urinary excretion in Abcc2<sup>−/−</sup> mice was significantly increased compared to wild-type mice, thereby reflecting increased plasma and kidney levels. In Abcg2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice however, despite 1.6-fold and 3.3-fold increased plasma AUCs and increased kidney concentrations of MTX (Figure 3A, Table 1S), the urinary excretion of MTX was not different from wild-type mice. This suggests that Abcg2 in the kidney is (at least partly) responsible for the increased urinary excretion of MTX in Abcc2<sup>−/−</sup> mice. Fecal excretion of MTX over 24 hr was not significantly different from wild-type mice in any of the knockout strains.

**Figure 4.** Biliary, direct intestinal, urinary and fecal excretion of MTX after iv administration of 50 mg/kg to female wild-type, Abcg2<sup>−/−</sup>, Abcc2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice. A, Biliary excretion of MTX in the first 60 min after iv administration to gall bladder cannulated mice of the various strains. B, small intestinal (S.I.) tissue and contents levels of MTX, 60 min after iv administration to gall bladder cannulated mice. MTX plasma and liver concentrations of the cannulated mice at t = 60 min are depicted in Figure 3S. C, urinary (left panel) and fecal (right panel) excretion in the first 24 hrs after iv administration to mice of the various strains (n = 5-9). Data are presented as means ± SD (*, P < 0.05, ***, P < 0.01, ANOVA).
Functionally overlapping roles of Abcg2 and Abcc2 in the elimination of 7OH-MTX.

ABCG2 and ABCC2 can transport 7OH-MTX, the main toxic metabolite of MTX, in vitro (20). Furthermore, Abcc2 influences 7OH-MTX pharmacokinetics in vivo (16). The Abcc2; Abcg2−/− mice were therefore used to analyze the relative roles of both transporters in the pharmacokinetics of 7OH-MTX after iv administration of MTX (Figure 5, Table 2S). As shown before (16), Abcc2−/− mice have a 6.2-fold increased 7OH-MTX plasma AUC compared to wild-type (Figure 5A, Table 2S). In Abcg2−/− mice on the other hand, the 7OH-MTX plasma AUC was not significantly different from wild-type, which would suggest that Abcg2 is not important for 7OH-MTX elimination in vivo. However, in Abcc2; Abcg2−/− mice the 7OH-MTX plasma AUC was 12.4-fold higher than in wild-type mice and 2.0-fold higher compared to Abcc2−/− mice (P = 0.03, Student’s t-test). This shows that when Abcc2 is absent, Abcg2 does influence the elimination of 7OH-MTX from the circulation.

Figure 5. Pharmacokinetics of 7OH-MTX after iv administration of 50 mg/kg MTX to female wild-type, Abcg2+/−, Abcc2+/− and Abcc2; Abcg2−/− mice. A, 7OH-MTX plasma concentration versus time curves of the various strains (n = 3-11). B, 7OH-MTX liver level, C, 7OH-MTX small intestinal (S.I.) tissue + contents level, D, 7OH-MTX kidney level versus time curves of the various strains (n = 3-9). Data are presented as means ± SD.
Already 7.5 min after MTX administration the 7OH-MTX liver levels (Figure 5B) were relatively high in all strains and significantly higher in Abcc2+/− and Abcc2; Abcg2+/− mice compared to wild-type, suggesting a rapid 7OH-MTX formation in the liver, which may even have been increased compared to wild-type in Abcc2-deficient mice due to overexpression of aldehyde oxidase 1 and 3 in livers of these mice (Figure 1S). Whereas in Abcc2−/− mice the 7OH-MTX liver levels decreased rapidly after 30 min (Figure 5B), the liver levels in Abcc2; Abcg2−/− mice stayed relatively high up to 60 min after administration, suggesting initially delayed elimination of 7OH-MTX from the liver in the Abcc2; Abcg2−/− mice.

There was little difference between Abcg2−/− and wild-type mice in 7OH-MTX small intestinal tissue and contents levels (Figure 5C), suggesting only a minor role for Abcg2 in the elimination of 7OH-MTX via the small intestine. As shown before (16), in the Abcc2−/− mice the small intestinal 7OH-MTX levels were markedly lower compared to wild-type at every time point (Figure 5C). In Abcc2; Abcg2−/− mice the small intestinal levels of 7OH-MTX were even more reduced, to only 11% of wild-type levels at 60 min (P = 3.1*10−5), suggesting that when Abcc2 is absent, Abcg2 is involved in excretion of 7OH-MTX into the small intestine, most likely via biliary excretion (see below).

7OH-MTX related toxicity in patients is suggested to mainly occur in the kidney, leading to renal failure (31;32). Therefore we analyzed the kidney levels of 7OH-MTX after iv administration of MTX (Figure 5D). These in general followed the plasma levels of 7OH-MTX. Thus, in Abcc2−/− and in Abcc2; Abcg2−/− mice they were significantly increased, up to 11-fold and 35-fold compared to wild-type mice at 60 min.

The effect of Abcg2 and Abcc2 on the biliary excretion of 7OH-MTX after iv injection of MTX is shown in Figure 6A. Clearly, Abcc2 is the main determinant for the biliary excretion of 7OH-MTX, but in the absence of Abcc2, Abcg2 can (partly) take over its function. In Abcc2; Abcg2+/− mice, despite increased liver levels (Figure 5B), the biliary 7OH-MTX output was decreased to only 5% of wild-type output (0.12 ± 0.04% versus 2.51 ± 1.53% of the dose, P = 0.04), illustrating the important role for both transporters in the biliary excretion of 7OH-MTX. The levels of 7OH-MTX in the small intestinal tissue and contents 60 min after MTX administration in the gall bladder cannulation experiment were negligible (not shown), indicating that direct intestinal excretion is not important for 7OH-MTX elimination.

The urinary excretion of 7OH-MTX during the first 24 hr after iv administration of MTX (Figure 6B) was quite low and not significantly different from wild-type in Abcg2+/− mice. As shown before (16), urinary excretion of 7OH-MTX was significantly increased in Abcc2−/− mice. Although in Abcc2; Abcg2−/− mice the urinary excretion of 7OH-MTX was significantly higher compared with wild-type, it was lower than in Abcc2−/− mice (n = 5-9, P < 0.001, ANOVA). As the AUC plasma of 7OH-MTX in the Abcc2; Abcg2−/− mice was 2-fold higher than in the Abcc2−/− mice
(Figure 5A), this suggests that Abcg2 is (at least partly) responsible for the urinary excretion of 7OH-MTX in the Abcc2−/− mice. The fecal excretion of 7OH-MTX was very low in all strains (<3% of the dose) and difficult to determine due to endogenous background peaks (not shown).

**Figure 6.** Biliary, urinary and fecal excretion of 7OH-MTX after iv administration of 50 mg/kg MTX to female wild-type, Abcg2−/−, Abcc2−/− and Abcc2;Abcg2−/− mice. A, 7OH-MTX biliary excretion in the first 60 min after iv administration of MTX to gall bladder cannulated mice of the various strains. Bile was collected in 15 min fractions over 1h (n = 3-5, *, P < 0.05, Student’s t-test). 7OH-MTX plasma and liver concentrations of the cannulated mice at t = 60 min are depicted in Figure 3S. B, 7OH-MTX urinary excretion in the first 24 hrs after iv administration of MTX to mice of the various strains (n = 6-9, *, P < 0.05, ***, P < 0.001, ANOVA). Data are presented as means ± SD.

**DISCUSSION**

To investigate the possibly overlapping roles of Abcg2 and Abcc2 in the elimination of shared endogenous and exogenous substrates in vivo, we generated Abcc2;Abcg2−/− mice, which are viable and fertile. The spontaneous phenotypes of the double knockout mice appear to be a combination of what we observed previously for Abcg2−/− and Abcc2−/− mice (9;12), suggesting separate roles of Abcg2 and Abcc2 in most physiological functions. We show here that Abcg2 and Abcc2 play functionally overlapping roles in the elimination of the anti-cancer drug MTX and its toxic metabolite 7OH-MTX. We conclude that Abcc2;Abcg2−/− mice are quite healthy overall and can be used to study overlapping Abcg2 and Abcc2 functions in vivo.

It was shown previously that in Abcg2−/− mice unconjugated bilirubin plasma levels were mildly increased (9), whereas in Abcc2−/− mice conjugated bilirubin plasma levels were increased (11;12). This was confirmed in the present study using HPLC. In the Abcc2;Abcg2−/− mice a combination of both was observed: 7-fold increased plasma concentrations of total bilirubin, due to significantly increased unconjugated as well as conjugated bilirubin. This is probably the consequence of reduced biliary excretion of conjugated and unconjugated bilirubin in the
Abcc2;Abcg2<sup>−/−</sup> mice. Combined, this suggests that Abcc2 is mainly involved in biliary excretion of bilirubin monoglucuronides, whereas Abcg2 excretes unconjugated bilirubin. However, if Abcg2 transports unconjugated bilirubin, it clearly has a low efficiency, as the amount excreted into bile is very small, also in wild-type mice. Even in Gunn rats, which have elevated serum unconjugated bilirubin due to absence of the conjugating enzyme UGT1A1, very little is excreted into bile (33). An alternative explanation for the observed changes is decreased unconjugated bilirubin uptake into the liver of Abcg2-deficient mice. An uptake system for unconjugated bilirubin in mice has, however, not been unambiguously identified yet (34), making this difficult to investigate. Note that in Abcc2;Abcg2<sup>−/−</sup> mice still substantial biliary output of conjugated bilirubin is present, suggesting that an alternative canicular transport mechanism transports bilirubin into bile. Nevertheless, the increased bilirubin plasma levels in the Abcc2;Abcg2<sup>−/−</sup> mice are still relatively low compared to normal bilirubin plasma levels in humans (5-30 ¦M). It is therefore unlikely that plasma bilirubin levels would markedly influence drug pharmacokinetics in these mice.

We have studied the overlapping roles of Abcc2 and Abcg2 in plasma- and tissue pharmacokinetics of MTX and 7OH-MTX. The MTX plasma pharmacokinetics in the different strains revealed additive effects of Abcg2 and Abcc2. As MTX-related toxicity and outcome in patients after high-dose MTX therapy appear to correlate with MTX serum levels (35-37), differences in expression or activity of ABCG2 and/or ABCC2 may have profound effects on MTX-induced toxicity in patients. Since many known ABCC2 and ABCG2 polymorphisms drastically reduce, or even abrogate the function of these proteins (2;6;7), this could be an important contributory factor to variable MTX efficacy and toxicity in patients. Interestingly, in psoriasis-patients treated with MTX, SNPs in ABCG2 were associated with a good response to therapy (38).

For the plasma pharmacokinetics of 7OH-MTX after MTX administration, we found Abcc2 to be the main determinant. Nevertheless, when Abcc2 is absent, Abcg2 still plays a significant role. This was best illustrated by the 12.4-fold increased 7OH-MTX plasma AUC in Abcc2;Abcg2<sup>−/−</sup> mice, versus 6.2-fold in Abcc2<sup>−/−</sup> mice.

Table 3S provides an overview of the overall excretion of MTX and 7OH-MTX in the strains analyzed. Analysis of the biliary excretion of MTX and 7OH-MTX revealed that Abcg2 and Abcc2 together dictate these processes. Abcc2 was the most important transporter in biliary excretion of both compounds after iv MTX administration. Although mRNA levels of aldehyde oxidase were increased in both Abcc2-deficient strains (Figure 1S), the hepatic MTX levels in these strains were not significantly different from wild-type levels. The reduced biliary excretion of MTX must therefore primarily be caused by reduced Abcc2/Abcg2-mediated transport of MTX into bile, and not by increased conversion of MTX to 7OH-MTX.

Table 3S provides an overview of the overall excretion of MTX and 7OH-MTX in the strains analyzed. Analysis of the biliary excretion of MTX and 7OH-MTX revealed that Abcg2 and Abcc2 together dictate these processes. Abcc2 was the most important transporter in biliary excretion of both compounds after iv MTX administration. Although mRNA levels of aldehyde oxidase were increased in both Abcc2-deficient strains (Figure 1S), the hepatic MTX levels in these strains were not significantly different from wild-type levels. The reduced biliary excretion of MTX must therefore primarily be caused by reduced Abcc2/Abcg2-mediated transport of MTX into bile, and not by increased conversion of MTX to 7OH-MTX.
It is interesting to note that males have higher hepatic Abcg2 levels than females (39). As our experiments were performed in female mice the effect of Abcg2 in the male liver may be underestimated here. Still, a clear effect of Abcg2 on biliary excretion in female Abcc2/− mice could be demonstrated, both for MTX and 7OH-MTX. This was reflected by drastically decreased levels of both compounds in the small intestine and its contents of Abcc2;Abcg2/− mice. Interestingly, Rau et al. (2006) (40) found an association between a frequent ABCC2 polymorphism and MTX pharmacokinetics, but only in females. This suggests that, as Abcg2 apparently can (partly) compensate for Abcc2 absence, in males ABCG2 expression is sufficient for effective compensatory MTX excretion, whereas in females it is not.

MTX-related toxicity in humans is often seen in bone marrow and the gastrointestinal tract, although this is generally rendered manageable by co-administration of leucovorin. As Abcc2 and Abcg2 are primarily responsible for the biliary excretion of MTX and 7OH-MTX, reduced expression or activity of ABCC2 and/or ABCG2 in patients may cause reduced biliary excretion of MTX and 7OH-MTX. This could lead to reduced exposure of the intestine to these compounds after iv administration, and therefore to reduced gastrointestinal toxicity.

Besides bone marrow and gastrointestinal toxicity, high-dose MTX treatment in patients may result in renal failure (41). This is thought to be caused by precipitation of MTX and especially 7OH-MTX, which is highly insoluble, in kidney tubules (31;32). As shown before (16), the urinary output of MTX and 7OH-MTX in Abcc2/− mice was significantly increased compared to wild-type in the first 24 hrs after MTX administration. Although in the Abcc2;Abcg2/− mice the plasma AUCs for MTX and 7OH-MTX were even higher than in the Abcc2/− mice, the urinary excretion of both compounds was reduced compared to Abcc2/− mice and comparable to wild-type. This suggests that in Abcc2/− mice, the increased urinary excretion of MTX and 7OH-MTX is mediated through Abcg2 in the kidney. Furthermore, whereas the Abcg2/− mice had (like the Abcc2/− mice) increased plasma MTX levels, they did not show increased urinary MTX excretion. This suggests that even in the wild-type situation Abcg2 may be involved in urinary MTX excretion. These findings are in line with the previously described effect of Abcg2 on the urinary excretion of another Abcg2 substrate, E3040S (42).

Given the increased kidney levels and urinary output of MTX and 7OH-MTX in Abcc2-deficient mice, reduced ABCC2 expression/activity may be a risk factor for kidney toxicity, which is primarily due to precipitation of these compounds in the urine (31;32). Interestingly, renal failure after high-dose MTX treatment in a patient with an ABCC2-mutation has been reported (43). In contrast, as Abcg2 appears to mediate excretion of MTX and especially 7OH-MTX into urine, one could speculate that decreased activity of ABCG2 may decrease the risk of nephrotoxicity after MTX treatment.
Since Abcc4 protein was 1.5-fold increased in the kidneys of \textit{Abcc2}^{-/-} and \textit{Abcc2;Abcg2}^{-/-} mice (Figure 1D, (12)), we cannot exclude a minor contribution of Abcc4 overexpression on the increased urinary excretion of MTX and/or 7OH-MTX in Abcc2-deficient mice. However, this does not explain the differences in urinary excretion between \textit{Abcc2}^{-/-} and \textit{Abcc2;Abcg2}^{-/-} mice, as both strains had increased kidney Abcc4. If any, the effects of increased Abcc4 on the urinary MTX and 7OH-MTX excretion are very small compared to the effect of Abcg2.

It should be noted that the iv bolus injection of 50 mg/kg MTX administered here is comparable to a dose of 154 mg/m\(^{2}\) in man (12;44), which is relatively low for high-dose cancer treatment, as doses of up to 12 g/m\(^{2}\) are given (29). Whether the effects of Abcc2 and/or Abcg2 found here are also valid for higher doses or longer infusion times remains to be elucidated. Interestingly, associations between ABCC2 and ABCG2 mutations/polymorphisms and MTX-pharmacokinetics or toxicities in patients were found previously after high-dose (1-5 g/m\(^{2}\)) MTX infusions (40;43;45), suggesting that our results are also relevant for higher doses and longer infusions.

Of course, as always, extrapolating data from mice to humans should be done with caution. For example, in humans, biliary excretion of MTX appears somewhat less important (up to 20% of the dose is excreted into bile, although this was measured in very few patients (46;47)), whereas urinary excretion of MTX appears somewhat more important compared to mice (46). Furthermore, other MTX/7OH-MTX transporters in human liver or kidney may be more significant in humans than in mice.

Overall, our data show that Abcg2 and Abcc2 are important transporters for plasma elimination as well as biliary and urinary excretion of MTX and its toxic metabolite 7OH-MTX. When one is absent, the other can (at least partly) compensate for its loss. These findings provide direct explanations for previously found correlations between ABCC2 and ABCG2 polymorphisms/mutations and MTX pharmacokinetics or toxicity in patients (38;40;43;48), and show the potential value of \textit{Abcc2;Abcg2}^{-/-} mice for studying the pharmacokinetics of endogenous and exogenous compounds \textit{in vivo}.

**ACKNOWLEDGEMENTS**

We thank our colleagues for critical reading of the manuscript, Rob Lodewijks, Enver Delic and Hans Tensen for excellent technical assistance, Martin van der Valk and Ji-Ying Song for histological analysis, and George Scheffer and Bruno Stieger for providing antibodies.
REFERENCES


**Table 1S.** Levels of MTX in tissues and plasma of female wild-type, *Abcg2<sup>-/-</sup>, Abcc2<sup>-/-</sup> and Abcc2;Abcg2<sup>-/-</sup> mice at different time points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th>Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2;Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (µg/ml)</td>
<td>7.5</td>
<td>23.3 ± 2.7</td>
<td>28.8 ± 2.0&lt;sup&gt;**&lt;/sup&gt;</td>
<td>36.2 ± 4.9&lt;sup&gt;***&lt;/sup&gt;</td>
<td>35.3 ± 4.8&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>9.1 ± 2.7</td>
<td>11.8 ± 1.3</td>
<td>18.9 ± 6.4</td>
<td>32.6 ± 13.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.3 ± 0.7</td>
<td>8.3 ± 1.4&lt;sup&gt;**&lt;/sup&gt;</td>
<td>11.9 ± 2.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>19.8 ± 6.8&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.4 ± 0.4</td>
<td>3.1 ± 0.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.7 ± 0.4</td>
<td>7.8 ± 1.4&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Plasma AUC&lt;sub&gt;0-120&lt;/sub&gt; (min·µg/ml)</td>
<td>0-120</td>
<td>444 ± 44</td>
<td>692 ± 56&lt;sup&gt;**&lt;/sup&gt;</td>
<td>870 ± 103&lt;sup&gt;***&lt;/sup&gt;</td>
<td>1446 ± 229&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (% of dose)</td>
<td>7.5</td>
<td>49.5 ± 4.4</td>
<td>53.4 ± 1.6</td>
<td>44.4 ± 4.9</td>
<td>49.4 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>36.0 ± 1.3</td>
<td>35.3 ± 3.7</td>
<td>28.1 ± 4.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>36.4 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>16.4 ± 5.3</td>
<td>36.5 ± 5.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>23.2 ± 3.7</td>
<td>25.0 ± 7.5&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4.8 ± 0.8</td>
<td>10.0 ± 1.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>4.0 ± 1.9</td>
<td>6.8 ± 0.2&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1.0 ± 0.4</td>
<td>1.7 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Kidney (% of dose)</td>
<td>7.5</td>
<td>3.3 ± 1.6</td>
<td>3.6 ± 0.6</td>
<td>5.3 ± 2.3</td>
<td>3.6 ± 0.3&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.9 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>2.6 ± 0.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.7 ± 0.6&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.5 ± 0.3&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.4 ± 1.3&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SI tissue + contents (% of dose)</td>
<td>7.5</td>
<td>5.1 ± 1.5</td>
<td>3.1 ± 0.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.5 ± 0.4&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>28.9 ± 2.8</td>
<td>19.1 ± 4.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>10.6 ± 4.3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.8 ± 1.0&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>39.5 ± 9.4</td>
<td>27.7 ± 1.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>14.8 ± 1.3</td>
<td>2.7 ± 0.3&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>47.7 ± 5.3</td>
<td>38.9 ± 3.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>24.5 ± 8.3&lt;sup&gt;***&lt;/sup&gt;</td>
<td>2.9 ± 0.4&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9) and MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). * P < 0.05, ** P<0.01, *** P<0.001 compared to wild-type mice at the same time point (Student’s t-test was used for statistical analysis).
Table 2S. Levels of 7OH-MTX in tissues and plasma of female wild-type, $Abcg2^{-/-}$, $Abcc2^{-/-}$ and $Abcc2;Abcg2^{-/-}$ mice at different time points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th>$Abcg2^{-/-}$</th>
<th>$Abcc2^{-/-}$</th>
<th>$Abcc2;Abcg2^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (µg/ml)</td>
<td>7.5</td>
<td>0.23 ± 0.07</td>
<td>0.18 ± 0.04</td>
<td>0.38 ± 0.16</td>
<td>0.57 ± 0.13***</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.25 ± 0.07</td>
<td>0.34 ± 0.07</td>
<td>1.08 ± 0.38***</td>
<td>1.60 ± 0.34***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.28 ± 0.10</td>
<td>0.29 ± 0.09</td>
<td>1.43 ± 0.37***</td>
<td>2.21 ± 0.61***</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.10 ± 0.03</td>
<td>0.22 ± 0.11†</td>
<td>1.00 ± 0.11***</td>
<td>2.75 ± 0.72***</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.05 ± 0.02</td>
<td>0.11 ± 0.06</td>
<td>0.46 ± 0.06</td>
<td>0.49 ± 0.13***</td>
</tr>
<tr>
<td>Plasma AUC$_{0-120}$ (min·µg/ml)</td>
<td>0-120</td>
<td>17 ± 3</td>
<td>25 ± 6</td>
<td>106 ± 11***</td>
<td>210 ± 36***</td>
</tr>
<tr>
<td>Liver (% of dose)</td>
<td>7.5</td>
<td>4.4 ± 1.0</td>
<td>3.6 ± 0.4</td>
<td>7.3 ± 1.9***</td>
<td>9.5 ± 1.4***</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.5 ± 1.0</td>
<td>4.6 ± 0.5</td>
<td>8.9 ± 0.7***</td>
<td>8.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.3 ± 0.9</td>
<td>3.4 ± 0.5†</td>
<td>6.2 ± 0.6***</td>
<td>7.3 ± 2.0**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.2 ± 0.2</td>
<td>2.9 ± 0.1†</td>
<td>4.8 ± 1.6***</td>
<td>8.5 ± 1.3***</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.2†</td>
<td>1.8 ± 0.7</td>
<td>1.1 ± 0.2**</td>
</tr>
<tr>
<td>Kidney (% of dose)</td>
<td>7.5</td>
<td>0.009 ± 0.003</td>
<td>0.007 ± 0.003</td>
<td>0.03 ± 0.02‡</td>
<td>0.03 ± 0.01***</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.01 ± 0.002</td>
<td>0.02 ± 3*10⁻³</td>
<td>0.11 ± 0.03***</td>
<td>0.13 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.02 ± 0.005</td>
<td>0.03 ± 0.003†</td>
<td>0.09 ± 0.01†</td>
<td>0.17 ± 0.02***</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.01 ± 0.008</td>
<td>0.03 ± 0.006‡</td>
<td>0.11 ± 0.01†</td>
<td>0.35 ± 0.20‡</td>
</tr>
<tr>
<td>SI tissue + contents (% of dose)</td>
<td>7.5</td>
<td>0.09 ± 0.02</td>
<td>0.03 ± 0.01***</td>
<td>0.02 ± 0.01***</td>
<td>0.01 ± 0.01***</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>0.2 ± 0.1‡</td>
<td>0.06 ± 0.02***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.1‡</td>
<td>0.14 ± 0.04***</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>3.0 ± 0.5</td>
<td>3.0 ± 0.5</td>
<td>0.8 ± 0.2‡</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Note: 7OH-MTX tissue levels are expressed as percentage of the MTX dose (means ± SD, n = 3-9) and 7OH-MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice at the same time point (Student’s t-test was used for statistical analysis).
Table 3S. Excretion of MTX and 7OH-MTX in female wild-type, Abcg2<sup>-/-</sup>, Abcc2<sup>-/-</sup> and Abcc2;Abcg2<sup>-/-</sup> mice after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biliary excretion (% of dose)</th>
<th>Direct intestinal excretion (% of dose)</th>
<th>Urinary excretion (% of dose)</th>
<th>Fecal excretion (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>45 ± 20</td>
<td>0.6 ± 0.4</td>
<td>47 ± 18</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>25 ± 10</td>
<td>0.8 ± 0.2</td>
<td>40 ± 26</td>
<td>18 ± 11</td>
</tr>
<tr>
<td>Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>12 ± 2&lt;sup&gt;□&lt;/sup&gt;, ↓</td>
<td>0.7 ± 0.3</td>
<td>75 ± 11&lt;sup&gt;**&lt;/sup&gt;, ↑</td>
<td>11 ± 9</td>
</tr>
<tr>
<td>Abcc2;Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>2.2 ± 0.5&lt;sup&gt;□&lt;/sup&gt;, ↓</td>
<td>2.2 ± 1.0↑</td>
<td>53 ± 19</td>
<td>21 ± 10</td>
</tr>
<tr>
<td><strong>7OH-MTX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.5 ± 1.5</td>
<td>nd</td>
<td>1.0 ± 0.4</td>
<td>nd</td>
</tr>
<tr>
<td>Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1.0 ± 0.4</td>
<td>nd</td>
<td>1.3 ± 0.4</td>
<td>nd</td>
</tr>
<tr>
<td>Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.4 ± 0.1&lt;sup&gt;□&lt;/sup&gt;, ↓</td>
<td>nd</td>
<td>6.9 ± 2.2&lt;sup&gt;**&lt;/sup&gt;, ↑</td>
<td>nd</td>
</tr>
<tr>
<td>Abcc2;Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.12 ± 0.04&lt;sup&gt;□&lt;/sup&gt;, ↓</td>
<td>nd</td>
<td>3.3 ± 1.2&lt;sup&gt;**&lt;/sup&gt;, ↑</td>
<td>nd</td>
</tr>
</tbody>
</table>

Note: Excretion is given as % of the administered dose. Biliary and direct intestinal (small intestinal contents) excretion were determined in the first 60 min after iv administration of MTX to anesthetized, gall bladder cannulated mice (n = 3-5, see Methods). Urinary and fecal excretion were determined over the first 24 hr after iv administration of MTX to unanesthetized mice (n = 5-9, see Methods). Data are presented as means ± SD, * P < 0.05, ** P < 0.01, *** P < 0.001, compared to wild-type mice in the same experiment (Student’s t-test was used for statistical analysis). ↑, increased compared to wild type, ↓, decreased compared to wild type, nd, not detected (background in this matrix was too high for reliable quantification).
**Figure 1S.** mRNA expression of aldehyde oxidase 1 (AOX1) (A), aldehyde oxidase 3 (AOX3) (B), Slco1a4 (C) and Slco1b2 (D) in liver of female wild-type, Abcg2−/−, Abcc2−/− and Abcc2;Abcg2−/− mice as measured by RT-PCR. Results are expressed as the -fold change in liver of knockout mice compared with wild-type mice. Data were normalized against the endogenous control glyceraldehyde-3-phosphate dehydrogenase. (n = 3, * P < 0.05, ** P < 0.01, Student’s t-test, statistical significance was determined by comparison of ΔCt values of knockout and wild-type mice). Data are presented as means ± SD.
Abcg2 and Abcc2 affect MTX and 7OH-MTX pharmacokinetics

Figure 2S. Transport of MTX by human and murine ABCC2/Abcc2 in vesicular uptake experiments. Membrane vesicles from Sf9 cells infected with baculovirus containing human ABCC2, murine Abcc2 or WT baculovirus were incubated with 12.5 µM [³H]MTX. ATP-dependent transport was calculated by subtracting the transport in the absence of ATP from that in the presence of 4 mM ATP. Data are presented as means ± SD (n = 3).

Figure 3S. MTX and 7OH-MTX in plasma (A) and liver (B) 60 min after iv administration of 50 mg/kg MTX to female gall bladder cannulated wild-type, Abcg2⁻/-, Abcc2⁻/- and Abcc2;Abcg2⁻/- mice. Data are presented as means ± SD.
Chapter 6

Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate \textit{in vivo}

Maria L.H. Vlaming, Anita van Esch, Zeliha Pala, Els Wagenaar, Koen van de Wetering, Olaf van Tellingen, Alfred H. Schinkel

\textit{To be submitted}
Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo.

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The multidrug transporters ABCC2, ABCC3 and ABCG2 can eliminate potentially toxic compounds from the body and have overlapping substrate specificities. To study the possibly overlapping functions of Abcc2, Abcc3 and Abcg2 in vivo, we generated and characterized Abcc3;Abcg2⁺/⁻ and Abcc2;Abcc3;Abcg2⁺/⁻ mice, which are viable and fertile. Using these mice, we investigated the relative impact of Abcc2, Abcc3 and Abcg2 on the pharmacokinetics of the anti-cancer drug methotrexate (MTX) and its main toxic metabolite 7-hydroxymethotrexate (7OH-MTX). Whereas in single and double knockout mice the plasma and liver concentrations of MTX and 7OH-MTX decreased quite rapidly within 120 min after iv MTX administration (50 mg/kg), in the Abcc2;Abcc3;Abcg2⁺/⁻ mice they remained relatively high. Sixty min after administration, 67% of the MTX dose was still present in livers of Abcc2;Abcc3;Abcg2⁺/⁻ mice as MTX or 7OH-MTX versus 7% in wild-type, showing dramatic liver accumulation of these toxic compounds when Abcc2, Abcc3 and Abcg2 were all absent. Furthermore, the respective urinary and fecal excretion of the nephrotoxic metabolite 7OH-MTX were 27- and 7-fold increased compared to wild-type. We conclude that Abcc2, Abcc3 and Abcg2 are very important for fast elimination of MTX and 7OH-MTX after iv administration, and that they can to a large extent compensate for absence of each other. Variations in expression or activity of these important proteins may therefore affect efficacy and toxicity of MTX treatment. The mouse models generated should provide useful tools for studies on the overlapping functions of Abcc2, Abcc3 and Abcg2 in the pharmacokinetics of many drugs.

INTRODUCTION

The ATP-binding cassette (ABC) transporters ABCC2, ABCC3 and ABCG2 are transmembrane proteins that are involved in the export of potentially toxic endogenous and exogenous compounds from the cell. Whereas ABCC2 and ABCG2
are expressed in the apical cell membrane of hepatocytes and epithelial cells of small intestine and kidney, pumping their substrates into bile, feces and urine, ABCC3 is expressed basolaterally in hepatocytes and intestinal epithelial cells, and pumps its substrates towards the blood (1-5). Due to their sites of expression, these ABC transporters are involved in the elimination of potential toxins from the body, thereby protecting the organism from these toxins.

ABCC2, ABCC3 and ABCG2 have very broad substrate specificities. They can influence the pharmacokinetics of a wide range of (anti-cancer) drugs and carcinogens, as well as potentially toxic endogenous compounds such as bile salts, bilirubin or porphyrins (1-6). The overlap in substrate specificity of these three proteins is relatively large. They can all transport the anti-cancer drugs etoposide and methotrexate (MTX), as well as a range of glucuronide conjugates of drugs and endogenous compounds (1-9).

The widely used anti-cancer and anti-rheumatic drug MTX is transported by many ABC transporters such as ABCB1, ABCC1-5 and ABCG2 in vitro (1;6;7;10;11). Using Abcc2;Abcg2−/− mice we have shown recently that Abcc2 and Abcg2 are the main determinants for the biliary excretion of MTX and its main toxic metabolite 7-hydroxymethotrexate (7OH-MTX) after iv administration of MTX (50 mg/kg) (12). Furthermore, using Abcc2−/− and Abcc2;Abcc3−/− mice we found, that when Abcc2 was absent, (upregulated) Abcc3 in the liver caused increased sinusoidal efflux of MTX and 7OH-MTX from liver to blood, leading to increased urinary excretion of both compounds (13). Using Abcc3−/− mice, Kitamura et al. (2008) recently showed that Abcc3 increases the systemic exposure to [3H]MTX after oral administration or continuous infusion in mice by mediating basolateral efflux in liver and duodenum. They found no difference between wild-type and Abcc4−/− mice in the oral plasma pharmacokinetics of [3H]MTX (14). Also in ABCC2-deficient rats an effect of ABCC2 on the biliary excretion of MTX was shown (15). Combining these literature data, there appear to be important and overlapping functions of especially Abcc2, Abcc3 and Abcg2 in the pharmacokinetics of MTX and 7OH-MTX. Also in humans it was shown that mutations or polymorphisms in ABCC2 or ABCG2 could influence the pharmacokinetics of MTX, in some cases associated with increased toxicity or efficacy in patients with loss-of-function mutations (16-20). No correlations between ABCC3 polymorphisms and MTX pharmacokinetics or toxicity have been reported yet.

Although quite extensive in vivo studies on the influence of MTX (and 7OH-MTX) have been performed already, it is still unclear what the relative effect of the different ABC transporters is. When using single (and even double) knockout mice the effects of the deleted gene(s) might be underestimated because other transporters may compensate for their loss. For example, we did not find an effect of Abcc3 on MTX pharmacokinetics after iv bolus administration when we analyzed
Abcc3⁻/⁻ mice, but when Abcc2 was additionally deleted, a clear effect of Abcc3 expression was found (13). The same was true for the effect of Abcg2 on 7OH-MTX pharmacokinetics, which only became apparent in the absence of Abcc2 (12).

In the present study we describe the generation and characterization of Abcc3;Abcg2⁻/⁻ and Abcc2;Abcc3;Abcg2⁻/⁻ mice. These mice were subsequently used to further unravel the overlapping and possibly compensatory functions of Abcc2, Abcc3 and Abcg2 in determining the pharmacokinetics of MTX and 7OH-MTX in vivo. We show here that these three transporters are the main determinants of fast liver and plasma elimination of MTX and 7OH-MTX and that other ABC transporters appear less important in these processes.

METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation of Abcc2⁻/⁻ (21), Abcc3⁻/⁻ (22), Abcg2⁻/⁻ (23), Abcc2;Abcc3⁻/⁻ (13;24) and Abcc2;Abcg2⁻/⁻ mice (12) was described before. Abcc3;Abcg2⁻/⁻ mice were generated by crossbreeding the two single knockout strains. Abcc2;Abcc3;Abcg2⁻/⁻ mice were generated by crossbreeding Abcc2;Abcc3⁻/⁻ and Abcc2;Abcg2⁻/⁻ mice. 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. MTX (Emthexate PF® 25 mg/ml) was from Pharmachemie (Haarlem, The Netherlands) and 7OH-MTX from Toronto Research Chemicals Inc. (North York, ON, Canada). Methoxyflurane (Metofane) was from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). MRPr1 and M₄I-80 were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands). A polyclonal antibody against mouse Abcc2 (25) was kindly provided by Prof. Dr. J.-M. Fritschy (University of Zürich, Zürich, Switzerland). RT-PCR primers were from QIAGEN (Venlo, The Netherlands).

Western analysis. Crude membrane fractions from tissues were prepared as described (23;26). Western blotting was performed as described (27). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. Abcc1 (Mrp1), Abcc2 (Mrp2) and Abcc4 (Mrp4) were detected with mAbs MRPr1 (dilution 1:1000), a polyclonal anti-murine Abcc2 antibody (dilution 1:1000) and M₄I-80 (dilution 1:400), respectively. Bound primary antibodies were detected by incubating the blot with HRP-labelled rabbit anti-rat IgG (1:1000, DAKO) or goat anti-rabbit IgG (1:1000, DAKO). Densitometric analysis was performed using the TINA 2.09 software program (Raytest, Staubenhardt).
**RT-PCR analysis.** RNA isolation, cDNA synthesis and real-time PCR (RT-PCR) analysis on livers of female mice (n = 3) were done as described (28).

**Histological, clinical-chemical and hematological analysis.** Histological analysis of tissues, standard clinical chemistry analyses on serum (twice within a time span of 1.5 years) and standard hematological analysis of male and female mice were performed as described (21).

**Plasma and tissue pharmacokinetic experiments.** MTX was administered to female wild-type, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice (n = 3-13) by injecting 5 µl/g body weight of a 10 mg/ml MTX solution in saline into the tail vein. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia at 7.5, 15, 30, 60 or 120 min after MTX administration and organs were removed at each time point.

**Fecal and urinary MTX excretion experiment.** Female wild-type, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice (n = 5-9) were individually housed in Ruco Type M/1 stainless steel metabolic cages (Valkenswaard, The Netherlands). They were allowed 24 hours to adapt before MTX was injected at 50 mg/kg into the tail vein as described above, and feces and urine were collected for 24 hours.

**HPLC analysis of MTX and 7OH-MTX.** Collected organs and feces were homogenized in an ice-cold 4% BSA solution and plasma was diluted in human plasma before HPLC analysis. Urine was diluted in water. MTX and 7OH-MTX concentrations in the different matrices were determined as described (29).

**Statistical analysis.** Unless otherwise indicated, the two-sided unpaired Student's t-test was used to assess the statistical significance of differences between wild-type and knockout mice. When statistical differences between more than 2 groups were analyzed, one-way ANOVA followed by Tukey's multiple comparison test was performed, as indicated. Results are presented as means ± standard deviations (SD). Differences were considered to be statistically significant when P < 0.05. Averaged concentrations for each time point were used to calculate the area under the plasma concentration versus time curve (AUC) from t = 0 to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (30).
RESULTS

Macroscopic and microscopic analysis of Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice.

Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice were viable, fertile and had normal life spans and body weights. Macroscopic and microscopic histological and pathological analysis did not reveal obvious specific aberrations in tissues of both knockout strains, including the liver. However, in Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice (but not in Abcc3;Abcg2<sup>-/-</sup> mice) the liver weight was 41-67% increased compared to wild-type (Figure 1A). Increased liver weight was previously shown in other Abcc2-deficient strains (12;13;21;31, Figure 1A). However, the liver weight in Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice (both male and female) was also significantly higher than in most Abcc2, Abcc3 and Abcg2 single and double knockout mice (ANOVA).

Protein and mRNA expression of other ABC transporters in tissues of Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice.

Protein expression of Abcc1 in liver of male wild-type, Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice was low and therefore hard to quantify (not shown). Abcc2 protein in liver of male and female Abcc3;Abcg2<sup>-/-</sup> mice was not different from wild-type mice (not shown). Abcc2 protein in kidney of female Abcc3; Abcg2<sup>-/-</sup> mice was not different from wild type either.

Abcc4 protein expression in kidney of female Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice was 1.6-fold increased compared to wild-type mice and similar to that of Abcc2;Abcg2<sup>-/-</sup> mice (Figure 1B). Abcc4 protein in livers of male Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice was low and therefore hard to quantify (not shown). In female Abcc3;Abcg2<sup>-/-</sup> mice liver Abcc4 protein was not different from wild type. In female Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice, Abcc4 liver expression appeared about 2-fold increased compared to wild-type, comparable to that in Abcc2;Abcg2<sup>-/-</sup> mice (12), although this was hard to quantify due to low expression levels in all strains (not shown). Quantitative RT-PCR analysis confirmed that in female Abcc2;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice Abcc4 mRNA expression in liver was significantly (14- to 19-fold) increased compared to wild-type mice (Figure 1C). Abcc4 mRNA levels in Abcc3;Abcg2<sup>-/-</sup> mice were not different from wild type (Figure 1C).

Expression levels of genes involved in methotrexate disposition.

Because we wanted to analyze the pharmacokinetics of MTX using Abcc3;Abcg2<sup>-/-</sup> and Abcc2;Abcc3;Abcg2<sup>-/-</sup> mice, we determined mRNA expression levels of other genes that may be involved in MTX metabolism and transport in livers of female knockout mice. We found that, similar to Abcc2<sup>-/-</sup> and Abcc2;Abcg2<sup>-/-</sup> mice (12), mRNA levels of the enzyme aldehyde oxidase 1, which is
Figure 1. Physiological characterization of Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice.

A, Liver weight (as percentage of body weight) of male (left panel) and female (right panel) wild-type, Abcc2−/−, Abcc2;Abcg2−/−, Abcc2;Abcc3−/−, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice (means ± SD, n = 3-12, **, P < 0.01, ***, P < 0.001, relative to wild-type, ANOVA). Abcc3−/− and Abcg2−/− mice had no aberrant liver weight (12;13). B, protein levels of Abcc4 in crude membrane fractions of kidney samples from two independent female wild-type, Abcc2;Abcc3;Abcg2−/− and (for comparison) Abcc2;Abcg2−/− mice (dilution experiment). Densitometric analysis of the blots showed that on average Abcc4 expression was 1.6-fold increased in Abcc2;Abcc3;Abcg2−/− mice compared to wild-type. The lane with the positive control (Sf-9 vesicles containing ABCC4 (39)), is indicated with “+”. Underglycosylation of ABCC4 in the Sf-9 cells causes the faster migration compared to the murine Abcc4. The amount of protein loaded in each lane (in µg) is noted above the respective lanes. C, mRNA expression of Abcc4 in liver of female wild-type, Abcc2;Abcg2−/−, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. Results are expressed as the fold change in liver of knockout mice compared with wild-type mice. Data were normalized against the endogenous control glyceraldehyde-3-phosphate dehydrogenase (n = 3, ***, P < 0.05, ns, not significant, ANOVA, statistical significance was determined by comparison of ΔCt values of different strains). Data are presented as means ± SD.
implicated in the conversion of MTX to 7OH-MTX (31), was 2.5-fold increased compared to wild-type mice (n = 3, P = 0.008, not shown). Aldehyde oxidase 3 mRNA expression in liver was not significantly different from wild-type (not shown). Furthermore, whereas we previously found that mRNA of the uptake transporter Slc01b2 was ~2-fold increased in Abcc2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice (12), mRNA levels of Slc01a4 and Slc01b2 were not significantly different from wild-type in Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice (not shown).

Table 1. Clinical chemical analysis of serum from wild-type, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Wild-type</th>
<th>Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcc2;Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Total Bilirubin</td>
<td>male</td>
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<td>2.7 ± 0.8</td>
<td>3.7 ± 0.5&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>(µM)</td>
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<td>3.0 ± 1.0</td>
<td>3.7 ± 1.9</td>
<td>4.2 ± 1.3&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
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<td>34 ± 5</td>
<td>36 ± 7</td>
<td>55 ± 14&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>female</td>
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<td>Creatinine (µM)</td>
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<td>17 ± 3</td>
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<td>Total protein (g/l)</td>
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<td>50 ± 2</td>
<td>52 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
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<td></td>
<td>female</td>
<td>49 ± 2</td>
<td>50 ± 3</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
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<td>1.7 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>3.4 ± 1.0&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>female</td>
<td>2.0 ± 0.7</td>
<td>2.0 ± 0.6</td>
<td>3.3 ± 0.8</td>
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Note: results are presented as means ± SD, n = 5-6, *P < 0.05, **P<0.01, ***P<0.001 compared to wild-type mice (Student’s t-test was used for statistical analysis). ALAT, alanine aminotransferase. 1Was not significant in this measurement (P = 0.13), but was significantly higher in a previous data set. All other measured parameters are shown in Supplementary Table 1.

Serum clinical chemistry and hematological analysis of Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice.

Table 1 shows clinical chemistry parameters of Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice that were different from wild-type mice. The results of all measured parameters are shown in Supplementary Table 1. Conjugated bilirubin levels were below the detection limit of the analyzer (2 µM). In the Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice the serum total bilirubin concentrations were mildly increased compared to wild-type, as was previously shown for Abcg2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice (12;23). In contrast to what was previously shown for Abcc2<sup>−/−</sup>
and Abcc2;Abcg2\textsuperscript{-/-} mice (12;21;33), we did not detect increased conjugated bilirubin levels in Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice. This is in line with the earlier observation that Abcc3 is responsible for transport of conjugated bilirubin from liver to blood when Abcc2 is absent (13). Besides bilirubin, also triglyceride serum concentrations were \( \approx \)2-fold increased in Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice (but not in Abcc3;Abcg2\textsuperscript{-/-} mice) (Table 1). Furthermore, total protein levels were mildly increased in serum of Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice (Table 1). In male (but not female) Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice also alanine aminotransferase (ALAT), creatinine and urea serum concentrations were mildly but significantly increased (Table 1).

Hematological analysis of Abcc3;Abcg2\textsuperscript{-/-} mice did not show consistent differences with wild-type mice (not shown). Female Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice had, like other Abcc2-deficient mice (12;21), mildly reduced hemoglobin levels compared to wild-type (7.2 \pm 0.2 mM vs. 7.8 \pm 0.3 mM, \( n = 6, \ P = 2.3 \times 10^{-3} \)). In males this was not found.

**Effect of Abcc2, Abcc3 and Abcg2 on MTX and 7OH-MTX pharmacokinetics.**

We have previously shown that Abcc2 and Abcg2 are the main determinants for the biliary excretion of MTX and 7OH-MTX after iv administration of MTX (50 mg/kg) (12). Furthermore, when Abcc2 is absent, (upregulated) Abcc3 in the liver causes increased efflux of MTX and 7OH-MTX from liver to blood, leading to increased urinary excretion of these compounds (13). To further investigate the functional overlap and importance of these ABC transporters, we administered MTX iv at a dose of 50 mg/kg to wild-type, Abcc3;Abcg2\textsuperscript{-/-} and Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice and analyzed the pharmacokinetics of MTX and 7OH-MTX. The results for all compound and single Abcc2, Abcc3 and Abcg2 knockout mice (12;13) are given in Supplementary Tables 2S (for MTX) and 3S (for 7OH-MTX). Figure 2 shows plasma and liver levels of MTX and 7OH-MTX in wild-type, Abcc3;Abcg2\textsuperscript{-/-} and Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice. Furthermore, the combined liver levels of MTX and 7OH-MTX are shown in Supplementary Figure 1. For comparison, also the pharmacokinetics of MTX and 7OH-MTX in Abcc2;Abcg2\textsuperscript{-/-} mice (12) are shown in each figure.

Whereas the AUC\textsubscript{plasma} for MTX was 1.6-fold increased in Abcg2\textsuperscript{-/-} mice (12), the plasma levels of MTX in Abcc3;Abcg2\textsuperscript{-/-} mice were similar to wild-type (Figure 2A, Supplementary Table 2S), suggesting that Abcc3 is necessary for the increased MTX plasma levels in Abcg2\textsuperscript{-/-} mice. The role of Abcc3 was additionally illustrated by reduced MTX plasma levels in Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice compared to Abcc2;Abcg2\textsuperscript{-/-} mice up to 60 min after administration (Figure 2A). Whereas in all other strains the MTX plasma levels rapidly decreased after 30 min, in Abcc2;Abcc3;Abcg2\textsuperscript{-/-} mice they stayed relatively high up to 120 min after administration, leading to a 1.4-fold increased AUC\textsubscript{plasma} compared to wild-type.
(Supplementary Table 2S). This suggests a significantly delayed overall elimination of MTX in these mice (Figure 2A).

Figure 2. Plasma and liver pharmacokinetics of MTX and 7OH-MTX after iv administration of MTX (50 mg/kg) to female wild-type, Abcc2;Abcg2−/−, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. A, MTX plasma concentration versus time curves of the different strains, semi-log plot (means ± SD, n = 3-13). B, MTX liver level versus time curves of the different strains (means ± SD, n = 3-9). C, 7OH-MTX plasma concentration versus time curves of the different strains (means ± SD, n = 3-13). D, 7OH-MTX liver level versus time curves of the different strains (means ± SD, n = 3-9).

As shown before (12), after initial high hepatic accumulation, elimination of MTX from the liver was quite rapid not only in wild-type, but also in Abcc2;(Abcg2)−/− mice, which was likely mediated by (upregulated) Abcc3 in these strains, leading to increased sinusoidal efflux (13). In Abcc3;Abcg2−/− mice on the other hand, MTX liver elimination was significantly delayed (Figure 2B), as was shown in Abcg2−/− mice before ((12), Supplementary Table 2S). After 120 min, the MTX liver levels in Abcc3;Abcg2−/− mice were back to wild-type levels. In
Abcc2;Abcc3;Abcg2−/− mice the liver levels of MTX were even more increased, up to 7-fold compared to wild-type at 120 min after administration (Figure 2B). This suggests that Abcc2, Abcc3 and Abcg2 together are the main transporters for elimination of MTX from the liver and that they can (at least to a large extent) compensate for the absence of each other.

We also investigated plasma and tissue levels of the toxic metabolite 7OH-MTX (Figure 2C and D). Whereas in the Abcc2;Abcg2−/− mice (due to reduced biliary excretion of MTX, 7OH-MTX, or both) the plasma levels of 7OH-MTX increased rapidly (12), this was not as pronounced in Abcc2;Abcc3;Abcg2−/− mice. This shows that part of the increased plasma 7OH-MTX concentrations in Abcc2;Abcg2−/− mice was dependent on the presence of (overexpressed) Abcc3 in the liver (Figure 2C, see also below). However, although Abcc3 was absent in the Abcc2;Abcc3;Abcg2−/− mice, substantial amounts of 7OH-MTX still reached the circulation, leading to increased plasma levels and a 9.6-fold increased AUCplasma compared to wild-type mice over the first 120 min (Figure 2C and Supplementary Table 3S).

Analysis of 7OH-MTX liver concentrations clearly showed the importance of Abcc2, Abcc3 and Abcg2 for the elimination of 7OH-MTX from the liver. The liver levels of 7OH-MTX were dramatically (up to 90-fold at t = 120 min) increased in the Abcc2;Abcc3;Abcg2−/− mice (Figure 2D), also compared to all double knockout strains (Supplementary Table 3S). At 60 min, the cumulative MTX and 7OH-MTX levels in wild-type as well as single and double knockout strains did not exceed 22 % of the given dose (ranging from 7.0 ± 0.9 % of the dose in wild-type to 22.0 ± 7.2 % of the dose in Abcc2;Abcc3−/− mice). However, in Abcc2;Abcc3;Abcg2−/− mice, the combined MTX and 7OH-MTX liver accumulation amounted to 67.1 ± 3.5 % of the dose (Supplementary Figure 1), illustrating the dramatically reduced liver elimination of both compounds in these mice. This demonstrates the overlapping functions of these three transporters in the fast elimination of MTX and 7OH-MTX from the liver. Nevertheless, despite the absence of these transporters, 7OH-MTX could still gradually exit from the liver, primarily towards plasma, as shown by the relatively high 7OH-MTX plasma levels at later time points in the triple knockout mice (Figure 2C).

MTX in the kidney in general reflected the plasma MTX levels, leading to 7- and 2-fold increased kidney levels in Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice, respectively, at 60 min after administration (Figure 3A, Supplementary Table 2S, (12)). The 7OH-MTX kidney levels also followed the plasma levels, resulting in substantial kidney accumulation in both Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice at 60 min after administration (Figure 3C).

We have shown before that the pronounced excretion of MTX and 7OH-MTX into the small intestine (up to about 50% of the dose in 60 min) is mainly
mediated by biliary excretion via Abcc2 and Abcg2 and that direct excretion across the intestinal wall is relatively low (12;13). Figure 3B shows that in Abcc2;Abcc3;Abcg2−/− mice the excretion of MTX into the small intestine was dramatically reduced, to only 15% of wild-type levels at 60 min after administration (Supplementary Table 2S). Despite markedly increased liver levels in this strain (Figure 2B), apparently relatively little MTX is excreted into the small intestine, again showing that Abcc2 and Abcg2 are very important for hepatobiliary excretion of MTX into the small intestine. Note that in Abcc2;Abcc3;Abcg2−/− mice somewhat higher small intestinal MTX levels compared to Abcc2;Abcg2−/− mice were found (P = 4.8*10^{-3} by Student’s t-test at 60 min), probably reflecting the higher liver MTX.
concentrations (Figure 2B). Clearly, although Abcc2 and Abcg2 are absent, still some (low-level) biliary excretion of MTX is present in the Abcc2;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice.

Figure 3D shows that, despite dramatically increased liver levels of 7OH-MTX in the Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice, the excretion into the small intestine was significantly lower than in wild-type. This confirms that Abcc2 and Abcg2 are the main transporters for (biliary) excretion of 7OH-MTX into the small intestine. However, like for MTX (see above), the small intestinal levels of 7OH-MTX were significantly higher at 60 min after administration in Abcc2;Abcc2;Abcg2<sup>−/−</sup> compared to Abcc2;Abcg2<sup>−/−</sup> mice (P < 0.01, ANOVA), suggesting that the increased liver and plasma concentrations in these mice led to somewhat increased (hepatobiliary and/or direct) excretion into the small intestine of 7OH-MTX, not mediated by Abcc2 or Abcg2.

**Figure 4.** Urinary and fecal excretion of MTX and 7OH-MTX 24 hrs after iv administration of MTX (50 mg/kg) to female wild-type, Abcc2;Abcg2<sup>−/−</sup>, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice. A, urinary (left panel) and fecal (right panel) excretion of MTX (as % of the dose). B, urinary (left panel) and fecal (right panel) excretion of 7OH-MTX (as % of the dose). C, cumulative urinary and fecal excretion of MTX (as % of the dose). D, cumulative urinary and fecal excretion of 7OH-MTX (as % of the dose). Data are presented as means ± SD (n = 5-9, *, P < 0.05, **, P < 0.01, ***, P < 0.001). Levels of 7OH-MTX in feces of wild-type mice were close to background and therefore hard to quantify (background ~3% of the dose). Levels of 7OH-MTX in feces of all strains may be slightly overestimated due to high background peaks in this matrix. Note the differences in axis scales.

**Fecal and urinary excretion of MTX and 7OH-MTX.**
The cumulative excretion of MTX and 7OH-MTX over 24 hours into urine and feces in wild-type, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice is shown in
Figure 4. Despite dramatically delayed hepatic elimination of MTX and 7OH-MTX early after administration (see above), especially in the triple knockout mice virtually all of the administered MTX was excreted as MTX or 7OH-MTX within 24 hours. The total urinary excretion of MTX was not different from wild-type in the compound knockout strains, showing that even if Abcc2, Abcc3 and Abcg2 are absent, MTX can still be excreted into urine. The fecal excretion of MTX was not significantly different from wild-type in the Abcc2;Abcc3;Abcg2−/− mice either. Interestingly, in Abcc3;Abcg2−/− mice, the fecal excretion of MTX was significantly increased compared to wild-type (Figure 4A). This may reflect reduced re-uptake of MTX from the intestinal lumen due to absence of Abcc3 (14).

The urinary excretion of 7OH-MTX in wild-type and Abcc3;Abcg2−/− mice was quite low, in line with the relatively low plasma and kidney concentrations in these strains. The fecal excretion of 7OH-MTX was, like that of MTX, somewhat increased in Abcc3;Abcg2−/− mice, perhaps due to the mildly increased 7OH-MTX liver concentrations up to 60 min after administration, and perhaps due to reduced re-uptake from the intestine (Supplementary Table 3S). In contrast, in Abcc2;Abcc3;Abcg2−/− mice the urinary and fecal excretion of 7OH-MTX were markedly increased (27.0- and 7.3-fold, respectively) compared to wild-type (Figure 4B). In fact, in Abcc2;Abcc3;Abcg2−/− mice the total amount of 7OH-MTX recovered in urine and feces represented ~43% of the total MTX dose, as opposed to less than 8% in wild-type and double knockout strains (Figure 4C and D). It is likely that the absence of Abcc2, Abcc3 and Abcg2 leads to dramatically increased retention of MTX in the liver and subsequent extensive 7OH-MTX formation and accumulation in the liver. This is subsequently eliminated over a relatively prolonged period via both urine and feces.

DISCUSSION
To investigate the possibly overlapping functions of Abcc2, Abcc3 and Abcg2 we generated and characterized Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. Despite the absence of up to three ABC transporters with overlapping or complementary functions in the protection from toxic compounds (1-3;34), both strains are viable, fertile and have a normal life span. They also do not display obvious physiological or pathological aberrations under standard housing conditions, except for an increased liver size and some mild changes in serum clinical chemistry. We therefore consider these double and triple knockout mice perfectly suitable for pharmacokinetic, toxicological and physiological research on the functions of Abcc2, Abcc3 and Abcg2. These models will be very useful tools for studies on the pharmacology of a wide range of drugs. We used the mice to evaluate the overlapping or complementary functions of Abcc2, Abcc3 and Abcg2 in the pharmacokinetics of the anti-cancer and anti-rheumatic drug MTX and its main toxic metabolite 7OH-MTX in vivo. We show here that Abcc2, Abcc3 and Abcg2
together are the primary ABC transporters responsible for the fast elimination of both MTX and 7OH-MTX from the liver and body, and that they can largely compensate for the loss of each other. Absence of all three ABC transporters leads to a dramatic accumulation of MTX and 7OH-MTX in the liver, as well as prolonged systemic exposure to both compounds after MTX administration.

In Abcc2;Abcc3;Abcg2−/− mice, some changes in clinical chemistry parameters of serum were found (Table 1). However, although the differences were consistent in two separate measurements, they were relatively small and extensive pathological analysis of liver and kidney of these mice did not reveal any signs of pathology. Furthermore, the mice had normal life spans. This suggests that there are no dramatic effects on the health of Abcc2;Abcc3;Abcg2−/− mice, showing that at least in the protective environment of the NKI mouse facility, Abcc2, Abcc3 and Abcg2 do not have overlapping physiological functions.

We found that after iv administration of MTX, Abcc2, Abcc3 and Abcg2 play important and clearly overlapping roles in the elimination of MTX and 7OH-MTX from the liver. Whereas in single and double knockout mice there was only relatively mild accumulation of both compounds in the liver (Supplementary Tables 2S and 3S), in the Abcc2;Abcc3;Abcg2−/− mice this was much more dramatic, especially for 7OH-MTX, and it lasted longer as well (Figure 2B and D, Supplementary Figure 1). Furthermore, whereas in all other strains the plasma levels of MTX and 7OH-MTX at 120 min after iv administration of MTX were quite low and comparable to wild-type (Supplementary Tables 2S and 3S), in Abcc2;Abcc3;Abcg2−/− mice they were still relatively high, suggesting prolonged systemic exposure to these toxic compounds when all three transporters were absent. This shows that Abcc2, Abcc3 and Abcg2 alone can to a large extent compensate for the absence of the two other transporters. Other ABC transporters, which can transport MTX in vitro, such as Abcc1, Abcc4, Abcc5 and Pgp (10;11;35;36), do not seem to play a significant role in the rapid elimination of MTX and 7OH-MTX in vivo, at least not after bolus iv administration of MTX at 50 mg/kg. Nevertheless, although Abcc2, Abcc3 and Abcg2 appear quite important for the elimination of MTX and 7OH-MTX early after administration, even when all three proteins were absent virtually all of the administered MTX was excreted as MTX or 7OH-MTX within the first 24 hr (Figure 4). This shows that other (lower capacity) elimination systems are still able to eliminate these compounds from the body.

The combined absence of Abcc2, Abcc3 and Abcg2 led to trapping of MTX in the liver due to reduced biliary (via Abcc2 and Abcg2) and sinusoidal (via Abcc3) elimination, and caused increased formation of the toxic metabolite 7OH-MTX in the liver (Figures 2B and D). This shows that Abcc2, Abcc3 and Abcg2 are very important for limiting the exposure of the body to the toxic metabolite 7OH-MTX after MTX treatment, both directly (by elimination of 7OH-MTX from the liver), as well as indirectly (by fast elimination of MTX from the liver), thus preventing
abundant 7OH-MTX formation. It is therefore possible that decreased activity and/or expression of ABCC2, ABCC3 and/or ABCG2 may lead to accumulation of MTX and 7OH-MTX in patients treated with MTX. This may cause increased risk of hepatotoxicity when patients with polymorphisms or mutations in one or more of these genes are treated with MTX.

It is worth observing that the functional overlap or complementarities between no less than 3 different genes that we observed here guarantees a considerable robustness in the protection from MTX, a xenobiotic toxin. This makes sense from both biological and clinical-therapeutic perspectives. Even though individual deficiencies in Abcc2, Abcc3 or Abcg2 can affect the clearance and systemic exposure of MTX to a certain extent, it is clear that the consequences would be much more dramatic in the absence of functions of all these proteins. Thus, overall protection against MTX toxicity is not entirely dependent on the function of one single gene and protein, but it is determined by at least three different genes. This means that the risk of dramatically increased toxicity due to genetic polymorphisms or coincidental inhibition affecting activity of a single gene or protein is much reduced. This is clearly advantageous in the natural protection from xenobiotic toxins. It also means that it is comparatively safe to use a drug like MTX in the clinic, as the risk of unpredictable toxicity due to dysfunctioning of one detoxifying protein is relatively limited. Clearly, drugs of which the toxicity is critically dependent on the function of just one detoxifying protein bear considerably higher risks, and they should probably be avoided in clinical practice. Nevertheless, as many drugs (and food components) may be substrates or inhibitors of these three ABC transporters, co-administration of MTX with drugs that are also ABCC2, ABCC3 and ABCG2 substrates should be done with caution.

Interestingly, in Abcc3;Abcg2/− mice, the fecal excretion of MTX and 7OH-MTX was significantly higher than in wild-type mice. The mildly increased liver accumulation of MTX and 7OH-MTX early after administration in these mice may have caused increased excretion of these compounds into the intestine over 24 hrs, most likely via Abcc2 (13). In addition, absence of Abcc3 in the intestinal wall of these mice may lead to reduced re-absorption of MTX and perhaps 7OH-MTX from the intestine (13). As a consequence, the intestine of Abcc3;Abcg2/− mice may possibly receive increased exposure to MTX and therefore these mice could be more prone to gastrointestinal toxicity. As this could mean that patients with decreased ABCC3 expression or activity in the intestine may be more susceptible to gastrointestinal toxicity (a common side effect of MTX treatment (37)), it would be interesting to investigate this in more detail in the future.

Another common toxicity in patients treated with high-dose MTX is kidney failure (38). This is thought to be primarily caused by formation of crystalline deposits of MTX and especially of the poorly water soluble metabolite 7OH-MTX in renal tubules (37). Since in the Abcc2;Abcc3;Abcg2/− mice the formation and
urinary excretion of 7OH-MTX was dramatically increased compared to wild-type mice (and the other (compound) knockout strains), this suggests that ABCC2, ABCC3 and ABCG2 together are very important in the protection of the kidneys from 7OH-MTX exposure and that they may prevent MTX and 7OH-MTX related toxicities in patients.

The data presented here data illustrate the dramatic changes in the pharmacokinetics of both MTX and 7OH-MTX when two or more ABC transporters are absent. In the future, these results may therefore be helpful in predicting responses to MTX treatment in patients based on individual expression or activity data of ABCC2, ABCC3 and/or ABCG2. Furthermore, the Abcc2;Abcc3;Abcg2-/- mice we generated will be excellent tools to determine the relative and overlapping effects of these ABC transporters on the pharmacokinetics of a wide range of existing and newly developed drugs.

ACKNOWLEDGEMENTS
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REFERENCES


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Supplementary Table 1S. Clinical chemical analysis of serum from wild-type, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Strain</th>
<th>Wild-type</th>
<th>Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcc2;Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wild-type</td>
<td>Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abcc2;Abcc3;Abcg2&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>male</td>
<td>2.2 ± 0.4</td>
<td>2.7 ± 0.8</td>
<td>3.7 ± 0.5&lt;sup&gt;***&lt;/sup&gt;</td>
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</tr>
<tr>
<td>(µM)</td>
<td>female</td>
<td>3.0 ± 1.0</td>
<td>3.7 ± 1.9</td>
<td>4.2 ± 1.3&lt;sup&gt;†&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Conjugated bilirubin</td>
<td>male</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
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<tr>
<td>(µM)</td>
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<td>&lt; 2</td>
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<tr>
<td>Alkaline phosphatase</td>
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<td>57 ± 5</td>
<td>56 ± 13</td>
<td>52 ± 10</td>
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<tr>
<td>(UI)</td>
<td>female</td>
<td>84 ± 22</td>
<td>68 ± 13</td>
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<tr>
<td>ASAT</td>
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<td>76 ± 13</td>
<td>75 ± 21</td>
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<tr>
<td>(UI)</td>
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<td>114 ± 51</td>
<td>77 ± 16</td>
<td>83 ± 38</td>
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<tr>
<td>ALAT</td>
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<td>34 ± 5</td>
<td>36 ± 7</td>
<td>55 ± 14&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>(UI)</td>
<td>female</td>
<td>27 ± 8</td>
<td>22 ± 9</td>
<td>33 ± 6</td>
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<tr>
<td>Creatinine</td>
<td>male</td>
<td>11 ± 2</td>
<td>19 ± 4&lt;sup&gt;**&lt;/sup&gt;</td>
<td>25 ± 4&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>(µM)</td>
<td>female</td>
<td>17 ± 4</td>
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<td>Urea</td>
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<td>10 ± 1</td>
<td>13 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>12 ± 1&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
<td>11 ± 1</td>
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<tr>
<td>Sodium</td>
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<td>162 ± 6</td>
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<td>162 ± 6</td>
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<td>(mM)</td>
<td>female</td>
<td>155 ± 9</td>
<td>155 ± 6</td>
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<tr>
<td>Potassium</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>4.5 ± 0.2</td>
<td>4.7 ± 0.6</td>
<td>4.3 ± 0.3</td>
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<tr>
<td>Calcium</td>
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<td>2.4 ± 0.1</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>2.5 ± 0.1</td>
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<tr>
<td>Phosphate</td>
<td>male</td>
<td>2.8 ± 0.4</td>
<td>2.8 ± 0.5</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>2.6 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>2.4 ± 0.1</td>
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<tr>
<td>Chloride</td>
<td>male</td>
<td>116 ± 6</td>
<td>116 ± 6</td>
<td>114 ± 4</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>112 ± 7</td>
<td>111 ± 5</td>
<td>113 ± 7</td>
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<tr>
<td>Total protein</td>
<td>male</td>
<td>48 ± 2</td>
<td>50 ± 2</td>
<td>52 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>(g/l)</td>
<td>female</td>
<td>49 ± 2</td>
<td>50 ± 3</td>
<td>53 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Albumin</td>
<td>male</td>
<td>29.8 ± 0.4</td>
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<tr>
<td>(g/l)</td>
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<td>32.0 ± 2.8</td>
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<tr>
<td>Triglyceride</td>
<td>male</td>
<td>1.7 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>3.4 ± 1.0&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>(mM)</td>
<td>female</td>
<td>2.0 ± 0.7</td>
<td>2.0 ± 0.6</td>
<td>3.3 ± 0.8&lt;sup&gt;†&lt;/sup&gt;</td>
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Note: results are presented as means ± SD, n = 5-6, * P < 0.05, ** P<0.01, *** P<0.001 compared to wild-type mice (Student’s t-test was used for statistical analysis). ASAT, aspartate aminotransferase, ALAT, alanine aminotransferase. <sup>†</sup>Was not significant in this measurement (P = 0.13), but was significantly higher in a previous data set.
Supplementary Table 2S. Levels of MTX in tissues and plasma of female mice at different time points after iv administration of MTX (50 mg/kg).

<table>
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<tr>
<th>Matrix</th>
<th>Time (min)</th>
<th>Strain</th>
<th>Wild-type</th>
<th>Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2;Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2;Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc3;Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>444 ± 44</td>
<td>870 ± 103***</td>
<td>368 ± 34</td>
<td>692 ± 56</td>
<td>435 ± 47</td>
<td>1446 ± 229</td>
<td>451 ± 26</td>
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<tr>
<td>Liver</td>
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<td>(min·µg/ml)</td>
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<td>7.5</td>
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<td>49.5 ± 4.4</td>
<td>44.4 ± 4.9</td>
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<td>15</td>
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<td>36.0 ± 1.3</td>
<td>28.1 ± 4.7</td>
<td>43.9 ± 2.0</td>
<td>36.3 ± 3.7</td>
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<td>16.4 ± 5.3</td>
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<td>19.4 ± 9.2</td>
<td>36.5 ± 5.9***</td>
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<td></td>
<td>4.8 ± 0.8</td>
<td>4.0 ± 1.9</td>
<td>6.2 ± 2.8</td>
<td>10.0 ± 1.2***</td>
<td>8.1 ± 2.6</td>
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<td>15.0 ± 2.6***</td>
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<td>S.I. + S.I.C.</td>
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<td>AUC</td>
<td>12.8 ± 1.1</td>
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<td>16.4 ± 2.5</td>
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<td>5.1 ± 1.5</td>
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<td>4.2 ± 0.8</td>
<td>3.1 ± 0.8**</td>
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<td>3.8 ± 1.2</td>
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<td>26.9 ± 2.8</td>
<td>10.6 ± 4.3**</td>
<td>19.4 ± 2.7</td>
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<td>14.7 ± 1.7**</td>
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<td>37.0 ± 14.8</td>
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<td>29.3 ± 1.2</td>
<td>2.7 ± 0.3**</td>
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<tr>
<td>Kidney</td>
<td></td>
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<td>(% of dose)</td>
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<tr>
<td></td>
<td>7.5</td>
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<td>3.3 ± 1.6</td>
<td>5.3 ± 2.3</td>
<td>3.1 ± 0.5</td>
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<td>15</td>
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<td></td>
<td>0.9 ± 0.3</td>
<td>2.6 ± 0.7**</td>
<td>1.6 ± 0.1**</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>2.7 ± 0.6**</td>
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<tr>
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<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2**</td>
<td>0.3 ± 0.2**</td>
<td>0.7 ± 0.1**</td>
<td>0.4 ± 0.03</td>
<td>1.5 ± 0.3**</td>
<td>0.5 ± 0.1</td>
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<tr>
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<td>60</td>
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<td>0.2 ± 0.1</td>
<td>0.3 ± 0.03**</td>
<td>0.2 ± 0.02</td>
<td>0.3 ± 0.1**</td>
<td>0.2 ± 0.02</td>
<td>1.4 ± 1.3**</td>
<td>0.2 ± 0.1</td>
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</table>

Note: MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9), MTX plasma AUCs are presented as min·µg/ml (means ± SD, n = 3-12, 0-120 min), and liver AUCs are presented as min·mg/g liver (means ± SD, n = 3-9, 0-120 min). *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild-type mice at the same time-point or time period (Student’s t-test was used for statistical analysis). S.I. + S.I.C., small intestinal tissue + contents.
**Supplementary Table 3S.** Levels of 7OH-MTX in tissues and plasma of female mice at different time points after iv administration of MTX (50 mg/kg).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th>Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2/Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2/Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc3/Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc2/Abcc3/Abcg2&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<tr>
<td>Plasma</td>
<td>AUC</td>
<td>17 ± 3</td>
<td>106 ± 11&lt;sup&gt;***&lt;/sup&gt;</td>
<td>12 ± 1</td>
<td>25 ± 6</td>
<td>73 ± 20&lt;sup&gt;**&lt;/sup&gt;</td>
<td>210 ± 36&lt;sup&gt;**&lt;/sup&gt;</td>
<td>20 ± 2</td>
<td>163 ± 17&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(min·µg/ml)</td>
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<tr>
<td>Liver</td>
<td>7.5</td>
<td>4.4 ± 1.0</td>
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<td>9.1 ± 0.5&lt;sup&gt;***&lt;/sup&gt;</td>
<td>9.5 ± 1.4&lt;sup&gt;***&lt;/sup&gt;</td>
<td>3.5 ± 0.4</td>
<td>11.3 ± 1.3&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>15</td>
<td>4.5 ± 1.0</td>
<td>8.9 ± 0.7&lt;sup&gt;***&lt;/sup&gt;</td>
<td>5.7 ± 0.8</td>
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<td>8.9 ± 1.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>8.8 ± 2.3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>6.2 ± 1.2</td>
<td>23.4 ± 1.1&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>2.3 ± 0.9</td>
<td>6.2 ± 0.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>2.2 ± 1.3</td>
<td>3.4 ± 0.5&lt;sup&gt;***&lt;/sup&gt;</td>
<td>12.3 ± 4.1&lt;sup&gt;***&lt;/sup&gt;</td>
<td>7.3 ± 2.0&lt;sup&gt;***&lt;/sup&gt;</td>
<td>3.6 ± 0.8&lt;sup&gt;***&lt;/sup&gt;</td>
<td>26.3 ± 3.6&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
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<td>60</td>
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<td>4.8 ± 1.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>1.6 ± 0.9</td>
<td>2.9 ± 0.1&lt;sup&gt;***&lt;/sup&gt;</td>
<td>13.9 ± 6.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>8.5 ± 1.3&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>120</td>
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<td>0.7 ± 0.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>8.0 ± 1.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>1.1 ± 0.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.4 ± 0.2</td>
<td>36.1 ± 1.6&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(min·mg/g)</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>AUC</td>
<td>2.3 ± 0.3</td>
<td>4.6 ± 0.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>2.3 ± 0.5</td>
<td>2.9 ± 0.2</td>
<td>9.6 ± 2.1&lt;sup&gt;***&lt;/sup&gt;</td>
<td>5.8 ± 0.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>3.1 ± 0.3</td>
<td>28.0 ± 1.5&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>S.I. + S.I.C.</td>
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<td>0.09 ± 0.02</td>
<td>0.02 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.06 ± 0.02</td>
<td>0.03 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.02 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.01 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.04 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.02 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>15</td>
<td>0.52 ± 0.11</td>
<td>0.15 ± 0.12&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.47 ± 0.07</td>
<td>0.66 ± 0.26</td>
<td>0.22 ± 0.04&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.31 ± 0.07&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>1.84 ± 0.59</td>
<td>0.63 ± 0.12&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>3.04 ± 0.54</td>
<td>1.82 ± 0.60&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.34 ± 0.07&lt;sup&gt;***&lt;/sup&gt;</td>
<td>3.39 ± 0.61</td>
<td>1.87 ± 0.47&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>Kidney</td>
<td>7.5</td>
<td>0.009 ± 0.003</td>
<td>0.03 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.007 ± 0.003</td>
<td>0.007 ± 0.003</td>
<td>0.011 ± 0.007</td>
<td>0.029 ± 0.009&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>0.02 ± 3*10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<td>0.13 ± 0.03&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.02 ± 0.01</td>
<td>0.05 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
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<td>0.030 ± 0.003</td>
<td>0.05 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.17 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.03 ± 0.01</td>
<td>0.11 ± 0.03&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>0.11 ± 0.01&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.008 ± 0.003</td>
<td>0.028 ± 0.006</td>
<td>0.07 ± 0.01</td>
<td>0.35 ± 0.20&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.02 ± 0.01</td>
<td>0.20 ± 0.03&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: 7OH-MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9), 7OH-MTX plasma AUCs are presented as min·µg/ml (means ± SD, n = 3-12, 0-120 min), and liver AUCs are presented as min·mg/g liver (means ± SD, n = 3-9, 0-120 min). *P < 0.05, **P<0.01, ***P<0.001 compared to wild-type mice at the same time-point or time period (Student’s t-test was used for statistical analysis). S.I. + S.I.C., small intestinal tissue + contents.
Supplementary Figure 1. Cumulative liver levels of MTX and 7OH-MTX after iv administration of MTX (50 mg/kg) to female wild-type, Abcc2;Abcg2<sup>−/−</sup>, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice.
Chapter 7

Impact of Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) on the oral pharmacokinetics of the anti-cancer drug methotrexate and its main metabolite 7-hydroxymethotrexate in mice

Maria L.H. Vlaming, Zeliha Pala, Anita van Esch, Els Wagenaar, Olaf van Tellingen and Alfred H. Schinkel

To be submitted
Impact of Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) on the oral pharmacokinetics of the anti-cancer drug methotrexate and its main metabolite 7-hydroxymethotrexate in mice

Maria L.H. Vlaming¹, Zeliha Pala², Anita van Esch¹, Els Wagenaar¹, Olaf van Tellingen³, Alfred H. Schinkel¹

Divisions of ¹Molecular Biology and ³Clinical Chemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands; ²Department of Pharmacology, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey.

The ATP-binding cassette (ABC) transporters ABCC2 (MRP2), ABCC3 (MRP3) and ABCG2 (BCRP) are involved in the efflux of potentially toxic compounds from the body. We have shown before that ABCC2, ABCC3 and ABCG2 together influence the pharmacokinetics of the anti-cancer and anti-rheumatic drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) after iv MTX administration. We now used female Abcc2;Abcc3;Abcg2²⁻ and corresponding single and double knockout mice to investigate the relative influences of these transporters on MTX and 7OH-MTX pharmacokinetics after oral MTX administration (50 mg/kg). The plasma areas under the curve (AUC_{plasma}) of Abcc2⁻² and Abcg2⁻² mice were 1.5- and 2.0-fold increased compared to wild-type, respectively. Abcc2;Abcg2²⁻ mice had a 3.3-fold increased AUC_{plasma}, suggesting additive effects of Abcc2 and Abcg2. The AUC_{plasma} in Abcc2;Abcc3;Abcg2²⁻ mice was not different from wild-type, suggesting that the Abcc3 protein is necessary for the increased plasma concentrations in the absence of Abcc2 and/or Abcg2. Furthermore, 2 hr after administration, MTX liver levels were increased in Abcg2-deficient strains and MTX kidney levels were 2.2-fold increased compared to wild-type in Abcc2;Abcg2⁻² mice. Absence of Abcc2 and/or Abcg2 furthermore led to significantly increased liver and kidney levels of 7OH-MTX 2 hr after MTX administration. Our results suggest that combined inhibition of ABCC2 and/or ABCG2 may increase the oral availability of MTX. Furthermore, SNPs or mutations in ABCC2 and/or ABCG2 that reduce expression or activity of these proteins may be risk factors for increased MTX-related toxicity in patients treated with oral MTX.
INTRODUCTION
The ATP-binding cassette (ABC) transporters ABCC2 (MRP2), ABCC3 (MRP3) and ABCG2 (BCRP) are membrane proteins that are involved in the efflux of potentially toxic endogenous and exogenous substrates from cells. They are expressed in epithelial cells of excretory organs, such as liver, kidney and small intestine, and can influence the pharmacokinetics of a wide range of (anti-cancer) drugs (1-5). Whereas ABCC2 and ABCG2 are present at the apical membranes of cells, transporting their substrates into bile, feces and urine, ABCC3 is located basolaterally, and it generally transports its substrates into the blood circulation (1,2, Supplementary Figure 1).

ABCC2, ABCC3 and ABCG2 have broad and substantially overlapping substrate specificities (1,2,4), but their relative impact on the pharmacokinetics of shared substrates is not clear yet. We have recently generated compound knockout mice for these transporters (6,7, Vlaming et al., submitted), which together with the previously generated single knockout mice for Abcc2 (8), Abcc3 (9) and Abcg2 (10) form a complete set of mouse models that can be used to elucidate the relative and possibly overlapping effects of these proteins on the pharmacokinetics of endogenous and exogenous substrates. Using this set of mouse strains we have recently shown that Abcc2, Abcc3 and Abcg2 have profound overlapping and additive effects on the iv pharmacokinetics of the widely used anti-cancer drug methotrexate (MTX) and its main toxic metabolite 7-hydroxymethotrexate (7OH-MTX) (6,7, Vlaming et al., submitted).

In cancer treatment, most drugs are given iv due to low and/or highly variable bioavailability, which can be caused by expression of ABC transporters in the intestine (5). However, because oral administration of drugs is more patient friendly as well as more cost effective, attempts are being made to improve the oral bioavailability of several drugs by co-administration of inhibitors of ABC transporters (5,11). Since ABCC2, ABCC3 and ABCG2 are all expressed in epithelial cells of the small intestine (1,3), they may, besides affecting the iv pharmacokinetics, also influence the oral pharmacokinetics of MTX (and 7OH-MTX). It was shown previously in Abcc2-deficient rats that after oral administration of MTX the plasma concentrations were significantly increased compared to wild-type rats (12,13). In mice, the effect of Abcc2 after oral MTX has not been investigated yet. Kitamura et al. (2008) (14) did show an effect of murine Abcc3 on plasma pharmacokinetics of \(^{3}\text{H}\)MTX after oral administration. Surprisingly, although the impact of Abcg2 on the oral pharmacokinetics of many drugs has been extensively studied (15,16), its effect on the disposition of MTX and 7OH-MTX after oral administration has not been investigated yet.

In the present study we have used the recently generated \textit{Abcc2;Abcc3;Abcg2}\(^{-}\) mice, as well as the corresponding single and double knockout mice, to investigate the relative effect of Abcc2, Abcc3 and Abcg2 on the
oral pharmacokinetics of MTX and its metabolite 7OH-MTX. We show here that
deletion of Abcc2 and/or Abcg2 increases the plasma concentrations of MTX after
oral administration, but that Abcc3 expression is necessary for this effect.
Furthermore, Abcc2, Abcc3 and Abcg2 clearly influence the tissue concentrations of
MTX and 7OH-MTX, also after oral MTX application.

MATERIALS AND METHODS

Animals. Mice were housed and handled according to institutional guidelines
complying with Dutch legislation. The generation of Abcc2+/− (8), Abcc3+/− (9), Abcg2−/−
(10), Abcc2;Abcc3−/− (6,17), Abcc2;Abcg2+/− (7), and Abcc3;Abcg2−/− and
Abcc2;Abcc3;Abcg2−/− mice (Vlaming et al., submitted) has been described. 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. MTX (Emthexate PF® 25 mg/ml) was from Pharmachemie (Haarlem,
The Netherlands), 7OH-MTX from Toronto Research Chemicals Inc. (North York,
ON, Canada) and methoxyflurane (Metofane) from Medical Developments Australia
Pty. Ltd. (Springvale, Victoria, Australia).

Plasma and tissue pharmacokinetic experiments. Before MTX administration,
mice were fasted for at least 4 hrs. MTX was administered to female wild-type,
Abcc2+/−, Abcc3+/−, Abcg2+/−, Abcc2;Abcc3−/−, Abcc2;Abcg2+/−, Abcc3;Abcg2−/− and
Abcc2;Abcc3;Abcg2−/− mice (n = 3-9) by dosing 10 µl/g body weight of 5 mg/ml
MTX in 5% glucose solution by gavage into the stomach. Blood samples (~60 µl)
taken from the tail vein in heparinised Microvette® CB 300 LH capillary tubes
(Sarstedt, Nümbrecht, Germany) at 7.5, 15, 30 and 60 min after administration. At
120 min, animals were killed by terminal bleeding through cardiac puncture under
methoxyflurane anesthesia and organs were removed. Small intestinal tissue and
contents (feces) were separated. Samples were stored at -20º C until analysis.

HPLC analysis of MTX and 7OH-MTX. Collected organs and feces were
homogenized in an ice-cold 4% BSA solution and plasma was diluted in human
plasma before HPLC analysis. MTX and 7OH-MTX concentrations in the different
matrices were determined as described (18).

Statistical analysis. Unless otherwise indicated, the two-sided unpaired Student's t-
test was used to assess the statistical significance of differences between wild-type
and knockout mice. When statistical differences between more than 2 groups were
analyzed, one-way ANOVA followed by Tukey’s multiple comparison test was
performed, as indicated. Results are presented as means ± standard deviations (SD). Differences were considered to be statistically significant when $P < 0.05$. Averaged concentrations for each time point were used to calculate the area under the plasma concentration versus time curve (AUC) from $t = 0$ to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (19). Because in many cases the plasma concentrations of MTX at 7.5 and 15 min could not be measured, for AUC calculations the concentrations at 30, 60 and 120 min after administration were used.

![Figure 1](image)

**Figure 1.** MTX plasma concentration versus time curve after oral administration of 50 mg/kg to female wild-type, *Abcc2*<sup>−/−</sup>, *Abcc3*<sup>−/−</sup>, *Abcg2*<sup>−/−</sup>, *Abcc2;Abcc3*<sup>−/−</sup>, *Abcc2;Abcg2*<sup>−/−</sup>, *Abcc3;Abcg2*<sup>−/−</sup> and *Abcc2;Abcc3;Abcg2*<sup>−/−</sup> mice (n = 3-9). Data are presented as means ± SD. Where no data points are given in the graph, the MTX plasma levels were below the lower limit of quantification (LLQ, 0.11 µg/ml). Additional data points (4 and 6 hrs) will be added to this graph in the near future.

**RESULTS**

**Impact of Abcc2, Abcc3 and Abcg2 on oral plasma pharmacokinetics of MTX.**

We have previously shown that the ABC transporters Abcc2, Abcc3 and Abcg2 have a profound impact on the plasma pharmacokinetics of MTX and its toxic
metabolite 7OH-MTX after iv bolus administration of MTX at 50 mg/kg (6,7,8, Vlaming et al., submitted). We now investigated the impact of these proteins on the pharmacokinetics of MTX and 7OH-MTX after oral administration of MTX at the same dose.

The plasma levels of MTX in all strains were relatively low (Figure 1): the AUCs\textsubscript{oral} of the different strains over 120 min were in the order of 10-fold lower than the previously determined AUCs\textsubscript{iv} (Table 1) (6,7, Vlaming et al., submitted). This suggests that at this (relatively high) dose of 50 mg/kg the oral bioavailability of MTX is quite low. However, as shown in Figure 1, both Abcc2 and especially Abcg2 did significantly affect the oral plasma pharmacokinetics of MTX. The plasma AUCs\textsubscript{oral} in \textit{Abcc2}\textsuperscript{-/-} and \textit{Abcg2}\textsuperscript{-/-} mice were 1.5- and 2.0-fold higher than in wild-type mice, respectively (Table 1). Furthermore, whereas in most other strains MTX was undetectable at 7.5 min after administration (Figure 1), in \textit{Abcg2}\textsuperscript{-/-} mice MTX was already clearly present in the blood. This suggests that already very early after administration Abcg2 reduces the oral uptake of MTX. The AUC\textsubscript{oral} of \textit{Abcc2;Abcg2}\textsuperscript{-/-} mice was even 3.3-fold increased compared to wild-type (Table 1), showing an additive effect of Abcg2 and Abcc2 on the oral plasma pharmacokinetics of MTX. Also in \textit{Abcc2;Abcg2}\textsuperscript{-/-} mice MTX could already be detected at 7.5 min (Figure 1), and tended to be somewhat higher than for \textit{Abcg2}\textsuperscript{-/-} mice (n = 3, P = 0.052), suggesting that Abcg2 is important early after administration, but Abcc2 may have a small additive effect as well.

It was shown recently by Kitamura et al. (2008) (14), that Abcc3 plays a role in [\textsuperscript{3}H]MTX plasma pharmacokinetics after oral administration of [\textsuperscript{3}H]MTX at 1 mg/kg (2.2 \textmu mol/kg). After administration of MTX at 50 mg/kg a similar tendency of reduced MTX plasma concentrations was found in \textit{Abcc3}\textsuperscript{-/-} mice (Figure 1). However, the AUC\textsubscript{oral} over 120 min of \textit{Abcc3}\textsuperscript{-/-} mice was not significantly different compared to wild-type mice (Table 1). The effect of Abcc3 on MTX pharmacokinetics became clearer when Abcc2 and/or Abcg2 were absent. Whereas in \textit{Abcc2}\textsuperscript{-/-}, \textit{Abcg2}\textsuperscript{-/-} and \textit{Abcc2;Abcg2}\textsuperscript{-/-} mice the AUCs\textsubscript{oral} were significantly increased compared to wild-type (see above), this was not the case in strains that additionally lacked Abcc3 (\textit{Abcc2;Abcc3}\textsuperscript{-/-}, \textit{Abcc3;Abcg2}\textsuperscript{-/-} and \textit{Abcc2;Abcc3;Abcg2}\textsuperscript{-/-} mice, Table 1). This shows that, like for iv administration (6,7, Vlaming et al., submitted, Table 1), Abcc3 expression (in intestine and/or liver) was necessary for the increased MTX plasma levels in \textit{Abcc2}\textsuperscript{-/-}, \textit{Abcg2}\textsuperscript{-/-} and \textit{Abcc2;Abcg2}\textsuperscript{-/-} mice after oral application of MTX.

7OH-MTX concentrations in plasma of mice after oral administration were, due to the small sample volumes, difficult to detect. Therefore, no conclusions on the effects of the different ABC transporters on 7OH-MTX plasma pharmacokinetics could be drawn.
Table 1. MTX plasma AUC_{0-120 min} of female mice after iv (Vlaming et al., submitted) and oral administration of MTX (50 mg/kg).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-type</th>
<th>Abcc2^{+/-}</th>
<th>Abcc3^{+/-}</th>
<th>Abcg2^{+/-}</th>
<th>Abcc2;Abcc3^{+/-}</th>
<th>Abcc2;Abcg2^{+/-}</th>
<th>Abcc3;Abcg2^{+/-}</th>
<th>Abcc2;Abcc3;Abcg2^{+/-}</th>
</tr>
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<tbody>
<tr>
<td>AUC_{iv} (min·µg/ml)</td>
<td>444±44</td>
<td>870±103***</td>
<td>368±34</td>
<td>692±56*</td>
<td>435±47</td>
<td>1446±229**</td>
<td>451±26</td>
<td>603±56*</td>
</tr>
<tr>
<td>AUC_{oral} (min·µg/ml)</td>
<td>23±4</td>
<td>35±6*</td>
<td>16±3</td>
<td>44±10*</td>
<td>25±6</td>
<td>74±11**</td>
<td>24±5</td>
<td>19±2</td>
</tr>
<tr>
<td>F (oral/iv) %</td>
<td>5±1.0</td>
<td>3.8±0.7</td>
<td>4.3±0.9</td>
<td>6.3±1.5</td>
<td>5.7±1.5</td>
<td>5.1±1.1</td>
<td>5.4±1.2</td>
<td>3.2±0.3*</td>
</tr>
</tbody>
</table>

Note: MTX plasma AUCs are presented as min·µg/ml and oral availabilities (F) are given as % of AUC_{iv} (means ± SD, n = 3-12, *P < 0.05, ** P<0.01, *** P<0.001 compared to wild-type mice over the same time period, Student’s t-test was used for statistical analysis). For oral administration additional data points (4 and 6 hrs) will be added in the near future.
Impact of Abcc2, Abcc3 and Abcg2 on tissue distribution of MTX and 7OH-MTX.

We further analyzed the levels of MTX and 7OH-MTX in tissues of the different strains at 120 min after oral administration of MTX (50 mg/kg). The liver levels of MTX were significantly increased compared to wild-type mice in most of the Abcg2-deficient strains (Figure 2A). In Abcg2+/− mice, liver levels were 1.7-fold increased and in Abcc2;Abcg2+/− mice they were 2.0-fold increased. This is very likely primarily a reflection of the increased plasma concentrations. In Abcc2+/− mice, however, despite increased plasma concentrations, the liver levels of MTX were not significantly different from wild-type and tended to be even somewhat lower (P = 0.07, Figure 2A), suggesting increased MTX liver elimination in Abcc2+/− mice, possibly by Abcc3, as we have previously shown after iv administration of MTX (6). In Abcc3;Abcg2+/− mice, although the plasma AUC was similar, liver levels of MTX were 1.6-fold increased compared to wild type, suggesting impaired liver elimination of MTX in Abcc3;Abcg2+/− mice (Figure 2A). A similar tendency was seen in Abcc2;Abcc3;Abcg2+/− mice (P = 0.07, Figure 2A).

The levels of MTX in small intestinal tissue and contents were relatively high in all strains (25-55% of the dose) and not significantly different from wild type in any of the strains, due to a high variation between individual mice (Figure 2B).

In most strains the MTX kidney levels were quite low and not significantly different from wild-type at 120 min after administration (Figure 2C). In Abcc2;Abcg2+/− mice, however, these were 2.2-fold increased compared to wild-type mice (Figure 2C). In Abcc3−/− mice, in line with a tendency of decreased plasma levels, MTX kidney levels were 2.0-fold lower than in wild-type (Figure 2C). Contrary to what we previously found after iv administration of MTX (6,7), the kidney levels did not simply follow the plasma levels: whereas in Abcc2+/− and Abcg2+/− mice the MTX plasma levels were respectively, 1.6- and 2.4-fold increased at 120 min, kidney levels were not different from wild type (Figure 2C).

7OH-MTX, the main, toxic metabolite of MTX, is mainly formed in the liver (20,21). The liver levels of 7OH-MTX at 120 min are shown in Figure 3A. This shows that in Abcc2−/− and Abcg2−/− mice the liver levels of 7OH-MTX were 2.3- and 2.1-fold increased compared to wild type. Furthermore, in Abcc2;Abcc3−/−, Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice the 7OH-MTX liver levels were 5.6-, 8.0- and 8.9-fold increased compared to wild type, respectively (Figure 3A). In Abcc3;Abcg2−/− mice on the other hand, 7OH-MTX liver levels were only mildly increased (Figure 3A). Apparently, absence of Abcc2 in particular, combined with either Abcc3 or Abcg2 deficiency leads to increased accumulation of 7OH-MTX in the liver.
Abcc2, Abcc3 and Abcg2 influence oral MTX pharmacokinetics

We have shown previously that Abcc2 is important for the biliary excretion of 7OH-MTX after iv administration of MTX, and that Abcg2 is also involved when Abcc2 is absent (6,7). After oral MTX administration this appears to be similar, as shown in Figure 3B. In nearly all Abcc2-deficient strains, despite increased liver levels (Figure 3A), levels of 7OH-MTX in the small intestinal tissue and contents were significantly decreased. In Abcc2;Abcc3−/− mice this was not the case, likely due to biliary excretion of 7OH-MTX by Abcg2 (7). Furthermore, in Abcg2−/− mice the levels of 7OH-MTX in small intestine were significantly higher than in wild-type mice, suggesting that Abcc2 expression, in combination with increased 7OH-MTX liver levels can cause increased biliary excretion of 7OH-MTX in these mice.

Figure 2. MTX tissue distribution 2 hr after oral administration of 50 mg/kg to female wild-type, Abcc2+/−, Abcc3+/−, Abcg2+/−, Abcc2;Abcc3+/−, Abcc2;Abcg2+/−, Abcc3;Abcg2+/− and Abcc2;Abcc3;Abcg2−/− mice (n = 5-9). A, MTX liver levels (as % of the dose) in the different strains. B, MTX small intestinal (SI) tissue and contents levels (as % of the dose) in the different strains (n = 5-9). C, MTX kidney levels (as % of the dose) in the different strains (n = 5-9). Data are presented as means ± SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to wild type, Student’s t-test).
Kidney levels of 7OH-MTX were very low (< 0.025% of the dose) in all strains and undetectable in all Abcc2 proficient mice (Figure 3C). In all Abcc2-deficient strains 7OH-MTX was detected, suggesting that absence of Abcc2 leads to increased exposure of the kidney to 7OH-MTX, possibly due to increased plasma levels of 7OH-MTX (not determined, see above). Furthermore, combined absence of Abcc2 and Abcg2 caused even further increased 7OH-MTX kidney levels (n = 4, P = 7*10⁻³) (Figure 3C).

**Figure 3.** 7OH-MTX tissue distribution 2 hr after oral administration of MTX (50 mg/kg) to female wild-type, Abcc2⁻/⁻, Abcc3⁻/⁻, Abcg2⁻/⁻, Abcc2;Abcc3⁻/⁻, Abcc2;Abcg2⁻/⁻, Abcc3;Abcg2⁻/⁻ and Abcc2;Abcc3;Abcg2⁻/⁻ mice (n = 5-9). A, 7OH-MTX liver levels (as % of the dose) in the different strains (n = 5-9). B, 7OH-MTX small intestinal (SI) tissue and contents levels (as % of the dose) in the different strains (n = 5-9). C, 7OH-MTX kidney levels (as % of the dose) in the different strains (n = 5-9) (nd, not detectable, detection limit 24 nM). Data are presented as means ± SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to wild type, Student’s t-test).
DISCUSSION
In this study we used the recently generated Abcc2;Abcc3;Abcg2-/- mice (Vlaming et al., submitted) to determine the relative effects of Abcc2, Abcc3 and Abcg2 on the oral pharmacokinetics of MTX and its main toxic metabolite 7OH-MTX. We show that Abcc2 and Abcg2 can both influence MTX plasma pharmacokinetics after oral application and that they have additive effects. Furthermore, we show that Abcc3 expression (either in the intestine or the liver) is necessary for the effects of Abcc2 and Abcg2 deletion on oral MTX plasma levels. Combined deletion of Abcc2 and Abcg2 furthermore led to increased concentrations of MTX and its toxic metabolite 7OH-MTX in liver and kidney. For MTX, this was not seen when Abcc3 was additionally deleted, illustrating the overlapping functions between the different transporters in determining the oral pharmacokinetics of MTX.

When MTX is used for cancer treatment, high doses (>15 mg/m²) are usually given (22). Because the oral bioavailability of MTX, especially at high doses, is unpredictable and relatively poor, it is given iv (22). However, oral application of MTX would be much more favourable, as this is more patient friendly and cost-effective in general (5,11). We show here that by combined deletion of Abcg2 and Abcc2 the plasma AUC after oral administration of MTX (50 mg/kg) can be increased by more than three-fold. However, our results also show that for this effect Abcc3 protein needs to be present, suggesting that Abcc3 expression is relatively important for the oral availability of MTX, as was previously shown by Kitamura et al. (2008) (14). This suggests that specific inhibition of ABCG2 and ABCC2 (without ABCC3 inhibition), may be an effective strategy to improve the oral pharmacokinetics of MTX. Inhibitors for ABCG2 have been developed and used in clinical trials to improve the oral bioavailability of drugs (5,23,24). However, no effective, specific inhibitors for ABCC2 are known at the moment, so before practical application of this option, more research will have to be performed.

Kitamura et al. (2008) have shown that single deletion of Abcc3 in mice led to a 3-fold decreased AUC over 4 hrs after oral MTX administration at a dose of 2.2 µmol/kg (1 mg/kg) (14). In the here described experiment, with a much higher dose, we did not find a significant effect on the AUC of MTX after deletion of Abcc3 alone, although there was a similar tendency of a reduced AUC (Figure 1). Possibly, the effect of Abcc3 is dependent on the dose, and other transporters are more important at higher MTX doses. We have shown previously that the effect of Abcc2 on the plasma elimination of [³H]MTX after iv administration was dose dependent as well (8). Possibly, at a lower dose of 1 mg/kg orally, the effect of Abcg2 and Abcc2 is less pronounced, making the effect of Abcc3 more clearly visible.

Although we show here that Abcc2 and Abcg2 limit the systemic exposure of MTX after oral application, it is not clear if this is mainly caused by limiting the uptake from the intestine or by their impact on the biliary excretion of MTX (or both). The AUCoral was 3.3-fold increased in Abcc2;Abcg2-/- mice compared to wild
type. However, we previously found that the AUC\textsubscript{iv}, with the same dose was 3.3-fold increased compared to wild type as well (most likely due to dramatically decreased biliary excretion of MTX) (7). It may therefore be that the increased plasma MTX levels in Abcc2;Abcg2\textsuperscript{-/-} mice after oral administration are primarily caused by decreased biliary excretion of MTX. On the other hand, especially in Abcg2-deficient mice, already 7.5 min after oral administration, increased plasma levels were found (Figure 1). When Abcc2 was additionally deleted, this difference became even bigger, although this may also be caused by increased Abcc3 expression in the liver of these mice (7). Furthermore, whereas after iv administration the effect of Abcc2 on the plasma elimination was more prominent, after oral administration this was more affected by absence of Abcg2. It will be interesting to study the effects of these ABC transporters after oral MTX application in more detail in the future.

Although we did find significant effects of Abcc2 and Abcg2 on the pharmacokinetics of MTX after oral application, still in all strains analyzed we found between 25-55% of the dose present in the small intestine after 2 hrs and this was not significantly different between the different strains, possibly due to high variation between individual mice. We have shown before after iv administration that deletion of Abcc2 and/or Abcg2 led to significantly reduced levels of MTX in the intestine at 1 hr after administration, due to dramatically decreased biliary excretion (7). The fact that we don’t see this effect after oral administration suggests that biliary excretion in this set up has little influence on the intestinal MTX levels and that the effect of Abcc2 and Abcg2 on decreasing the intestinal uptake after oral administration is limited compared to the total amount of MTX in the small intestine, for example due to saturation of specific uptake transporters for MTX in the intestine.

Oral MTX administration is often used in the treatment of rheumatoid arthritis as well as psoriasis. We show here that Abcg2 and Abcc2 can influence the pharmacokinetics of MTX after oral administration. Interestingly, in a patient study with oral MTX, correlations between 3 SNPs in ABCC2 and MTX toxicity have been found recently (25). Furthermore, in a study with psoriasis patients 2 ABCG2 SNPs positively correlated with efficacy of MTX therapy (26). Our results show that deletion of Abcc2 or Abcg2, in combination with expression of Abcc3, increases MTX levels in the circulation, but also in liver and kidney. Furthermore, absence of Abcc2 and/or Abcg2 leads to increased exposure of liver and kidney to the toxic metabolite 7OH-MTX. The effects found in patients are therefore likely caused by direct effects of reduced ABCC2 and/or ABCG2 activity. When patients are treated with oral MTX it may therefore be advisable to check for mutations in ABCC2, ABCG2 and ABCC3 in order to predict possible adverse effects.
REFERENCES


Supplementary Figure 1. Localization of ABCC2, ABCC3 and ABCG2 in intestine (A), liver (B) and kidney (C).
Chapter 8

Impact of ABC transporters on the disposition of the dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and its mutagenic metabolites in vivo


To be submitted
Impact of ABC transporters on the disposition of the dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and its mutagenic metabolites in vivo

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The multidrug transporters MDR1, BCRP, MRP2 and MRP3 are involved in the elimination of potentially toxic compounds from the body and they influence the pharmacokinetics of many drugs and carcinogens. The food derived carcinogen [¹⁴C]PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is transported by MDR1, BCRP and MRP2. To investigate the combined 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 recently generated Bcrp1;Mrp2;Mrp3−/− mice, were used to determine the combined effects of multidrug transporters on the pharmacokinetics of PhIP and its metabolites. 30 min after oral or iv administration of PhIP (1 mg/kg), the PhIP levels in small intestine were 4-6-fold reduced compared to wild-type in Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice. Fecal excretion of PhIP over 24 h was 8-20-fold lower in knockouts. Biliary PhIP excretion over 1 hr was even 41-fold reduced in Bcrp1;Mdr1a/b;Mrp2−/− mice. Biliary and small intestinal levels of PhIP-metabolites were also reduced in Bcrp1;Mrp2-deficient mice. Furthermore, in both knockout strains kidney levels and urinary excretion of some carcinogenic PhIP-metabolites were significantly increased, suggesting that reduced biliary excretion of PhIP and its metabolites leads to increased urinary excretion of its metabolites. In Bcrp1;Mrp2;Mrp3−/−, but not Bcrp1;Mdr1a/b;Mrp2−/− mice, the potentially carcinogenic metabolites N-OH-PhIP and PhIP-N-sulphate accumulated in liver, suggesting that Mrp3 is involved in the elimination of these compounds from liver. Our results show that Bcrp1, Mdr1a/b, Mrp2 and Mrp3 together are important for the biliary and fecal elimination of PhIP and its carcinogenic metabolites and may thus affect PhIP-induced carcinogenesis.
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 all expressed in excretory organs such as liver, kidney and small intestine, where they are 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. Furthermore, these proteins are found in human tumors, where they can confer multidrug resistance to anti-cancer agents [1-3]. 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 [1-3].

Besides endogenous compounds and a wide range of (anti-cancer) 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 [3-9]. 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 results in colon, prostate and mammary gland tumors [10-12]. PhIP is rapidly taken up after ingestion and is heavily metabolized, leading to both detoxification products as well as activated, potentially carcinogenic metabolites (Supplementary Figure 1) [10-13]. Furthermore, part of the carcinogenic potential of PhIP is possibly explained by the fact that PhIP possesses oestrogenic activity and may therefore be involved in the stimulation of cell proliferation [12].

Using Bcrp1 or Mrp2 deficient mice and rats it was previously shown that Bcrp1 and Mrp2 significantly influence the pharmacokinetics of $[^{14}\text{C}]\text{PhIP}$ in vivo [4-6;9]. However, as in most studies radioactivity was measured, no differentiation between parent PhIP and its mutagenic metabolites could be made. In Mrp2-deficient TR^-^ rats it was shown that rat Mrp2 influences the biliary excretion of PhIP, 4’-OH-PhIP and some glucuronide conjugates [8]. Furthermore, in Bcrp1^-^- mice it was shown that Bcrp1 restricts the brain and/or testis penetration of PhIP, N-OH-PhIP and 4’-OH-PhIP [7]. Murine Mdr1a could transport $[^{14}\text{C}]\text{PhIP}$ in vitro, but in vivo no effect of murine Mdr1a/b on $[^{14}\text{C}]\text{PhIP}$ plasma elimination was found [5]. This is possibly due to the overlapping activity of Bcrp1 and/or Mrp2 in Mdr1a/b^-^- mice. The effect of other ABC transporters on the pharmacokinetics of PhIP and its metabolites has not been investigated yet.

To investigate the possibly overlapping or complementary roles of Bcrp1, Mdr1a/b, Mrp2 and Mrp3 in vivo, we have recently generated a set of compound...
knockout mice deficient in up to three of these ABC transporters [6;14-18, Vlaming et al., submitted]. These compound ABC transporters proved to be very useful tools for pharmacokinetic studies. To also determine the combined effects of the apically located transporters Mdr1a/b, Bcrp1 and Mrp2 on physiology and pharmacology, we have generated \( Bcrp1;Mdr1a/b;Mrp2^{+/+} \) mice, as described here. Furthermore, we have recently developed a fast and sensitive LC-MS/MS assay for the carcinogen PhIP and most of its main metabolites (Teunissen et al., in preparation). We used the \( Bcrp1;Mdr1a/b;Mrp2^{+/+} \) mice to investigate the combined effect of the ABC transporters Bcrp1, Mdr1a/b and Mrp2 on the elimination of PhIP and its metabolites \textit{in vivo}. Furthermore, as the basolateral transporter Mrp3 is often upregulated when Mrp2 is absent and this may function as a compensatory mechanism for decreased apical efflux from the liver [6;19;20], we additionally investigated the pharmacokinetics of PhIP and its metabolites in \( Bcrp1;Mrp2;Mrp3^{+/+} \) mice.

**MATERIALS AND METHODS**

**Animals.** Mice were housed and handled according to institutional guidelines complying with Dutch legislation. \( Bcrp1;Mdr1a/b;Mrp2^{+/+} \) mice were generated by cross-breeding \( Bcrp1;Mdr1a/b^{+/+} [14] \) and \( Bcrp1;Mrp2^{+/+} [18] \) mice. The generation of \( Bcrp1;Mrp2;Mrp3^{+/+} \) mice was described before (Vlaming et al., submitted). 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 \textit{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 (N-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 albumine, dimethylsulfoxide (DMSO) and formic acid were from Merck (Darmstadt, Germany). Methanol originated 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 [6].

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 intravenous 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 (feces) and tissue were separated.

Biliary excretion of PhIP and its metabolites. Gall bladder cannulations in male 
mice were performed as described [5]. After cannulation, PhIP was administered iv 
at 1 mg/kg as described above. Bile was collected in 15 min fractions for 60 min. 
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 were snap-frozen. 
Tissue and feces were weighed before snap-freezing. The complete organ or the total 
volume of sampled feces was homogenized in a 4% (m/v) BSA solution using a 
Polytron blender.

Sample processing. A 100 µl aliquot of plasma, bile, tissue or feces homogenate 
was processed immediately after thawing by addition of 300 µl internal standard 
(PhIP-D3) solution in acetonitrile. The mixture was vortexed for 10 sec followed by 
centrifugation for 10 min at 10,500 rpm. 100 µl of the clear supernatant was 1:1 
diluted 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 3.5 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 110Å column (150 x 2.0 mm I.D., 4 µm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.2 ml/min using a block gradient (0-4 min: 30% B, 4-8min: 80% B, 8-13min: 30% B) in a total run time of 13 min.

The LC eluate was let into an API 3000 triple quadrupole MS equipped with an electrospray ion source operating in the positive ion mode. For quantification multiple reaction monitoring chromatograms were acquired and processed (integrated). Calibration curves of analyte/internal standard peak area ratio versus respectively PhIP and N-OH-PhIP concentrations were constructed and a weighted $1/x^2$ (the reciprocal of the squared concentration) linear regression was applied to the data. Quantification of metabolites was performed based on the calibration curve of PhIP. PhIP-glucuronides and OH-PhIP-glucuronide metabolites (Supplementary Figure 1) could not be unequivocally distinguished, as reference standards were not in all cases available. Therefore, in this manuscript, “PhIP-glucuronides” stands for the sum of PhIP-N2-glucuronide and PhIP-N3-glucuronide. Likewise, “OH-PhIP-glucuronides” in this manuscript is defined as the sum of all OH-PhIP-glucuronides detected (Supplementary Figure 1).

**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. When more than two groups were compared, one-way ANOVA followed by Tukey’s multiple comparison test was used, as indicated in the text and/or figure legends. 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+/− and Bcrp1;Mrp2;Mrp3+/− mice.**

$Bcrp1;Mdr1a/b;Mrp2+/−$ and $Bcrp1;Mrp2;Mrp3+/−$ mice were viable, fertile and 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 (4.8 ± 0.2% of body weight in wild-type vs. 7.0 ± 0.5% of body weight in $Bcrp1;Mdr1a/b;Mrp2+/−$ mice, n = 5, $P = 2.8*10^{-5}$). As previously described, the liver weight of male $Bcrp1;Mrp2;Mrp3+/−$ was ~70% higher than that of wild-type mice (Vlaming et al.,
submitted). A (milder) increase in liver weight was previously shown for other Mrp2-deficient mouse strains [6;15;18;21]. Despite the markedly increased liver size, detailed microscopic analysis of liver sections did not reveal obvious pathological changes.

**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 [18]. 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 shown in other Mrp2-deficient mouse strains [6;18]. Other measured parameters did not consistently show differences with wild-type mice (not shown).

**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 [10-12], we developed a quantitative LC-MS/MS assay to detect most of the currently known metabolites in mouse matrices (Methods and Teunissen et al., in preparation). We used this method to determine the plasma and tissue concentrations of PhIP and its carcinogenic metabolites 30 min after oral or iv administration of PhIP (1 mg/kg) to wild-type, Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice. Overviews of all metabolites detected in the tissues of the three strains are presented in Supplementary Tables 1 (iv) and 2 (oral). Most metabolites, for example all glucuronide conjugates are considered detoxifying metabolites and do not have carcinogenic potential (Supplementary Figure 1) [10-12]. Also the formation of 4'-OH-PhIP and subsequent formation of PhIP-4'-sulphate (Supplementary Figure 1) is considered a detoxification pathway [10-12]. On the other hand, besides PhIP itself, N-OH-PhIP and PhIP-N-sulphate are considered (pre)carcinogenic [10-12]. 5-OH-PhIP and PhIP-5-sulphate are considered surrogate markers for carcinogenesis, as they are formed after formation of DNA-adducts (Supplementary Figure 1) [22].

PhIP and its main metabolite N-OH-PhIP were detected in plasma of all mouse strains 30 min after iv or oral administration of PhIP. However, no significant differences between the strains were found (Supplementary Tables 1 and 2). After iv administration PhIP-N-sulphate was additionally detected in plasma of both knockout strains but not in wild-type plasma, suggesting that Bcrp1 and/or Mrp2
influence the plasma elimination of this carcinogenic metabolite (Supplementary Table 1). After oral administration on the other hand, the detoxification product 4’-OH-PhIP was found in plasma of all strains, but no differences between strains were found (Supplementary Table 2).

**Figure 1.** Levels of PhIP and its primary carcinogenic metabolites in liver of male wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> and Bcrp;Mrp2;Mrp3<sup>-/-</sup> mice 30 min after iv (n = 4-11) or oral (n = 5) administration of PhIP (1 mg/kg). A, parent PhIP in liver of the different strains after iv or oral administration of PhIP. B, Levels of N-OH-PhIP and PhIP-N-sulphate in liver of the different strains after iv administration of PhIP. nd, not detected, detection limits for N-OH-PhIP and PhIP-N-sulphate were 0.02% and 0.01% of the dose, respectively. C, N-OH-PhIP levels in liver of the different strains after oral administration of PhIP. Data are means ± SD (n = 4-11, **, P < 0.01).
Figure 2. PhIP and N-OH-PhIP levels in the intestinal tract (tissue and contents) of male wild-type, Bcrp1;Mdr1a/b;Mrp2\textsuperscript{-/-} and Bcrp;Mrp2;Mrp3\textsuperscript{-/-} mice 30 min after iv (n = 4-11) or oral (n = 5) administration of PhIP (1 mg/kg). A, parent PhIP in the intestinal tract of the different strains after oral administration of PhIP. B, parent PhIP in the intestinal tract of the different strains after iv PhIP administration. C, N-OH-PhIP in the small intestine of the different strains after iv (left panel) or oral (right panel) PhIP administration. N-OH-PhIP detection limit was 0.02% of the dose. D, PhIP-N-sulphate in the small intestine of the different strains after iv (left panel) or oral (right panel) PhIP administration. PhIP-N-sulphate detection limit was 0.02% of the dose. Data are means ± SD (n = 4-11, *, P < 0.05, **, P < 0.01, ***, P < 0.001), nd, not detected, S.I., small intestine.
30 min after oral administration, in livers of all strains 2.7-4.5% of the given dose was found as unchanged PhIP (Figure 1A). Similar amounts of PhIP (1.8-2.5%) were found in liver after iv administration (Figure 1A). There were no significant differences between the three strains. Interestingly, in Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice both after iv and oral administration the precarcinogen N-OH-PhIP accumulated in the liver (Figures 1B and C). This was not the case in the wild-type and Bcrp1;Mdr1a/b;Mrp2<sup>+/+</sup> mice, suggesting that Mrp3 prevents accumulation of this metabolite in the liver. After iv administration, the same was found for the potential carcinogen PhIP-N-sulphate (Figure 1B), which was not detected in the liver (and plasma, see above) after oral administration.

Figure 2 shows the levels of the parent compound PhIP in the intestinal tract after oral (Figure 2A) or iv (Figure 2B) application of PhIP (1 mg/kg). For both administration routes the small intestinal levels (tissue and contents) of PhIP in Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> and Bcrp1;Mrp2;Mrp3<sup>-/-</sup> 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. As there was no difference between the Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> and Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice, Mdr1a/b and Mrp3 do not seem to be involved here. Also in the cecum and colon (the latter for iv application only) of the compound knockout strains PhIP concentrations were reduced (Figure 2A and B). Small intestinal levels of the main PhIP-metabolites N-OH-PhIP and PhIP-N-sulphate were significantly reduced compared to wild-type in Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> and Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice, both after oral and iv 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 potentially carcinogenic metabolites. After iv administration, N-OH-PhIP levels in small intestine of Bcrp1;Mdr1a/b;Mrp2<sup>-/-</sup> mice were not detectable and thus even lower than in the Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice. This is most likely a reflection of the far higher N-OH-PhIP liver levels in the Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice (Figures 1B and C). However, we cannot exclude reduced reabsorption of N-OH-PhIP due to the intestinal Mrp3 deficiency in the Bcrp1;Mrp2;Mrp3<sup>-/-</sup> mice. Another possible explanation is an additional effect of Mdr1a/b on the intestinal elimination of N-OH-PhIP. Besides PhIP and N-OH-PhIP, various other PhIP metabolites (glucuronide and sulphate conjugates) were detected in wild-type small intestine, both after oral and iv administration (Supplementary Tables 1 and 2). 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 intestinal elimination of these metabolites as well.

PhIP levels in kidney 30 min after oral or iv administration were not significantly different between the analyzed strains (Figure 3A). However, in the kidneys of the compound knockout mice a significant accumulation of various PhIP-metabolites, including the (pre)carcinogens N-OH-PhIP and PhIP-N-sulphate, was
seen (Figures 3B and C). Apparently, combined absence of Bcrp1 and Mrp2 leads to the accumulation of PhIP metabolites in the kidney.

**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 oral and iv administration were significantly reduced in mice deficient in Bcrp1 and Mrp2, we hypothesized that this was mainly caused by reduced biliary excretion of these compounds. To investigate this, we performed gallbladder cannulations in male wild-type and $Bcrp1;Mdr1a/b;Mrp2^{-/-}$ mice and analyzed the biliary excretion of PhIP and its metabolites in the first 60 min after iv administration of PhIP (1 mg/kg). The cumulative biliary excretion of PhIP over 60 min was 41-fold lower in compound knockout compared to wild-type mice (Figure 4A), showing that Bcrp1, Mrp2 and (possibly, to a minor extent) Mdr1a/b are the most important 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 N-OH-PhIP was clearly detected in plasma and many tissues after oral and iv administration (Figures 1-3), in bile of the mice this could not be detected. This suggests that, in contrast to PhIP, for this metabolite, which is mainly formed in liver but also extrahepatically [13], 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. This shows that concentrations of the carcinogen PhIP-N-sulphate as well as OH-PhIP-glucuronide were significantly increased in plasma of $Bcrp1;Mdr1a/b;Mrp2^{-/-}$ mice 60 min after iv administration of PhIP during gallbladder cannulation, thereby likely reflecting the reduced biliary excretion of these compounds. N-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 abrogated biliary excretion (Figure 4B), 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, n = 5, P = 5.2*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 knockouts, but there was no significant difference ($4.4 \pm 2.1\%$ of the dose in knockout versus $2.9 \pm 1.0 \%$ of the dose in wild-type, n = 5, P = 0.18). Other PhIP metabolites were not detected in livers of the mice after the gall bladder cannulation experiment.
Figure 3. PhIP and metabolites in kidney of male wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp;Mrp2;Mrp3<sup>−/−</sup> mice 30 min after iv (n = 4-11) or oral (n = 5) administration of PhIP (1 mg/kg). A, parent PhIP in kidney of the different strains after iv or oral administration of PhIP. B, PhIP metabolites in kidney of the different strains after iv administration of PhIP. nd, not detected (below 0.02% of the dose). PhIP-4’-sulphate and PhIP-glucuronide were below the LLQ (lower level of quantification), which was 0.005% of the dose for both metabolites. C, PhIP metabolites in kidney of the different strains after oral administration of PhIP. Data are means ± SD (n = 4-11, *, P < 0.05, **, P < 0.01, ***, P < 0.001), nd, not detected. PhIP-X-sulphate is a sulphate metabolite of PhIP, other than PhIP-4-sulphate or PhIP-N-sulphate, and likely PhIP-5-sulphate (see Supplementary Figure 1).
Figure 4. Biliary excretion of PhIP and its metabolites after iv administration of PhIP (1 mg/kg) to male gall bladder cannulated wild-type and Bcrp1;Mdr1a/b;Mrp2\textsuperscript{-/-} mice. Bile was collected in 15 min fractions over 1h. A, cumulative biliary excretion of parent PhIP in the first 60 min after iv administration. B, cumulative biliary excretion of PhIP metabolites in the first 60 min after iv administration of PhIP. PhIP-N-sulphate and PhIP-4'\textsuperscript{-}-sulphate were not detected (nd) in Bcrp1;Mdr1a/b;Mrp2\textsuperscript{-/-} bile. LLQ for both compounds in bile was 1*10\textsuperscript{-4} % of the dose. N-OH-PhIP was not detected in bile of the mice. PhIP-glucuronide in Bcrp1;Mdr1a/b;Mrp2\textsuperscript{-/-} bile: 1.6*10\textsuperscript{-4} ± 1.4*10\textsuperscript{-4} % of the dose. C, plasma concentrations of PhIP and its metabolites 60 min after iv administration of PhIP. Data are means ± SD (n = 5, *, P < 0.05, **, P < 0.01, ***, P < 0.001).
Effect of ABC transporters on urinary and fecal excretion of PhIP and its metabolites.

As Bcrp1 and/or 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 iv administration of PhIP at 1 mg/kg to wild-type, Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice. As shown in Figure 5A, as already observed 30 min after iv (and oral) administration (Supplementary Tables 1 and 2) for the plasma concentrations of PhIP, the urinary excretion of PhIP was not altered in the compound knockout mice. This suggests that these three transporters are not involved in urinary PhIP excretion (Figure 5A). However, consistent 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). We found no significant difference in fecal PhIP excretion between Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice (P > 005, ANOVA), suggesting that the differences with wild-type were mainly caused by absence of Bcrp1 and Mrp2. However, there was a tendency of a somewhat lower fecal excretion of PhIP in the Bcrp1;Mdr1a/b;Mrp2−/− mice versus Bcrp1;Mrp2;Mrp3−/− mice, suggesting that additional deletion of Mdr1a/b may have had a minor additional effect on fecal PhIP excretion.

Although urinary excretion of PhIP was not altered in the compound knockout mice, the urinary excretion of some metabolites clearly was (Figure 5B). The urinary excretion of the (pre)carcinogenic metabolites N-OH-PhIP, PhIP-N-sulphate and PhIP-X-sulphate (which is likely PhIP-5-sulphate, a marker for DNA adduct formation [22]), as well as the detoxification product OH-PhIP-glucuronide, was substantially (3-10-fold) increased in both knockout strains (Figure 5B). This was in line with increased kidney accumulation of these compounds (Figure 3B and C). There were no significant differences in urinary excretion of PhIP and its metabolites between Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice. Unfortunately, PhIP metabolite concentrations in the feces of the mice were below the limit of quantification.
Figure 5. Fecal and urinary excretion of PhIP and its metabolites in the first 24 hr after iv administration of PhIP (1 mg/kg) to male wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp;Mrp2;Mrp3<sup>−/−</sup> 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 of the mice were below the limit of detection and could therefore not be quantified.

DISCUSSION

The apical multidrug transporters Bcrp1, Mdr1a/b and Mrp2 have very broad and substantially overlapping substrate specificities [1-3]. To study these, we developed Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice, which are viable and fertile, and show no obvious phenotypic aberrations other than an increased liver weight, and increased plasma bilirubin levels, as was previously shown for Bcrp1;Mrp2<sup>−/−</sup> mice [18]. We therefore consider this new model perfectly amenable for studies on the combined in vivo effects of Bcrp1, Mdr1a/b and Mrp2. These mice should be very useful tools for detailed studies on the relative effects of Bcrp1, Mdr1a/b and Mrp2 on the pharmacokinetics of shared substrates in vivo, especially in combination with the previously generated single and double knockout mice [6;14;16-18]. In this first study with Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> mice, combined with the recently generated Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice (Vlaming et al., submitted), we show that Bcrp1 and Mrp2 are the main transporters for the biliary and fecal excretion of PhIP, as well as for the biliary excretion of its carcinogenic metabolite PhIP-N-sulphate and some other PhIP metabolites. Furthermore, combined absence of Bcrp1 and Mrp2 leads to increased exposure of liver and kidney to potentially carcinogenic PhIP metabolites and increased urinary excretion of some of these compounds. We additionally found that Mrp3 appears important for the sinusoidal elimination of N-OH-PhIP from the liver. Our results have potential implications for the roles of ABC transporters in PhIP-mediated carcinogenesis.

The carcinogen PhIP is heavily metabolized (Supplementary Figure 1, [10-13]), and it can be converted to various detoxification products such as 4'-OH-PhIP, PhIP-4-sulphate, PhIP-glucuronides and OH-PhIP-glucuronides. However, besides
its potential intrinsic carcinogenicity, presumably due to its oestrogenic activity [12], it can also be metabolized to potentially mutagenic metabolites, such as N-OH-PhIP and PhIP-N-sulphate. These metabolites can form DNA adducts, eventually leading to the metabolite PhIP-5-sulphate (Supplementary Figure 1). As these potentially carcinogenic compounds may be excreted from the body by ABC transporters, we were interested in the effect of the transporters on the fate of the metabolites, in particular the ones with carcinogenic potential. With LC-MS/MS we managed to detect (besides parent PhIP) a large set of PhIP-metabolites, amongst which the potential carcinogens N-OH-PhIP and PhIP-N-sulphate. We furthermore detected, besides PhIP-4-sulphate and PhIP-N-sulphate, a third PhIP-sulphate, which we termed PhIP-X-sulphate as we were not able to unequivocally determine its structure. However, we consider it quite likely that this compound is PhIP-5-sulphate, which is formed in vivo after formation of DNA adducts, and therefore may be used as a surrogate marker for mutagenesis [22]. Whether this compound is indeed PhIP-5-sulphate will be further investigated in the near future. Interestingly, we found that in the Bcrp1 and Mrp2 deficient mice, the urinary excretion of PhIP-X-sulphate was significantly increased (Figure 5B). Whether this means that in these mice more DNA adducts were formed remains to be investigated, as this increase may also be caused by decreased fecal excretion in the knockout strains. This will be investigated in the near future.

We found that Bcrp1 and Mrp2 together are the main transporters for biliary, intestinal and fecal excretion of the parent compound PhIP. In mice deficient for both transporters we found 41-fold decreased biliary excretion and up to 20-fold decreased fecal excretion of PhIP. As the urinary excretion of parent PhIP was not altered in the knockout mice (and therefore the total PhIP excretion over 24 hrs was reduced), this suggests that when Bcrp1 and/or Mrp2 are absent, PhIP accumulates in the body and may be converted to (carcinogenic) metabolites. We further show that Bcrp1 and/or Mrp2 are responsible for the biliary and intestinal excretion of a range of PhIP metabolites, amongst which PhIP-N-sulphate and PhIP-X-sulphate (see above). This suggests that Bcrp1 and Mrp2 are very important for the rapid elimination of PhIP and its carcinogenic metabolites from the body and may therefore be involved in protecting the body from carcinogenesis by these compounds.

Besides the detoxification products PhIP-4-sulphate and OH-PhIP-glucuronides, we detected the mutagenic metabolite PhIP-N-sulphate in bile of wild-type mice but not in bile of Bcrp1;Mdr1a/b;Mrp2−/− mice (Figure 4B). This metabolite was also detected in the small intestine of wild-type but not in Bcrp1;Mrp2-deficient mice, both after oral and iv administration, and it accumulated in plasma of these mice after iv administration (Supplementary Tables 1 and 2). This suggests that Bcrp1 and/or Mrp2 are involved in the biliary excretion of PhIP-N-sulphate. Furthermore, we found that in the Bcrp1;Mrp2;Mrp3−/− mice PhIP-N-
sulphate accumulated in the liver after iv administration, which was not seen in wild-type and Bcrp1;Mdr1a/b;Mrp2−/− mice (Figure 1B). This suggests that Mrp3 is involved in sinusoidal liver elimination of PhIP-N-sulphate. We additionally found substantially increased kidney levels and urinary excretion of PhIP-N-sulphate (Figures 3B, C and 5B), suggesting that reduced biliary excretion due to absence of Bcrp1 and/or Mrp2 leads to increased urinary excretion of this compound. As the overall urinary excretion was not different between Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice (Figure 5B) we conclude that although Mrp3 seems important for short term sinusoidal liver elimination of PhIP-N-sulphate, it does not affect its urinary excretion over 24 hrs. Possibly other basolateral ABC transporters such as Mrp4 can also gradually transport this compound into the circulation (albeit more slowly than Mrp3), leading to increased urinary excretion.

Like Dietrich et al. (2001) concluded for rat Mrp2, we show that murine Bcrp1, Mrp2 and Mdr1a/b are not essential for the urinary excretion of PhIP and its metabolites [8]. In mice lacking Bcrp1 and Mrp2 (and in addition Mdr1a/b), the urinary excretion of PhIP was comparable to wild-type mice and for many metabolites this was even increased in the knockouts perhaps due to increased plasma concentrations of these compounds. Dietrich et al. (2001) previously postulated that the increased urinary excretion of PhIP-glucuronides and sulphates in Mrp2-deficient rats may be a consequence of Mrp3 activity in the basolateral membrane of the liver [8]. Although Mrp3 appears to affect the liver elimination of some PhIP metabolites (see above), we did not find differences in long-term cumulative urinary excretion of metabolites between Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/−, suggesting that for excretion of PhIP metabolites within 24 hrs Mrp3 is not essential.

This study shows that murine Bcrp1, Mrp2 and/or Mdr1a/b are very important for the biliary excretion of PhIP and the primary carcinogenic PhIP metabolites, and therefore may protect the body from carcinogenesis by reducing systemic exposure to these carcinogens. The expression of ABC transporters in stem cells [2] may further be highly important for reducing the risk of carcinogenesis in various tissues in the body. On the other hand, as PhIP was shown to cause intestinal tumors in mice and rats [10-12], one could speculate that these ABC transporters may even increase the carcinogenic potential of PhIP in vivo by exposing the intestine to high concentrations of PhIP and its mutagenic metabolites. Thus, our results suggest that ABC transporters could also influence the location of tumor formation. As many polymorphisms and mutations in BCRP, MRP2, MRP3 and P-gp are known, in many cases leading to reduced function [23;24], and as PhIP is a very abundant carcinogen, it will be of great interest to investigate the effect of these ABC transporters on the carcinogenic potential of PhIP. Our newly generated Bcrp1;Mdr1a/b;Mrp2−/− and Bcrp1;Mrp2;Mrp3−/− mice should therefore not only be useful for pharmacokinetic studies of drugs, but also become valuable tools for
studies on the effect of the different ABC transporters on limiting (or possibly increasing) xenobiotic-induced carcinogenesis in vivo.

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REFERENCES


PhIP pharmacokinetics in compound transporter knockout mice

Supplementary Table 1. Levels of PhIP and its metabolites in tissues and plasma of male wild-type, Bcrp1;Mdr1a/b;Mrp2Δ/Δ and Bcrp1;Mrp2;Mrp3Δ/Δ mice 30 min after iv administration of PhIP (1 mg/kg).

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<th>PhIP</th>
<th>N-OH-PhIP</th>
<th>PhIP-N-sulphate</th>
<th>PhIP-X-sulphate</th>
<th>PhIP-4'-sulphate</th>
<th>PhIP-glucuronide</th>
<th>OH-PhIP-glucuronide</th>
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<td>Plasma (ng/ml)</td>
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<td>2.8 ± 1.7</td>
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<tr>
<td>Liver (% of dose)</td>
<td>Wild-type</td>
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<tr>
<td></td>
<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
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<tr>
<td>S.I. (% of dose)</td>
<td>Wild-type</td>
<td>5.4 ± 2.0</td>
<td>0.18 ± 0.11</td>
<td>0.17 ± 0.06</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.10</td>
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<tr>
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<td>Bcrp1;Mdr1a/b;Mrp2Δ/Δ</td>
<td>0.9 ± 0.3</td>
<td>nd (&lt; 0.02)</td>
<td>nd (&lt; 0.02)</td>
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</tr>
<tr>
<td></td>
<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
<td>1.0 ± 0.4</td>
<td>0.06 ± 0.01</td>
<td>nd (&lt; 0.02)</td>
<td>nd</td>
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<tr>
<td>Kidney (% of dose)</td>
<td>Wild-type</td>
<td>2.0 ± 0.4</td>
<td>0.01 ± 0.01</td>
<td>0.009 ± 0.003</td>
<td>nd (&lt; 0.002)</td>
<td>0.003 ± 0.003</td>
<td>0.001 ± 0.001</td>
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<tr>
<td></td>
<td>Bcrp1;Mdr1a/b;Mrp2Δ/Δ</td>
<td>1.5 ± 0.6</td>
<td>0.06 ± 0.01***</td>
<td>0.029 ± 0.004***</td>
<td>0.005 ± 0.002</td>
<td>0.007 ± 0.002*</td>
<td>0.007 ± 0.002***</td>
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<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
<td>1.9 ± 0.6</td>
<td>0.02 ± 0.01</td>
<td>0.073 ± 0.056***</td>
<td>0.011 ± 0.005</td>
<td>0.03 ± 0.03***</td>
<td>0.007 ± 0.001***</td>
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<td>Brain (% of dose)</td>
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<td>Bcrp1;Mdr1a/b;Mrp2Δ/Δ</td>
<td>0.13 ± 0.03***</td>
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<tr>
<td></td>
<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
<td>0.06 ± 0.03**</td>
<td>0.03 ± 0.01</td>
<td>nd</td>
<td>nd</td>
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<td>Testis (% of dose)</td>
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<td>Bcrp1;Mdr1a/b;Mrp2Δ/Δ</td>
<td>0.09 ± 0.05***</td>
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<tr>
<td></td>
<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
<td>0.05 ± 0.01*</td>
<td>nd</td>
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<tr>
<td>Spleen (% of dose)</td>
<td>Wild-type</td>
<td>0.06 ± 0.02</td>
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<td></td>
<td>Bcrp1;Mdr1a/b;Mrp2Δ/Δ</td>
<td>0.06 ± 0.03</td>
<td>nd</td>
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<td>Bcrp1;Mrp2;Mrp3Δ/Δ</td>
<td>0.05 ± 0.03</td>
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Means ± SD (n = 5), *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis), nd, not detected, §, below LLQ. S.I., small intestine (tissue + contents). PhIP-X-Sulphate is a sulphate metabolite of PhIP, other than PhIP-N-Sulphate or PhIP-4'-Sulphate. It is likely to be PhIP-5-Sulphate. 4'-OH-PhIP was not detected in plasma or tissues of the mice.
**Supplementary Table 2.** Levels of PhIP and its metabolites in tissues and plasma of male wild-type, Bcrp1;Mdr1a/b;Mrp2<sup>−/−</sup> and Bcrp1;Mrp2;Mrp3<sup>−/−</sup> mice 30 min after oral administration of PhIP (1 mg/kg).

<table>
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<tr>
<th>Matrix</th>
<th>Strain</th>
<th>Analyte</th>
<th>Plasma (ng/ml)</th>
<th>Liver (% of dose)</th>
<th>S.I. (% of dose)</th>
<th>Kidney (% of dose)</th>
<th>Brain (% of dose)</th>
<th>Testis (% of dose)</th>
<th>Spleen (% of dose)</th>
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<tr>
<td></td>
<td></td>
<td>PhIP</td>
<td>N-OH-PhIP</td>
<td>PhIP-N-sulphate</td>
<td>PhIP-X-sulphate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PhIP-4′-sulphate</td>
<td>4′-OH-PhIP</td>
<td>PhIP-glucuronides</td>
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<tr>
<td>Plasma</td>
<td>Wild-type</td>
<td>34.7 ± 23.8</td>
<td>12.2 ± 6.6</td>
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<td>9.2 ± 1.9</td>
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<tr>
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<td>Bcrp1;Mdr1a/b;Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>32.4 ± 17.4</td>
<td>5.9 ± 2.2</td>
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<td>nd</td>
<td>9.3 ± 2.1</td>
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<tr>
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<td>Bcrp1;Mrp2;Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>40.6 ± 22.6</td>
<td>5.2 ± 2.0</td>
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<td>9.0 ± 1.4</td>
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<td>Liver</td>
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<td>0.03 ± 0.01</td>
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<tr>
<td></td>
<td>Bcrp1;Mdr1a/b;Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.7 ± 1.3</td>
<td>0.03 ± 0.01</td>
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<td></td>
<td>Bcrp1;Mrp2;Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4.5 ± 2.7</td>
<td>0.22 ± 0.12**</td>
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<td>S.I.</td>
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<td>8.2 ± 2.0</td>
<td>0.46 ± 0.18</td>
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<tr>
<td></td>
<td>Bcrp1;Mdr1a/b;Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.7 ± 0.7***</td>
<td>0.032 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
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<tr>
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<td>Bcrp1;Mrp2;Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.1 ± 0.7***</td>
<td>0.099 ± 0.049**</td>
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<td>Kidney</td>
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<td>0.004 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.006 ± 0.005&lt;sup&gt;f&lt;/sup&gt;</td>
<td>nd</td>
<td>0.003 ± 0.003&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Bcrp1;Mdr1a/b;Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.6 ± 0.8</td>
<td>0.035 ± 0.027*</td>
<td>0.02 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
<td>0.010 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.026 ± 0.014**</td>
<td>0.04 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.017 ± 0.007**</td>
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<td>0.10 ± 0.05</td>
<td>0.03 ± 0.02</td>
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<td>Bcrp1;Mrp2;Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>0.03 ± 0.01</td>
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<td>0.037 ± 0.016</td>
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<td>0.035 ± 0.014&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>Wild-type</td>
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<tr>
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<td>Bcrp1;Mdr1a/b;Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.07 ± 0.04</td>
<td>nd</td>
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<td>Bcrp1;Mrp2;Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.07 ± 0.04</td>
<td>nd</td>
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Means ± SD (n = 5), *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis), nd, not detected, §, below LLQ, S.I., small intestine (tissue + contents), <sup>a</sup>PhIP-X-Sulphate is a sulphate metabolite of PhIP, other than PhIP-N-Sulphate or PhIP-4′-Sulphate. It is likely to be PhIP-5-Sulphate. 4′-OH-PhIP was not detected in plasma or tissues of the mice.
Supplementary Figure 1. Overview of PhIP metabolism in vivo. The main bioactivation pathway of PhIP resulting in mutagenic compounds is highlighted in grey. In brief, PhIP can be converted to the potentially carcinogenic PhIP-N-sulphate directly by SULT1A1*2 or after conversion to N-OH-PhIP by CYP1A1 or CYP1A2, followed by SULT1A1*2 mediated conversion to PhIP-N-sulphate. PhIP-N-sulphate can subsequently, by heterolytic cleavage, form a radical cation, which forms DNA adducts. PhIP-DNA adducts subsequently spontaneously degrade to 5-OH-PhIP, which can then be converted to PhIP-5-sulphate by SULT1A1*2. All other metabolic pathways are considered “detoxification pathways”, as these do not lead to carcinogenic metabolites [10;12;13;22;25;26].
Chapter 9

Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice

Maria L.H. Vlaming, Jurjen S. Lagas and Alfred H. Schinkel

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Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice

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* These authors contributed equally to this work.

The multidrug transporter ABCG2 (BCRP/MXR/ABCP) can actively extrude a broad range of endogenous and exogenous substrates across biological membranes. ABCG2 limits oral availability and mediates hepatobiliary and renal excretion of its substrates, and thus influences the pharmacokinetics of many drugs. Recent work, relying mainly on the use of Abcg2−/− mice, has revealed important contributions of ABCG2 to the blood-brain, blood-testis and blood-fetal barriers. Together, these functions indicate a primary biological role of ABCG2 in protecting the organism from a range of xenobiotics. In addition, several other physiological functions of ABCG2 have been observed, including extrusion of porphyrins and/or porphyrin conjugates from hematopoietic cells, liver and harderian gland, as well as secretion of vitamin B2 (riboflavin) and possibly other vitamins (biotin, vitamin K) into breast milk. However, the physiological significance of these processes has been difficult to establish, indicating that there is still a lot to learn about this intriguing protein.

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   2.3. Functional role of ABCG2/Abcg2 at the blood-testis barrier.
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4. Concluding remarks
1. Introduction
The ATP-binding cassette (ABC) transporter ABCG2 (BCRP) is located at the apical membrane of hepatocytes and epithelial cells of intestine and kidney where it pumps a wide variety of endogenous and exogenous compounds out of the cell. Due to its activity in these excretory organs, ABCG2 can have a profound effect on the pharmacokinetics of many drugs and their metabolites, by enhancing their excretion and limiting their uptake from the intestinal lumen after oral administration. In addition, ABCG2 can confer multidrug resistance to tumor cells (1-5). ABCG2 might further be important for the pharmacological sanctuary properties of several tissues, due to its expression in the blood-brain, blood-placental and blood-testis barriers, where it could limit the penetration of its substrates into these critical tissues (1-7). ABCG2 is also found in stem cell-enriched cell populations and progenitor cells of a number of tissues, where it might potentially protect these important cells from insult by a variety of toxic or carcinogenic xenobiotics (2). A schematic overview of ABCG2 tissue distribution is shown in Figure 1. In general, ABCG2 appears to have a xenobiotic protective function, reducing levels of noxious compounds in individual cells, in certain organs, and in the body as a whole. Nevertheless, recently also high expression of ABCG2 in the lactating mammary gland was demonstrated, in the luminal membrane (Figure 1). Here ABCG2 concentrates its (often toxic) substrates into the milk (8), leading to the question whether ABCG2 may have additional, yet unrecognized physiological functions. The physiological and pharmacological roles of ABCG2/Abcg2 elucidated thus far have been extensively described in earlier reviews (1-5). Characterization of Abcg2 knockout (Abcg2<sup>−/−</sup>) mice has greatly contributed to the knowledge of in vivo ABCG2 functions (9;10). This review will focus on some of the more recently gained insights into the physiological and pharmacological functions of Abcg2, obtained using these valuable mouse models.

2. Recently established pharmacological functions of ABCG2/Abcg2
2.1 Functional role of Abcg2 at the blood-brain barrier; value of compound transporter knockout mice.

P-glycoprotein (P-gp, MDR1/ABCB1) was the first ABC multidrug transporter that was found to be highly expressed at the blood-brain barrier (BBB), where it efficiently restricts the entry of a wide variety of compounds into the brain (Figure 1) (11;12). More recently, ABCG2 was also identified at the BBB of humans, pigs and rodents, where it colocalizes with P-gp at the luminal side of endothelial cells of brain capillaries (13-17). Mdr1a knockout mice have proven to be a valuable tool to unravel the dominant function of P-gp at the BBB (11). In contrast, for ABCG2 it was not as straightforward to unequivocally establish a functional role at the BBB, despite the availability of mouse models deficient in either P-gp or Abcg2 (9-11). This was illustrated by a number of studies, with
sometimes contradicting outcomes. Shortly after the discovery of ABCG2 expression at the BBB, Cisternino et al. performed in situ brain perfusion experiments with the prototypic ABCG2 substrates prazosin and mitoxantrone (18). To exclude involvement of P-gp, they used Mdr1a−/− mutant CF-1 mice and compared these to wild-type mice. Brain uptake of both compounds was not affected by the absence of P-gp, indicating that P-gp does not restrict the uptake of these compounds into the brain. Furthermore, elacridar, a dual P-gp and ABCG2 inhibitor, significantly increased the brain uptake of the studied compounds in wild-type and Mdr1a−/− CF-1 mice. Interestingly, elacridar increased the brain uptake of prazosin and mitoxantrone more in Mdr1a−/− mutant mice than in wild-type mice, which appeared to correlate with the observation that Mdr1a−/− mutant CF-1 mice had a threefold higher Abcg2 mRNA expression in their brain capillaries. From these somewhat indirect experiments it was inferred that Abcg2, and not P-gp, restricts the uptake of prazosin and mitoxantrone into the brain.

Contradicting results, however, were reported by Lee et al. (16), who performed similar in situ brain perfusion experiments with the ABCG2 model substrates mitoxantrone and dehydroepiandrosterone sulfate (DHEAS), using knockout mice in an FVB strain background (16). Mdr1a/b−/− mice displayed moderately but significantly higher brain uptake compared to wild-type mice for mitoxantrone and DHEAS, suggesting that brain penetration of both compounds is limited by P-gp. However, when Abcg2−/− mice were compared to wild-type mice, no difference in brain uptake of mitoxantrone and DHEAS was found. Furthermore, co-perfusion of the brains with elacridar resulted in a comparable and significantly increased brain uptake of mitoxantrone and DHEAS in all three genotypes. Based on these results, the authors concluded that one or more elacridar sensitive transporters are involved in the efflux of mitoxantrone and DHEAS at the BBB, but that no evidence was found for a functional role of Abcg2 at the BBB. Expression of ABC transporters in the BBB of mice (for instance, Abcc2) can sometimes vary markedly between different mouse strains (19). If this is also true for Abcg2, this could perhaps explain the contradicting outcomes of the described experiments. Whether indeed other ABC transporters than P-gp or Abcg2 are involved in limiting transport of mitoxantrone across the BBB may possibly be investigated in the future using (compound) knockout mice for different ABC transporters.

In another study addressing the functional role of Abcg2 at the BBB, Breedveld et al. used a different approach (20). Imatinib, a tyrosine kinase inhibitor anticancer drug and in vitro substrate of both P-gp and Abcg2 (21), was intravenously applied to wild-type, Mdr1a/1b−/− and Abcg2−/− mice and brain concentrations were determined 2 hours after administration. Consistent with a previous study (22), Mdr1a/1b−/− mice displayed 3.6-fold higher brain penetration compared to wild-type mice. Interestingly, for Abcg2−/− mice, which have functional P-gp at the BBB, a 2.5-fold higher imatinib brain uptake was found. Furthermore,
pharmacological inhibition of both P-gp and Abcg2 with the dual inhibitor elacridar resulted in a 4.2-fold increased brain penetration in wild-type mice. In addition, co-administration of imatinib with the Abcg2 inhibitor pantoprazole slightly increased the brain uptake in wild-type and Mdr1a/1b<sup>−/−</sup> mice, but did not affect brain penetration in Abcg2<sup>−/−</sup> mice. Collectively, these observations suggest that, in addition to P-gp, Abcg2 restricts the brain penetration of imatinib.

**Figure 1.** Schematic overview of ABCG2 expression throughout the body. Bold dark grey lines indicate the location of ABCG2. At all expression sites where small arrows indicate the direction of ABCG2-mediated transport, *in vivo* functionality of ABCG2/Abcg2 has been demonstrated. Wide arrows indicate net body excretion of ABCG2 substrates. For testis the situation in humans is depicted, where ABCG2 is found in both myoid cells of the seminiferous tubules and in blood capillary endothelial cells. However, only the Abcg2 barrier function of testis endothelial cells as demonstrated in mice is indicated with arrows. Expression of ABCG2 in endothelial cells of blood capillaries and veins, or in "side population" cells throughout many tissues in the body is not indicated. This figure was modified from reference (2).
The roles of P-gp and Abcg2 in limiting the brain uptake of imatinib were also studied in a recent series of in situ brain perfusion experiments (23). In this study, brain uptake of imatinib was not different between wild-type and Abcg2<sup>2−/−</sup> mice when it was perfused at non-saturating concentrations. However, when imatinib was perfused at higher (>1 μM) perfusate concentrations, increased brain penetration was found in both wild-type and Abcg2<sup>2−/−</sup> mice, which suggests that saturation of one or more efflux processes occurred. The fact that this saturation phenomenon did not occur in Mdr1a/1b<sup>−/−</sup> mice, pointed towards saturation of P-gp. Interestingly, at imatinib concentrations exceeding ~20 μM, brain uptake was substantially more increased in Abcg2<sup>2−/−</sup> than in wild-type mice. This suggests that, when P-gp is saturated in wild-type mice, the contribution of Abcg2 in reducing imatinib passage across the BBB becomes detectable. Saturation of P-gp might also explain the higher imatinib brain uptake that Breedveld et al. found in Abcg2<sup>2−/−</sup> mice (20). Initial high plasma concentration in Abcg2<sup>2−/−</sup> mice of ~15 mg/L (~25 μM), as applied in this study, indeed exceeded the saturation cut-off of ~20 μM that was found by Bihorel et al. (23).

The above summarized studies, employing mice that are deficient in either P-gp or Abcg2, suggest that P-gp is a dominant transporter at the BBB, which can even restrict brain penetration of comparatively poor P-gp substrates. Therefore, if one uses Abcg2<sup>2−/−</sup> mice, in which P-gp is still present, it can be difficult to unequivocally demonstrate a functional role for Abcg2 at the BBB for shared P-gp/ABCG2 substrates. This was recently further supported by Enokizono et al., who found that the brain uptake clearance of the dietary carcogen and shared ABCG2 and P-gp substrate PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in brain perfusion experiments was not different between wild-type and Abcg2<sup>2−/−</sup> mice (7), whereas the ABCG2 (but not P-gp) substrates dantrolene and daidzein did show increased brain uptake clearance. Interestingly, however, when continuous infusion into the systemic circulation was applied, Abcg2<sup>2−/−</sup> mice did show increased brain-to-plasma ratios even for the common ABCG2 and P-gp substrates PhIP and prazosin (7), although the differences were modest (1.5- to 2.5-fold). This discrepancy might be explained by the fact that in the brain perfusion experiments the compounds were perfused for only 1 minute, whereas in the continuous infusion experiments the substrates were applied for 150 minutes before brain concentrations were measured. Consequently, prolonged exposure of the brain to relatively high plasma concentrations of these compounds may result in saturation of P-gp at the BBB and therefore a marked detectable effect on brain penetration of PhIP and prazosin in Abcg2<sup>2−/−</sup> mice. Using these same continuous infusion conditions, the drug dantrolene and the phytoestrogens daidzein, genistein and coumestrol (Figure 3), which are ABCG2 but not P-gp substrates, did show very marked (up to 10-fold) increased brain penetration in Abcg2<sup>2−/−</sup> mice (7;24).
Considering the complications described above, an elegant model to study the overlapping functions of P-gp and ABCG2 at the BBB for shared substrates is the compound P-gp and Abcg2 knockout mouse (Mdr1a/1b/Abcg2+/−) (25). Recently, de Vries et al. used this model to study the brain uptake of the anticancer drug topotecan, which is a good substrate of ABCG2 and a weaker P-gp substrate (26). Compared to wild-type mice, brain-to-plasma AUC ratios were 2.0-fold higher in Mdr1a/1b+/− mice, not significantly different in Abcg2+/− mice, and 3.2-fold higher in Mdr1a/1b/Abcg2+/− mice. This study shows that, although topotecan appears to be a better ABCG2 substrate in vitro and in the mouse intestine (27), P-gp still dominates at the BBB. Nonetheless, Abcg2 also has a functional role and restricts the brain uptake of topotecan when P-gp is absent. Similarly, Oostendorp et al. recently used the Mdr1a/1b/Abcg2+/− mouse model to establish the restricting roles of P-gp and Abcg2 for the brain penetration of imatinib (28). The brain-to-plasma ratios of imatinib were highly (>10-fold) increased in Mdr1a/1b/Abcg2+/− mice, whereas for Mdr1a/1b+/− mice a modest (~2-fold) increase and for Abcg2+/− mice no increase in brain-to-plasma ratios was found. These latter two studies illustrate the value of compound transporter knockout mouse models to study the overlapping roles of Abcg2 and P-gp in the brain. To further illustrate the usefulness of compound knockout mice, recently also Abcg2/Abcc4−/− mice have been generated and these could be similarly used to elucidate the overlapping functions of both transporters in the disposition of the purine analogue drug PMEA (9-(2-(phosphonomethoxy)ethyl)-adenine) to various tissues (29). However, in this case brain penetration was not affected by the combination knockout in comparison to the single knockouts.

Taken together, Abcg2 appears to play a significant role at the BBB, where it can effectively restrict the brain penetration of potentially harmful compounds, especially those that are good ABCG2 substrates and not P-gp substrates. For shared substrates, Abcg2 and P-gp can (partially) take over each other's function at the BBB, although P-gp often dominates, whereas absence of both transporters can result in a drastically increased brain penetration.

2.2 Functional role of ABCG2/Abcg2 in the placenta and fetal membranes.
One of the main expression sites for ABCG2/Abcg2 is the placenta, where it is, like P-gp and MRP2 (ABCC2), expressed at the apical membrane of syncytiotrophoblasts (Figure 1) (6). The syncytiotrophoblast cellular layer forms the main barrier between the maternal and fetal blood circulations, and virtually all exchange of nutrients and waste products between mother and fetus occurs across these cells. Here ABCG2, which faces the maternal blood, could be involved in transport of its substrates from fetal to maternal blood, likely protecting the fetus from toxic compounds in the maternal circulation (6). Interestingly, the expression of Abcg2 changes with gestational age. In mice and rats Abcg2 mRNA expression
peaks at mid-gestation (day 12-15) and decreases thereafter (6). However, from gestation day 9.5 on, protein levels of murine Abcg2 did not change significantly over time, although there was a tendency of a decrease after mid-gestation (30). Whether this is similar in humans is not completely clear, as contradictory results have been reported (6). The physiological function of the variable RNA expression of Abcg2 during gestation is not known (6). If the protein levels remain similar, it may simply reflect a lower turnover rate of placental Abcg2 protein at later gestational stages.

The first evidence that ABCG2 in the placenta limited fetal penetration of drugs was found in Mdr1a/b−/− mice that were treated with the Abcg2 and P-gp inhibitor elacridar. The fetal penetration of the anti-cancer drug topotecan was 2-fold increased in Mdr1a/b−/− mice that were treated with elacridar compared to vehicle-treated mice (27). Using Abcg2−/− and/or Mdr1a/b/Abcg2−/− mice, the effect of Abcg2 on limiting fetal exposure has recently been more directly demonstrated for the drugs topotecan (9), nitrofurantoin (31) and glyburide (32), as well as for the phytoestrogen genistein (24). In these studies absence of Abcg2 led to 2- to 5-fold increased fetal-to-maternal plasma ratios of the tested compounds, indicating the relatively important role for Abcg2 in protecting the fetus from potential toxins. The functional role of placental ABCG2 in humans has been investigated in vitro by several studies on the anti-diabetic drug glyburide, which is used in gestational diabetes. Using right-side out vesicles that were derived from human placental brush border membranes a 1.4-fold increase of glyburide uptake was shown after adding the ABCG2 inhibitor novobiocin (33). Furthermore, an ex vivo placental perfusion study revealed that co-administration of the ABCG2-inhibitor nicardipine led to 2-fold increased fetal-to-maternal concentration ratios of glyburide as measured in the respective perfusates (34). These data suggest a barrier function of ABCG2 in the human placenta as well. However, it cannot be excluded that the used inhibitors may have inhibitory or stimulating effects on other efflux and/or uptake transporters. This complicates the interpretation of these results. Furthermore, ex-vivo studies may not completely reflect what happens in vivo. To address such issues, the complementary use of Abcg2−/− mice for studies on Abcg2-mediated transplacental transport remains invaluable.

Recently, expression of Abcg2 and other ABC transporters in murine fetal membranes, in particular the visceral yolk sac, has been detected. mRNA expression was higher than in the placental membrane and, like for placental expression of murine Abcg2, peaked at mid-gestation (35). Protein levels of Abcg2 in the yolk sac could only be measured after gestational day 12 and did not change up to 18 days after gestation (30). Similar high expression of ABCG2 protein and RNA was recently demonstrated in human fetal membranes (36). For the mouse, immunolocalization of Abcg2 in the yolk sac indicated that it faces the maternal side, leading to the speculation that it might have a protective function for the fetus,
In analogy to the placental Abcg2 (30). Interestingly, the multidrug transporter Mrp2/Abcc2 colocalizes with Abcg2 at the apical (maternal) side of the visceral yolk sac membrane (35). However, whether Abcg2/ABCG2 expression at this site has any physiological or pharmacological significance remains to be investigated in functional studies.

Altogether, there is strong evidence that placental ABCG2/Abcg2 can have a marked protective effect for the fetus against numerous xenobiotics, in analogy to the function of placental P-gp. A possible protective contribution of ABCG2/Abcg2 in fetal membranes remains to be established.

2.3 Functional role of ABCG2/Abcg2 at the blood-testis barrier.

The testis is another sanctuary site where ABCG2 is highly expressed (Figure 1). Immunohistochemical analysis of human testis sections revealed that ABCG2, like P-gp, is expressed in the luminal membranes of endothelial cells of blood capillaries (Figure 2). In analogy to endothelial cells at the blood-brain barrier, testis endothelial cells form tight junctions with each other, providing a continuous cellular layer that could contribute to the blood-testis barrier (37). However, testis capillary endothelial cells (at least in the rat) are not as tightly linked as those in the brain, which has raised doubts about an optimal barrier function (38). Obviously, when the physical barrier is not completely closed, the impact of drug transporters may be limited. Furthermore, ABCG2 and P-gp are strongly expressed in the apical membranes of myoid cells surrounding the seminiferous tubules (Figure 2) (39). The transport direction of both transporters in myoid and endothelial cells is outward from the seminiferous tubuli, which suggests that ABCG2 and P-gp protect the developing germ cells by restricting testicular penetration of potentially harmful substrates (Figure 2). It should be noted, though, that myoid cells too are not consistently joined by tight junctions (at least, in the rat), which might hamper their function as an effective barrier (38). In addition, the ABC multidrug transporter MRP1/ABCC1 is expressed in the basal membrane of the Sertoli cells of the seminiferous tubules (Figure 2), where it can protect the seminiferous tubules from drug induced damage, as was shown for the anticancer agent etoposide (40). Sertoli cells are consistently joined by tight junctions. It is noteworthy, however, that the progenitor spermatogenic cells (i.e., germ line cells) are located just outside the Sertoli cells (41) and are therefore not protected by MRP1 (Figure 2). Theoretically there is therefore a need for an additional protective barrier between blood and these critical germline cells, which could be situated in the myoid and/or capillary endothelial cells expressing ABCG2 and P-gp.
Figure 2. Schematic representation of a cross-section through a seminiferous tubule and a blood capillary in the testis. The strategic localization of drug efflux transporters in the blood-testis barrier is depicted. In humans ABCG2 and P-gp (ABCB1) are found in the apical (luminal) membrane of blood capillary endothelial cells and in the apical membranes of myoid cells surrounding the seminiferous tubules. In addition, MRP1 (ABCC1) is expressed in the basal membrane of Sertoli cells. Note that the myoid cells are not consistently connected by tight junctions, whereas the endothelial cells of the testis blood capillaries are connected by tight junctions, but not as consistently as in brain capillaries. Sertoli cells are consistently joined by tight junctions.

Also in the mouse testis, Abcg2 expression was recently demonstrated in the luminal membranes of endothelial cells of blood capillaries (24). In contrast to the human testis, however, Abcg2 expression in the apical membranes of myoid cells was not observed (24). In the same study, expression of ABCG2/Abcg2 in the murine and human epididymis was investigated. The epididymis is part of the male reproductive system that is attached to the testis and used for maturation and storage of spermatozoa after production in the testis (42). In the body region of the mouse epididymis (i.e., somewhat distal from the testis), Abcg2 was observed in the endothelial cells of the blood capillaries, whereas in the epididymis head region (i.e., most proximal to the testis) Abcg2 was expressed in both the luminal and abluminal membranes of the ducts that contain spermatozoa. Expression of Abcg2 in the blood capillaries of the epididymis head and in the abluminal membranes of the ducts in the epididymis body might restrict penetration of potentially harmful Abcg2 substrates and thus protect the spermatozoa in the ducts. However, expression of Abcg2 in the luminal membrane of the ducts in the epididymis head seems paradoxical, because the transport direction is towards the spermatozoa in the lumen. A physiological function cannot be excluded, but male fertility (and hence sperm function) in Abcg2<sup>−/−</sup> mice does not seem to be compromised (9;10). In the human epididymis, mRNA expression of BCRP was demonstrated, but no immunohistochemical analysis was reported yet (24).
Using Abcg2−/− mice, it was recently demonstrated that penetration of a number of exogenous compounds (all Abcg2 substrates) into the testis was indeed efficiently restricted by Abcg2 (7;24). When these compounds were systemically infused under steady state conditions, the testis-to-plasma ratios were markedly (up to 15-fold) higher in Abcg2−/− mice compared to WT mice. This was shown for PhIP, dantrolene, prazosin and for the phytoestrogens daidzein, genistein and coumestrol (7;24). In addition, genistein penetration into the epididymis was also higher in Abcg2−/− mice (2.5-fold). Together, these data indicate a prominent role of ABCG2 in reducing testis and epididymis exposure to numerous xenobiotics.

As pointed out by Enokizono et al. (7), the pronounced effect of Abcg2 knockout on testis penetration of a range of substrates provides strong evidence for a substantial role of testis capillary endothelial cells in the blood-testis barrier, at least in the mouse, as these cells are the only location where testicular Abcg2 could be detected. The physiological function of Abcg2 at the blood-testis and blood-epididymis-borders might be particularly important with respect to (phyto)estrogens, many of which have been identified as Abcg2 substrates (Figure 3) (24;43;44), because these compounds are known to influence reproductive functions, e.g. reducing testicular weight and sperm count (45;46).
In conclusion, at all three of the above discussed blood-tissue barriers, ABCG2 appears to play an important role in restricting the uptake of (potentially harmful) substrates. It is interesting to note that for shared substrates of ABCG2 and P-gp, the restricting role of P-gp at the BBB appears more pronounced than that of ABCG2. Even for very good ABCG2 substrates in vitro and in the intestine, such as topotecan (27), penetration into brain is predominantly limited by P-gp and an effect of ABCG2 could only be demonstrated under P-gp deficient conditions (9;26). Nonetheless, deficiency of both P-gp and Abcg2 results in higher brain penetration than observed for P-gp deficient animals (9;26). This illustrates that Abcg2 can be an important backup mechanism for shared substrates, which can partially take over the function of P-gp at the sanctuary site barriers.

3. Physiological functions of ABCG2/Abcg2

3.1.1 Abcg2 pumps vitamins into milk.
In addition to its expression in excretory organs such as liver, kidney and small intestine, and its function in protecting the brain, testis and fetus from xenobiotics, it was recently found that Abcg2 is also expressed in the lactating mammary gland of mice, sheep, cows and humans (Figure 1) (8;47). Abcg2 localizes here to the apical side of alveolar epithelial cells, the main site of milk production. Mammary Abcg2 expression is strongly induced during pregnancy and lactation, and only falls back upon weaning of the pups (8). As a consequence, (potentially toxic) Abcg2 substrates in the maternal circulation are actively pumped into the milk, leading to the exposure of suckling infants and dairy consumers to a wide range of xenobiotics. This intriguing finding therefore has major pharmacological and toxicological consequences for breast-feeding infants and dairy consumers (and producers), which are discussed in more detail in a recent review (2). The fact that Abcg2/ABCG2 is expressed in the lactating mammary gland led to the hypothesis that besides a xenobiotic efflux transporter, Abcg2 may also be involved in the transfer of nutrients from mother to pup via the milk (2).

Recently, it was indeed found that an important nutrient, namely riboflavin (vitamin B\textsubscript{2}) (Figure 4A), is a transported substrate for mouse and human Abcg2/ABCG2 in vitro (48). Riboflavin is necessary to form the enzyme cofactors FMN and FAD (Figure 4A), which function as electron carriers in many essential redox reactions in the body (49). Using Abcg2\textsuperscript{2/-} mice it was shown that Abcg2 on the one hand limits the tissue distribution and plasma concentrations of dietary riboflavin, but on the other hand also actively pumps riboflavin into the milk. In fact, steady state milk secretion of riboflavin in Abcg2\textsuperscript{2/-} mice was 60-fold reduced compared to wild-type mice. Additionally, the (already low) concentrations of FMN were ~6-fold reduced in milk of Abcg2\textsuperscript{2/-} mice, suggesting that Abcg2 also pumps FMN into the milk. Nevertheless, pups from Abcg2\textsuperscript{2/-} mothers do not display any phenotype associated with riboflavin-deficiency (e.g. growth retardation, skin
lesions or neurodegenerative changes (50)), suggesting that Abcg2-mediated transport of riboflavin into milk is not essential for the health of suckling pups (48). This is most likely explained by the fact that substantial amounts of the cofactor FAD can still enter the milk independently of Abcg2 (48). FAD can be converted to riboflavin in the intestine before being absorbed, and this is probably sufficient to compensate for the riboflavin deficiency in the milk of Abcg2<sup>−/−</sup> mice, at least in the protective environment of the lab, with ample supplies of riboflavin-rich food. It could well be that under more natural conditions, such as a variable food supply, reduced dietary intake, or increased need for vitamins due to stress or disease, Abcg2-mediated transport of riboflavin into the milk will be essential for an optimal nutritional and health state of suckling pups.

After the mammary transport of riboflavin by Abcg2 was discovered, the milk of Abcg2<sup>−/−</sup> mice was analyzed for levels of other vitamins (48). In addition to riboflavin, the concentration of biotin (vitamin H, vitamin B<sub>7</sub>) (Figure 4B) was reduced in milk of Abcg2<sup>−/−</sup> mice, although the difference was smaller than for riboflavin (~3.5-fold decrease). This suggests that Abcg2 may also be involved in the secretion of biotin into the milk. Direct transport of biotin by Abcg2/ABCG2, however, has not been shown yet, so decreased milk concentrations of biotin may also be caused by indirect effects of Abcg2 deficiency. Furthermore, no signs of biotin deficiency (dermatitis, hair loss and neurological signs (51)) in suckling Abcg2<sup>−/−</sup> pups have been reported. This may be due to the fact that a significant amount of biotin is still present in the milk of Abcg2<sup>−/−</sup> mothers (48), which may be sufficient for the pups, at least in the protective environment of the lab where the mice receive an excess of nutrients through the diet.

The fact that Abcg2/ABCG2 has a prominent effect on the elimination or transport of a variety of exogenous and endogenous porphyrins, such as pheophorbide a (9), PPIX (9;52;53) and PPIX glycoconjugates (53) and its interaction with hemin (54) (see also below), would suggest that vitamin B<sub>12</sub>, a porphyrin-containing vitamin, could be transported into the milk by Abcg2. However, the analysis of milk of Abcg2<sup>−/−</sup> mice did not show decreased levels of this vitamin, suggesting that this is not the case, or at least that Abcg2 does not make a quantitatively important contribution to vitamin B<sub>12</sub> secretion into milk (48).

Folic acid (folate, vitamin B<sub>9</sub>) is another vitamin that is directly transported by ABCG2 in vitro (55). It was therefore hypothesized that ABCG2 would transport folic acid into the milk. Nevertheless, studies under both steady-state conditions (48) as well as after i.v. administration of 1 mg/kg [3]<sup>1</sup>H]folic acid (8) to lactating dams did not reveal any difference in folate levels between milk of wild-type and Abcg2<sup>−/−</sup> mice. This indicates that alternative, and quantitatively more important pathways of folate secretion into milk must exist in the mammary gland. Some other ABC transporters are also capable of folic acid transport, at least in vitro (55), and these might perhaps contribute, but various other transport processes may also be relevant.
In fact, very little is known yet about milk secretion mechanisms of various vitamins, and this may be an interesting area for future research.

**Figure 4.** Vitamins of which the milk secretion can be affected by Abcg2. (A) Riboflavin (vitamin B$_2$) (R = R1) and FMN (R = R2), but not FAD (R = R3) are concentrated into milk by Abcg2. (B) Biotin (vitamin B$_7$) levels in milk are 3.5-fold decreased in *Abcg2*$_{-/-}$ mice. (C) Vitamin K$_1$ (R = R1) levels in milk are not affected by Abcg2 and vitamin K$_2$ (R = R2) or K$_3$ (R = R3) concentrations in *Abcg2*$_{-/-}$ milk have not been determined yet. Vitamin K$_3$ is transported by ABCG2 *in vitro* and therefore may be concentrated into milk by ABCG2/Abcg2.

It was recently shown that ABCG2 expression in HEK 293 cells conferred resistance to vitamin K$_3$ (menadione, 2-methyl-1,4-naphtoquinone) (*Figure 4C*) *in vitro*, and that ABCG2-dependent efflux of mitoxantrone could be inhibited by vitamin K$_3$ (56). This suggests that this vitamin is a substrate for ABCG2 as well. Vitamin K$_3$ is a synthetic precursor of vitamin K$_2$ (menaquinone) (*Figure 4C*), and can be converted to K$_2$ in the body, although the biological significance of this conversion is unclear (57). Vitamin K$_1$ (phylloquinone) (*Figure 4C*) levels in milk were not different between *Abcg2*$_{-/-}$ and wild-type mice, suggesting that this compound is not a substrate of Abcg2, or that other transport processes dominate its milk secretion (48). Levels of vitamins K$_2$ and K$_3$ in milk of *Abcg2*$_{-/-}$ mice have not been analyzed thus far. Based on the *in vitro* experiments with vitamin K$_3$ this may be interesting to investigate, although also vitamin K deficiency (reflected by haemorrhagic disease (57)) has never been reported for *Abcg2*$_{-/-}$ pups. This might of course be explained by the fact that vitamin K$_1$ secretion into milk is normal in *Abcg2*$_{-/-}$ mothers, which could be sufficient to meet the required vitamin K intake of pups, at least in the favorable environment of the lab (see above).
Although these recent reports indicate that Abcg2 contributes to transport of some important nutrients into the milk, the question remains why pups fed by Abcg2<sup>+/−</sup> mothers do not show any abnormalities. Challenging Abcg2<sup>+/−</sup> mice with different types of diets containing low amounts of nutrients may shed more light on this. It appears that for many of these nutrients multiple (possibly redundant) pathways are available for transport into the milk. Investigation of other mechanisms of nutrient secretion into milk may also provide more insight into the relative contribution of Abcg2 to this process.

3.1.2. Secretory function of the multidrug resistance transporter ABCG2/Abcg2 in the mammary gland: a conundrum?

What is the biological meaning of ABCG2 function in the mammary gland? Clearly, the use of a multispecific xenobiotic transporter to secrete a number of vitamins into milk poses problems, both biological and conceptual. Based on the tissue distribution of ABCG2 (Figure 1), its extremely wide substrate specificity, and extensive functional studies in Abcg2<sup>−/−</sup> mice, there can be no doubt that one of the main biological functions of ABCG2 is protection from naturally occurring dietary xenobiotics. It limits oral uptake and bioavailability, mediates hepatobiliary and renal excretion, and restricts penetration of its substrates into critical tissues such as brain, testis, and fetus, as well as individual cells (e.g. hematopoietic progenitors) expressing ABCG2. It thus limits exposure to its substrates at the organismal (systemic), organ and cellular levels. This is all well and good for noxious xenobiotics, but at the same time ABCG2 will also limit uptake and availability of transported vitamins such as riboflavin, folate, and vitamin K<sub>3</sub>. In theory this might compromise vitamin supply to the organism, to critical tissue and cell compartments, and to the developing fetus. We indeed observed higher levels of riboflavin in Abcg2<sup>−/−</sup> than in wild-type mice (48), but the differences were modest. It is therefore reasonable to assume that for critical nutrients that are ABCG2 substrates, efficient uptake and retention mechanisms exist in all relevant barriers (intestine, placenta and other blood-tissue barriers, cell membranes) that largely overrule the extruding function of ABCG2. This would make the vitamin extrusion function of ABCG2 tolerable for the organism.

Conversely, the active secretion of potentially noxious xenobiotics into the milk by ABCG2 seems difficult to reconcile with optimal protection of the newborn offspring. Altogether, the combination of properties of ABCG2 (tissue distribution, substrate specificity, established functions) seems highly paradoxical. Here we will consider some hypotheses on ABCG2 function in the mammary gland that might begin to explain this paradox.

A. ABCG2 expression in mammary gland is coincidental. This hypothesis assumes that there is no positive biological need for mammary gland ABCG2. This would be
consistent with the lack of obvious deficiency phenotypes observed so far in pups nursed by $Abcg2^{-/-}$ mothers. The mammary gland expression of ABCG2 during pregnancy and lactation would merely be a consequence of the presence of transcriptional regulation elements that are necessary for proper expression and regulation of ABCG2 at other developmental stages or elsewhere in the body. The possible negative effects of pumping noxious dietary xenobiotics into the milk are mitigated by the fact that the nursing mother can itself prevent high systemic accumulation of dietary ABCG2 substrates through her own ABCG2 functions (in liver, intestine, kidney). Only a limited amount of noxious ABCG2 substrates would therefore be secreted into the milk. The riboflavin and other vitamin secretion into milk would be a coincidental consequence of the very wide substrate specificity of ABCG2. Although we cannot exclude this coincidental hypothesis, we feel it is less likely. Mammary gland ABCG2 is consistently induced during pregnancy and lactation in at least four widely divergent mammalian species (mouse, man, sheep, cow) (8;47). This evolutionary conservation suggests some functional need. Moreover, Abcg2 expression elsewhere in the mouse body (liver, kidney, small intestine) is not altered during pregnancy and lactation (58), so there is also at least one tissue-specific factor in the mammary gland that allows specific induction of ABCG2. Altogether, the conserved presence of so many regulatory factors that allow specific mammary gland induction of ABCG2 in a variety of mammalian species suggests there is some biological need for it.

B. ABCG2 activity in the mammary gland is useful as a xenobiotic clearance mechanism for the mother. This hypothesis assumes that mammary gland Abcg2 is not necessary for transport of nutrients, but functions primarily as an “overflow mechanism”. Lactating mothers are in general more vulnerable and may therefore need extra elimination routes for potentially toxic compounds. Abcg2 expression in the mammary gland may be used for this. Still then the question remains why it would be acceptable to expose suckling pups to these potentially toxic compounds. Abcg2 protein expression has been detected in the intestinal submucosa of the fetus recently (30). Abcg2 in the intestinal wall of the suckling pups may therefore be sufficient to restrict intestinal uptake of Abcg2 substrates from the milk. Even though we have observed that sometimes substantial fractions (up to 15% of the dose) of i.v. administered drugs are cleared via the milk (59), we consider this hypothesis unlikely as well. If the mother is compromised by xenobiotic exposure, so will the pup, and it is likely that the rapidly growing and developing pup is more susceptible to adverse effects of toxins than the mother. Moreover, there are highly efficient alternative clearance pathways available to the mother (liver, kidney, intestine). If need be, it would make more biological sense for the mother to optimize those clearance pathways, for instance by upregulating ABCG2 in these tissues, rather than endangering the well-being of the pups through the milk.
C. Xenobiotic exposure of suckling pups prepares them for the switch to solid food. This hypothesis assumes that deliberate transfer of moderate levels of dietary xenobiotics through the milk will induce proper detoxification systems in the pup (48). Very likely, upon weaning the pups will be eating the same solid food as the mother, and therefore be exposed to the same dietary xenobiotics. It would make biological sense to preemptively upregulate the detoxification systems in the pup that are suited to handling these xenobiotics (e.g., xenobiotic-metabolizing, -conjugating and -transporting proteins), instead of challenging a "virgin" detoxification system. The preinduction through the milk xenobiotics, for instance via activation of the nuclear xenobiotic receptors PXR and CAR, will help the pups in dealing immediately with the solid food xenobiotics, and thus improve their overall fitness. We feel this hypothesis is conceptually attractive, and pilot experiments to test it are ongoing. A limitation that we have encountered here is, however, that there are not yet many ABCG2 substrates known that are also good activators of PXR and CAR, which would seem to be the most obvious mediators for preemptive upregulation of detoxification systems in pups (60).

D. Milk transfer of xenobiotics may reduce the chance of developing allergy against these molecules later in life. This hypothesis is based on the recent demonstration that, upon milk transfer of an allergen to which the lactating mother is exposed, the chance that the suckling pups will develop allergy against this compound later in life is reduced (61). This oral tolerance induction in the pups was dependent on the presence of TGF-β, which is normally present in breast milk. The presence of this protein suggests that oral tolerance induction is a natural function of milk. Milk transfer by ABCG2 of potentially allergenic compounds (either exogenous or endogenous) to which the mother is exposed might thus reduce the chance for the pups of developing allergy against these compounds later in life. A limitation of this hypothesis is that it would mainly apply to relatively small molecules (i.e., transported by ABCG2) whereas the experimental demonstration concerned a protein (ovalbumine). Proteins and protein conjugates are more usual allergens than small molecules. However, small molecules can also be highly allergenic (penicillin is a well-known example) (62), and true allergies to various other drugs (cephalosporins, sulfa drugs, anticonvulsants, neuromuscular blocking agents, novocaine) have been described (62-64). This hypothesis could be tested when a suitable allergenic small molecule is identified as an ABCG2 substrate.

E. Mammary gland ABCG2 is necessary for nutrient transfer in the milk. This assumes that we have not yet applied the proper conditions to nursing Abcg2<sup>−/−</sup> mice to reveal a strong need for ABCG2 in providing vitamins and perhaps other nutrients in milk. Testing this hypothesis may depend on finding the proper conditions in the
lab, or perhaps identifying additional nutrients that might be transported by ABCG2 into the milk. Nevertheless, this hypothesis still begs the question why mammals didn't evolve dedicated transport systems to pump the various nutrients into milk, rather than a multispecific xenobiotic transporter. This would circumvent the risk of xenobiotic exposure of pups through milk. Still, nature tends to be pragmatic, and it may have been relatively easy (in an evolutionary sense), sufficiently efficient and with acceptable risk to the pups to apply a multidrug transporter for simultaneously pumping a variety of nutrients into milk.

Of course other, even more tentative hypotheses can be formulated, but here we restrict ourselves to the most obvious ones. We further note that some possibilities are not mutually exclusive. For instance, it is quite possible that mammary gland ABCG2 is important for both preemptively inducing detoxification mechanisms in suckling pups (C), and in providing them with some nutrients (E). Even the allergen hypothesis (D) might apply in parallel to hypotheses C and E. We finally note that there may still be other, unrecognized functions for mammary gland ABCG2 that would clarify its presence in the breast. Clearly, much more work will need to be done to resolve these complex but intriguing questions.

3.2 Abcg2 is expressed in the harderian gland and involved in transport of conjugated protoporphyrin IX.

The tubulo-alveolar epithelial cells of the harderian gland have recently been identified as another site of high Abcg2 expression in the mouse (53). The harderian gland is a lipid-secreting exocrine gland located behind the eye, which is especially well developed in rodents and many other vertebrates (occupying about one third of the eye socket in mice), but appears to be absent in humans and other primates. Perhaps related to its absence in humans, the physiological function of the harderian gland is still unclear. Situated next to the tear gland, its secreta are released at the inner corner of the eye. In rodents it secretes lipids as well as large amounts of porphyrins, which are, amongst others, suggested to function as phototransducers capable of absorbing UV-light (65). Due to its high concentration and excretion of porphyrins, the harderian gland is an interesting organ for studies on porphyrin excretion mechanisms. It may be worth observing that substantial amounts of these porphyrins, which have a reddish colour, end up in the coat of rats through grooming. In white-coated animals they are responsible for the typical reddish coat glow that is often seen. Conversely, in sick animals harderian gland secreta accumulate in the eye corner due to lack of grooming, causing typical red crusts. A possible function of harderian gland secreta in rodent coat care or function should not be excluded.

One of the main porphyrins, protoporphyrin IX (PPIX), is a direct precursor of heme (Figure 5), which is an important cofactor in many essential biological
processes (O2 transport by hemoglobin, electron transfer in cytochrome P450s, etc). Nevertheless, porphyrins are highly efficient photosensitizers and an excess can cause severe cellular damage (66), so their synthesis and distribution must be carefully regulated. When harderian glands of Abcg2+/− mice were macroscopically investigated, it was found that they had a deep red-brown colour instead of the pale colour seen in wild-type mice. Fluorescence microscopy showed that this could be attributed to a vast increase in intracellular porphyrin levels, which suggested that Abcg2 is normally involved in secretion of porphyrins into the tubulo-alveolar lumen of the harderian gland (53).

The involvement of Abcg2/ABCG2 in endogenous porphyrin transport has previously been suggested based upon the fact that Abcg2+/− mice had increased erythrocyte PPIX levels (9). Subsequently, it was shown that progenitor cells from Abcg2+/− mice were more affected by hypoxic conditions (leading to increased intracellular heme concentrations) compared to wild-type progenitor cells, and that ABCG2 specifically could bind to hemin, a heme analogue (Figure 5) (54). This binding could be intensified by the presence of ABCG2 substrates. Presence of hemin on the other hand also increased ABCG2-mediated transport of estrone-3-sulfate (54). Furthermore, Zhou et al. (52) showed that ABCG2 overexpression in K562 cells led to reduced cellular PPIX levels, which could be reversed by the ABCG2 inhibitor Ko143. Also treatment of mature erythrocytes expressing ABCG2 with Ko143 could increase PPIX accumulation (52). These studies clearly suggested that ABCG2 is capable of transporting PPIX and that it can interact with heme under excess conditions. However, they did not distinguish between PPIX and its various conjugates.

Analysis of harderian gland extracts from Abcg2+/− mice showed that levels of the PPIX-glycoconjugates protoporphyrin-1-O-acyl-β-xyloside (penta) and to a minor extent protoporphyrin-1-O-acyl-β-glucoside (hexa) (Figure 5) were dramatically increased. No unconjugated PPIX was detected in harderian glands of Abcg2+/− mice, which suggested that PPIX is mainly excreted from harderian gland as a penta conjugate (53). Indeed, only the penta conjugate of PPIX (but not unconjugated PPIX) was detectable in tear fluid collected from wild-type mice. Furthermore, although Abcg2+/− mice still showed penta secretion in tear fluid, the tear/gland ratio of penta was 9-fold lower than in wild-type. Subsequent in vitro cellular accumulation experiments with MDCKII cells expressing murine Abcg2 or human ABCG2 suggested that the harderian gland PPIX glycoconjugates are indeed transported efficiently by Abcg2/ABCG2 (53).

Also in the liver of Abcg2+/− mice an accumulation of protoporphyrin-1-O-acyl-β-xyloside (penta) was found, but under normal conditions the total amounts of PPIX and its conjugates were only slightly increased in livers of Abcg2+/− mice. However, when a high dose of PPIX was administered i.v. to wild-type and Abcg2+/− mice, the biliary excretion of total PPIX was dramatically reduced in the Abcg2+/−
mice compared to wild-type (53). This suggests that although Abcg2/ABCG2 is likely an efficient transporter of PPIX-glycoconjugates, it is probably also a low-affinity (but high capacity) transporter for PPIX, which may primarily be important under excess PPIX conditions.

![Diagram of porphyrin structures](image)

**Figure 5.** Physiological and exogenous porphyrins that interact with and/or are transported by ABCG2/Abcg2. Hemin, an analogue of heme, interacts with ABCG2 *in vitro* (54). The heme-precursor PPIX is likely transported by ABCG2 *in vitro* (52) and PPIX levels in erythrocytes of *Abcg2*⁻/⁻ mice are elevated (9). The PPIX-glycoconjugates “penta” and “hexa” are likely transported *in vitro* and *in vivo* by Abcg2/ABC2 (53). The exogenous porphyrin pheophorbide a is transported by Abcg2/ABC2 *in vitro* and causes severe phototoxicity in *Abcg2*⁻/⁻ mice (9).

The physiological function of Abcg2-mediated transport of PPIX(-conjugates) remains unclear, also as no functional abnormalities have been described in *Abcg2*⁻/⁻ mice that could be directly attributed to the altered PPIX levels. One possibility is that Abcg2 helps to reduce porphyrin toxicity in liver and other cells in situations of PPIX excess. Also, the PPIX-conjugates that are excreted from the harderian gland by Abcg2 will likely have a (so far unknown) exocrine or
endocrine function. Considering that the whole heme biosynthetic pathway up till PPIX (i.e., the last step before insertion of Fe to generate heme, Figure 5) must be highly activated in the harderian gland in order to obtain the normal levels of PPIX conjugate secretion, it is difficult to imagine that there would not be a biological need for it. Be that as it may, the actual biological function of PPIX conjugate secretion (and thus of Abcg2) in the harderian gland remains a mystery.

3.3 Abcg2 is expressed at the murine blood-retinal barrier where it might protect the retina from circulating phototoxins.

Abcg2 is expressed in blood capillary endothelial cells of many so-called tissue sanctuaries (see above). Recently, also the murine blood-retinal barrier (BRB) has been identified as a site of Abcg2 expression, where it was detected at the luminal membrane of retinal capillary endothelial cells (67). Intrinsic to its function as a light-sensing organ, the retina is highly photosensitive and vulnerable to damage caused by circulating phototoxic compounds such as pheophorbide a and PPIX, which are both Abcg2 substrates (see also above) (9;53;68). Abcg2 expression was also detected in conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB2 cells), in which the accumulation of pheophorbide a increased significantly by treatment with the Abcg2-inhibitor Ko143, suggesting that Abcg2 expression in these cells is functional (67). The physiological function of the Abcg2 expression in the BRB may be to protect the retina from phototoxicity induced by circulating PPIX(-conjugates), or phototoxins derived from the diet. It should be noted, though, that ABCG2 is generally expressed in endothelial cells of small veins and capillaries throughout the body, also in blood vessels where there are no tight junctions between endothelial cells (69). It is therefore uncertain whether endothelial ABCG2 expression always has to indicate a blood-tissue barrier function. Whether Abcg2 really limits the penetration of drug or phototoxic substrates into the retina should therefore be determined in future studies, possibly using Abcg2\(^{-/-}\) mice.

4. Concluding remarks

The last 4-5 years have seen a significant increase in insights into possible pharmacological and physiological functions of ABCG2, many based on analyses of Abcg2\(^{-/-}\) mice. These include a definitive demonstration of the impact of ABCG2 in protecting brain, testis, and fetus from various xenobiotics, and elucidation of the function of mammary gland ABCG2 in pumping various drugs, carcinogens and toxins into milk. These findings can have a wide range of clinical applications. For example, when pregnant women are treated with drugs that are potentially harmful to the fetus, it might be preferable to select drugs that are ABCG2 substrates, so the fetus is protected. Conversely, if a drug should enter the fetus for therapeutic purposes, it might be better to select non-, or poor ABCG2 substrates. A similar consideration would apply to drugs that should or should preferably not enter the
brain. On the other hand, when drugs that are potentially dangerous to suckling infants are used for treatment of lactating mothers, it would be wise to select drugs that are not ABCG2 substrates, in order to reduce the amount of drug that is secreted into the milk. Of course, whether ABCG2 functions as efficiently at these sites in humans as in mice will first have to be investigated in more detail, before extrapolating directly from the mouse to humans. Nevertheless, we consider that Abcg2-/- and combination knockout mice are valuable tools to determine the in vivo effects of ABCG2 for many drugs that are currently used in the clinic, as well as for the characterization of newly discovered drugs.

As for the physiological functions of ABCG2, even though we have learnt many new aspects of both physiological ABCG2 substrates and new expression sites and functions, it seems that a straightforward picture is still lacking. Although we are convinced that one main physiological function of ABCG2 is protection from noxious dietary xenobiotics, there are many indications that there must be various additional physiological functions. Given the multispecificity of ABCG2, it may simply be that the body has applied this protein for a multitude of different functions, without an essential underlying common theme, except for the transport function. However, it would not surprise us if we are still lacking many pieces of the ABCG2 puzzle, which may one day come together to yield a comprehensive picture of the functions of this intriguing protein. We are convinced that Abcg2-/- mice will provide very useful tools in this process.

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Chapter 10

Impact of Abcg2 (Bcrp1) expression in mother and pup on riboflavin levels in suckling pups


To be submitted
Chapter 10

Impact of Abcg2 (Bcrp1) expression in mother and pup on riboflavin levels in suckling pups

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The multidrug transporter ABCG2 (BCRP) is expressed in excretory organs and transports its substrates into bile, feces and urine. Furthermore, ABCG2 is strongly induced in the mammary gland during lactation and pumps riboflavin (vitamin B₂) into milk. However, pups nurtured by Abcg2⁻/⁻ dams did not suffer from riboflavin deficiency. We now investigated the separate effects of Abcg2 in mammary glands of lactating mothers and Abcg2 in the suckling pups on riboflavin distribution in pups. Newborn Abcg2⁻/⁻ pups were fostered with wild-type dams and vice versa. We found that stomach riboflavin levels were solely dependent on Abcg2 of the lactating mother. Small intestinal riboflavin levels, however, were mainly determined by Abcg2 of the mother and to a minor extent by the genotype of the pups, suggesting that Abcg2 in suckling pups excretes riboflavin into the intestinal lumen. Furthermore, in plasma, cecum and colon of pups the riboflavin levels were mainly determined by Abcg2 of the pup. We show here for the first time that Abcg2 in newborn pups appears to reduce the systemic exposure to a substrate - in this case, surprisingly, a vitamin. Furthermore, Abcg2 of mother and pup appear to co-operate in increasing riboflavin concentrations in the intestinal lumen of pups. We therefore hypothesize that Abcg2 in mother and pup might be somehow beneficial for the optimal gastrointestinal development of suckling pups by increasing intestinal riboflavin levels.

INTRODUCTION

The ATP binding cassette transporter ABCG2 (BCRP) is a multidrug transporter that can actively transport a wide range of endogenous and exogenous compounds over biological membranes. It is present in the apical membrane of hepatocytes and epithelial cells of kidney and small intestine, where it excretes its substrates into bile, urine and feces. Furthermore, ABCG2 is localized in brain, testis and placenta, where it limits the penetration of its substrates into these so-called tissue sanctuaries (1;2). One main function of ABCG2 is thought to be the protection of cells, tissues and the systemic circulation from accumulation of potentially toxic endogenous and
exogenous compounds. Because of its broad substrate specificity, ABCG2 influences the pharmacokinetics of a wide range of drugs (1,2).

ABCG2 has recently also been detected in the lactating mammary gland, where it pumps its substrates into milk (3). The physiological function of this expression site is not clear yet, raising the intriguing question of why a xenotoxin efflux system would be localized in the lactating mammary gland, thereby exposing vulnerable pups to potentially toxic compounds (1-3). Interestingly, besides many toxic compounds, ABCG2 has recently been shown to transport a range of nutrients, amongst which one of the main vitamins present in milk, riboflavin (vitamin B₂) (1,4). ABCG2 can transport this vitamin in vitro and in vivo, and endogenous levels of riboflavin in the milk of Abcg2⁻/⁻ dams were dramatically lower than in wild-type milk (4). However, although riboflavin is an essential vitamin, pups nurtured by Abcg2⁻/⁻ dams did not show any signs of riboflavin deficiency (4). Furthermore, although the milk concentrations of riboflavin were 63-fold decreased, the plasma levels of (Abcg2⁻/⁻) pups fed by Abcg2⁻/⁻ dams were only 1.6-fold lower than plasma levels of Abcg2²⁺/⁺ pups fed by wild-type dams.

The finding that pups fed by Abcg2⁻/⁻ dams do not suffer from riboflavin deficiency could be explained by the fact that another biologically active form of riboflavin, the co-factor FAD (flavin adenine dinucleotide) (5), is still present in milk of Abcg2⁻/⁻ dams (4). FAD can be converted to riboflavin in the intestine of the pup and subsequently taken up into the body (6). Puzzlingly, Abcg2 expression in the intestine and/or liver of the pups may reduce riboflavin plasma levels by pumping this vitamin into bile and the intestinal lumen. Abcg2 expression in intestinal submucosa and liver of the fetus has recently been detected (7), but the effect of Abcg2 on the pharmacokinetics of its substrates in newborn pups has not been investigated yet. So far, the biological significance of riboflavin transport by Abcg2 into mother milk, as well as the influence of Abcg2 expression in pups remains unclear (1).

We wanted to investigate the effect of Abcg2 on endogenous riboflavin levels in newborn pups (as well as the levels of its biologically active forms FAD and FMN (flavin mononucleotide)) in more detail and to thus obtain more insight into the physiological role of Abcg2. We therefore compared the relative influence of Abcg2 expression in mammary glands of lactating mothers versus the influence of Abcg2 expression in suckling pups.

MATERIALS AND METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used were Abcg2⁻/⁻ (8) and wild-type mice, all of >99% FVB background. Animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet containing 12-14 mg/kg riboflavin (AM-II, Hope Farms, Woerden, The
Netherlands) and acidified water *ad libitum*. 

**Chemicals.** Riboflavin, FMN and FAD were from Sigma Chemical Co. (St. Louis, MO). Methoxyflurane (Methofane) was obtained from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia).

**Foster experiments.** Newborn pups from wild-type dams were fostered with *Abcg2<sup>-/-</sup>* dams and vice versa at the first day after birth. Subsequently, at day 5 or 12 after birth, pups were sacrificed by decapitation and blood was collected in heparinized Microvette® CB 300 LH capillary tubes (Sarstedt, Nümbrecht, Germany). At 21 days of age, pups were sacrificed by cardiac puncture under methoxyflurane anesthesia, followed by cervical dislocation. Stomach, small intestine, cecum and colon of the pups were also collected (all tissues including contents). All samples were protected from light and stored at -80°C until extraction.

**HPLC analysis of riboflavin, FMN and FAD.** Samples (gastrointestinal tissues including contents) were homogenized in 3 volumes of PBS. To 200 µl of each homogenate 600 µl methanol was added to precipitate proteins. To plasma samples four times the sample volume of methanol was added. Riboflavin, FMN and FAD were determined by HPLC as described previously (9), with modifications as described (4). All samples were protected from light.

**Statistical analysis.** All values are given as means ± standard deviations (SD). One-way ANOVA followed by Tukey’s multiple comparison test was performed to assess the significance of differences between data sets. Differences were considered to be statistically significant when the P-value was < 0.05.

**RESULTS**

Genotype of the foster mother determines riboflavin contents in the stomach of suckling pups.

To separate the effects of *Abcg2* expression in mammary gland of the lactating mother and *Abcg2* expression in the intestine of suckling pups, *Abcg2<sup>-/-</sup>* pups were fostered with wild-type mothers and wild-type pups with *Abcg2<sup>-/-</sup>* mothers at the day of birth (day 1). We subsequently used pups from these two groups, as well as control groups (wild-type pups with wild-type mothers and *Abcg2<sup>-/-</sup>* pups with *Abcg2<sup>-/-</sup>* mothers) at day 5 (when the pups solely ingest mother milk), day 12 (when the pups are about to start eating solid food, in addition to drinking milk) and day 21 (when the pups are weaned and are exclusively eating solid food) after birth to collect plasma, stomach, small intestine and cecum and colon. The levels of riboflavin, FMN and FAD in the different matrices were analyzed. In all cases gastrointestinal tissues including contents were measured.
The results for the stomach levels of the pups at different ages are shown in Figure 1A. This clearly shows that the amount of riboflavin ingested by suckling pups is completely dependent on Abcg2 expression in the mammary gland of the foster mother. Pups with an Abcg2\(^{+/+}\) foster mother had 6-15-fold less riboflavin in the stomach than pups of the same genotype with a wild-type foster mother, both at 5 and 12 days of age, consistent with the drastically reduced riboflavin levels in milk of Abcg2\(^{+/+}\) dams (4). When the pups were no longer drinking mother milk, but solely eating (riboflavin-rich) solid food (21 days), the difference between pups nursed by Abcg2\(^{+/+}\) and wild-type dams disappeared (Figure 1A). At this age (day 21) stomach riboflavin levels in all groups were comparable to stomach riboflavin levels of 5-day old pups nursed by wild-type dams. This shows that the amount of riboflavin ingested on this standard diet is comparable to the amount ingested by pups drinking wild-type milk. The results show that riboflavin levels in the stomach of suckling pups are solely dependent on Abcg2 expression in the mammary gland of the mother and not on Abcg2 expression in the pups. Nevertheless, also pups nurtured by Abcg2\(^{+/+}\) mice did have some riboflavin in the stomach, suggesting an alternative (but far less effective) mechanism for riboflavin excretion into milk, as shown before (4).

Levels of FMN in the stomach of pups from all groups were relatively low (< 0.25 µg/g organ) in all groups and there were no consistent differences between the groups (not shown). Levels of FAD in the stomach of all groups were substantial and comparable to riboflavin levels in pups with wild-type foster mothers (2.0-4.4 µg/g organ). There were no consistent differences between the four groups analyzed (not shown).

**Genotype of the foster mother is the main determinant for riboflavin levels in the small intestine of pups.**

The riboflavin levels in the small intestines of the pups showed similar patterns as in the stomach, but the differences were much less pronounced (Figure 1B). This suggests that the genotype of the foster mother is still a determinant for riboflavin levels in the small intestine of pups. However, it seems that Abcg2 expression in the pup may lead to somewhat higher intestinal riboflavin levels, suggesting that Abcg2 in the pup can actively pump riboflavin into the small intestinal lumen (Figure 1B). At 21 days of age there were no differences in small intestinal levels between the different groups anymore, except for a minor difference between Abcg2\(^{+/+}\) pups with either wild-type or Abcg2\(^{+/+}\) foster mothers, which is (based on the data for the stomach) likely coincidental.

Levels of FMN in small intestine of the pups were relatively low (< 0.45 µg/g) and not significantly different between the groups analyzed (not shown). Levels of FAD were comparable to riboflavin levels in the small intestine of the pups with wild-type foster mothers (8-13 µg/g), but not different between the four groups analyzed (not shown).
Figure 1. Endogenous levels of riboflavin in 5, 12 or 21 days old wild-type (WT) and Abcg2\(^{-/}\) pups nurtured by wild-type or Abcg2\(^{-/}\) foster mothers. A, riboflavin in stomach (tissue + contents) of the different groups. B, riboflavin in the small intestine (SI, tissue + contents) of the different groups. C, riboflavin in cecum and colon (cecc + col, tissue + contents) of the different groups. Data are presented as means ± SD (n = 4-13, ns, not significant, *, P < 0.05, **, P < 0.01, ***, P < 0.001, ANOVA). Note the difference in axis scales between the different graphs.
Genotype of foster mother and pup together determine riboflavin levels in cecum and colon of pups.

In the cecum and colon of most 5- and 12-day old pups, the levels of riboflavin were much higher than in the small intestine (Figure 1C). The levels of riboflavin in this part of the intestinal tract were clearly determined both by Abcg2 expression in the mammary gland of the foster mother, and by Abcg2 in the intestine and/or liver of the pups. Pups with wild-type foster mothers had high concentrations of riboflavin in the cecum and colon (wild-type pups with wild-type foster mothers: 24 ± 6 µg/g, Abcg2<sup>2−/−</sup> pups with wild-type foster mothers: 12 ± 3 µg/g, both at age 12 days) (Figure 1C), suggesting that high levels of riboflavin in the stomach also leads to high levels in cecum and colon. Nevertheless, wild-type pups that were fed by Abcg2<sup>2−/−</sup> foster mothers, and therefore ingested relatively low levels of riboflavin (Figure 1A), still had quite high riboflavin levels in cecum and colon (32 ± 6 µg/g at age 12 days), whereas Abcg2<sup>2−/−</sup> pups with Abcg2<sup>2−/−</sup> foster mothers did not (1.1 ± 0.6 µg/g at age 12 days) (Figure 1C). Furthermore, as Abcg2<sup>2−/−</sup> pups with wild-type foster mothers at 12 days of age had 2-fold lower levels of riboflavin in cecum and colon than their wild-type counterparts (see above, Figure 1C), this suggests that Abcg2 in the pups concentrates riboflavin in the intestinal lumen.

Riboflavin concentrations in cecum and colon of 21 days old pups of all groups were, despite comparable stomach levels (Figure 1A), much lower than in the younger wild-type pups and comparable to levels in suckling Abcg2<sup>2−/−</sup> pups with Abcg2<sup>2−/−</sup> foster mothers (Figure 1C). Furthermore, we found no significant differences between the four groups at this age. Levels of FMN in cecum and colon of all pups were quite low in all groups (<1.4 µg/g tissue) and showed similar patterns to the riboflavin levels in cecum and colon (although the differences between the groups were not in all cases significant (not shown)). This suggests that FMN levels in cecum and colon of pups of all ages were directly related to the levels of riboflavin in these pups. This is likely due to the fact that riboflavin can be converted to FMN in the intestine (5). FAD levels in cecum and colon of the pups were relatively low as well (<4.5 µg/g tissue) and no consistent differences between the groups were found (not shown).

Riboflavin plasma concentrations are mainly influenced by the genotype of the pup.

The plasma levels of riboflavin in all groups analyzed are shown in Figure 2. This shows that in suckling pups (5 and 12 days old) plasma levels were mostly dependent on the genotype of the pups. Abcg2<sup>2−/−</sup> pups (also the ones with Abcg2<sup>2−/−</sup> foster mothers and therefore low riboflavin intake (Figure 1A)) had significantly (1.3-5.5 fold) higher riboflavin plasma concentrations than the wild-type pups. This shows that Abcg2 expression in pups already early after birth reduces plasma levels of riboflavin. Also an effect of riboflavin intake (and therefore genotype of the foster
mother) was found: suckling $Abcg2^{-/-}$ pups with wild-type foster mothers (and therefore higher riboflavin intake) had 2-fold higher plasma levels than $Abcg2^{-/-}$ pups with $Abcg2^{-/-}$ foster mothers. However, this was only seen in $Abcg2$ deficient pups. In wild-type pups the genotype of the foster mother did not affect plasma levels of the pups (Figure 2). Collectively, these data suggest that $Abcg2$ expression in newborn pups strongly reduces the plasma levels of riboflavin, especially when riboflavin intake is high. On the other hand, $Abcg2$ in the mammary gland of the mother only has a modest effect on the riboflavin plasma levels of pups. In 21 days old pups, in contrast to what was previously found in adult $Abcg2^{-/-}$ males (4), we did not find significant differences between the groups analyzed (Figure 2).

FMN levels in plasma of all groups analyzed were relatively low (<0.04 µg/ml) and appeared to correlate with riboflavin levels in plasma of the pups, although the differences between groups were less pronounced and not always significant (not shown). As shown before for adult mice (4), FAD levels in plasma of wild-type and $Abcg2^{-/-}$ pups were also low (0.01-0.02 µg/ml) and not significantly different between genotypes (not shown).

**Figure 2.** Endogenous levels of riboflavin in plasma of 5, 12 or 21 days old wild-type (WT) and $Abcg2^{-/-}$ pups nurtured by wild-type or $Abcg2^{-/-}$ mothers. Data are presented as means ± SD (n = 4-5, ns, not significant, *, P < 0.05, **, P < 0.01, ***, P < 0.001, ANOVA).
DISCUSSION

By fostering Abcg2−/− pups with wild-type mothers and vice versa we were in this study able to separate the effects of Abcg2 in the mammary gland of the mother, pumping riboflavin into milk, and Abcg2 in intestine and liver of the suckling pups, potentially pumping riboflavin into the intestinal lumen of the pup, thereby reducing the riboflavin plasma levels. We found that the levels of riboflavin in stomach and small intestine of suckling pups are primarily determined by Abcg2 expression in the mammary gland of the mother. On the other hand, riboflavin levels in cecum and colon of pups were dependent both on Abcg2 in the mammary gland of the mother and Abcg2 in liver and/or intestine of the pup itself. Furthermore, riboflavin plasma levels (and thereby the systemic exposure of suckling pups to riboflavin) were primarily determined by Abcg2 expression in the pups. This shows that, although Abcg2 actively pumps riboflavin into milk, this mainly increases riboflavin levels in the intestine of pups, but not in the circulation. However, Abcg2 in the pups appears to actively extrude riboflavin from the circulation into the intestine, thereby efficiently reducing riboflavin exposure of suckling pups. This observation is most evident in the wild-type and Abcg2−/− pups with Abcg2−/− foster mothers: in spite of a similarly low intake of riboflavin through milk, the riboflavin levels are far lower in cecum and colon of the Abcg2−/− pups (Figure 1C), whereas the plasma riboflavin levels are higher (Figure 2). This is to our knowledge the first demonstration of potential Abcg2 activity in newborn pups.

The finding that Abcg2 was expressed in the lactating mammary gland where it could actively pump its substrates, of which many are potential toxins, into the milk, led to the question what purpose this would serve (1-3). The subsequent finding that Abcg2 could transport the vitamin riboflavin seemed to partly explain this paradox (4). However, it was still not clear why a multispecific transporter is used for pumping such an important vitamin into the milk, at the expense of exposing vulnerable pups to all kinds of other potential toxins. Furthermore, it was unclear why Abcg2−/− pups did not suffer from riboflavin deficiency (4). The fact that the co-factor FAD is still abundantly present in the milk of Abcg2−/− mothers may explain this (4). Our results additionally suggest another explanation, namely that Abcg2 in the wild-type pups already early after birth plays an important role in pumping its substrates (including riboflavin) out of the body. Actually, as we show here, Abcg2−/− pups, even though they receive very low amounts of riboflavin via the milk (4), have relatively high riboflavin plasma levels due to absence of Abcg2 (Figure 2). In wild-type pups that receive high amounts of riboflavin, it appears that a large fraction of this riboflavin is effectively pumped back into the intestinal lumen by Abcg2 (Figure 1C). This suggests that Abcg2 in the mammary gland of lactating mothers is not very effective in increasing the systemic level of riboflavin in wild-type pups. The fact that Abcg2 actively pumps riboflavin into the milk, while at the same time Abcg2 in the pups actively pumps this vitamin into the
intestinal lumen (either directly or via the liver), leads to the hypothesis that high concentrations of riboflavin may be important in the lumen of the intestine of suckling and developing pups.

That riboflavin in the intestinal lumen may be important for the development of pups was previously also suggested by Yates et al. (2003), who showed that specific absence of riboflavin in the intestinal lumen (but not the systemic levels) of weanling rats led to disruption of normal gastrointestinal development (10). The mechanism behind this remains unclear. Although Yates et al. showed that riboflavin in the lumen is especially important for the development of the duodenum of pups, whereas our results suggest an effect of Abcg2 in riboflavin concentration especially in the cecum and colon, these findings may be related. In other words, it could well be that the biological function of Abcg2 in the mammary gland is to deliver high amounts of riboflavin to the pup, which subsequently remain high in the intestinal lumen of the pup by active (back-)transport via Abcg2 in intestine and liver. Whether this is indeed the case should be investigated in the future.

It is interesting to note that these high levels of riboflavin in small intestine, cecum and colon that we found here in wild-type pups (especially the ones fed by wild-type foster mothers, but also when fed by Abcg2<sup>−/−</sup> foster mothers) were only seen in pups up to 12 days old (Figure 1B and C). In 21 days old pups the riboflavin levels in plasma were quite comparable to the younger pups, but in the intestinal tract they were much lower than in the younger pups (Figures 1 and 2), suggesting that the effect of Abcg2 in retaining high intestinal riboflavin levels, is especially important in pups early after birth. Interestingly, whereas in adult mice the liver levels of Abcg2 are much lower in females than in males, in 10 days old pups Abcg2 expression in liver is similar between male and female pups (11). This suggests that early after birth, Abcg2 may (also in female pups) play an important role in pumping its substrates, like riboflavin, into bile. Merino et al. (2005) further showed that at 5 weeks of age the expression of Abcg2 in liver of female mice was already much lower than in males (11), suggesting that at this age the effect of Abcg2 in liver may already be less important. However, many other explanations for the differences between the 21 days old pups and the suckling pups can be suggested, as it is likely that when pups start dealing with solid food many physiological and anatomical changes occur in the intestines of pups.

The finding that Abcg2 in the newborn pups, as we show here, is already very active and important for the pharmacokinetics of riboflavin further explains why it might not be so problematic to have Abcg2 expression in the mammary gland of lactating females, pumping potential toxins into the milk. As Abcg2 in the pup is quite active already early after birth, the pups are, like adults, probably quite well protected from toxins in milk that are Abcg2 substrates, and will only be exposed to low systemic concentrations of these compounds. The majority of the toxins that are
ingested via the milk is likely efficiently excreted from the body of the pups by Abcg2.

Our results suggest that at least one physiological effect of Abcg2 functioning is to maintain high levels of riboflavin in the intestinal lumen of developing pups. However, although this mouse strain has been carefully studied in the past years (1;2), no severe problems with the intestinal development of Abcg2−/− pups have been reported. It could be that in the protective environment of laboratory mouse facilities, on a standard diet with high riboflavin content, an effect of Abcg2 may be missed. Possibly, under more natural conditions, with lower amounts of riboflavin in the diet, it may be much more important to have Abcg2 expression in lactating mammary gland as well as in the intestine and liver of suckling pups. It would be interesting to investigate this possibility in more detail in future studies.

ABCG2 is also expressed in the mammary gland of lactating humans (3), and many ABCG2 polymorphisms in humans are known, in some cases leading to altered function of the protein (12). Furthermore, many compounds (either drugs or dietary components) have been identified that can inhibit the function of ABCG2 (12). As we show here that Abcg2 appears important in suckling pups (and therefore likely also in suckling infants) for reducing the systemic exposure to ABCG2/Abcg2 substrates, it might be important to investigate whether polymorphisms in ABCG2 of infants can lead to increased systemic exposure to toxins in the mother milk. Furthermore, it would be interesting to investigate whether reduced activity in ABCG2 of mother and/or suckling infant may lead to problems with gastrointestinal development of children due to low riboflavin levels in the intestinal lumen.

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Summary
SUMMARY
The body possesses many systems for the protection from potentially toxic compounds to which it is continuously exposed. These toxins are for example present in the diet, but also drugs and some endogenous compounds can be harmful. One well-known mechanism for the protection of the body from these toxins is the expression of ABC (ATP-binding cassette) multidrug efflux transporters in the cellular membrane. These proteins can actively extrude their substrates from cells. They are often located in epithelial cells of excretory organs such as liver, kidney and intestine, and excrete their substrates out of the body, and into bile, urine and feces. Many ABC transporters are additionally present in the blood-tissue barriers of so-called pharmacological sanctuaries such as brain, testis and placenta, where they protect these important organs from entry of potential toxins. ABC transporters are also often detected in human tumors, where they can confer multidrug resistance (MDR). Due to their function in the excretion of substrates from the body, they often influence the pharmacokinetics of drugs. As in cancer therapy the therapeutic window (the range between efficacious and toxic concentrations of a drug) is usually narrow, variation in expression or activity of these transporters can determine whether a drug is toxic and/or effective in reducing the tumor. Since the expression and activity of ABC transporters varies between individuals, and ABC transporters have substantially overlapping functions and substrate specificities, it is important to investigate the relative functions of the various transporters in vivo.

The in vivo functions of ABC transporters have been extensively investigated using transporter deficient (knockout) mouse models. However, due to the largely overlapping substrate specificities of ABC transporters, it is often difficult to distinguish their separate roles and functional overlap using single knockout mice. For example, when one transporter is absent, another one may partly or completely compensate for its loss. As a consequence, often no or only minor effects of the single deletion are seen. Chapters 1 to 8 of this thesis focus on the generation and characterization of ABC transporter compound knockout mice, and the use of these models for pharmacological studies. An overview of the recently generated compound knockout mice and recent findings obtained with these mice is described in Chapter 1. This shows that for most of the ABC transporters that are primarily involved in determining the pharmacokinetics of drugs compound knockout mice have now been generated. These compound knockout mice have provided very useful insights in the overlapping function of ABC transporters in vivo. From the results obtained so far it is clear that many transporters can compensate for the absence of each other, and that the relative effect of each transporter is highly dependent not only on the substrate, but also on the given dose and the tissue distribution of the transporter.

One ABC transporter that can affect the pharmacokinetics of many drugs is ABCC2 (MRP2). Whereas for other ABC transporters single knockout mice had
already been generated before, for Abcc2 this was not the case. To make compound knockout mice in which also Abcc2 was deleted, we therefore first generated \textit{Abcc2}⁻/⁻ mice, as described in Chapter 2. Using these mice we showed that, like human and rat ABCC2/Abcc2, murine Abcc2 is involved in the biliary excretion of the endogenous compounds glutathione and bilirubin glucuronide. Furthermore, we showed that Abcc2 influences the pharmacokinetics of the food-derived carcinogens \([^{14}\text{C}]\text{PhIP} \ (2\text{-amino-1-methyl-6-phenylimidazo}[4,5-b]\text{pyridine}), \ [^{14}\text{C}]\text{IQ} \ (2\text{-amino-3-methylimidazo}[4,5-f]\text{quinoline})\) as well as the anti-cancer and anti-rheumatic drug \([^3\text{H}]\text{methotrexate}\ (\text{MTX})\). We subsequently crossed the \textit{Abcc2}⁻/⁻ mice with knockout mice for another ABC transporter that influences the pharmacokinetics of drugs, ABCB1 (P-gp, MDR1) to obtain \textit{Abcb1a/1b;Abcc2}⁻/⁻ (\textit{Mdr1a/1b;Mrp2}⁻/⁻) mice. With this model we showed that Abcc2 and \textit{Abcb1a/1b} together are the main transporters for the biliary excretion of the anti-cancer drug doxorubicin, although \textit{Abcb1a/1b} was most important. For the tested compound, Abcc2 and \textit{Abcb1a/1b} had mostly additive effects and they hardly compensated for the loss of each other.

Chapter 3 describes in more detail the generation and characterization of the \textit{Abcb1a/1b;Abcc2}⁻/⁻ (\textit{Mdr1a/1b;Mrp2}⁻/⁻) mice. Furthermore, in this chapter the compound knockout mice were used to determine the relative effects of Abcc2 and \textit{Abcb1a/1b} on the pharmacokinetics of the anti-cancer drug paclitaxel. We found that \textit{Abcb1a/1b} were the main transporters for excretion of paclitaxel into the intestine, whereas Abcc2 was the main transporter for its hepatobiliary excretion. Therefore, whereas Abcc2 and \textit{Abcb1a/1b} together influenced the pharmacokinetics of paclitaxel after iv administration, after oral administration only \textit{Abcb1a/1b} played a role. This showed that besides affinity for a substrate, also the tissue localization and drug administration route determine the relative effect of ABC transporters on the pharmacokinetics of drugs.

In Chapter 4 we describe the generation and characterization of \textit{Abcc2;Abcc3}⁻/⁻ (\textit{Mrp2;Mrp3}⁻/⁻) mice. We found that Abcc3 can compensate for the loss of Abcc2 in the elimination of bilirubin glucuronide, as was previously hypothesized, by pumping this compound from liver into the circulation, thereby facilitating increased urinary excretion of bilirubin glucuronide in \textit{Abcc2}⁻/⁻ mice. We obtained similar results for the impact of Abcc3 on MTX pharmacokinetics when Abcc2 was absent. Furthermore, we showed that, besides for biliary MTX excretion, Abcc2 is highly important for the biliary excretion of 7-hydroxymethotrexate (7OH-MTX), the main toxic metabolite of MTX. When Abcc2 was absent, Abcc3 again partly mediated sinusoidal liver elimination of 7OH-MTX, as was shown by substantial liver accumulation of 7OH-MTX in \textit{Abcc2;Abcc3}⁻/⁻ compared to \textit{Abcc2}⁻/⁻ mice.

Chapter 5 describes the generation and characterization of \textit{Abcc2;Abcg2}⁻/⁻ (\textit{Bcrp1;Mrp2}⁻/⁻) mice. Using these mice we showed that Abcc2 and Abcg2 together are the main transporters for the biliary excretion of MTX and that they have
additive effects. Abcc2 was the main transporter for biliary 7OH-MTX excretion, but when Abcc2 was absent, Abcg2 could partly compensate. We further found that when Abcc2 was absent, and Abcc3 expression caused increased plasma levels of MTX and 7OH-MTX (as described in Chapter 4), Abcg2 in the kidney subsequently pumped these compounds into the urine as an alternative elimination pathway. As MTX and 7OH-MTX are considered nephrotoxic, this may have implications for the effects of reduced expression or activity of ABCC2, ABC3 and ABCG2 in MTX-related toxicity of patients treated with high-dose MTX.

Chapter 6 continues on these findings, as here the generation and characterization of Abcc2;Abcc3;Abcg2−/− mice are described. Surprisingly, although 3 important hepatic and intestinal transporters are deleted, these mice are viable and show no specific aberrations under standard housing conditions. From this we conclude that these transporters (at least in the protective environment of the NKI mouse facility) do not have overlapping vital physiological functions. However, the overlapping functions of these transporters in the elimination of MTX and 7OH-MTX from the body were clearly demonstrated. Whereas in all single and double knockout mice for Abcc2, Abcc3 and/or Abcg2 only moderate effects on the pharmacokinetics of MTX and 7OH-MTX after iv administration were seen (Chapters 4 and 5), in Abcc2;Abcc3;Abcg2−/− a dramatic increase in the liver accumulation of MTX and 7OH-MTX was found. Also, due to the delayed liver elimination of MTX, much more of the toxic metabolite 7OH-MTX was formed and excreted into urine and feces of these mice. These results therefore show that Abcc2, Abcc3 and Abcg2 together are the main transporters involved in the fast elimination of MTX and 7OH-MTX after iv administration, and that they can to a large extent compensate for the absence of each other. As decreased expression or activity of all three transporters in patients is quite unlikely, this suggests that treated patients are usually quite well protected from MTX-related toxicity.

Although in cancer treatment drugs are often given iv in order to obtain high and reproducible plasma concentrations, it would be more favorable to give a drug orally. This is more cost-effective and also more convenient for the patient. As the oral bioavailability of drugs is often limited by ABC transporters, we used the Abcc2;Abcc3;Abcg2−/− and corresponding single and double knockout mice to investigate the relative influences of Abcc2, Abcc3 and Abcg2 on the pharmacokinetics of MTX and 7OH-MTX after oral administration of MTX. The results are described in Chapter 7. We found that the absence of especially Abcg2, but also of Abcc2 caused increased plasma levels of MTX already early after administration. Absence of both transporters led to a more than 3-fold increased plasma AUC_0-120 min (area under the plasma concentration-time curve). Interestingly, these effects were only seen when Abcc3 was present, showing that Abcc3 protein (in liver or intestine) is important for the oral bioavailability of MTX. This implies that combined inhibition of ABCC2 and ABCG2 (without ABC3 inhibition) may
be a useful strategy to increase MTX plasma levels after oral administration of this drug in patients.

Chapter 8 describes the generation and characterization of Abcb1a/1b;Abcc2;Abcg2<sup>−/−</sup> (Bcrp1;Mdr1a/1b;Mrp2<sup>−/−</sup>) mice. We used these mice, together with the Abcc2;Abcc3;Abcg2<sup>−/−</sup> (Bcrp1;Mrp2;Mrp3<sup>−/−</sup>) mice to determine the relative roles of the ABC transporters Abcb1a/1b, Abcg2, Abcc2 and Abcc3 in the pharmacokinetics of the dietary carcinogen PhIP and its metabolites, of which a subset is mutagenic. We found that Abcc2 and Abcg2 together are the main transporters for the biliary, intestinal and subsequent fecal excretion of PhIP. We also found that Abcc2 and Abcg2 together mediate the biliary excretion of mutagenic PhIP metabolites. Absence of Abcc2 and Abcg2 together led to increased urinary excretion of these metabolites. Furthermore, Abcc3 appeared very important for liver elimination of the potentially carcinogenic PhIP metabolite N-OH-PhIP. These results have potential implications for the roles of ABC transporters in the protection from carcinogenesis, as well as in determining the location of tumor formation by PhIP and its metabolites.

Chapters 9 and 10 describe the physiological and pharmacological functions of the intriguing ABC transporter ABCG2 (BCRP). In Chapter 9 we give an overview of the studies performed with Abcg2<sup>−/−</sup> (Bcrp1<sup>−/−</sup>) mice so far. These studies have shown that besides excretion of toxic compounds from the body, ABCG2/Abcg2 is also present in the lactating mammary gland and pumps potentially toxic compounds into breast milk. An explanation for this phenomenon has not been found yet. However, it was discovered recently that besides toxic compounds, ABCG2 can also transport some vitamins, amongst which riboflavin (vitamin B<sub>2</sub>), into milk. Still, the function of this riboflavin transport into the milk is not clear, as pups fed by Abcg2<sup>−/−</sup> dams did not suffer from riboflavin deficiency. Some hypotheses to explain the function of Abcg2 in the mammary gland are discussed in Chapter 9.

In Chapter 10 we investigated the separate effects of Abcg2 in the lactating mammary gland of the mother and Abcg2 in the pup on the endogenous riboflavin levels in suckling pups. We separated the effects of both processes by fostering Abcg2<sup>−/−</sup> pups with wild-type dams and vice versa. We found that Abcg2 expression in the mammary gland of the foster mother provides high levels of riboflavin in the stomach and small intestine of the pup. Surprisingly, Abcg2 expression in the intestine and/or liver of the pups appeared to subsequently reduce systemic riboflavin plasma levels, presumably by pumping this vitamin into bile and/or the intestinal lumen of the pups. We therefore hypothesize that the active secretion of riboflavin into the milk and subsequent excretion of riboflavin into the intestinal lumen of pups by Abcg2 may have a biological meaning, for example to aid the intestinal development of pups. Whether this is indeed true, should be investigated in the future.
Combined, the mouse models generated and studied in this thesis, in combination with previously generated knockout mice, should provide useful tools for studies on the *in vivo* physiological and pharmacological functions of ABC transporters. Hopefully, this will lead to a better understanding of the separate effects of these ABC transporters, which can be used to optimize drug treatment of patients, while minimizing side effects.
Samenvatting
SAMENVATTING
Het lichaam wordt continu blootgesteld aan potentieel giftige stoffen (toxines) en het bezit dan ook vele systemen om zich daar tegen te beschermen. Deze toxines zijn bijvoorbeeld aanwezig in het voedsel, maar ook geneesmiddelen en bepaalde lichaamseigen stoffen kunnen giftig zijn. Een bekend mechanisme voor de bescherming van het lichaam tegen toxines is de expressie van zogenaamde "ABC (ATP-binding cassette) multidrug efflux transporters" in het membraan van cellen. Deze eiwitten kunnen actief hun substraten de cel uit pompen. Ze zijn veel aanwezig in epitheelcellen van excretieorganen zoals lever, nier en darm en pompen hun substraten naar gal, urine en feces en daarmee het lichaam uit. Veel ABC transporters zijn daarnaast aanwezig in de bloed-weefsel barrières van zogeheten farmacologische "sanctuaries" zoals hersenen, testis en placenta, waar ze deze belangrijke organen beschermen tegen het binnenkomen van potentieel giftige stoffen. ABC transporters worden ook vaak gevonden in humane tumoren, waar ze multidrug resistentie (MDR) kunnen veroorzaken. Door hun functie in de uitscheiding van substraten beïnvloeden ze in veel gevallen de farmacokinetiek van geneesmiddelen. Aangezien in kankertherapie het “therapeutisch venster” (het bereik tussen effectieve en toxische concentraties van een geneesmiddel) vaak erg smal is, kan variatie in expressie of activiteit van ABC transporters de toxiciteit en effectiviteit van een antikanker-geneesmiddel bepalen. Aangezien de expressie en activiteit van ABC transporters varieert tussen individuen en aangezien ABC transporters substantiële overlap hebben in functie en substraat-specificiteit, is het belangrijk de relatieve effecten van de verschillende transporters in vivo te onderzoeken.

De in vivo functies van ABC transporters zijn uitgebreid onderzocht met behulp van transporter deficiënte (knock-out) muismodellen. Ondanks dat is het door de grote overlap in substraat-specificiteiten van ABC transporters vaak moeilijk om de verschillende effecten en overlappende functies van elkaar te onderscheiden met muizen die een enkele transporter missen. Als één transporter afwezig is, kan het bijvoorbeeld zo zijn dat een andere transporter zijn functie geheel of gedeeltelijk overneemt. Dit heeft tot gevolg dat vaak geen of maar kleine effecten van de afwezigheid van één transporter worden gezien. Hoofdstuk 1 t/m 8 van dit proefschrift focussen zich op het genereren en karakteriseren van ABC transporter combinatie knock-out muizen en het gebruik van deze modellen voor farmacologische studies. Een overzicht van de recent gemaakte combinatie knock-out muizen en recente bevindingen opgedaan met deze modellen wordt gegeven in Hoofdstuk 1. Dit hoofdstuk laat zien dat voor de meeste ABC transporters die een belangrijke rol spelen in het bepalen van de farmacokinetiek van geneesmiddelen nu combinatie knock-out muizen gegenereerd zijn. Deze combinatie knock-out muizen hebben veel nuttige inzichten in de overlappende functies van ABC transporters in vivo opgeleverd. De resultaten die tot nu toe ver zameld zijn laten duidelijk zien dat
het relatieve effect van iedere transporter niet alleen sterk afhankelijk is van het substraat, maar ook van de gegeven dosis en de weefseldistributie van de transporter.

Eén van de ABC transporters die de farmacokinetiek van vele geneesmiddelen kan beïnvloeden is ABCC2 (MRP2). Terwijl voor andere ABC transporters al enkele knock-out muizen waren gegenereerd, was dit voor Abcc2 niet het geval. Om combinatie knock-out muizen te kunnen maken waarin ook Abcc2 afwezig is, hebben we daarom eerst Abcc2+/− muizen gegenereerd. Dit is beschreven in Hoofdstuk 2. Met deze muizen hebben we aangetoond dat, net als humaan en rat ABCC2/Abcc2, ook muis Abcc2 een rol speelt in de galexcretie van de endogene stoffen glutathion en bilirubine glucuronide. Daarnaast hebben we laten zien dat Abcc2 de farmacokinetiek van de in het voedsel aanwezige carcinogenen [14C]PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), [14C]IQ (2-amino-3-methylimidazo[4,5-f]quinoline) en van het anti-kanker en antireumatisch geneesmiddel [³H]methotrexaat (MTX) beïnvloedt. We hebben deze Abcc2+/− muizen vervolgens gekruist met knock-out muizen voor een andere ABC transporter die de farmacokinetiek van geneesmiddelen beïnvloedt, ABCB1 (P-pg, MDR1) om Abcb1a/1b;Abcc2+/− (Mdr1a;1b;Mrp2+/−) muizen te verkrijgen. Met dit model konden wij laten zien dat Abcc2 en Abcb1a/1b samen de belangrijkste transporters voor de galexcretie van het antikanker geneesmiddel doxorubicine zijn, waarbij Abcb1a/1b de grootste bijdrage leverde. Voor de geteste stof bleken Abcc2 en Abcb1a/1b vooral additieve effecten te hebben en amper te compenseren voor het verlies van de ander.

Hoofdstuk 3 beschrijft het genereren en karakteriseren van de Abcb1a/1b;Abcc2+/− (Mdr1a;1b;Mrp2+/−) muizen meer gedetailleerd. Daarnaast werden in dit hoofdstuk de combinatie knock-out muizen gebruikt om de relatieve effecten van Abcc2 en Abcb1a/1b op de farmacokinetiek van het antikanker geneesmiddel paclitaxel te bepalen. We ontdekten dat Abcb1a/1b de belangrijkste transporters voor de excretie van paclitaxel naar de darm waren, terwijl Abcc2 het belangrijkst was voor de galuitscheiding van paclitaxel. Vandaar dat Abcc2 en Abcb1a/1b samen de farmacokinetiek van paclitaxel na iv toediening beïnvloedden, terwijl na orale toediening alleen Abcb1a/1b een rol bleek te spelen. Dit liet zien dat, naast de affiniteit voor een substraat, ook de weefsellokalisatie en de manier van toediening van een geneesmiddel het relatieve effect van ABC transporters op de farmacokinetiek van geneesmiddelen bepalen.

In Hoofdstuk 4 beschrijven we het genereren en karakteriseren van Abcc2;Abcc3+/− (Mrp2;Mrp3+) muizen. We liken zien dat, zoals eerder was gehypothetiseerd, Abcc3 kan compenseren voor het verlies van Abcc2 in de eliminatie van bilirubine glucuronide, door deze stof vanuit de lever de bloedcirculatie in te pompen en daarmee verhoogde urine uitscheiding van bilirubine glucuronide in Abcc2+/− muizen te faciliteren. We vonden vergelijkbare resultaten
Samenvatting

voor het effect van Abcc3 op de farmacokinetiek van MTX wanneer Abcc2 afwezig was. Verder lieten we zien dat, naast galuitscheiding van MTX zelf, Abcc2 ook zeer belangrijk is voor de galexcretie van 7-hydroxymethotrexaat (7OH-MTX), de belangrijkste, toxische metaboliet van MTX. In afwezigheid van Abcc2 mediëerde Abcc3 weer gedeeltelijk de sinusoidale lever eliminatie van 7OH-MTX, zoals te zien was aan substantiële leveraccumulatie van 7OH-MTX in Abcc2; Abcc3−/− vergeleken met Abcc2−/− muizen.

Hoofdstuk 5 beschrijft het genereren en karakteriseren van Abcc2; Abcg2−/− (Bcrp1; Mrp2−/−) muizen. Met deze muizen lieten we zien dat Abcc2 en Abcg2 samen de belangrijkste transporters voor de galexcretie van MTX zijn en dat zij additieve effecten hebben. Abcc2 was de belangrijkste transporter voor galexcretie van 7OH-MTX, maar wanneer Abcc2 afwezig was kon Abcg2 hiervoor gedeeltelijk compenseren. We vonden daarnaast dat, wanneer Abcc2 afwezig was en Abcc3 verhoogde plasma spiegels van MTX en 7OH-MTX veroorzaakte (zie Hoofdstuk 4), Abcg2 in de nier deze stoffen vervolgens naar de urine pompt als een alternatieve eliminatieroute. Aangezien MTX en 7OH-MTX toxisch voor de nierlijken te zijn, zou dit implicaties kunnen hebben voor de effecten van verlaagde expressie of activiteit van ABCC2, ABC3 en ABCG2 op MTX-gerelateerde toxiciteit in patiënten die worden behandeld met hoge doses MTX.

Hoofdstuk 6 gaat verder op deze bevindingen, aangezien we hier het genereren en karakteriseren van Abcc2; Abcc3; Abcg2−/− muizen beschrijven. Verbazingwekkend genoeg zijn deze muizen levensvatbaar en vruchtbaar en vertonen zij geen specifieke afwijkingen onder standaard huisvestingsomstandigheden, ondanks dat zij 3 belangrijke hepatische en intestinale transporters missen. Hieruit concluderen wij dat deze transporters (in ieder geval in de beschermde omgeving van de muizenfaciliteit van het NKI) geen overlappende, vitale, fysiologische functies hebben. Toch konden we de overlappende functies van deze transporters in de eliminatie van MTX en 7OH-MTX duidelijk aantonen. Terwijl in de enkele en dubbele knock-out muizen voor Abcc2, Abcc3 en/of Abcg2 maar matige effecten op de farmacokinetiek van MTX en 7OH-MTX na iv toediening werden gezien (Hoofdstukken 4 en 5), vonden we in Abcc2; Abcc3; Abcg2−/− muizen een dramatische toename in de leveraccumulatie van beide stoffen. Verder werd, door vertraagde levereliminatie van MTX, veel meer van de toxische metaboliet 7OH-MTX gevormd en uitgescheiden in urine en feces van deze muizen. Deze resultaten laten daarom zien dat Abcc2, Abcc3 en Abcg2 samen de belangrijkste transporters zijn voor de snelle eliminatie van MTX en 7OH-MTX na iv toediening en dat zij voor een groot deel voor afwezigheid van elkaar kunnen compenseren. Aangezien verminderde expressie of activiteit van alle drie de transporters in patiënten vrij onwaarschijnlijk is, suggereert dit dat behandelde patiënten normaal gesproken vrij goed beschermd zijn tegen MTX-gerelateerde toxiciteit.
Hoewel voor de behandeling van kanker geneesmiddelen vaak iv worden toegediend om hoge en reproduceerbare plasmaniveaus te bereiken, zou het doorgaans wenselijker zijn om een geneesmiddel oraal te geven. Dit is minder duur en ook prettiger voor de patiënt. Aangezien de orale biobeschikbaarheid van geneesmiddelen vaak gelimiteerd wordt door ABC transporters, hebben we de Abcc2;Abcc3;Abcg2<sup>−/−</sup> en corresponderende enkele en dubbele knock-out muizen gebruikt om de relatieve invloed van Abcc2, Abcc3 en Abcg2 op de farmacokinetiek van MTX en 7OH-MTX na orale toediening van MTX te bepalen. De resultaten hiervan zijn beschreven in Hoofdstuk 7. We vonden dat afwezigheid van vooral Abcg2, maar ook van Abcc2, al vrij snel na toediening verhoogde plasmaspiegels van MTX veroorzaakte. Afwezigheid van beide transporters leidde tot een meer dan 3-voudig verhoogde plasma AUC<sub>0-120 min</sub> (oppervlakte onder de plasmaconcentratie versus tijd curve). Interessant genoeg waren deze effecten alleen zichtbaar wanneer Abcc3 aanwezig was. Dit laat zien dat Abcc3 (inlever of darm) belangrijk is voor de orale beschikbaarheid van MTX. Dit impliceert dat de gecombineerde remming van ABCC2 en ABCG2 (zonder remming van ABCC3) een nuttige strategie zou kunnen zijn om MTX plasmaspiegels na orale toediening in patiënten te verhogen.

Hoofdstuk 8 beschrijft het genereren en karakteriseren van Abcb1a/1b;Abcc2;Abcg2<sup>−/−</sup> (Bcrp1;Mdr1a/1b;Mrp2<sup>−/−</sup>) muizen. We hebben deze muizen, samen met de Abcc2;Abcc3;Abcg2<sup>−/−</sup> (Bcrp1;Mrp2;Mrp3<sup>−/−</sup>) muizen, gebruikt om de relatieve effecten van de ABC transporters Abcb1a/1b, Abcg2, Abcc2 en Abcc3 op de farmacokinetiek van het in voedsel aanwezige carciogen PhIP en zijn metabollen (waarvan een aantal mutageen is) te bepalen. We ontdekten dat Abcc2 en Abcg2 samen de belangrijkste transporters voor de biliaire, intestinale en vervolgens fecale uitscheiding van PhIP zijn. Verder vonden wij dat Abcc2 en Abcg2 samen de galexcretie van mutagene PhIP metabollen verzorgen. Afwezigheid van Abcc2 en Abcg2 samen leidde tot verhoogde urine uitscheiding van deze metabollen. Daarnaast lijkt Abcc3 erg belangrijk voor de levereliminatie van de potentieel carcinogene PhIP metaboliet N-OH-PhIP. Deze resultaten hebben potentiële implicaties voor de rol van ABC transporters in de bescherming tegen carcinogenese en in het bepalen van de locatie van tumorvorming door PhIP en haar metabollen.

De hoofdstukken 9 en 10 beschrijven de fysiologische en farmacologische functies van de intrigerende ABC transporter ABCG2 (BCRP). In Hoofdstuk 9 geven wij een overzicht van de onderzoeken tot nu toe die gedaan zijn met Abcg2<sup>−/−</sup> (Bcrp1<sup>−/−</sup>) muizen. Deze studies hebben laten zien dat naast haar detoxificerende functie, ABCG2/Abcg2 ook aanwezig is in de lacterende borstklier en daar potentieel toxische stoffen de moedermelk in pomp. Een sluitende verklaring voor dit fenomeen is er nog niet, maar er is recent ontdekt dat naast toxische stoffen, ABCG2 ook enkele vitamineen (waaronder riboflavine, vitamine B<sub>2</sub>) de melk in transporteert. Toch blijft de functie van dit riboflavine transport naar de melk
onduidelijk, aangezien pups die door Abcg2\(^{-/-}\) moeders gevoed worden geen verschijnselen van riboflavinedeficiëntie vertonen. Een aantal hypotheses om de functie van Abcg2 in de borstklier te verklaren wordt in Hoofdstuk 9 besproken.

In Hoofdstuk 10 onderzochten we de verschillende effecten van Abcg2 in de lacterende borstklier van de moeder en Abcg2 in de pup zelf op de endogene riboflavine spiegels in zogende pups. We konden beide processen van elkaar scheiden door Abcg2\(^{-/-}\) pups te laten adopteren door wild type moeders en vice versa. We ontdekten dat Abcg2 in de borstklier van de adoptiemoeder zorgt dat grote hoeveelheden riboflavine in de maag en dunne darm van de pup terecht komen. Verbazingwekkend genoeg bleek Abcg2 in de darm en/of lever van de pups de riboflavine plasmaspiegels te verlagen door de vitamine naar gal en/of darminhoud (terug) te pompen. We hypothetiseren daarom dat de actieve uitscheiding van riboflavine in de melk en vervolgens in het intestinale lumen van de pup door Abcg2 een biologisch nut heeft, bijvoorbeeld voor het bevorderen van de ontwikkeling van de darmen van pups. Of dit werkelijk het geval is zal in de toekomst uitgezocht moeten worden.

De muismodellen die in dit proefschrift gegenereerd en bestudeerd zijn, in combinatie met eerder gegenereerde knock-out muizen, zijn waarschijnlijk zeer nuttige modellen voor het bestuderen van de in vivo fysiologische en farmacologische functies van ABC transporters. Hopelijk zal dit leiden tot een beter begrip van de verschillende effecten van deze ABC transporter. Dit kan vervolgens gebruikt worden voor het optimaliseren van behandeling van patiënten met geneesmiddelen en het tegelijkertijd minimaliseren van bijverschijnselen veroorzaakt door geneesmiddelen.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>7OH-MTX</td>
<td>7-hydroxymethotrexate</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ALAT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ASAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the concentration versus time curve</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCRP/Bcrp</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>BDG</td>
<td>bilirubin diglucuronide</td>
</tr>
<tr>
<td>BMG</td>
<td>bilirubin monoglucuronide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ID</td>
<td>internal diameter</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LTC4</td>
<td>leukotriene C4</td>
</tr>
<tr>
<td>Hygro</td>
<td>hygromycin</td>
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<tr>
<td>IQ</td>
<td>2-amino-3-methylimidazo[4,5-f]quinoline</td>
</tr>
<tr>
<td>Iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>ko</td>
<td>knockout</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MRP/Mrp</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>PMEA</td>
<td>9-(2-(phosphonomethoxy)ethyl)-adenine</td>
</tr>
<tr>
<td>PPIX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>small intestine</td>
</tr>
<tr>
<td>SIC</td>
<td>small intestinal contents</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>SULT</td>
<td>sulfotransferase</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>UCB</td>
<td>unconjugated bilirubin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
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</tbody>
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LIST OF PUBLICATIONS


Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and 7-hydroxymethotrexate in vivo. Clin Cancer Res 2009; in press.

Vlaming MLH, van Esch A, Pala Z, Wagenaar E, van de Wetering K, van Tellingen O, Schinkel AH. Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. Submitted.

Vlaming MLH, Lagas JS, Schinkel AH. Compound transporter knockout mice: powerful tools to unravel the pharmacological and physiological functions of ATP binding cassette drug transporters. To be submitted.

Vlaming MLH, Pala Z, van Esch A, Wagenaar E, van Tellingen O, Schinkel AH. Impact of Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) on the oral pharmacokinetics of the anti-cancer drug methotrexate and its main metabolite 7-hydroxymethotrexate in mice. To be submitted.


Vlaming MLH, van Herwaarden AE, van Esch A, Rosing H, Beijnen JH, Schinkel AH. Impact of Abcg2 (Bcrp1) expression in mother and pup on riboflavin levels in suckling pups. To be submitted.
CURRICULUM VITAE

Maria (Marijn) Louise Hubertina Vlaming was born on August 10th, 1979 in Laren, the Netherlands. In 1997 she received her VWO diploma at Scholengemeenschap Laar en Berg in Laren, The Netherlands. In the same year she started her studies of Biomedical Engineering at the University of Technology Eindhoven in Eindhoven, The Netherlands. During her studies she performed internships at the department of Macromolecular and Organic Chemistry of the University of Technology Eindhoven under the supervision of Prof. Dr. E.W. Meijer and Dr. M. Merkx, and at the Burnham Institute in La Jolla (CA, USA) under the supervision of Dr. D. Hanein. In 2003 she obtained her Masters degree and started as a PhD-student on the project titled “ABC transporter compound knockout mice: physiological and pharmacological characterization”, which led to the forthcoming of this thesis. The research described in this thesis was performed at the departments of Experimental Therapy and Molecular Biology of The Netherlands Cancer Institute in Amsterdam, The Netherlands, under the supervision of Dr. A.H. Schinkel and Prof. Dr. P. Borst. Since February 2009 she works as project leader/researcher at the department of Biosciences of TNO Quality of Life in Zeist, The Netherlands.
DANKWOORD

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Dankwoord

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