ABC transporter compound knockout mice: physiological and pharmacological characterization
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Summary
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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 Abcc2<sup>−/−</sup> 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 <sup>14</sup>CPhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), <sup>14</sup>CIQ (2-amino-3-methylimidazo[4,5-f]quinoline) as well as the anti-cancer and anti-rheumatic drug <sup>3</sup>Hmethotrexate (MTX). We subsequently crossed the Abcc2<sup>−/−</sup> mice with knockout mice for another ABC transporter that influences the pharmacokinetics of drugs, ABCB1 (P-gp, MDR1) to obtain Abcb1a/1b;Abcc2<sup>−/−</sup> (Mdr1a/1b;Mrp2<sup>−/−</sup>) mice. With this model we showed that Abcc2 and Abcb1a/1b together are the main transporters for the biliary excretion of the anti-cancer drug doxorubicin, although Abcb1a/1b was most important. For the tested compound, Abcc2 and 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 Abcb1a/1b;Abcc2<sup>−/−</sup> (Mdr1a/1b;Mrp2<sup>−/−</sup>) mice. Furthermore, in this chapter the compound knockout mice were used to determine the relative effects of Abcc2 and Abcb1a/1b on the pharmacokinetics of the anti-cancer drug paclitaxel. We found that 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 Abcb1a/1b together influenced the pharmacokinetics of paclitaxel after iv administration, after oral administration only 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 Abcc2;Abcc3<sup>−/−</sup> (Mrp2;Mrp3<sup>−/−</sup>) 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 Abcc2<sup>−/−</sup> 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 Abcc2;Abcc3<sup>−/−</sup> compared to Abcc2<sup>−/−</sup> mice.

Chapter 5 describes the generation and characterization of Abcc2;Abcg2<sup>−/−</sup> (Bcrp1;Mrp2<sup>−/−</sup>) 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, ABCC3 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$_{0-120\text{ min}}$ (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 ABCC3 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 $\text{Abcb1a/1b;Abcc2;Abcg2}^{-/-}$ ($\text{Bcrp1;Mdr1a/1b;Mrp2}^{-/-}$) mice. We used these mice, together with the $\text{Abcc2;Abcc3;Abcg2}^{-/-}$ ($\text{Bcrp1;Mrp2;Mrp3}^{-/-}$) 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 $\text{Abcg2}^{-/-}$ ($\text{Bcrp1}^{-/-}$) 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$_2$), into milk. Still, the function of this riboflavin transport into the milk is not clear, as pups fed by $\text{Abcg2}^{-/-}$ 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 $\text{Abcg2}^{-/-}$ 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 \textit{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.