ABC transporter compound knockout mice: physiological and pharmacological characterization
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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

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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
A. **LIVER**

(hepatocytes)

- P-gp
- ABCG2
- ABC3
- ABC4
- ABCG2

B. **SMALL INTESTINE**

(enterocytes)

- P-gp
- ABCC1
- ABC2
- ABC3
- ABCG2

C. **KIDNEY**

(proximal tubular cells)

- P-gp
- ABCC1
- ABC2
- ABC3
- ABCC4
- ABCG2

D. **BRAIN**

(capillary endothelial cells)

- P-gp
- ABC2
- ABC4
- ABCG2

**Fig. 1.** Tissue distribution of the human ATP binding cassette (ABC) transporters ABCB1, ABCG2 and ABCC1-4. A) ABC transporters in the liver. P-gp, ABCG2 and ABCC2 are located in the canicular (apical) membrane of hepatocytes, pumping their substrates into bile. ABCC3 and ABCC4 are present in the sinusoidal (basolateral) membrane of hepatocytes, and pump their substrates towards the blood circulation. Notably, it was recently shown that Abcc1 is present in activated rat hepatic stellate cells (HSCs), but not in hepatocytes (73). B) ABC transporters in the small intestine. P-gp, ABCG2 and ABCC2 in the luminal (apical) membrane of enterocytes pump their substrates into the intestinal lumen. ABCC1 and ABCC3, located in the basolateral membrane of enterocytes pump their substrates towards the blood circulation. C) ABC transporters in the kidney. P-gp, ABCG2, ABCC2 and ABCC4 are localized in the apical 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 amphipathic 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−/− 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−/− 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−/− 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−/− 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−/− 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−/− 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 C4 (LTC4), 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−/− mice (25;26), and both mutants had normal viability, fertility and life span. However, Abcc1−/− mice
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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<sup>−/−</sup>, Abcg2<sup>−/−</sup> and Abcb1a/1b;Abcg2<sup>−/−</sup> 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<sup>−/−</sup> mice may be useful to further address these issues.

Abcb1a/1b;Abcg2<sup>−/−</sup> 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<sup>−/−</sup> mice than in their wild-type counterparts [reviewed in (28)]. However, when the brain penetration in compound Abcb1a/1b;Abcg2<sup>−/−</sup> mice was compared to that in Abcb1a/1b<sup>−/−</sup> 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<sup>−/−</sup> mice, whereas these ratios were 2.0-fold higher in Abcb1a/1b<sup>−/−</sup> mice and 3.2-fold higher in Abcb1a/1b;Abcg2<sup>−/−</sup> mice (39). Although topotecan appears to be a better substrate for ABCG2 than for P-gp <em>in vitro</em> 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{-/-}} 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{-/-}} 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{-/-}} 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{-/-}} mice, not altered in \textit{Abcb1a/1b\textsuperscript{-/-}} mice and 9.3-fold higher in \textit{Abcb1a/1b;Abcg2\textsuperscript{-/-}} 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{-/-}} 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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Compound knockout strain(s)</th>
<th>Findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoposide</td>
<td><em>Abcb1a/1b;Abcc1</em></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>
</tr>
<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>
</tr>
<tr>
<td>Imatinib</td>
<td><em>Abcb1a/1b;Abcg2</em></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>
</tr>
<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><em>Abcb1a/1b;Abcc2</em></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>
</tr>
<tr>
<td>Paclitaxel</td>
<td><em>Abcb1a/1b;Abcc2</em></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>
</tr>
<tr>
<td>PMEA</td>
<td><em>Abcc4;Abcg2</em></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>
<td>9</td>
</tr>
<tr>
<td>Methotrexate</td>
<td><em>Abcc2;Abcc3</em> and Abcc2;Abcg2*</td>
<td>Abcc2 is the main transporter for biliary excretion. Abcc2 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;Abcc2;Abcg2*</td>
<td>Abcc2 is the main transporter for biliary excretion. Abcc2 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*</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</td>
<td>Abcc2 mediates the biliary excretion. Abcc3 mediates the sinusoidal excretion from the liver.</td>
<td>13</td>
</tr>
</tbody>
</table>
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 Abcb1a/1b<sup>−/−</sup> and Abcg2<sup>−/−</sup> mice, Abcb1a/1b;Abcg2<sup>−/−</sup> mice have proven to be a valuable tool to study the separate and combined impact of P-gp and ABCG2 at the BBB.

**Abcb1a/1b;Abcc2<sup>−/−</sup> 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 *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 Abcc2 (EHBR and TR<sup>−</sup>) 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 *in vivo* (8;52), and ABCC2 thus has a substantial overlap in substrate specificity with P-gp. Recently, we and others independently generated Abcc2<sup>−/−</sup> mice (52;53), and we additionally crossed our Abcc2<sup>−/−</sup> mice with Abcb1a/1b<sup>−/−</sup> mice (4) to obtain compound Abcb1a/1b;Abcc2<sup>−/−</sup> mice (8;52). Similar to single Abcc2<sup>−/−</sup> mice, compound Abcb1a/1b;Abcc2<sup>−/−</sup> 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, Abcb1a/1b;Abcc2<sup>−/−</sup> mice appear in many respects very similar to Abcc2<sup>−/−</sup> mice and are likely as amenable to pharmacological analysis. We used the Abcb1a/1b;Abcc2<sup>−/−</sup> 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 Abcc2<sup>−/−</sup> mice resulted in a 1.3-fold higher area under the plasma concentration-time curve (AUC) upon intravenous paclitaxel administration. Interestingly, the AUC<sub>i.v.</sub> for paclitaxel in Abcb1a/1b<sup>−/−</sup> 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_{i.v.} in \textit{Abcb1a/1b;Abcc2^{-/-}} 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^{-/-}} 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^{-/-}} mice, Abcg2 expression is increased in spleen and brain, whereas thymus and spleen from \textit{Abcg2^{-/-}} mice had increased \textit{Abcc4^{-/-}} expression (9), suggesting compensatory changes in these tissues when either one of the transporters is absent.

\textit{Abcc4} and \textit{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, \textit{Abcc4} and \textit{Abcg2} also transport endogenous compounds, such as cGMP (10) and steroid conjugates (61;62).

Recently, two research groups independently generated \textit{Abcc4;Abcg2^{-/-}} 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^{-/-}} mice in C57BL6;129SVJ background did not reveal any specific aberrations due to absence of both transporters (9), suggesting that \textit{Abcc4} and \textit{Abcg2} do not have any vital, overlapping physiological functions. However, these mice appeared very useful to investigate the overlapping pharmacological functions of \textit{Abcc4} and \textit{Abcg2}. Intravenous administration of the purine nucleoside phosphonate analogue $[^{3}\text{H}]$PMEA to \textit{Abcc4^{-/-}} mice resulted in accumulation in the spleen but not in other tissues, suggesting that \textit{Abcc4} plays a modest role in limiting the tissue accumulation of PMEA. In contrast, in \textit{Abcg2^{-/-}} mice $[^{3}\text{H}]$PMEA accumulated in liver, kidney, brain and ovary, showing a significant effect of \textit{Abcg2} on the tissue accumulation of the drug. In \textit{Abcc4;Abcg2^{-/-}} mice, however, $[^{3}\text{H}]$PMEA concentrations in liver, kidney and heart were even more
increased than in *Abcg2*<sup>−/−</sup> 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*<sup>−/−</sup> and *Abcc4;Abcg2*<sup>−/−</sup> mice, indicating that Abcg2 does not affect PMEA concentrations in this organ. This first pharmacological experiment employing *Abcc4;Abcg2*<sup>−/−</sup> 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<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> mice of both backgrounds, the bile flow was significantly decreased compared to wild-type (12;13), as shown previously for *Abcc2*<sup>−/−</sup> 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<sup>−/−</sup> mice indeed showed that Abcc3 in the liver of Abcc2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice have already proven to be useful models for studying the overlapping and compensatory roles of Abcc2 and Abcc3 <i>in vivo</i>.

<i>Abcc2;Abcg2<sup>−/−</sup> mice, a model to study the functional overlap of ABCG2 and ABCC2 in hepatobiliary excretion</i>
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 can not 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.
Chapter 1

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