ABC transporter compound knockout mice : physiological and pharmacological characterization

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Chapter 3

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

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[1]Division of Experimental Therapy and [2]Division of Clinical Chemistry, The Netherlands Cancer Institute, Amsterdam; [3]Slotervaart Hospital, Department of Pharmacy and Pharmacology, Amsterdam**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 $AUC_{i.v.}$ 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 AUC_{oral} 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 AUC_{oral} 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*$^{-/-}$ 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®) (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*$^{-/-}$ and *Mdr1a/1b*$^{-/-}$ 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*$^{-/-}$ mice (20) and crossed them with *Mdr1a/1b*$^{-/-}$ mice (15) to obtain *Mdr1a/1b/Mrp2*$^{-/-}$ 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). [^{3}H]Paclitaxel (4.8 Ci/mmol) was from Moravek Biochemicals (Brea, CA, USA). Paclitaxel metabolites 3'*p*-hydroxypaclitaxel and 6α-hydroxypaclitaxel 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/1b*$^{-/-}$ (15), *Mrp2*$^{-/-}$ (20), *Mdr1a/1b/Mrp2*$^{-/-}$ 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 [^{3}H]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^-, Ca^{2+}, 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 noncompartimental 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 ($t_{½,\,el}$) 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 $Cl = Dose/AUC_{i.v.}$ and the oral bioavailability (F) was calculated by the formula $F = AUC_{oral} / AUC_{i.v.} \times 100\%$. 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.

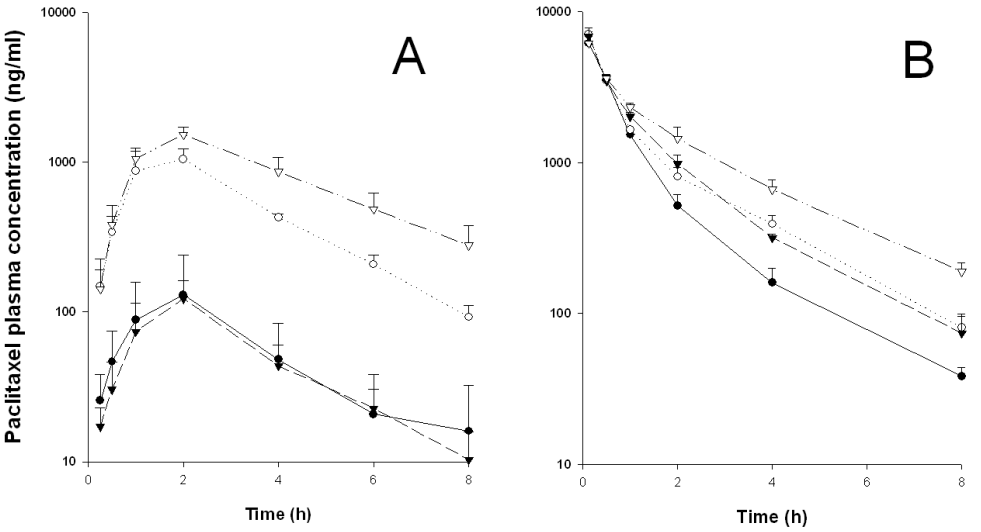

Fig. 1. Plasma concentration-time curves of paclitaxel in male FVB wild-type (●), *Mdr1a/1b*$^{-/-}$ (o), *Mrp2*$^{-/-}$ (▼) and *Mdr1a/1b/Mrp2*$^{-/-}$ (∇) mice, after oral (*A*) and i.v. (*B*) administration of paclitaxel at a dose of 10 mg/kg. Data represent mean concentrations ± SD, n = 5-6 for oral 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 (AUC_{oral}) were not different between *Mrp2*$^{-/-}$ and wild-type mice (Fig. 1A and Table 1). For *Mdr1a/1b*$^{-/-}$ mice the AUC_{oral} 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*$^{-/-}$ mice the AUC_{oral} was increased another 1.7-fold compared to *Mdr1a/1b*$^{-/-}$ mice (and 14.2-fold compared to wild-type mice), the C_{max} 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 $AUC_{i.v.}$ was 1.3-fold higher in *Mrp2*$^{-/-}$ mice than in wild-type mice (Fig. 1B, Table 1). A similar 1.3-fold increase in $AUC_{i.v.}$ was found for *Mdr1a/1b*$^{-/-}$ 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 $AUC_{i.v.}$ 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.

	Strain			
	Wild-type	*Mdr1a/1b*$^{-/-}$	*Mrp2*$^{-/-}$	*Mdr1a/1b/Mrp2*$^{-/-}$
Oral				
$AUC_{(0-8)}$, hr.mg/l	0.44 ± 0.19	3.75 ± 0.38***	0.40 ± 0.08	6.23 ± 0.60***/†
C_{max}, mg/l	0.13 ± 0.11	1.05 ± 0.19***	0.12 ± 0.04	1.53 ± 0.19***/†
$t_{½, el}$, hr	1.96 ± 0.28	1.69 ± 0.16	1.74 ± 0.11	2.42 ± 0.28*/†
i.v.				
$AUC_{(0-8)}$, hr.mg/l	5.57 ± 0.26	7.08 ± 0.31*	7.33 ± 0.34*	9.41 ± 0.57**
C_{max}, mg/l	6.17 ± 0.21	7.09 ± 0.30	6.89 ± 0.89	6.17 ± 0.68
$t_{½, el}$, hr	1.65 ± 0.11	1.79 ± 0.10	1.61 ± 0.11	2.08 ± 0.12*
Cl, l/hr.kg	1.80 ± 0.08	1.41 ± 0.06*	1.36 ± 0.06*	1.06 ± 0.06**
F, %	7.9 ± 3.4	53.0 ± 5.8**	5.5 ± 1.1	66.2 ± 7.5**

$AUC_{(0-8)}$, area under plasma concentration-time curve up to 8 hr; C_{max}, maximum plasma levels; $t_{½, el}$, 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*$^{-/-}$ 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.

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

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 *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 *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 *Mdr1a/1b*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ mice, less than 2% was excreted in the feces. For *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 *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 6α-hydroxypaclitaxel in intact mice after i.v. administration of [^{3}H]paclitaxel at 10 mg/kg.

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

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 6α-hydroxypaclitaxel. In *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 [^{3}H]paclitaxel at 5 mg/kg.

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

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*$^{-/-}$, and especially *Mrp2*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ 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. [^{3}H]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*$^{-/-}$ mice did not show a significant reduction in biliary excretion of paclitaxel, in line with previous findings (5, 15). In contrast, in *Mrp2*$^{-/-}$ mice biliary excretion of paclitaxel was reduced by 80% compared to wild-type mice, whereas in *Mdr1a/1b/Mrp2*$^{-/-}$ 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*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ 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*$^{-/-}$ mice about 20% of the radioactive dose was recovered in bile, whereas this was only ~4% in *Mrp2*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ 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*-hydroxypaclitaxel and 6α-hydroxypaclitaxel, 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 (*ABCB1*) were evaluated, but could not explain the substantial interindividual variability in paclitaxel pharmacokinetics

(31). 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.

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