Multidrug Resistance Protein 2 Is an Important Determinant of Paclitaxel Pharmacokinetics

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Abstract

Purpose: P-glycoprotein (P-gp; *ABCB1*) efficiently transports lipophilic amphipathic drugs, including the widely used anticancer drug paclitaxel (Taxol). We found previously that human multidrug resistance protein 2 (MRP2; *ABCC2*) also transports paclitaxel *in vitro*, and although we expected that paclitaxel pharmacokinetics would be dominated by P-gp, the effect of Mrp2 was tested *in vivo*.

Experimental Design: We generated and characterized *Mdr1a/1b/Mrp2*$^{-/-}$ mice, allowing assessment of the distinct roles of Mrp2 and Mdr1a/1b P-gp in paclitaxel pharmacokinetics.

Results: Surprisingly, the effect of Mrp2 on i.v. administration of paclitaxel was as great as that of P-gp. The area under plasma concentration-time curve $(AUC)_{i.v.}$ in both *Mrp2*$^{-/-}$ and *Mdr1a/1b*$^{-/-}$ mice was 1.3-fold higher than in wild-type mice, and in *Mdr1a/1b/Mrp2*$^{-/-}$ mice, a 1.7-fold increase was found. In spite of this similar effect, 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: Thus far, 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 anticancer drugs, even in the presence of other efficient transporters. Variation in MRP2 activity might thus directly affect the effective exposure to paclitaxel, on i.v. administration, but also on oral administration, especially when P-gp activity is inhibited.

ATP-binding cassette multidrug transporters, such as P-glycoprotein (P-gp; *ABCB1*), BCRP (*ABCG2*), and multidrug resistance protein 2 (MRP2; *ABCC2*), can have an important effect 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 done, 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 gastrointestinal 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 or GF120918 (Elacridar; refs. 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

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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 show 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 effect 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). [^{3}H]Paclitaxel (4.8 Ci/mmol) was from Moravek Biochemicals (Brea, CA). Paclitaxel metabolites 3′-*p*-hydroxypaclitaxel and 6α-hydroxypaclitaxel were purified from patients' feces as described (21) or purchased from Gentest Corp. (Woburn, MA). Ketamine (Ketanest-S) was from Pfizer (Cappelle a/d IJssel, the Netherlands). Xylazine was from Sigma Chemical Co. (St. Louis, MO). Methoxyflurane (Metofane) was from Medical Developments Australia (Springvale, Victoria, Australia). Heparin (5,000 IE/mL) was from Leo Pharma BV (Breda, the Netherlands). Bovine serum albumin, Fraction V, was from Roche (Mannheim, Germany). The organic solvents methanol, acetonitril [both high-performance liquid chromatography (HPLC) grade], and diethyl ether were from Merck (Darmstadt, Germany). Drug-free 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 (WT) mice, all with 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 cycle 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, 6 mg/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 minutes and 1, 2, 4, 6, and 8 hours using heparinized capillary tubes (Oxford Labware, St. Louis, MO). Blood samples were centrifuged at 2,100 × *g* for 10 minutes at 4°C, and the plasma fraction was collected, completed to 200 μL with human plasma, and stored at −20°C until analysis. For i.v. 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 minutes and 1, 2, 4, and 8 hours after paclitaxel administration, with three to four animals per time point. Blood samples were centrifuged at 2,100 × *g* for 10 minutes at 4°C, and 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/animal), was injected into a tail vein. Feces and urine were collected over a 24-hour period; urine was diluted 5-fold with human plasma and feces were homogenized in 4% bovine serum albumin (1 mL/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 anesthetized by i.p. injection of a combination of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), in a volume of 4.33 μL/g body weight. After opening the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex Ltd., Hythe, United Kingdom), with an inner diameter of 0.28 mm, was inserted into the incised gall bladder and fixed with an additional ligation. Bile was collected for 60 minutes after i.v. injection of paclitaxel. For gall bladder cannulation experiments, 5 mg/kg were 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% bovine serum albumin; intestinal contents were separated from intestinal tissues before 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 liquid chromatography-mass spectrometry/mass spectrometry 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 mol/L 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 WT, *Mdr1a/1b*$^{-/-}$, *Mrp2*$^{-/-}$, and *Mdr1a/1b/Mrp2*$^{-/-}$ mice (n = 6, males and females) were done 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.

Hematologic analysis. Hemoglobin, haematocrit, mean corpuscular volume, RBC, WBC, lymphocytes, monocytes, granulocytes, and platelets were determined in EDTA blood on a Beckman Coulter (Miami, FL) Ac·T Diff analyzer.

Pharmacokinetic calculations and statistical analysis. Pharmacokinetic variables were calculated by noncompartimental methods using the software package WinNonlin Professional, version 5.0. The area under plasma concentration-time curves (AUC) were calculated using

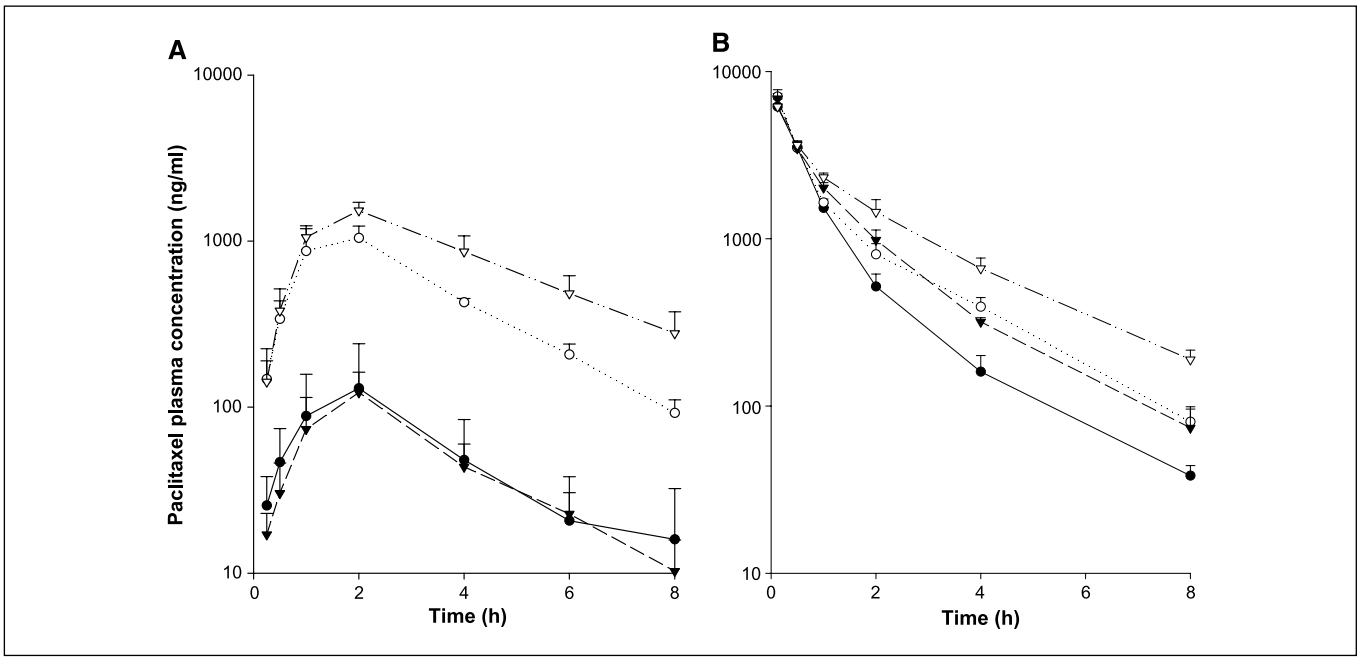

Fig. 1. Plasma concentration-time curves of paclitaxel in male FVB WT (●), *Mdr1a/1b*$^{-/-}$ (○), *Mrp2*$^{-/-}$ (▼), and *Mdr1a/1b/Mrp2*$^{-/-}$ (▽) mice after oral (*A*) and i.v. (*B*) administration of paclitaxel at a dose of 10 mg/kg. Points, mean concentrations for oral (n = 5-6) and i.v. administration (n = 3-4); bars, SD.

the trapezoidal rule, without extrapolating to infinity. Elimination half-lives ($t_{1/2,\ el}$) were calculated by linear regression analysis of the log-linear part of the plasma concentration-time curves. Plasma clearance after i.v. paclitaxel administration was calculated by the formula plasma clearance = 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 with data obtained with WT mice, unless stated otherwise. Differences were considered statistically significant when $P < 0.05$. Data are presented as mean ± 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*$^{-/-}$ versus 4.8 ± 0.3% in WT, 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% to 50% of WT 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 with WT mice, which could be attributed to elevated levels of conjugated bilirubin (3.2 ± 1.6 μmol/L in males and 2.7 ± 0.8 μmol/L in females, n = 6). Conjugated bilirubin levels in WT plasma were below the detection limit (<1 μmol/L). Conjugated and total bilirubin levels in *Mdr1a/1b/Mrp2*$^{-/-}$ mice were not significantly different from those in *Mrp2*$^{-/-}$ mice. The other clinical-chemical variables measured in plasma (see Materials and Methods) showed no significant differences between WT and *Mdr1a/1b/Mrp2*$^{-/-}$ mice.

Hemoglobin levels were moderately but significantly decreased in both male and female *Mdr1a/1b/Mrp2*$^{-/-}$ mice [males, 7.0 ± 0.1 mmol/L in knockout mice versus 7.4 ± 0.1 mmol/L in WT mice, n = 3-4 (P = 0.017); females, 7.2 ± 0.5 mmol/L in knockout mice versus 7.6 ± 0.1 mmol/L in WT mice, n = 5-6 (P = 0.016)]. These results are qualitatively similar to those for *Mrp2*$^{-/-}$ mice. None of the other hematologic variables measured revealed significant differences between WT 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 pharmacologic analyses.

Effect 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 i.v. plasma pharmacokinetics in WT, *Mrp2*$^{-/-}$, *Mdr1a/1b*$^{-/-}$, and *Mdr1a/1b/Mrp2*$^{-/-}$ mice. On oral administration of 10 mg/kg paclitaxel, plasma concentrations and AUC_{oral} were not different between *Mrp2*$^{-/-}$ and WT mice (Fig. 1A; Table 1). For *Mdr1a/1b*$^{-/-}$ mice, the AUC_{oral} was ~8.5-fold higher, in line with previous results (5, 6), but the $t_{1/2,\ el}$ 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 with *Mdr1a/1b*$^{-/-}$ mice (and 14.2-fold compared with WT mice), the maximum plasma level (C_{max}) was 1.5-fold increased, and a 1.4-fold extended $t_{1/2,\ el}$ was found ($P < 0.01$ for each variable; 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 effect on oral paclitaxel plasma pharmacokinetics.

The relative effect of Mrp2 versus P-gp was even more pronounced after i.v. administration of paclitaxel. The $AUC_{i.v.}$ was 1.3-fold higher in *Mrp2*$^{-/-}$ mice than in WT mice (Fig. 1B; Table 1). A similar 1.3-fold increase in $AUC_{i.v.}$ was found for

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

	Strain			
	WT	*Mdr1a/1b*$^{-/-}$	*Mrp2*$^{-/-}$	*Mdr1a/1b/Mrp2*$^{-/-}$
Oral				
$AUC_{(0\text{-}8)}$ (h.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*†
$t_{1/2,\ el}$ (h)	1.96 ± 0.28	1.69 ± 0.16	1.74 ± 0.11	2.42 ± 0.28†‡
i.v.				
$AUC_{(0\text{-}8)}$ (h.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
$t_{1/2,\ el}$ (h)	1.65 ± 0.11	1.79 ± 0.10	1.61 ± 0.11	2.08 ± 0.12‡
Cl (L/h.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§

NOTE: $t_{1/2,\ el}$ is calculated from 2 to 8 hours for both oral and i.v. administration. Data are mean ± SD, n = 5-6 for oral and n = 3-4 for i.v. administration.
Abbreviations: $AUC_{(0\text{-}8)}$, AUC up to 8 hours; Cl, plasma clearance; *F*, oral bioavailability.
*P < 0.001, compared with WT mice.
†P < 0.01, compared with *Mdr1a/1b*$^{-/-}$ mice.
‡P < 0.05, compared with WT mice.
§P < 0.01, compared with WT mice.

Mdr1a/1b$^{-/-}$ mice, consistent with our previous results (5, 6). This similarity in effect of Mrp2 and P-gp on paclitaxel plasma levels after i.v. administration is striking because 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 WT mice and a significantly prolonged $t_{1/2,\ el}$ (Fig. 1B; Table 1).

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 hours after i.v. administration of paclitaxel at 10 mg/kg were below the limits of detection in WT 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 hours (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.

Effect 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). In WT mice, 68.2% of the radioactivity was recovered from the feces. 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 variable). For urinary excretion of radioactivity, a reverse pattern was found, ranging from 3.3% in WT mice to 27.1%

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

Biological matrix	Compound	Strain			
		WT	*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
	6-α-Hydroxypaclitaxel	ND	0.6 ± 0.7	1.0 ± 0.7	4.4 ± 1.5
Liver (% 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

NOTE: 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).
Abbreviation: ND, not detectable.
*P < 0.01, compared with WT mice.
†P < 0.05, compared with WT mice.
‡P < 0.001, compared with WT mice.

Table 3. Cumulative fecal and urinary excretion (0-24 hours) of paclitaxel, 3′-*p*-hydroxypaclitaxel, and 6α-hydroxypaclitaxel in intact mice after i.v. administration of [^{3}H]paclitaxel at 10 mg/kg

Biological matrix	Compound	Strain			
		WT	*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*
	6-α-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
	6-α-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*

NOTE: Excretion is given as percentage of the dose (mean ± SD, n = 5).
Abbreviation: ND, not detectable.
*P < 0.001, compared with WT mice.
†P < 0.01, compared with WT mice.
‡P <0.05, compared with WT mice.

in *Mdr1a/1b/Mrp2*$^{-/-}$ mice. The combined radioactivity data revealed a shift from almost exclusively fecal excretion in WT mice to roughly equal fecal and urinary excretion in *Mdr1a/1b/Mrp2*$^{-/-}$ mice.

HPLC-UV analyses showed that fecal excretion of unmodified paclitaxel in WT mice was 49% of the administered dose (Table 3). In *Mdr1a/1b*$^{-/-}$ and *Mdr1a/1b/Mrp2*$^{-/-}$ mice, <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 (~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.

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). WT 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 with WT 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 WT 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 seemed to be responsible for nearly all of the fecal excretion of the metabolites in the *Mdr1a/1b*$^{-/-}$ mice.

In the urine of *Mdr1a/1b*$^{-/-}$ mice 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.

Effect of Mrp2 and P-gp on biliary and direct intestinal excretion of paclitaxel and its hydroxylated metabolites. We did 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 WT 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 with WT 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 because, in WT and *Mdr1a/1b*$^{-/-}$ mice, ~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-hour gall

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

Biological matrix	Compound	Strain			
		WT	*Mdr1a/1b*$^{-/-}$	*Mrp2*$^{-/-}$	*Mdr1a/1b/Mrp2*$^{-/-}$
Plasma*	Paclitaxel	546 ± 43	534 ± 65	825 ± 128†	837 ± 99†
	[^{3}H] label	936 ± 94	1,068 ± 160	1,324 ± 126†	1,532 ± 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
	6-α-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§
	6-α-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‡
SIC	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‡
	6-α-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‡

NOTE: Levels are given as percentage of the dose (mean ± SD, n = 4-6).
Abbreviations: ND, not detectable; SIC, 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.
$^{\dagger}P < 0.01$, compared with WT mice.
$^{\ddagger}P < 0.001$, compared with WT mice.
$^{\S}P < 0.05$, compared with WT mice.

bladder cannulation experiments. Because 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 WT 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, whereas 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 effect 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 physiologic abnormalities, such as increased liver weight, mild conjugated hyperbilirubinemia, reduced bile flow, and a modest decrease in blood hemoglobin levels. No severe deficiencies due to the combination of *Mrp2* and *Mdr1a/1b* knockout were observed. Consequently, the *Mdr1a/1b/Mrp2*$^{-/-}$ mice seem as suitable for pharmacologic analyses as the separate *Mrp2*$^{-/-}$ and *Mdr1a/1b*$^{-/-}$ mice (15, 20). These mice thus provide a powerful tool to study not only redundant or overlapping but also complementary functions of Mrp2 and P-gp in pharmacology, toxicology, and physiology.

Although we had shown previously that paclitaxel is transported by human MRP2 (16), we were surprised to find that the effect of Mrp2 on the pharmacokinetics of paclitaxel after i.v. 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 on oral administration of the drug. However, on i.v. 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 i.v., variation in MRP2 activity might directly affect their effective paclitaxel exposure.

The pronounced effect of P-gp on (oral) paclitaxel pharmacokinetics seems 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 on 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 effect 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, whereas 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 hours, 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 $AUC_{i.v.}$. ($1.3 \times 1.3 = 1.69$, 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 anticancer drug methotrexate (20, 29). Our data show that Mrp2 can also be a major determinant of the pharmacokinetic behavior of a highly lipophilic anticancer drug, even in the presence of other very efficient transporters for this drug. As it is now clear that several other nonanionic and lipophilic (anticancer) drugs, including docetaxel and 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 anticancer 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*, and *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, such as hepatic diseases, renal failure, or exposure to certain drugs, can result in interindividual 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 anticancer drugs than previously realized and this should be taken into account during chemotherapy treatment of patients.

Our study shows that Mrp2 has a marked effect 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 coadministered with paclitaxel or other MRP2 substrate drugs to counteract multidrug resistance in tumors or to improve the oral bioavailability of the anticancer 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 effect of MRP2 on the pharmacokinetic behavior of many anticancer drugs when P-gp is inhibited.

In principle, simultaneous inhibition of P-gp and MRP2 might be used to further increase the oral availability of paclitaxel when desirable. However, to date, no compounds are identified that specifically inhibit the transport of lipophilic amphipathic drugs by MRP2. For instance, although for organic anions several studies in rats show that biliary excretion via Mrp2 can be inhibited by probenecid (33, 34), we found previously that the *in vitro* transport of lipophilic amphipathic anticancer drugs and HIV protease inhibitors by MRP2 was rather stimulated in the presence of probenecid (16, 30).

The mouse models we have generated will provide useful tools to qualitatively assess the pharmacokinetic effect of MRP2 and P-gp 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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