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**P-glycoprotein and Mrp1 collectively protect the bone marrow from vincristine-induced toxicity in vivo**

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Keywords: stem cells; knockout mice; pharmacokinetics

ABC transporter proteins may protect haematopoietic progenitor cells from chemotherapy-induced toxicity. By using an in vitro colony-forming assay, we found that bone marrow of Mdr1ab, Mrp1, Mdr1ab/Mrp1 knockout (KO) mice was two-, five- to 10- and 25-fold, respectively, more sensitive to vincristine than wild-type mice bone marrow. To study the impact of ABC transporters on in vivo bone marrow sensitivity without the added complication of altered pharmacokinetics, we created chimeras of wild-type mice transplanted with bone marrow from wild-type, Mrp1, Mdr1ab or Mdr1ab/Mrp1 KO donor mice. Following a single bolus injection of vincristine, the chimeras transplanted with wild-type or Mdr1ab KO marrow cells showed no reductions in WBC. A significant reduction was observed in Mrp1 KO chimeras, but the most pronounced effect was observed in mice receiving bone marrow from Mdr1ab/Mrp1 KO mice. A pharmacokinetic analysis in wild-type and KO mice showed that the absence of P-gp reduced the body clearance of vincristine, but that no further reduction occurred when Mrp1 was also absent. However, the tissue accumulation of vincristine in tissues of these Mdr1ab/Mrp1 KO mice was further increased. This study demonstrates that the presence of multiple drug transporters protects the bone marrow, and probably other tissues as well, against chemotherapeutic insults.

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**ABC transporters such as P-glycoprotein (P-gp) provide efficient protection against xenobiotics.** As demonstrated by using knockout (KO) mouse models, P-gp and Breast cancer resistance protein (Bcrp1) limit the uptake from the gastrointestinal tract of substances, such as drugs (Sparreboom et al, 1997; Jonker et al, 2000) or potentially toxic dietary substances (Jonker et al, 1994, 1996). Multidrug resistance-associated protein (Mrp1) KO mice were hypersensitive to etoposide (Lerico et al, 1997; Wijnholds et al, 1997). Mrp1 protects the oropharyngeal mucosal layer and the testicular tubules (Wijnholds et al, 1998) and plays a role in the blood–cerebrospinal fluid barrier (Rao et al, 1999; Wijnholds et al, 2000).

**Myelotoxicity is a common complication when treating cancer patients with chemotherapeutic drugs.** Already in 1991, Chaudhary and Roninson (1991) showed that P-gp was present in haematopoietic progenitor cells, suggesting that this transporter might also be involved in the protection of bone marrow stem cells. The role of drug transporters in bone marrow-derived cells under in vitro conditions was further addressed in several studies in vitro. Mdr1ab P-gp contributed to the extrusion of the fluorophore rhodamine 123 from haematopoietic progenitor cells (Schinkel et al, 1997). Moreover, it was recently shown that Bcrp1 is the transport protein responsible for the extrusion of the marker dye Hoechst 33342 (Zhou et al, 2001; Kim et al, 2002; Scharenberg et al, 2002). Flow cytometry cell sorting of the fraction of Hoechst 33342 dull cells resulted in a highly enriched fraction of primitive haematopoietic progenitor cells. Subsequent RT-PCR analyses of RNA from these so-called side-population (SP) cells revealed the presence of Mrp1, Mrp3 and Mrp4 but not Mrp2 (Zhou et al, 2001).

The role of these drug transporters in vivo is currently under investigation. Bcrp1 appears to protect the bone marrow from mitoxantrone-induced toxicity (Zhou et al, 2002). Johnson et al (2001) showed that mice with compound disruptions of the Mdr1a, Mdr1b and the Mrp1 genes (Mdr1ab/Mrp1 triple KO mice) were about 128-fold more sensitive for i.p. vincristine. Their results suggested that the bone marrow might be involved in the toxicity profile, but it remained unclear whether the toxicity was directly due to the absence of these drug transporters in the haematopoietic progenitor cells or whether it was also, or even merely, a consequence of a reduced drug clearance in these Mdr1ab/Mrp1 triple KO mice. The pharmacokinetics of vincristine has not been documented, but it is well established that the absence of P-gp reduces the clearance of substrate drugs, such as vinblastine (van Asperen et al, 1996).

In the present study, we have investigated the role of P-gp and Mrp1 in the protection of the bone marrow in vitro and in vivo. We have used a bone marrow transplantation model to avoid complications in data interpretation due to differences in drug clearance. Wild-type mice receiving whole-body irradiation at a dose that was lethal to the bone marrow were transplanted with...
bone marrow from donor mice deficient in \( Mdr1ab \) and/or \( Mrp1 \) genes. Animals transplanted with wild-type bone marrow were used as control group. After their full recovery, the mice were exposed to the anticancer drug vincristine and the bone marrow toxicity was determined by serial analyses of haematology parameters. In parallel experiments, the pharmacokinetics and toxicology of vincristine in the wild-type and \( Mdr1ab/Mrp1 \) triple KO mice were established using a selective high-performance liquid chromatographic (HPLC) assay.

**MATERIALS AND METHODS**

**Animals**

Male wild-type, \( Mrp1 \) knockout (Wijnholds et al, 1997), \( Mdr1ab \) double knockout (\( Mdr1ab \) DKO; animals with compound deletion of \( Mdr1a \) and \( Mdr1b \) genes) (Schinkel et al, 1997) and \( Mdr1ab/ Mrp1 \) triple knockout (\( Mdr1ab/Mrp1 \) TKO) were used. The latter were obtained by crossingbreeding of \( Mrp1 \) KO and \( Mdr1ab \) DKO mice. All mouse strains were backcrossed for at least seven generations to obtain a more than 99% homogeneous FVB background. The animals were given food (Hope Farms BV, Woerden, The Netherlands) and acidified water \( ad \ libitum \). They were handled according to the institutional guidelines, which are based on Dutch law and conform the standards required by the UKCCCR guidelines (Workman et al, 1998). The animal experiment committee of the Institute approved the experiments described in this paper.

**Determination of the maximum tolerated dose (MTD)**

Vincristine (Pharmachemie, Haarlem, The Netherlands; 1 mg ml\(^{-1} \)) was diluted in saline and administered i.v. to wild-type and \( Mdr1ab/Mrp1 \) TKO mice, aged 10–14 weeks, at dose levels ranging between 0.125 and 4 mg kg\(^{-1} \). Animals were monitored daily and killed when they lost more than 20% of their initial body weight. The MTD was defined as one dose step below the dose where more than one animal in that group had to be killed. Necropsies were performed in wild-type and \( Mdr1ab/Mrp1 \) TKO mice receiving vincristine at or near the MTD and killed 2 days later.

**Pharmacokinetics**

The pharmacokinetic behaviour of vincristine in wild-type, \( Mdr1ab \) KO and \( Mdr1ab/Mrp1 \) TKO mice, 10–14 weeks of age, was established at a dose of 1 mg kg\(^{-1} \). Animals were killed at 5, 15, 30 min, 1, 2 and 4 h after drug administration for collection of plasma. At 1 and 4 h, a range of tissues was also collected. Vincristine levels in plasma and tissues were analysed by HPLC developed by us previously (Boven et al, 1999). Plasma AUC\(_{0\rightarrow 4h} \) (area under the curve) values were calculated by the linear trapezoidal rule using standard equations (van Asperen et al, 1996) and the clearance was calculated as Dose/AUC\(_{0\rightarrow 4h} \). Statistical tests were performed with SPSS v11.0 (SPSS Inc., Chicago, IL, USA).

**In vitro bone marrow toxicity**

The toxicity of vincristine in haematopoietic progenitor cells was tested by an in vitro colony forming unit (CFU) assay. Mouse bone marrow progenitor cells were obtained from the femurs of FVB mice flushed with Dulbecco’s phosphate-buffered saline solution. After centrifugation (10 min, 200 g, ambient temperature), the cell pellet was resuspended in Iscoves medium (MM) (StemCell Technologies Inc., Vancouver, BC, Canada) with 2% (v/v) fetal calf serum (FCS). Nucleated bone marrow cells were seeded in Methocult GF M3534 at a density of 2.5 × 10\(^{5} \) cells ml\(^{-1} \) and vincristine was added at concentrations ranging from 2.5 to 100 ng ml\(^{-1} \). Aliquots of 1 ml were plated in duplicate in uncoated six-wells culture plates (Greiner, Alphen a/d Rijn, The Netherlands) and incubated for 5–6 days at 37°C in 5% CO\(_{2} \) in humidified air. CFUs were scored by phase-contrast light microscopy. We have used the concentration that reduces the number of colonies by more than 90% relative to untreated controls (IC\(_{50} \)) instead of IC\(_{50} \) because the decreasing size of the colonies at higher drug concentrations makes accurate scoring of their numbers difficult. Hardly any colonies were present at the IC\(_{50} \) making this parameter easier to determine.

**In vivo bone marrow toxicity**

Wild-type mice of about 5–6 weeks of age received whole-body irradiation of 6.8 Gy (HP320 Radiobiology Constant Potential X-ray Unit, Pantak, East Haven, CT, USA). The next morning each mouse was i.v. injected with 1.5 to 3 × 10\(^{6} \) nucleated bone marrow cells from donor mice of wild-type, \( mrp1 \) KO, \( Mdr1ab \) DKO or \( Mdr1ab/Mrp1 \) TKO genotype. After a recovery period of 6 weeks, the mice received an i.v. bolus injection of 2 mg kg\(^{-1} \) of vincristine via the tail vein. To minimise the effects of mild dehydration occurring at this dose level of vincristine, mice were supported by daily i.p. administrations of 1 ml of saline: dextrose 5% (1 : 1; v/v) for 3 days. At days 0 (before vincristine), 2, 4, 7 and 12, peripheral blood was sampled from the tail and haematologic parameters (WBC and Haemoglobin (Hb)) were determined on a Cell Dyn 1200 analyzer (Abbott Laboratories, Santa Clara, CA, USA). Experiments were performed on six different occasions. At some of these occasions, the mice receiving bone marrow of TKO mice were also challenged with vincristine at lower dose levels (e.g. 0.5 or 1 mg kg\(^{-1} \)). Bone marrow was obtained at the end of the experiments from a number of randomly selected mice and used to determine the in vitro sensitivity of the grafted bone marrow using the in vitro bone marrow toxicity test.

**PCR analyses**

As a random test, the genotype of the engrafted bone marrow was verified by PCR analyses in about 50% of all animals. DNA was prepared from whole-blood samples of recipient animals using the DNeasy kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s protocol. Disruption of genes in the KO mice strains was achieved by replacement of relevant genomic fragments by a selection gene, for example, hygromycin in case of \( Mdr1a \) (4). As a result, PCR verification of the \( Mdr1a \) KO genotype was verified by the presence and absence of bands for hygromycin and \( Mdr1a \), respectively.

**RESULTS**

We first examined the relative sensitivities of bone marrow derived from wild type, \( Mrp1 \) KO, \( Mdr1ab \) DKO and \( Mdr1ab/Mrp1 \) TKO mice using an in vitro CFU assay. Cell kill occurred in a dose-dependent manner. The bone marrow of wild-type animals was most resistant to vincristine (Table 1). \( Mdr1ab \) DKO bone marrow cells were about two-fold more sensitive, whereas \( Mrp1 \) KO cells were about five to 10-fold more sensitive. The absence of both drug transporters in cells from the \( Mdr1ab/Mrp1 \) TKO mice resulted in an about 25-fold higher susceptibility towards vincristine. These experiments were repeated several times and although the absolute IC\(_{50} \) values varied with different batches of culture medium, the relative differences in IC\(_{50} \) between the bone marrow of the various genotypes were consistent throughout these experiments.

We next investigated the MTD of vincristine in our mouse strains (Table 2). Based on our previous experience, we selected a few dose levels in wild-type animals (1, 2 and 4 mg kg\(^{-1} \)). The MTD in wild-type mice was 2 mg kg\(^{-1} \), where the mice experienced mild but clear
Toxic effects of vincristine in wild-type and Mdr1ab/Mrp1 TKO mice were assessed by full necropsy of the animals 2 days after they received vincristine at their respective MTD. In Mdr1ab/Mrp1 TKO mice receiving 0.5 mg kg\(^{-1}\), two out of four animals had to be killed (at days 4 and 5) because their body weight dropped by more than 20%, whereas the two others survived. These results indicate that there is a moderate increase in vincristine toxicity in animals lacking both these drug-transporting proteins.

We further investigated the pharmacokinetics of vincristine given at a dose of 1 mg kg\(^{-1}\) to wild-type, Mdr1ab DKO and Mdr1ab/Mrp1 TKO mice by sampling of blood and tissues for up to 4 h after drug administration. Although this dose level was above the MTD in Mdr1ab/Mrp1 TKO mice, it was selected because toxic effects are relatively mild during the first 4 h and because of the detection limit of the assay (5 ng ml\(^{-1}\) for a 200 µl plasma sample). In line with the toxicity results there was only a moderate reduction in the plasma clearance of vincristine (Figure 1), and this appears to be due mainly to the absence of P-gp, since the plasma concentration–time curves of Mdr1ab DKO and Mdr1ab/Mrp1 TKO mice are overlapping. The plasma clearance in wild-type mice was 16.2 ± 1.0 µg ml\(^{-1}\) h and was significantly \((P<0.01)\) reduced to 12.0 ± 0.6 and 12.1 ± 0.5 µg ml\(^{-1}\) h in Mdr1ab DKO and Mdr1ab/Mrp1 TKO mice, respectively. Similar as in plasma, the vincristine levels observed in tissues of Mdr1ab DKO were significantly higher than in wild-type mice (Figure 2). Interestingly, however, the levels of vincristine in several tissues of Mdr1ab/Mrp1 TKO were again significantly higher than those in Mdr1ab DKO, despite equivalent plasma levels, suggesting that Mrp1 offers additional protection against vincristine accumulation in these tissues. The vincristine levels in the brain were below the limit of detection in all animals.

To avoid the complicating effects of these transporters on the pharmacokinetics of vincristine, we investigated the consequences of the absence of the various transporters on the bone marrow.

**Table 1** Toxicity of bone marrow progenitor cells \((in vitro)\)

| Conc. (ng ml\(^{-1}\)) | Wild type | Mrp1 KO | Mdr1ab DKO | Mdr1ab/Mrp1 TKO |
|------------------------|-----------|---------|-------------|-----------------|
| 100                    | −         | −       | −           | −               |
| 50                     | +         | −       | −           | −               |
| 25                     | +         | +       | −           | −               |
| 10                     | +         | +       | ND          | −               |
| 5                      | ND        | +       | ND          | ±               |
| 2                      | ND        | ND      | ND          | +               |
| 1                      | ND        | ND      | ND          | +               |

Bone marrow cells harvested from wild-type, Mrp1 KO, Mdr1ab DKO and Mdr1ab/Mrp1 TKO mice were incubated continuously with various concentrations of vincristine. Colonies were scored by day 5. Wells were scored with – when the number of colonies was reduced by more than 90% relative to control wells without the drug \((IC_{90})\). ± indicates a borderline value, whereas clearly less than 90% reduction is marked by a + sign. ND means not determined.

**Table 2** Toxicity of vincristine in mice

| Dose (mg kg\(^{-1}\)) | Number of animals | Toxic deaths | Decrease in body weight |
|-----------------------|-------------------|--------------|------------------------|
| **Mdr1ab/Mrp1 TKO**   |                   |              |                        |
| 0.125                 | 9                 | 0            | 1.9 ± 0.9              |
| 0.25                  | 13                | 0            | 3.0 ± 0.7              |
| 0.5                   | 9                 | 0            | 10.3 ± 1.4             |
| 1                     | 4                 | 2            | 20.5 ± 2.7             |
| **Wild type**         |                   |              |                        |
| 1                     | 5                 | 0            | 4.6 ± 0.6              |
| 2                     | 5                 | 1            | 12.1 ± 0.9             |
| 4                     | 5                 | 5            | 23.1 ± 2.0             |

Animals received vincristine by i.v. injection. Body weight was determined daily. Animals experiencing more than 20% body weight loss were killed and counted as toxic deaths. The size of, especially, the highest dose groups was kept as low as possible in order to minimise the number of animals experiencing major discomfort.
**DISCUSSION**

This study using an *in vivo* model shows that the ABC-transporters P-gp and Mrp1 protect haematopoietic progenitor cells in the bone marrow from vincristine toxicity. The most significant effects in WBC and Hb values were observed in mice grafted with bone marrow from *Mdr1ab/Mrp1* TKO donor mice. This is probably due to the high sensitivity of the PCR assay, which detects small quantities of host genomic DNA in the blood. It was estimated that up to 5% of circulating nucleated cells were of wild-type origin.

**Figure 2** Tissue accumulation of vincristine. Mice receiving 1 mg kg\(^{-1}\) were killed at 1 and 4 h after drug administration. Drug levels were determined by HPLC. Each bar represents at least four animals and the error bar depicts the s.e. Statistical analyses were performed by ANOVA using Bonferroni post hoc test for multiple comparisons. \* *P* < 0.05 relative to wild-type; \**P* < 0.005 relative to *Mdr1ab* DKO, otherwise not significant.
or wild-type donor mice. Thus, in the case of vincristine, the two transporters appear to function in concert in bone marrow progenitor cells and the alternate transporter can partly or completely compensate the loss of function of the other. Overall, the lethality in Mrp1 KO or wild-type bone marrow. This shows that the increased haematotoxicity after single high-dose vincristine treatment was not a dose-limiting event per se.

A pharmacokinetic analysis of vincristine showed that, relative to wild-type mice, the plasma clearance of vincristine was reduced in Mrp1 KO but not further reduced in Mrp1 TKO mice. This result is in line with the location of P-gp in apical membranes of excretory organs (liver, intestines, kidneys) (Thiebaut et al, 1987), where it is involved in detoxification by extrusion of substrates from the body, whereas Mrp1 is mainly located in basolateral membranes and has relatively little direct effect on drug excretion from the body (Borst et al, 1999; Hipfner et al, 1999). However, this study shows that the accumulation of vincristine in tissues from Mrp1 TKO mice was clearly enhanced relative to Mrp1 DKO mice showing that Mrp1 offers protection to tissues. A previous study in Mrp1 KO mice did not find any effects on tissue distribution of etoposide (Wijnholds et al, 1997). This discrepancy may be (partly) due to the presence of P-gp in these mice, which may be such a dominant factor that it conceals the effects of a loss of Mrp1. However, it is also possible that the analytical methodology (determination of total radioactivity after administration of radiolabelled drug) was not suited to find these differences, because it cannot discern unchanged substrate drug from (radiolabelled) metabolites and/or degradation products. We have used a selective HPLC to determine unchanged vincristine levels. The higher drug levels in tissues correlates with previous reports of local organ toxicities when Mrp1 KO mice were challenged with chemotherapeutics (Wijnholds et al, 1998). It thus seems likely that P-gp located at the apical side and Mrp1 at the basolateral side of epithelia cooperate in limiting the accumulation of compounds that are substrates of both transporters, such as vincristine, whereas they are less efficient for compounds that are substrate of only one of them. Moreover, their concerted activity has also been shown in cells expressing these transporters in a nonpolarised fashion. By using immortalised fibroblast cell lines, it was found that both Mrp1 and Mrp1 are implicated in innate resistance to cytotoxic substrates (Allen et al, 2000; Lin et al, 2002), but the strongest effects were found in cells lacking both transporters. Our findings of a markedly enhanced toxicity by vincristine in the in vitro bone marrow toxicity assays are in line with these results.

The dose-limiting toxicity of vincristine in Mrp1 TKO mice appears to be related to the effects on the gastrointestinal tract. We found that the Mrp1 TKO mice were only about four-fold more sensitive to vincristine than wild-type mice, which is much less than the 128-fold reported previously (Johnson et al,
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2001). It is unlikely that the different routes of administration in the two studies (i.p. vs i.v.) would explain this discrepancy. In our experience, the clearance of hydrophobic drugs from the peritoneal cavity of mice occurs rapidly. Moreover, both wild-type and Mdr1ab/Mrp1 TKO mice received vincristine by the same route, and the results for wild-type mice were consistent between the two studies. The difference may be explained by differences in drug elimination between animals in the two studies. In addition to elimination by excretion, metabolism is also a very important route for many drugs including vincristine. It has previously been shown that Mdr1a KO mice maintained in our institute have a higher expression of cytochrome P450 isozymes than mice of similar genotype kept in the United States (Schuetz et al., 2000). Consequently, clearance of vincristine might occur more rapidly in our populations of Mdr1ab DKO and Mdr1ab/Mrp1 TKO mice, rendering them less susceptible to vincristine.

To eliminate the effects of reductions in body clearance of vincristine on bone marrow toxicity, we have used a bone marrow transplantation model yielding chimeras that were of identical wild-type genotype, except for their bone marrow progenitor cells. The course of the WBC counts in the Mdr1ab/Mrp1 TKO chimeras closely resembled the pattern that is usually observed in patients experiencing chemotherapy-induced myelotoxicity. The nadir and return to baseline levels occurred somewhat more abruptly, but this is most likely due to the fact that most physiological processes in mice proceed at a higher velocity than in humans. Interestingly, the nadir was about similar in Mdr1a KO chimeras, but there was a very strong rebound in WBC counts by day 7. This difference between Mdr1 KO and Mdr1ab/Mrp1 TKO chimeras may be due to the fact that the relative expression of ABC transporters appears to vary along the lineage from uncommitted stem cells to mature blood cells. In mice, CD34+ sorted SP cells appear to represent a more primitive subpopulation of progenitor cells than CD34+ cells (Osawa et al., 1996) and these cells contain relatively high levels of Bcrp1 and Mdr1 mRNA, whereas Mrp1 appears to be higher in the murine CD34+ subpopulation of cells (Zhou et al., 2001). The absence of Mrp1 will make this population of CD34+ progenitor cells more vulnerable to vincristine. A single bolus of a high dose of vincristine given to Mrp1 KO chimeras may significantly reduce the numbers of these precursor cells, thus eliminating part of the maturing blood cells from the pipeline. The decline in WBC will trigger signalling to induce a compensatory wave of haematopoiesis and because the population of primitive CD34+ precursor cells is probably not so much affected due to protection by P-gp, this can still occur effectively. In Mdr1ab/Mrp1 TKO chimeras, however, this more primitive population of CD34+ cells is no longer protected by P-gp and may therefore be more vulnerable to vincristine, thus causing further delay in WBC recovery.

In this study, we have shown that a single dose of vincristine resulted in a clear but nonlethal bone marrow toxicity, which was Mrp1 and P-gp dependent. Repeated dosing or continuous infusion of this G2–M cell cycle specific drug might have resulted in greater bone marrow toxicity. More pronounced cytotoxic effects on these primitive precursor cells have been shown in a recent study, where irradiated mice that were transplanted with mixtures of wild-type and Bcrp1 KO donor bone marrow were challenged with mitoxantrone (Zhou et al., 2002). After five daily repeated injections of 2 mg of mitoxantrone per kilogram, the relative contribution of Bcrp1 null cells in the peripheral blood myeloid and lymphoid compartment declined to very low values and remained low during the many weeks afterwards. In this case with repeated dosing of mitoxantrone, it appears that the majority of primitive Bcrp1 null stem cells have been eradicated and have been replaced by wild-type cells. Given that mitoxantrone is also a substrate of P-gp, we expect that the effect of this drug may be even greater in bone marrow cells with a compound deletion of the Mdr1ab and Bcrp1 alleles.

In conclusion, although the physiological function of P-gp, Mrp1 and other ABC-transporters in haematopoietic progenitor stem cells is still conjectural, it is clear that they protect the cells against chemotherapy-induced injury. Inhibition of their function, for example, as part of drug regimens aimed to sensitize drug-resistant tumour cells, may thus result in enhanced myelotoxicity. However, the fact that multiple transporters with (partly) overlapping substrate specificities are present appears to be a safety mechanism that may render this a relatively infrequent complication.

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