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The Pharmacological Phenotype of Combined Multidrug-Resistance *mdr1a/1b*- and *mrp1*-deficient Mice

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ABSTRACT

Two major classes of plasma membrane proteins that actively extrude a wide range of structurally diverse hydrophobic amphipathic antineoplastic agents from cells, with different mechanisms of action, lead to multidrug resistance. To study the importance of these ATP-binding cassette transporters to the toxicity of cancer chemotherapy agents, we have used mice genetically deficient in both the *mdr1a* and *mdr1b* genes [*mdr1a/1b(−/−)* mice], the *mrp1* gene [*mrp1(−/−)* mice], and the combined genes *mdr1a/1b* and *mrp1* [*mdr1a/1b(−/−)*, *mrp1(−/−)* mice] and embryonic fibroblasts derived from wild-type mice and from the three gene knockout animals. The consequences of export pump deficiencies were evaluated primarily using vincristine and etoposide. Mice deficient in the three genes, *mdr1a/1b* and *mrp1*, exhibited a 128-fold increase in toxicity to vincristine and a 3–5-fold increase in toxicity to etoposide; increased toxicity to embryonic fibroblast cells from triple knockout mice also occurred with vincristine and etoposide. Vincristine, which normally does not express toxicity to the bone marrow and to the gastrointestinal mucosa when used at therapeutic doses, caused extensive damage to these tissues in *mdr1a/1b(−/−)*, *mrp1(−/−)* mice. The findings indicate that the P-glycoprotein and mrp1 are compensatory transporters for vincristine and etoposide in the bone marrow and the gastrointestinal mucosa and emphasize the potential for increased toxicities by the combined inhibition of these efflux pumps.

INTRODUCTION

Resistance of tumor cells to multiple chemotherapeutic agents (MDR)[3] is a major obstacle to the treatment of most human cancers. The phenomenon of MDR confers upon malignant cells the ability to withstand exposure to lethal doses of many structurally unrelated antineoplastic agents. Multidrug resistance has been characterized by the overexpression of membrane-associated glycoproteins; the two most studied of these ABC transporters that have a role in drug efflux are the P-gly, discovered in 1976 by Juliano and Ling (1), and the MRP1, first reported in 1992 by Cole *et al.* (2), which represents a family of ABC transporters. There are at least five additional members of this family, including MRP2 (cMOAT, $ABCC_2$), MRP3 (MOAT-D, $ABCC_3$), MRP4 (MOAT-B, $ABCC_4$), MRP5 (MOAT-C, $ABCC_5$), and MRP6 (MOAT-E, $ABCC_6$). Structural homology within the MRP family is greatest for MRP1, MRP2, MRP3, and MRP6, whereas the structures of MRP4 and MRP5 are similar (Refs. 3–5 and references therein).

The P-gly, expressed by the *MDR1* gene in humans and two closely related genes, *mdr1a* and *mdr1b*, in the mouse, and MRP1 play central roles in export pump-mediated resistance through the active extrusion of a wide range of structurally diverse antineoplastic agents including the *Vinca* alkaloids, the epipodophyllotoxins, and the anthracyclines (3–6). Although the P-gly transports free drugs, MRP1 can be considered to be an organic anion transporter capable of transporting a broad spectrum of organic anion conjugates of glutathione, glucuronic acid, and sulfate. In addition, we have shown with etoposide (7) and Loe *et al.* (8) with vincristine that mrp1 also functions as a cotransporter of xenobiotics and glutathione. In keeping with these findings, we have also demonstrated that levels of GSH in *mrp1(−/−)* mice were elevated by 25–90% in most tissues, especially in those tissues that are known to express high levels of mrp1 (9). That tissue increases in GSH in *mrp1(−/−)* mice were not attributable to the increased synthesis of GSH was supported by the finding that the levels of γ-glutamylcysteine synthase, the rate-limiting enzyme in the synthesis of GSH, was not significantly different in any of the tissues of *mrp1(+/+)* and *mrp1(−/−)* mice (9). Prior to these findings, a variety of studies had provided evidence that GSH was required for the transport of chemotherapeutic agents (see Ref. 3 for appropriate references).

Schinkel *et al.* (10, 11) have derived mice deficient in the *mdr1a* gene [*mdr1a(−/−)*] and, in addition, have derived mice deficient in the *mdr1b* gene [*mdr1b(−/−)*], and in both the *mdr1a* and *mdr1b* genes [*mdr1a/1b(−/−)*]. All three of these gene knockout animals are normal in all measured physiological parameters, displaying normal viability, fertility, and life span, as well as normal levels of a range of serum enzymes, proteins, electrolytes, and hematological parameters.

In clinical trials, the P-gly has often been shown to be elevated in the hematological malignancies, particularly after the failure of multiple drug therapy (12). Thus, the MDR phenotype as a cause of resistance in acute myelocytic leukemia and multiple myeloma and possibly in the late stages of non-Hodgkin's lymphoma and acute lymphocytic leukemia has been documented. The role of *MDR1* gene expression in the clinical resistance of solid tumors, however, is currently not firmly established (13). Nonetheless, in several malignancies, such as acute myelocytic leukemia, various childhood cancers, and advanced breast cancer, overexpression of the *MDR1* gene has been shown to correlate with a poor response in patients receiving cancer chemotherapeutic agents (reviewed in Ref. 14). In colon cancer, renal cell carcinoma, primary breast cancer, and osteosarcoma, clinical studies have shown that P-gly positivity is associated with aggressive tumor behavior and is a strong predictor of treatment outcome. Whether in these instances the P-gly is a marker for drug resistance, tumor aggressiveness, or both is currently unknown.

We (9) and Wijnholds *et al.* (15) have shown that disruption of *mrp1* did not affect the viability or fertility of mice, nor were hematological parameters or levels of serum enzymes, proteins, and electrolytes different in *mrp1(−/−)* and *mrp1(+/+)* mice. However, *mrp1(−/−)* mice were hypersensitive to a relatively large number of anticancer drugs (9, 15, 16). The demonstration that the lack of mrp1 in *mrp1(−/−)* mice led to toxicity to the oropharyngeal mucosa and the seminiferous tubules of the testis in etoposide phosphate-treated animals indicates that mrp1 protects these tissues against damage from mrp1 substrates (17). The expression of *MRP1* has been detected in a number of human cancers and shown to be associated with drug

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[3] The abbreviations used are: MDR, multidrug resistance; ABC, ATP binding cassette; P-gly, P-glycoprotein; MRP1, multidrug resistance (-associated) protein; GSH, glutathione; *mrp1(−/−)*, *mrp1* gene deficiency; *mdr1a/1b(−/−)*, *mdr1a/1b* gene deficiency; VBL, vinblastine; *mdr1a/1b(−/−)*, *mrp1(−/−)*, combined *mdr1a/1b*, *mrp1* gene deficiency; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2*H*-tetrazolium; MTD, maximum tolerated dose.

resistance or reduced patient survival in a variety of tumor types, including lung, breast, colon, and gastric cancers, as well as in the childhood cancers, neuroblastoma and retinoblastoma (18–28).

The P-gly and MRP1 have been targets for the development of inhibitors of transport in an effort to restore sensitivity to neoplastic cells using these export pumps to extrude tumoricidal agents (reviewed in Refs. 29–32). The identification of several agents that have the capacity to inhibit both the P-gly and MRP1 make it conceivable that new serious toxicities may also result from the use of such agents (9, 33–40).

The present report is an effort to further understand the consequences of multiple ABC transporter gene disruption and its impact on antineoplastic agent toxicity. The findings demonstrate that the P-gly and MRP1 are compensatory transporters for vincristine and etoposide and that a functional deficiency in these transporters can produce unexpected serious new toxicities.

MATERIALS AND METHODS

Breeding and Genotyping. *mdr1a/1b(−/−), mrp1(−/−)* triple knockout mice were bred, and their genotypes were confirmed as follows. *mdr1a/1b(−/−)* mice developed by Schinkel *et al.* (10) and obtained from Taconic Farms, Inc. (Germantown, NY) were cross-bred with *mrp(−/−)* mice generated previously in our laboratory (9). The resulting F_1 heterozygous animals were bred, and the F_2 offspring were genotyped for *mrp1* status by Southern blotting, essentially as described previously (9). Briefly, genomic DNA was isolated from peripheral blood (Wizard Genomic DNA Purification kit; Promega Corp., Madison, WI), digested with *Sac*I, subjected to electrophoresis in 1% agarose, then transferred onto a nylon membrane (Biodyne B; Life Technologies, Inc., Gaithersburg, MD), and probed using a 400-bp probe (*Xba*I-*Bam*HI) directed 5′ to the *mrp1*-targeted construct. This procedure yields a diagnostic 5-kb band in the setting of a disrupted *mrp1* allele. Homozygously disrupted mice at the *mrp1* locus were further genotyped to determine their *mdr1a/1b* status by PCR at Taconic Biotechnology, a division of Taconic Farms, Inc. (Rensselaer, NY) using the primer pair 5′-CAGCTCCATCCAACAACTTC-3′ and 5′-GACACAGGTACTGTCCACAG for the *mdr1a* wild-type reaction, generating a 411-bp PCR product, and 5′-ATGTCCTGCGGGTAAATAGC-3′ and 5′-CGTCAGGACATTGTTGGAGC-3′ for the *mdr1a* knockout reaction, yielding a 481-bp PCR product. *mdr1b* genotyping was completed in a single reaction using the primers 5′-GAGAAACCATGTCCTTCCAG-3′, 5′-AAGCTGTGCATGATTCTGGG-3′, 5′-TGTCAAGACCGACCTGTCCG-3′, and 5′-TATTCGGCAAGCAGGCATCG-3′, generating a 540-bp *mdr1b* wild-type allele and a 453-bp PCR product in the presence of a knockout *mdr1b* allele.

Western Blotting. Western blot analyses of mrp1 and mdr1a/1b were performed on protein obtained from cultured mouse embryo fibroblasts using the monoclonal murine mdr1 antibody C219 (Signet, Dedham, MA) at a 1:80 dilution and the monoclonal rat mrp1 antibody MRPr1 (Signet) at a concentration of 0.3 μg/ml. Fibroblasts were grown to near confluence in 25-cm^2 flasks, harvested, and resuspended in Tris-buffered saline [10 mM Tris-HCl (pH 7.6), 0.1 M NaCl, and 1 mM EDTA] containing a mixture of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, and 1 μg/ml of aprotinin), lysed by adding an equal volume of 2× SDS containing gel-loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol], placed in a boiling water bath for 5 min, and vortexed vigorously. Extracts were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBST [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.01% Tween 20] for 30 min, incubated overnight with the respective antibody in TBST containing 5% milk, washed with three changes of TBST for a total of 30 min, and then incubated with horseradish peroxidase-conjugated sheep antimouse IgG or rabbit antirat IgG for 1 h and washed for 30 min with TBST (three changes). The immunoreactive proteins were visualized by the enhanced chemiluminescence method (Amersham Corp., Arlington Heights, IL).

Embryo Fibroblast Cultures. Embryo fibroblast lines were prepared from wild type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice on the 13th or 14th day of pregnancy. The embryos were dissected free of associated membranes, and placentas and were rinsed thoroughly in PBS containing 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. In a new sterile Petri dish, the embryos were finely minced in 2 ml of trypsin/EDTA, incubated at 37°C for 30 min, and then further disrupted by repeated aspiration in a 1-ml syringe. Fifteen ml of DMEM containing 15% FCS were added, the suspensions were transferred to a 50-ml polypropylene tube, and the larger pieces were allowed to settle for 1–2 min. The supernatants were then transferred to clean tubes and centrifuged at 600 × *g* for 5 min, and the resulting pellet was resuspended in 7.5 ml of DMEM containing 15% FCS, plated in 25-cm^2 tissue culture flasks, and incubated at 37°C with 5% CO_2. Cells typically grew to confluency after ~48 h with a change in medium at 24 h to remove cellular debris.

***In Vitro* Drug Sensitivities of Embryo Fibroblasts.** Embryo fibroblasts were grown from frozen stocks and grown as passage 0 in DMEM supplemented with 15% FCS. The cultures were subsequently passaged every 3–4 days, and toxicity experiments were performed on cells up to passage four. Sensitivities to vincristine, etoposide, and paclitaxel (Biomol Plymouth Meeting, PA) were measured in culture using the CellTiter 96 Cell Proliferation Assay (Promega Corp., Madison, WI). Briefly, confluent embryonic fibroblasts were seeded in 96-well plates at 3000 cells/well in complete DMEM and incubated for 24 h, after which drug was added such that the final volume of medium/well was 100 μl. After 96 h of exposure to either vincristine or paxlitaxel or 72 h to etoposide, 20 μl of MTS tetrazolium solution (Promega) were added, and incubations were continued for an additional 2 h at 37°C. The soluble MTS tetrazolium formazan product formed by the dehydrogenase activity of viable cells was quantified at 490 nm using a microplate reader, and the sensitivity to drugs was expressed as a percentage of untreated controls.

***In Vivo* Toxicity of Vincristine and Etoposide.** *In vivo* etoposide phosphate (generously provided by the Bristol-Myers Squibb Co., Princeton, NJ) toxicity using male mice and vincristine toxicity using female mice were determined as follows. Groups of three to six wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* male mice were treated with single i.p. doses of etoposide phosphate ranging from 50 to 200 mg/kg of body weight. In a similar manner, groups of three or six wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* female mice were treated with single i.p. doses of vincristine ranging from 0.03125 to 10 mg/kg of body weight. The survival of treated animals was followed for 30 days. In both cases, six mice were used in treatment groups that were one dose above and below the MTD. The MTD was expressed as the highest dose of drug at which all of the animals of a given genotype survived. The sensitivity ratio was calculated as the ratio of the MTD for wild-type animals divided by the MTD for each respective genotype and was expressed as a hypersensitivity index.

Peripheral WBC Counts after Vincristine Administration. Peripheral WBCs of wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice given a single i.p. injection of 1 mg/kg of vincristine were determined at various times after treatment with the *Vinca* alkaloid using a Coulter Multisizer II.

Necropsy. Necropsy, including histological evaluation, was performed on female mice, including untreated control and drug-treated animals from each of the four genotypes [wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)*] 3 days after a single i.p. injection of 2 mg/kg of vincristine. Toxicity was further evaluated in male mice 3 days after a single i.p. injection of 1 mg/kg of vincristine.

Blood Chemistries. Peripheral blood chemistries (Antech Diagnostics, Farmingdale, NY) including glucose, urea nitrogen, creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, cholesterol, calcium, phosphorous, sodium, potassium, chloride, albumin:globulin ratio, blood urea nitrogen:creatinine ratio, globulin, lipase, amylase, triglycerides, creatine phosphokinase, gamma glutamyl transpeptidase, magnesium, and calculated osmolality were measured. These values were obtained from pools of blood collected by cardiac puncture (three mice each) of wild-type and combined *mdr1a/1b(−/−), mrp1(−/−)* male and female mice ranging in age from 7 to 9 weeks. Mice were anesthetized with sodium pentobarbitol (70 mg/kg) prior to the collection of blood.

RESULTS

To characterize the impact of the P-gly on the tissue toxicity of antineoplastic agents transported by the ABC transporters, we have

used the *mdr1a/1b(−/−)* mice developed by Schinkel *et al.* (10). Triple knockout mice with disruptions in both alleles of the three transport protein genes *mrp1*, *mdr1a*, and *mdr1b* were generated by crossing the F_1 offspring resulting from matings between male *mdr1a/1b(−/−), mrp1(+/+)* mice (10), purchased from Taconic Farms, and female *mdr1a/1b(+/+), mrp1(−/−)* mice (9), developed in this laboratory. A representative *mrp1* Southern blot resulting from this cross is shown in Fig. 1*A*. The presence of the 5-kb band in the absence of a 2-kb band indicates homozygosity for the disrupted *mrp1* allele. Because all of the F_1 animals were heterozygous at all three genetic loci, some of the offspring resulting from an $F_1 \times F_1$ cross contained disruptions in both alleles of all three genes. Generation F_2 offspring testing positive for the homozygous disruption of *mrp1* were further tested for *mdr1a/1b(−/−)* status by PCR (Fig. 1*B*). Mouse numbers 2, 6, 8, and 10 are representative animals displaying homozygous disruption of the *mrp1*, *mdr1a*, and *mdr1b* genes. Ten of 29 *mrp1(−/−)* mice (5 females and 5 males), evaluated for mdr1a/1b status, tested positive for the homozygous disruption of *mdr1a* and *mdr1b*. Male and female mice with the *mrp1(−/−), mdr1a/1b(−/−)* genotype were mated to establish the triple knockout colony.

To document the disruption of *mdr1a/1b* and *mrp1* at the protein level, Western blot analyses were performed on embryonic fibroblasts derived from wild-type; *mrp1(−/−); mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* animals (Fig. 2). Using the murine monoclonal antibody C219 to detect mdr1a/1b, the presence of mdr1a and mdr1b was confirmed in wild-type and *mrp1(−/−)* embryonic fibroblast lines and its absence documented in *mdr1a/1b(−/−)* and *mdr1a/1b(−/−), mrp1(−/−)* fibroblast lines. Similarly, using the rat monoclonal antibody MRPr1, the presence of mrp1 in wild-type and *mdr1a/1b(−/−)* animals was observed, and its absence in the *mrp1(−/−)* and *mdr1a/1(−/−), mrp1(−/−)* mice was documented.

Vincristine and etoposide are substrates for both mrp1 and the P-gly, whereas paclitaxel is transported only by the P-gly (8). The results of measurements of the sensitivity of wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* embryonic fibroblasts to these three antineoplastic agents are shown in Fig. 3. Cell viability was expressed as a percentage of control over a range of drug concentrations and from which increased sensitivity in comparison to wild-type cells, based upon comparative IC_{50}s, was calculated for each genotype. In the case of vincristine, *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* embryonic fibroblasts exhibited increased sensitivities of approximately 1.7-, 3.5- and 12-fold, respectively, following exposure to the *Vinca* alkaloid for 96 h. These findings were suggestive of a synergistic effect in the setting of the triple gene deficiency. For etoposide, after a 72-h incubation, *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* animals showed increased sensitivities of 3.1-, 1.4- and 4.3-fold, respectively. With paclitaxel exposure for 96 h, both *mdr1a/1b(−/−)* and *mdr1a/1b(−/−), mrp1(−/−)* cells demonstrated an equivalent degree of enhanced sensitivity of about 25-fold over wild-type cells, whereas *mrp1(−/−)* cells displayed no increased sensitivity, a finding consistent with paclitaxel not being a substrate for mrp1.

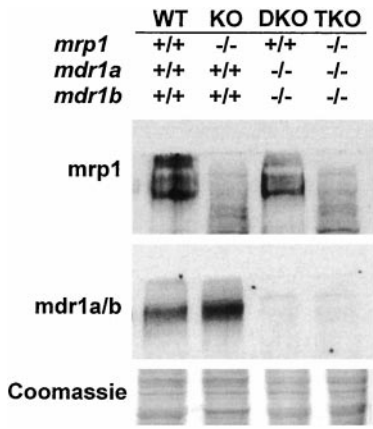

Fig. 2. Western blot analyses of mrp1 and mdr1a and/or mdr1b in cultured embryonic fibroblast lines prepared from mouse embryos. The rat monoclonal antibody MRPr1 was used to detect mrp1. mdr1a and mdr1b were detected using the mouse monoclonal C219 antibody. Embryonic fibroblasts were prepared from mice with the three transport protein genes: intact wild-type (*WT*), with disruptions in both alleles of the *mrp1* gene (*KO*); of each of the two *mdr* genes (*DKO*); and of all three transport protein genes (*TKO*). The *bottom panel* is a section of an identical gel stained with Coomassie blue to show approximately equal loading.

The *in vivo* toxicity of etoposide phosphate to wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice is shown in Table 1. The maximum tolerated single i.p. doses of etoposide phosphate for wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice were 175, 100, 100, and 50 mg/kg, respectively. Although both the *mrp1(−/−)* and *mdr1a/1b(−/−)* mice tolerated the 100 mg/kg dose, at the next higher dose tested four of the six *mrp1(−/−)* animals survived *versus* two of the six *mdr1a/1b(−/−)* mice. The hypersensitivity factor for these animals toward etoposide phosphate was 1.75 for both *mrp1(−/−)* and *mdr1a/1b(−/−)* and 3.5 for the triple knockout mice as illustrated in Fig. 4. Analogous vincristine mortality data are presented in Table 2. The maximum tolerated single i.p. doses of vincristine for wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice were 4, 1, 0.25 and 0.03125 mg/kg, respectively. The hypersensitivity

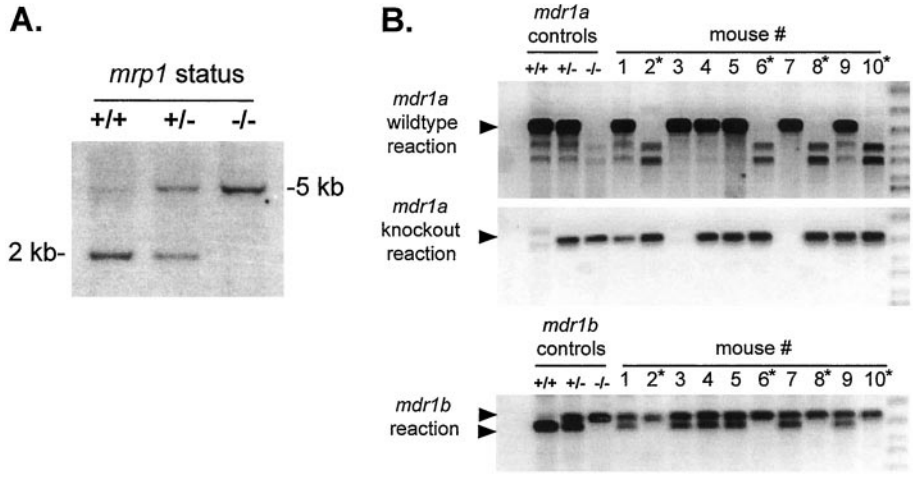

Fig. 1. Identification of mice with disruptions in both alleles of the three transport protein genes *mrp1*, *mdr1a*, and *mdr1b*. *A*, Southern blot analyses of *Sac*I-digested genomic DNA from mice resulting from a cross of the F_1 offspring of *mrp1(−/−), mdr1a/1b(+/+)* × *mrp1(+/+), mdr1a/1b(−/−)* mice. For identification of mice with a targeted disruption at the *mrp1* locus, the blot was hybridized with the 0.4-kb *Xba*I-*Bam*HI probe positioned immediately 5′ of the targeted construct. The presence of a 5-kb band instead of a 2-kb band is diagnostic of a mutated allele. *B*, mice identified as having disruptions in both alleles at the *mrp1* locus were further analyzed by PCR (Taconic Biotechnology) for disruptions at the *mdr1a* (*top two panels*) and *mdr1b* (*bottom panel*) loci. The *mdr1a* analysis requires two PCR reactions because the molecular weight of the knockout and wild-type signals are similar and are not resolved when the heterozygotes are analyzed. *Arrowheads*, the positions of the relevant bands. ∗, mice with disruptions in both alleles of all three genes.

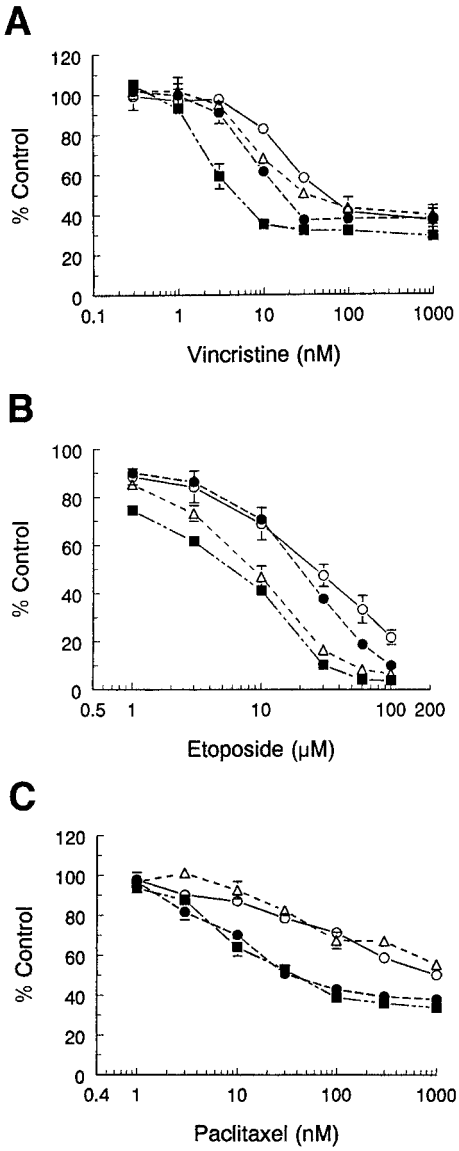

Fig. 3. Sensitivities of wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* embryonic fibroblasts to vincristine (*A*), etoposide (*B*), and paclitaxel (*C*). Wild-type (○); *mrp1(−/−)* (△); *mdr1a/1b(−/−)* (●); and *mdr1a/1b(−/−), mrp1(−/−)* (■) embryonic fibroblasts were exposed to various concentrations of vincristine or paclitaxel for 96 h or to etoposide for 72 h. Drug sensitivities were evaluated using the MTS/PES colorimetric assay. Data points represent means from three independent experiments; *bars*, SE.

Table 1. *Toxicity of etoposide phosphate to wild-type (WT); mrp1 (−/−),* (KO); *mdr1a/1b (−/−),* (DKO); and *mdr1a/1b (−/−), mrp1(−/−),* (TKO) mice

	Survival (%)			
Dosage (mg/kg)	WT	KO	DKO	TKO
50	ND	ND	ND	100
75	100	100	100	83
100	100	100	100	50
125	100	67	33	0
150	100	17	0	0
175	100	17	ND[a]	ND
200	0	ND	ND	ND

[a] ND, not done.

factors for the various genotypes for the *Vinca* alkaloid were significantly more pronounced than for etoposide [4-, 16-, and 128-fold for the *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* mice, respectively (Fig. 5)]. The increase in sensitivity toward vincristine observed in mice with disruptions in the three transport genes was consistent with the enhanced sensitivity of embryonic fibroblasts to this agent observed *in vitro*.

Peripheral WBC counts determined daily for 5 consecutive days after a single 1 mg/kg i.p. dose of vincristine are shown in Fig. 6. After an approximately 10–20% decrease in WBC counts by day 3, the levels of WBCs in wild-type and *mrp1(−/−)* mice began to recover, essentially returning to baseline by posttreatment day 4. The nadir in the WBC count for the *mdr1a/1b(−/−)* mice occurred on the second day after treatment with the *Vinca* alkaloid, with values just under the 50% level, followed by a progressive recovery in the white cell count to >70% of normal by posttreatment day 5 for three of six surviving mice. The *mdr1a/1b(−/−), mrp1(−/−)* mice showed a decrease in the WBC counts to approximately 20–25% of normal by posttreatment day 3 and remained depressed without evidence of recovery until the deaths of all mice.

Examination of male and female triple knockout mice did not reveal any major anatomical or histopathological abnormalities that

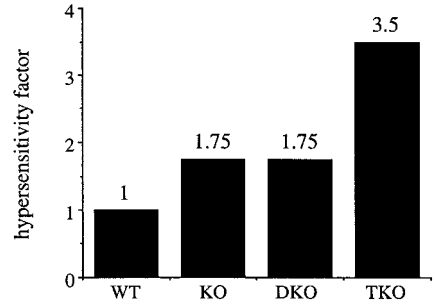

Fig. 4. Calculated hypersensitivity factor for etoposide. The hypersensitivity factor is the MTD for wild-type mice (*WT*) divided by the MTD for *mrp1(−/−)* (*KO*); *mdr1a/1b(−/−)* (*DKO*); or *mdr1a/1b(−/−), mrp1(−/−)* (*TKO*) animals. MTD is the maximum dosage tolerated by all of the mice in a test group.

Table 2. *Toxicity of vincristine to wild-type (WT); mrpl (−/−),* (KO); *mdr1a/1b (−/−),* (DKO); and *mdr1a/1b (−/−), mrpl (−/−),* (TKO) mice

	Survival (%)			
Dosage (mg/kg)	WT	KO	DKO	TKO
0.03125	ND[a]	ND	100	100
0.0625	ND	ND	100	33
0.125	ND	ND	100	0
0.25	ND	ND	100	0
0.5	ND	ND	67	0
1	ND	100	0	0
2	100	83	0	0
4	100	0	0	0
6	50	0	0	0
8	0	0	ND	0

[a] ND, not done.

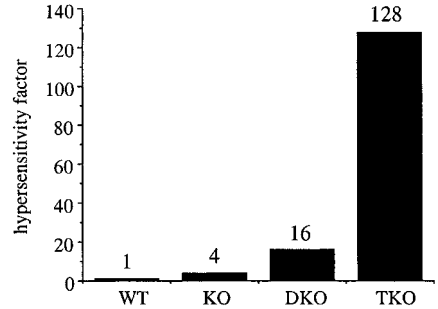

Fig. 5. Calculated hypersensitivity factor for vincristine. The hypersensitivity factor is the MTD for wild-type mice (*WT*) divided by the MTD for *mrp1(−/−)* (*KO*); *mdr1a/1b(−/−)* (*DKO*); or *mdr1a/1b(−/−), mrp1(−/−)* (*TKO*) animals. MTD is the maximum dosage tolerated by all of the mice in a test group.

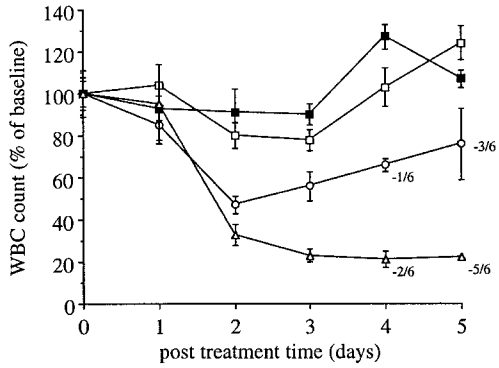

Fig. 6. WBC counts in wild-type (■); *mrp1(−/−)* (□); *mdr1a/1b(−/−)* (○); and *mdr1a/1b(−/−), mrp1(−/−)* (△) mice after a single i.p. injection of 1 mg/kg of vincristine. Data are the means from six mice/treatment group; *bars*, SE. Mice dying of drug toxicity during the study are indicated within the graph.

could be attributed to the combined functional absence of all three transporters. Retinal degeneration involving the photoreceptor layer was observed in both *mdr1a/1b(−/−)* and *mdr1a/1b(−/−), mrp1(−/−)* mice, but this abnormality is common in the FVB mouse (41). Routine blood chemistries of male and female triple knockout animals did not appear to be significantly different from control wild-type mice. However, necropsy and histological examination of animals after vincristine administration at 1 and 2 mg/kg demonstrated phenotypic correlatable manifestations of gastrointestinal and bone marrow toxicity, consistent with the known toxicity of vincristine to rapidly dividing cells, which was most extensive in *mdr1a/1b(−/−), mrp1(−/−)* mice (37). The gastrointestinal tract showed epithelial necrosis in both the *mdr1a/1b(−/−)* and *mdr1a/1b(−/−), mrp1(−/−)* animals treated for 72 h with 1 mg/kg of vincristine. These manifestations of gastrointestinal toxicity were more extensive in triple knockout animals. At the 2 mg/kg dose of the *Vinca* alkaloid, more extensive necrosis occurred in the base of the intestinal crypts in both the *mdr1a/1b(−/−)* and *mdr1a/1b(−/−), mrp(−/−)* genotypes, with a complete destruction of the epithelial lining being observed in portions of the small intestine of the triple knockout animals. No significant gastrointestinal lesions were observed in either the wild-type or *mrp1(−/−)* animals.

In an analogous manner, histological examination of the bone marrow after vincristine administration demonstrated enhanced toxicity with increasing gene disruption. Thus, 72 h after a 1-mg/kg dose of the *Vinca* alkaloid, changes in the bone marrow of *mrp1(−/−)* mice occurred, characterized by a relatively modest depletion of myeloid elements. Moderate depletion of myeloid elements was also produced in *mdr1a/1b(−/−)* animals, whereas triple knockout mice exhibited severe marrow destruction, with almost complete loss of myeloid elements, frank necrosis, hemorrhage, and fibrin deposition. Representative H&E-stained sections of bone marrow from the femurs of untreated animals and from those treated 72 h earlier with 2 mg/kg of vincristine are shown in Fig. 7. No significant change from that of untreated animals was observed in vincristine-treated marrow from wild-type animals. Expansion of the blood-filled medullary sinusoids because of the modest loss of myeloid elements was observed in *mrp1(−/−)* mice, whereas a more severe reduction of hematopoietic precursors, with many expanded blood-filled vascular spaces and prominent stroma replacing hematopoietic precursors, was seen in *mdr1a/1b(−/−)* animals. Bone marrow from triple knockout mice exposed to the *Vinca* alkaloid exhibited extensive acute necrosis and hemorrhage with loss of almost all of the hematopoietic precursors.

In *mdr1a/1b(−/−), mrp1(−/−)* mice treated with 2 mg/kg of the *Vinca* alkaloid for 72 h, marked necrosis of pancreatic exocrine tissue and scattered necrosis of the endometrial crypt epithelium of the uterus was also seen.

DISCUSSION

Previous studies have shown that the complete abrogation of *mrp1* expression did not affect the viability of mice, nor their fertility (9, 10). The litters of *mrp1(−/−)* couples were of the same size as litters from wild-type mice, and the growth and behavior of *mrp1(−/−)* mice were normal. Furthermore, hematological parameters and the levels of a range of serum enzymes, proteins, and electrolytes did not differ between wild-type and *mrp1* knockout mice. Gross anatomical and microscopic examination of most organs and tissues did not reveal any abnormalities. Several potential physiological substrates of

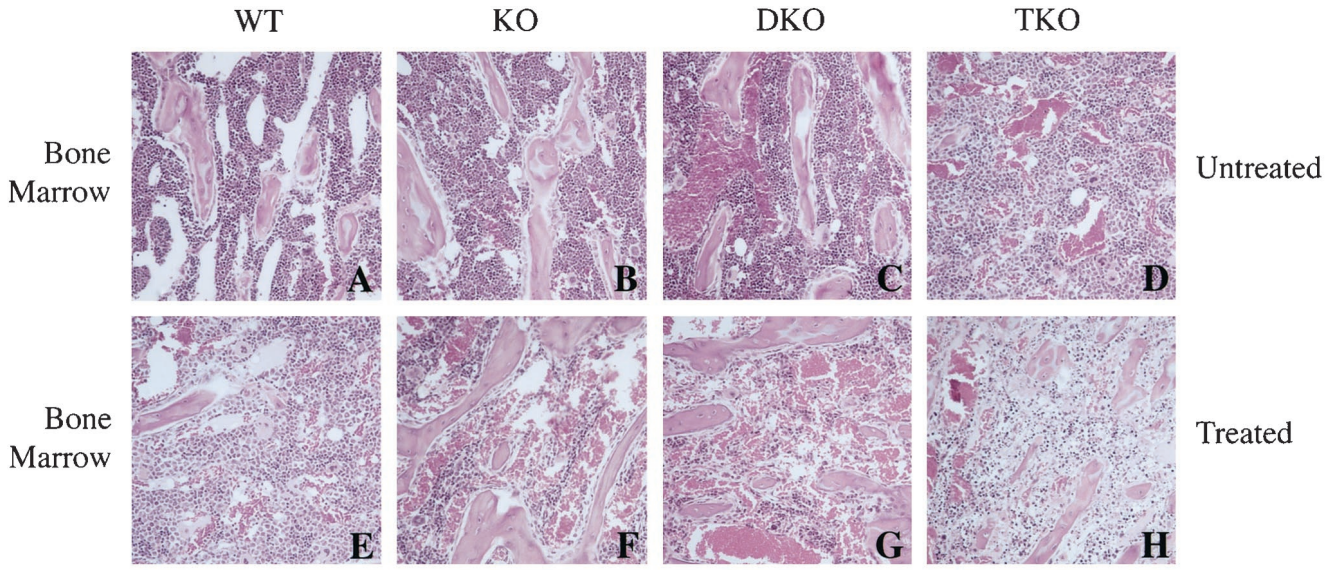

Fig. 7. H&E-stained sections of bone marrow from wild-type (*WT*); *mrp1(−/−)* (*KO*); *mdr1a/1b(−/−)* (*DKO*); and *mdr1a/1b(−/−), mrp1(−/−)* (*TKO*) mice 72 h after i.p. treatment with 2 mg/kg of vincristine. *A–D*, untreated bone marrow sections from wild-type; *mrp1(−/−)* (*KO*); *mdr1a/1b(−/−)* (*DKO*); and *mdr1a/1b(−/−), mrp1(−/−)* (*TKO*) mice, respectively. *E–H*, bone marrow sections from vincristine treated wild-type; *mrp1(−/−)* (*KO*); *mdr1a/1b(−/−)* (*DKO*); and *mdr1a/1b(−/−), mrp1(−/−)* (*TKO*) animals, respectively. ×200.

mrp1, including leukotriene C4 and 17β-estradiol-17(β-D-glucuronide), have been identified by studies using membrane vesicles from mrp1-overexpressing cells (38–40). Despite these possible functions of mrp1, no major biological anomalies have been observed in *mrp1(−/−)* mice. In an analogous fashion, despite the relatively large number of functions speculatively attributed to the P-gly, no physiological abnormalities were found in *mdr1a/1b(−/−)* mice, the only clear difference in phenotype being alterations in tissue distribution, cellular accumulation, and excretion of several drugs (10). It is conceivable that for the biological functions of mrp1 and P-gly, other protein systems exist that can substitute when one or both of these transporters are not available. Alternatively, it is possible that they do not play a major role in physiological functions and that their only role is to protect the organism against naturally occurring toxins. However, the finding that *in vitro*, in the absence of exposure to any xenobiotic, mrp1 mediates the export of GSH into the extracellular medium (7, 8) argues against the latter possibility and suggests that, together with GSH, mrp1 cotransports one or more physiological substrates, the nature of which are at present unknown.

mdr1a(−/−) mice have a complete loss of the P-gly present in the brain capillaries and the intestinal epithelium, demonstrating that the *mdr1a* gene is responsible for the P-gly found in these tissues and that the absence of the mdr1a P-gly does not lead to the activation of mdr1b P-gly in these or in most other tissues (11). However, an up-regulation of *mdr1b* occurred in the liver and kidneys of *mdr1a(−/−)* mice, suggesting that a compensatory mechanism was operative in these tissues. The most striking effect of the treatment of wild-type and *mdr1a(−/−)* mice with a single dose of 6 mg/kg of VBL was a 12-fold higher concentration of VBL in the brain of the *mdr1a(−/−)* mice than in this tissue in wild-type animals at 4 h after the drug, and this differential in the brain increased with time because of slower elimination of VBL in *mdr1a(−/−)* mice. The lethal dose of VBL was ~3-fold lower in *mdr1a(−/−)* mice than in their wild-type counterparts, and the signs of general toxicity were similar in parental and knockout animals.

mdr1b(−/−) mice did not exhibit any significant differences from wild-type animals in the tissue distribution of [^{3}H]digoxin, and this finding presumably corresponded to the compensatory use of the *mdr1a* gene in the major organs of *mdr1b(−/−)* mice (10). In contrast, *mdr1a/1b(−/−)* mice exhibited increased plasma levels of [^{3}H]digoxin and marked increases in this agent in the brain and in the testes of mice compared with that of parental mice. In addition, [^{3}H]digoxin accumulation in adrenal glands and ovaries was increased over that of plasma levels and that present in most of the other tissues of the *mdr1a/1b(−/−)* mice compared with *mdr1a/1b(+/+)* mice. Studies of the elimination rate of [^{3}H]digoxin in *mdr1a/1b(−/−)* mice demonstrated that the liver has a substantial [^{3}H]digoxin excretion capacity that is distinct from the mdr1-type P-gly and that the urinary excretion of [^{3}H]digoxin was not impaired in *mdr1a/1b(−/−)* mice, indicating that the mdr1-type P-gly is not essential for the excretion of this agent by the kidney. In contrast, the direct elimination of [^{3}H]digoxin from the intestine in *mdr1a/1b(−/−)* animals was markedly decreased compared with *mdr1a/1b(+/+)* mice. Comparable findings were also demonstrated for the antineoplastic agent paclitaxel in *mrp1a/1b(−/−)* mice, suggesting the presence of a compensatory transporter(s) in the liver and the kidneys.

The P-gly substrate rhodamine 123 has been used to measure the importance of the P-gly to hematopoietic stem cells by determining the rate of efflux of this dye from partially purified hematopoietic progenitor cells from the bone marrow of wild-type and knockout mice (10). The rate of rhodamine efflux from these hematopoietic cells from *mdr1a(−/−)* and *mdr1b(−/−)* mice was decreased relative to these cells from wild-type animals, whereas a supra-additive decrease was observed in these cells from *mdr1a/1b(−/−)* mice, demonstrating that both *mdr1a* and *mdr1b* genes contribute substantially to drug efflux from hematopoietic progenitor cells.

To ascertain whether the baseline expression of *mrp1* protects mice from the toxic effects of xenobiotics, limited toxicity tests have been conducted with etoposide and etoposide phosphate (Etopophos), a water-soluble etoposide ester that is completely and rapidly dephosphorylated to etoposide in plasma (9). Etoposide phosphate, injected i.p. as a single dose, was twice as toxic to *mrp1(−/−)* mice than to *mrp1(+/+)* mice, with calculated LD_{50}s of 95 and 190 mg/kg, respectively. One of the main toxicities of etoposide in humans, as well as in mice, is to the bone marrow. To determine whether treatment with etoposide phosphate resulted in differential bone marrow toxicity to *mrp1(+/+)* and *mrp1(−/−)* mice, in a previous study we measured the total WBC count at different times after the i.p. injection of 150 mg/kg of etoposide phosphate (9). After a rapid initial drop in the WBC count, a nadir was reached between days 2 and 3 in both *mrp1(+/+)* and *mrp1(−/−)* mice. Subsequently, the leukocyte count recovered in wild-type animals but not in *mrp1* knockouts. This result implied that etoposide phosphate exerted a differential toxicity to the bone marrow of wild-type and *mrp1* knockout mice. These findings were corroborated by a pathological examination of the bone marrow and spleen of wild-type and *mrp1* knockout mice 5 days after treatment with 150 mg/kg of etoposide phosphate. Although in *mrp1* knockout animals the bone marrow exhibited a severe depletion of nucleated cells and the spleen exhibited a depletion of myeloid activity in the red pulp, in wild-type animals the bone marrow and spleen were either normal or hypercellular (data not shown). In contrast, although Wijnholds *et al.* (15) demonstrated that *mrp1(−/−)* mice were hypersensitive to etoposide, expressed as increased loss of body weight and increased mortality, these authors found no difference in leukopenia and thrombocytopenia between *mrp1(−/−)* and *mrp1(+/+)* mice.

In the present report, we demonstrate that mice lacking the three genes *mdr1a*, *mdr1b*, and *mrp1* develop normally and are without physical dysmorphology, internal anatomical abnormality, and known endogenous biochemical abnormality. These animals reproduce and show normal viability and, in the absence of pharmacological challenge, are indistinguishable from wild-type; *mrp1(−/−)*; or *mdr1a/1b(−/−)* mice. These findings corroborated those of Wijnholds *et al.* (42), who have independently developed *mdr1a/1b(−/−), mrp1(−/−)* mice. Retinal degeneration was observed in some of the triple knockout mice; this finding is consistent with the retinal degeneration known to arise on the genetic background of the *mdr1a/1b(−/−)* strain used to breed the combined *mdr1a/1b(−/−), mrp1(−/−)* mice (41) and therefore was assumed to be pathogenically unrelated to the combined triple gene knockout. It is provocative, however, to note that mutations in another ABC transporter (ABCR) have been associated with retinal pathology (43).

The consequences of combined mdr1a/1b, mrp1 export pump deficiency in the face of pharmacological challenge were evaluated with vincristine, etoposide, and paclitaxel *in vitro* in embryonic fibroblasts from triple knockout mice, as well as with vincristine and etoposide *in vivo*. *In vitro* studies with vincristine using embryonic fibroblasts demonstrated a picture of increasing sensitivity correlated with increased transporter deficiency, yielding enhanced sensitivities of 1.7-, 3.5- and 12-fold over wild-type; *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mdr1a/1b(−/−), mrp1(−/−)* embryonic fibroblasts, respectively. Studies with etoposide in embryonic fibroblasts indicated that triple knockout embryonic fibroblasts were 4.3-fold more sensitive than wild-type fibroblasts; this hypersensitivity appeared to be accounted for primarily by a deficiency in mrp1, with mdr1a/1b contributing little if any to the observed increase in sensitivity to the epipodophyl-

lotoxin. Paclitaxel, which is preferentially transported by the P-gly, was no more cytotoxic to embryonic fibroblasts lacking both the P-gly and mrp1 than fibroblasts deficient in the P-gly alone, with both genotypes demonstrating an ~25-fold enhanced sensitivity compared with wild-type and *mrp1(−/−)* cells. These results corresponded to those reported recently by Allen *et al.* (44), who found increased sensitivity to vincristine and etoposide in independently developed triple knockout fibroblast cell lines. An unexplained finding was a 28-fold increase in sensitivity to vincristine with only a 7.1-fold increase in sensitivity to VBL over that of wild-type fibroblasts.

Vincristine had an even more pronounced effect *in vivo*, with mortality data indicating that the triple knockout mice were 128 times more sensitive to the *Vinca* alkaloid in terms of lethality than were wild-type animals, whereas 16- and 4-fold enhanced sensitivities were observed with *mdr1a/1b(−/−)* and *mrp1(−/−)* genotypes, respectively. The *in vivo* toxicity of vincristine also exhibited genotype-related trends in the time of death at a given dosage of drug, such that at 4 mg/kg of the *Vinca* alkaloid, all of the wild-type mice survived, whereas the average survival times for *mdr1a/1b(−/−)*, *mrp1(−/−)*; *mdr1a/1b(−/−)*; and *mrp1(−/−)* animals were 4, 5, and 7 days, respectively. Qualitative assessment of vincristine-treated mice also demonstrated evidence of unsteady gait and tremor, which appeared to be the most severe in the triple knockout animals.

A change in peripheral WBC counts that correlated with genotype was observed after treatment with 1 mg/kg of vincristine. Thus, the WBC count in triple knockout mice dropped markedly lower than that occurring with the other genotypes and exhibited little recovery. *mdr1a/1b(−/−)* animals showed an intermediate response between that of the triple knockout and *mrp1(−/−)* mice, which was apparent between posttreatment days 2 and 5; this finding is consistent with the known expression of the P-gly in pluripotent stem cells of the bone marrow (45). These observations were further supported by the extensive histological manifestations of bone marrow toxicity after vincristine administration to triple knockout mice. Taken together, the *in vivo* histology and mortality data, as well as the *in vitro* toxicity with the *Vinca* alkaloid, present a picture of enhanced drug sensitivity in animals with the combined disruption of the three transport genes, such that the functional absence of these three transporters resulted in greater than additive toxicities compared with those observed in the absence of either mdr1a/1b or mrp1 alone. This phenomenon implies that the P-gly and mrp1 are compensatory transporters in the bone marrow and intestinal mucosa, as well as in other tissues. Furthermore, the relative importance of these transporters was clearly drug and tissue dependent, as illustrated by the *in vivo* sensitivity to etoposide, as well as by the *in vitro* response of embryonic fibroblasts to paclitaxel. Thus, although the same degree of enhanced toxicity to etoposide was not observed across the transporter-deficient genotypes, as was seen with vincristine, the triple knockout mice exhibited a 3.5-fold increased sensitivity to the epipodophyllotoxin, whereas a 1.75-fold enhanced sensitivity occurred in both the *mdr1a/1b(−/−)* mice and *mrp1(−/−)* mice. The enhanced sensitivity to etoposide corresponded with that described by Wijnholds *et al.* (42), who also showed increased accumulation of etoposide in the colon, brown adipose tissue, salivary gland, heart, and the female urogenital system of triple knockout mice.

The findings also permit the suggestion that the therapeutic differential toxicity of antineoplastic agents effluxed by the P-gly and/or MRP1 to malignant cells relative to normal tissues, such as the bone marrow and the gastrointestinal mucosa, may be attributable, at least in part, to the presence of ABC transporters in the normal tissues.

It is conceivable that additional transporters other than the P-gly and mrp1 may be present in sensitive tissues such as the bone marrow and/or the intestinal mucosa. Such a possibility may explain, at least in part, the markedly different hypersensitivity factors seen with vincristine and etoposide in Figs. 4 and 5. Thus, one could speculate that the P-gly and mrp1 are the major transporters of vincristine in these and other sensitive tissues and other ABC transporters that may be present are not involved in the export of this agent. In contrast, transport systems other than the P-gly and mrp1 may play a greater role in the efflux of etoposide, resulting in the much lower increase in the hypersensitivity to the epipodophyllotoxin in *mdr1a/1b(−/−)*, *mrp1(−/−)* knockout animals that has been observed.

It cannot be stated unequivocally from the etoposide and vincristine mortality data that death was secondary to bone marrow toxicity, as opposed to another cause, such as systemic shock related to gastrointestinal toxicity. However, the degree of apparent bone marrow compromise, as manifested by the peripheral WBC count and by histological examination, was marked. Concern over therapy-limiting bone marrow toxicity in the setting of ABC transporter inhibition has been raised by others (4). The findings presented in the this report regarding the triple knockout genotype amplify these concerns, particularly in light of the observed enhanced hypersensitivity obtained with vincristine, which normally does not express overt toxicity to the bone marrow or the gastrointestinal mucosa. Importantly, the degree of toxicity to these tissues varied with the class of drug, further supporting the concern that attempts at inhibiting the P-gly and/or MRP1 in resistant tumors in humans need to be conducted with caution because the associated toxicities are likely to vary, not only with respect to the therapeutic agent being used, but also with respect to the inhibitor and the extent to which drug export via the P-gly and MRP1 is reduced.

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