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

Dennis R. Johnson, Rick A. Finch, Z. Ping Lin, Caroline J. Zeiss, and Alan C. Sartorelli

Department of Pharmacology and Developmental Therapeutics Program, Cancer Center and Section of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520

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 fibroblasts 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) 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, ABCC2), MRP3 (MOAT-D, ABCC3), MRP4 (MOAT-B, ABCC4), MRP5 (MOAT-C, ABCC5), and MRP6 (MOAT-E, ABCC6). 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-glycoprotein, expressed by the MDR1 gene in humans and two closely related genes, mdr1a and mdr1b, in the mouse, and MRPL1 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, MRPL1 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 mdr1b(-/-) 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 mdr1b(-/-) 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 mdr1b(+/+) and mdr1b(-/-) 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 Wijnschols 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 mdr1b(-/-) mice led to toxicity to the orpharyngeal mucosa and the seminiferous tubules of the testis in etoposide phosphate-treated animals indicates that mrp1 protects these tissues against damage from mdr1b substrates (17). The expression of MRP1 has been detected in a number of human cancers and shown to be associated with drug
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 MRPI 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 MRPI 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 MRPI 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 mrp1(−/−) mice generated previously in our laboratory (9). The resulting F1 heterozygous animals were bred, and the F2 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 Sphl, and 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 (XbaI/Sphl) from the 5′-end of the mrp1 gene. The 5′-end of the gene was radiolabeled with [α-32P]dATP using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA) and detected on autoradiographic film.

Peripheral WBC Counts after Vincristine Administration. Peripheral WBCs of wild-type; mdr1a/1b(−/−); mrp1(−/−); and mdr1a/1b(−/−), mrp1(−/−) mice given a single i.p. injection of 1 mg/kg of vincristine were determined after 3 days. Groups of three or six wild-type; mdr1a/1b(−/−); mrp1(−/−); and mdr1a/1b(−/−), mrp1(−/−) 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; mdr1a/1b(−/−); mrp1(−/−); and mdr1a/1b(−/−), mrp1(−/−) female mice were treated with single i.p. doses of vincristine ranging from 0.03125 to 0.1 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; mdr1a/1b(−/−); mrp1(−/−); 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; mdr1a/1b(−/−); mrp1(−/−); 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, amylose, 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 pentobarbital (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/lb(-/-) mice developed by Schinkel et al. (10). Triple knockout mice with disruptions in both alleles of the three transport protein genes mpr1, mdr1a, and mdr1b were generated by crossing the F1 offspring resulting from matings between male mdr1a/lb(-/-), mpr1(+/+) mice (10), purchased from Taconic Farms, and female mdr1a/lb(+/+) mpr1(+-/-) mice (9), developed in this laboratory. A representative mpr1 Southern blot resulting from this cross is shown in Fig. 1A. The presence of the 5-kb band in the absence of a 2-kb band indicates homozygosity for the disrupted mpr1 allele. Because all of the F1 animals were heterozygous at all three genetic loci, some of the offspring resulting from an F1 × F1 cross contained disruptions in both alleles of all three genes. Generation F2 offspring testing positive for the homozygous disruption of mpr1 were further tested for mdr1a/lb(-/-) status by PCR (Fig. 1B). Mouse numbers 2, 6, 8, and 10 are representative animals displaying homozygous disruption of the mpr1, mdr1a, and mdr1b genes. Ten of 29 mpr1(-/-) mice (5 females and 5 males), evaluated for mdr1a/lb(-/-) status, tested positive for the homozygous disruption of mdr1a and mdr1b. Male and female mice with the mpr1(-/-), mdr1a/lb(-/-) genotype were mated to establish the triple knockout colony.

To document the disruption of mdr1a/lb and mpr1 at the protein level, Western blot analyses were performed on embryonic fibroblasts derived from wild-type; mpr1(-/-); mdr1a/lb(-/-); and mdr1a/lb(-/-), mpr1(-/-) animals (Fig. 2). Using the murine monoclonal antibody C219 to detect mdr1a and mdr1b was confirmed in wild-type and mpr1(-/-) embryonic fibroblast lines and its absence documented in mdr1a/lb(-/-) and mdr1a/lb(-/-), mpr1(-/-) fibroblast lines. Similarly, using the rat monoclonal antibody MRPr1, the presence of mpr1 in wild-type and mdr1a/lb(-/-) animals was observed, and its absence in the mpr1(-/-) and mdr1a/lb(-/-), mpr1(-/-) mice was documented.

Vincristine and etoposide are substrates for both mpr1 and the P-gly, whereas paclitaxel is transported only by the P-gly (8). The results of measurements of the sensitivity of wild-type; mpr1(-/-); mdr1a/lb(-/-); and mdr1a/lb(-/-), mpr1(-/-) 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 IC50s, was calculated for each genotype. In the case of vincristine, mpr1(-/-); mdr1a/lb(-/-), mpr1(-/-) mice demonstrated an equivalent degree of enhanced sensitivity of about 25-fold over wild-type cells, whereas mpr1(-/-) cells displayed no increased sensitivity, a finding consistent with paclitaxel not being a substrate for mpr1.

In vivo toxicity of etoposide phosphate to wild-type; mpr1(-/-); mdr1a/lb(-/-); and mdr1a/lb(-/-), mpr1(-/-) mice is shown in Table 1. The maximum tolerated single i.p. doses of etoposide phosphate for wild-type; mpr1(-/-); mdr1a/lb(-/-); and mdr1a/lb(-/-), mpr1(-/-) mice were 175, 100, 100, and 50 mg/kg, respectively. Although both the mpr1(-/-) and mdr1a/lb(-/-) mice tolerated the 100 mg/kg dose, at the next higher dose tested four of the six mpr1(-/-) animals survived versus two of the six mdr1a/lb(-/-) mice. The hypersensitivity factor for these animals toward etoposide phosphate was 1.75 for both mpr1(-/-) and mdr1a/lb(-/-) 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; mpr1(-/-); mdr1a/lb(-/-); and mdr1a/lb(-/-), mpr1(-/-) mice were 4, 1, 0.25 and 0.03125 mg/kg, respectively. The hypersensitivity

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**Fig. 1.** Identification of mice with disruptions in both alleles of the three transport protein genes mpr1, mdr1a, and mdr1b. A. Southern blot analyses of SacI-digested genomic DNA from mice resulting from a cross of the F1 offspring of mpr1(-/-), mdr1a/lb(+/+), mdr1b(+/+) mice. For identification of mice with a targeted disruption at the mpr1 locus, the blot was hybridized with the 0.4-kb XbaI-BamHI 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 having disruptions in both alleles at the mpr1 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 knock out 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.
The increased 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

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

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**Table 2. Toxicity of vincristine to wild-type (WT); mrpl (−/−), (KO); mdr1a/1b (−/−), (DKO); and mdr1a/1b (−/−), mrp1 (−/−), (TKO) mice**

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<th>Dosage (mg/kg)</th>
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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(-/-), mdr1b(-/-), mdr1b(-/-) 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(-/-), mdr1b(-/-) mice (37). The gastrointestinal tract showed epithelial necrosis in both the mdr1a/1b(-/-) and mdr1a/1b(-/-), mdr1b(-/-), mdr1b(-/-) 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(-/-), mdr1b(-/-), mdr1b(-/-) 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 mdr1(-/-) 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 mdr1(-/-) 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 fumurs 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 mdr1(-/-) 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(-/-), mdr1b(-/-) 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 mdr1 expression did not affect the viability of mice, nor their fertility (9, 10). The litters of mdr1(-/-) couples were of the same size as litters from wild-type mice, and the growth and behavior of mdr1(-/-) 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 mdr1 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); mdr1(-/-) (KO); mdr1a/1b(-/-) (DKO); and mdr1a/1b(-/-), mdr1b(-/-) (TKO) mice 72 h after i.p. treatment with 2 mg/kg of vincristine. A-D, untreated bone marrow sections from wild-type; mdr1(-/-) (KO); mdr1a/1b(-/-) (DKO); and mdr1a/1b(-/-), mdr1b(-/-) (TKO) mice, respectively. E-H, bone marrow sections from vincristine treated wild-type; mdr1(-/-) (KO); mdr1a/1b(-/-) (DKO); and mdr1a/1b(-/-), mdr1b(-/-) (TKO) animals, respectively. ×200.
mrp1, including leukotriene C4 and 17β-estradiol-17β-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(−/−) 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.

mdrla(−/−) mice have a complete loss of the P-gly present in the brain capillaries and the intestinal epithelium, demonstrating that the mdrla gene is responsible for the P-gly found in these tissues and that the absence of the mdrla 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 mdrla(−/−) mice, suggesting that a compensatory mechanism was operative in these tissues. The most striking effect of the treatment of wild-type and mdrla(−/−) mice with a single dose of 6 mg/kg of VBL was a 12-fold higher concentration of VBL in the brain of the mdrla(−/−) 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 mdrla(−/−) mice. The lethal dose of VBL was ~3-fold lower in mdrla(−/−) 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 [3H]digoxin, and this finding presumably corresponded to the compensatory use of the mdrla gene in the major organs of mdr1b(−/−) mice (10). In contrast, mdr1alb(−/−) mice exhibited increased plasma levels of [3H]digoxin and marked increases in this agent in the brain and in the testes of mice compared with that of parental mice. In addition, [3H]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 mdr1alb(−/−) mice compared with mdr1alb(+/+) mice. Studies of the elimination rate of [3H]digoxin in mdr1alb(−/−) mice demonstrated that the liver has a substantial [3H]digoxin excretion capacity that is distinct from the mdr1-type P-gly and that the urinary excretion of [3H]digoxin was not impaired in mdr1alb(−/−) 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 [3H]digoxin from the intestine in mdr1alb(−/−) animals was markedly decreased compared with mdr1alb(+/+) mice. Comparative findings were also demonstrated for the antineoplastic agent paclitaxel in mdr1alb(−/−) 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 mdr1alb(−/−) mice, demonstrating that both mdrla 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 (Etopos), 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 mdr1a(−/−) mice than to mrp1(+/-) mice, with calculated LD₅₀ 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 mdrla, 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 mdr1alb(−/−) mice. These findings corroborated those of Wijnholds et al. (42), who have independently developed mdr1alb(−/−), 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 mdr1alb(−/−) strain used to breed the combined mdr1alb(−/−), 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 mdr1alb/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(−/−); mdr1alb(−/−); and mdr1alb(−/−), 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 mdr1alb/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. In contrast, the other genotypes 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 MRPl 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 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 MRPl 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 MRPl is reduced.

REFERENCES


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