Chemotherapy and Chemosensitization of Transgenic Mice Which Express the Human Multidrug Resistance Gene in Bone Marrow: Efficacy, Potency, and Toxicity

Gerald H. Mickisch,1 Thomas Licht, Glenn T. Merlino, Michael M. Gottesman, and Ira Pastan2

ABSTRACT

A common form of multidrug resistance in human cancer results from expression of the \textit{MDR1} gene which encodes a plasma membrane energy-dependent multidrug efflux pump. We have engineered transgenic mice which express this multidrug transporter in their bone marrow cells and demonstrated that peripheral WBC of these animals provide a rapid and reliable system for assessing the bioactivity of agents that reverse multidrug resistance. Immunocytochemical analysis of bone marrow smears suggests that the activation of the \textit{MDR1} transgene has occurred at a very early stage of bone marrow differentiation since most bone marrow cells express the transporter. Expression of this transgene in bone marrow produces about 10-fold resistance to leukopenia induced by taxol compared to normal bone marrow. Chemosensitization of \textit{MDR1} mice to daunorubicin and taxol, measured by a fall in WBC, is detectable at a dose as low as 0.01 mg/kg R-verapamil. A dose of 0.5 mg/kg R-verapamil reduces the WBC by nearly 50%. Chemosensitization of \textit{MDR} transgenic mice with 5 mg/kg R-verapamil, which is highly effective in reversing MDR and readily tolerated by mice, necessitates a reduction of the maximum tolerated dose of most chemotherapeutic agents by only 20%. In addition, detailed histopathological examination shows that treatment of mice with chemotherapeutic drugs and R-verapamil does not change the organ-related toxicity pattern but only moderately accentuates inherent toxic side effects of the chemotherapeutic agents. We conclude that \textit{MDR1}-transgenic mice represent a valid model for evaluating efficacy, potency, and toxicity associated with chemotherapy and chemosensitization of multidrug-resistant cells in animals.

INTRODUCTION

Classical chemotherapy of metastatic disease has routinely produced cures only in testicular cancer, some leukemias and lymphomas, Hodgkin's disease, and some childhood sarcomas. In solid carcinomas such as breast carcinoma, treatment is at most palliative, remissions are of transient duration, and the overall benefit for the patient is sometimes questionable. A broad-spectrum chemoresistance, termed multidrug resistance, is one major obstacle to more favorable results (1). Recent approaches which are being pursued to improve therapeutic results include the search for new and better chemotherapeutic agents and the search for methods of enhancing the efficacy of classical chemotherapy (2).

It was previously shown that a \textit{M}, 170,000 plasma membrane protein, termed P-glycoprotein, which is encoded in humans by the \textit{MDR1} gene (3), is sufficient to produce MDR in human cancer cells (1-4). P-glycoprotein functions as a multidrug transporter (2) that rapidly extrudes many types of natural-product chemotherapeutic agents from the target cancer cell before the drugs can exert their designated effects (5).

Clinical interest in this gene was stimulated when it became evident that most drug-resistant human tumors express the \textit{MDR1} gene (6), that \textit{MDR1} RNA levels are elevated in many cancers which have failed to respond to chemotherapy (6-8), and that the presence of \textit{MDR1} gene expression may predict poor results in chemotherapy using agents affected by the multidrug transporter (9, 10). Hence, it is believed that interference with the underlying mechanism conferring MDR, termed chemosensitization, could potentiate the efficacy of classical chemotherapy (11) and that the number of human cancers amenable to chemotherapy may also be increased.

Numerous agents which inhibit the activity of the multidrug transporter, such as verapamil, have been described (11), and the majority of these appear to be substrates for the transporter which compete with anticancer drugs for their transport (5, 12). An initial report on a limited number of patients with drug-resistant multiple myeloma suggests that verapamil can reverse multidrug resistance in a clinical trial (13), and several other trials are now under way. However, the inherent and potentially toxic cardiovascular activities of verapamil necessitate the search for new and better resistance modifiers (14). The development of preclinical models for the rapid testing of agents which circumvent MDR is, therefore, a high priority of drug resistance research. We have previously investigated transgenic mice which express the human \textit{MDR1} gene in their bone marrow cells. We demonstrated that measurements of their WBC provide a simple and reliable system of screening for chemosensitzers in vivo (15) and help to determine whether drugs that appear, on the basis of tissue culture samples, to be useful in overcoming MDR would act in a similar manner in an animal (16). The present studies focus on the bone marrow cell type-specific expression of transgenic P-glycoprotein, indicate the degree of chemoresistance afforded by the human \textit{MDR1} transgene in vivo, determine the potency of chemosensitization possible in our \textit{MDR}-transgenic mouse model, and evaluate the toxicity associated with chemotherapy and chemosensitization in this novel animal system.

MATERIALS AND METHODS

\textbf{MDR-transgenic Mice.} The development and characterization of transgenic mice expressing the human \textit{MDR1} gene in their bone marrow has been published elsewhere (15, 17). A plasmid carrying the full-length \textit{MDR1} complementary DNA under the control of a chicken \textit{\beta}-actin promoter was injected into fertilized C57Bl/6 × SJL F1 mouse embryos, and these transgenic embryos were implanted in foster mice. After establishing a homozygous line (MDR 39), males were backcrossed to MDR-negative C57Bl/6 × SJL F1 females. The heterozygous descendants were analyzed for the MDR transgene using DNA obtained from tail samples (5) which were hybridized with the MDR-specific probe pHDR5A (18). In these studies, only 6-8-week-old sex-matched, MDR-heterozygous littermates were used.
Transgenic Mice. The MDR-transgenic mice mainly express P-glycoprotein (Centocor) at a 1:2 dilution containing 10% bovine serum albumin (BSA). R-verapamil was provided by courtesy of the manufacturer (St. Louis, MO). The drugs were administered by a single i.p. injection into the lower right quadrant of the abdominal cavity.Drug concentrations were adjusted so that a maximum volume of 400 μl was injected per experiment. Each experiment included a minimum of 3 animals/group and was repeated at least once. The results were highly reproducible, with less than 10% variation in WBC. Peripheral blood was collected by periorbital bleeding with heparinized microhematocrit capillary tubes (Fisher) and diluted 1:20 (v/v) in 3% acetic acid solution for erythrocyte lysis. The refractile, viable WBC were counted in an ultraplane Neubauer's hemocytometer (Hausser).

Detection of P-Glycoprotein in Bone Marrow Cells. Marrow was flushed from long bones with PBS/1 mM EDTA using a syringe with a 25-gauge needle. Bone marrow cells were separated from the matrix core by manual pipetting, filtered through gauze, and washed twice in PBS/1% albumin. After rewashing in PBS, cells were attached to a positively charged adhesive slides (Biorad) and allowed to settle for 20 min in a humidified chamber. Nonvital cells, which had lost their membrane charge, were removed by washing with PBS. Cells were then fixed in acetone for 8 min and air dried. The samples were incubated with the commercially available C219-fluorescein isothiocyanate conjugate (Centocor) at a 1:2 dilution containing 10% bovine serum albumin for 1 h. An irrelevant antibody conjugate provided by the manufacturer was used as a negative control. Experiments were performed with cells from the bone marrow of normal control mice and of MDR-transgenic mice. MDR Chinese hamster ovary cells and human normal adrenal gland served as positive control samples. More than 1500 bone marrow cells/experiment were first analyzed by bright-field illumination and then reidentified by dark-field microscopy using immunofluorescence staining. In parallel, specimens from the same bone marrow preparation were morphologically evaluated using standard May-Grünwald-Giemsa staining (Sigma).

Histopathology. To assess the toxicity associated with chemotherapy and chemosensitization, groups of mice were challenged with the maximum tolerated dose of each chemotherapeutic agent as detailed in Table 1. Simultaneously, R-verapamil was administered at 5 mg/kg. On day 7, animals were sacrificed by cervical dislocation, and organs were immediately placed in PUS buffered formalin (10%) and sent for pathology. Histological specimens were prepared and stained with cells from the bone marrow of normal control mice and of MDR-transgenic mice. MDR Chinese hamster ovary cells and human normal adrenal gland served as positive control samples. More than 1500 bone marrow cells/experiment were first analyzed by bright-field illumination and then reidentified by dark-field microscopy using immunofluorescence staining. In parallel, specimens from the same bone marrow preparation were morphologically evaluated using standard May-Grünwald-Giemsa staining (Sigma).

RESULTS

Most Bone Marrow Cells Express P-glycoprotein in the MDR-Transgenic Mice. The MDR-transgenic mice mainly express the MDR1 gene in their bone marrow cells (17). We have recently reported that this transgene confers in vivo resistance to all chemotherapeutic agents of the MDR family of drugs and that apparently all major types of peripheral WBC are protected as indicated by differential WBC (15). When expression of P-glycoprotein is directly determined in bone marrow smears of MDR-transgenic and of normal control mice using fluorescein-conjugated monoclonal antibody C219, virtually all of the bone marrow components of the MDR-transgenic mice were found to express P-glycoprotein, as indicated by immunofluorescent staining at their cell surface (Fig. 1A, top). In addition, the distribution of cells in the bone marrow of MDR-transgenic mice was analyzed (Fig. 1B). Examination of a total of 1500 bone marrow cells revealed that all of the expected types of normal cells were present. Myeloid and erythroid cell populations were abundant, whereas lymphatic and monocytic cells were present in fewer numbers. Occasionally, megakaryocytes were seen. The distribution and maturation of erythropoiesis and granulopoiesis were normal. There was no numerical enhancement of blast cells.

These results suggest that the expression of the MDR transgene occurs in all major bone marrow cells and that this expression must be activated at a very early stage of bone marrow differentiation. This explains why there is no reduction in the number of peripheral leukocytes, thrombocytes, and erythrocytes in these transgenic mice after chemotherapy (15).

Determination of Extent of Resistance to Taxol in MDR-Transgenic Mice. To establish the degree of resistance induced by the MDR transgene, groups of mice were challenged with increasing amounts of taxol. Taxol is a novel investigational antimicrotubule agent which seemed suitable for our studies due to its high bone marrow specificity (19). Fig. 2 demonstrates that taxol acts on bone marrow in a dose-dependent manner and that a dose as low as 2 mg/kg leads to a significant decrease in WBC (Fig. 2A). In accordance with previous studies, a reduction of the WBC of >50% was judged to be a significant change. In MDR-transgenic mice, however, the same amount of taxol (Fig. 2B) or a dose as high as 18 mg/kg (Fig. 2C) exerted no effect, because the bone marrow is drug resistant. At 20 mg/kg, a minor drop in the WBC was noted (Fig. 2C). Higher doses yield significant bone marrow suppression (Fig. 2D), which is similar to that produced by low doses of taxol in normal control mice (Fig. 2A). At 24 mg/kg (Fig. 2D), taxol lowered the WBC in MDR-transgenic mice by >50%. These data imply that the inserted MDR transgene results in an approximately 10-fold resistance of bone marrow cells to taxol in vivo.

Determination of Maximum Tolerated Doses of Chemotherapy in MDR-Transgenic Mice. To address the question of whether the MDR transgene generally diminishes the toxicity of chemotherapy and thus permits mice to tolerate higher doses, we determined the MTD of six commonly used MDR drugs. In these experiments, groups of 7-week-old female mice, which included a minimum of 6 animals/dose point, received chemotherapy as a bolus injection i.p. and were observed for 14 days.

Table 1 Determination of the maximum tolerated dose of various cytotoxic drugs in MDR-transgenic mice in the absence and presence of R-verapamil at 5 mg/kg

<table>
<thead>
<tr>
<th>Chemotherapeutic drug</th>
<th>Chemotherapeutic drug alone (mg/kg)</th>
<th>Surviving animals</th>
<th>Higher dose (mg/kg)</th>
<th>Surviving animals</th>
<th>+ 5 mg/kg R-verapamil (mg/kg)</th>
<th>Surviving animals</th>
<th>Higher dose (mg/kg)</th>
<th>Surviving animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>24</td>
<td>6 (6)</td>
<td>30</td>
<td>2 (6)</td>
<td>14</td>
<td>6 (6)</td>
<td>16</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>14</td>
<td>6 (6)</td>
<td>16</td>
<td>3 (6)</td>
<td>10</td>
<td>24 (24)</td>
<td>12</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>15</td>
<td>6 (6)</td>
<td>20</td>
<td>2 (6)</td>
<td>12</td>
<td>6 (6)</td>
<td>15</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>7</td>
<td>6 (6)</td>
<td>10</td>
<td>2 (6)</td>
<td>6</td>
<td>6 (6)</td>
<td>7</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>27</td>
<td>6 (6)</td>
<td>30</td>
<td>5 (6)</td>
<td>20</td>
<td>6 (6)</td>
<td>25</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>1.4</td>
<td>6 (6)</td>
<td>1.5</td>
<td>3 (6)</td>
<td>1.2</td>
<td>6 (6)</td>
<td>1.4</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>
The MTD was defined as a dose that did not kill any of the mice, whereas any higher amount led to the chemotherapy-related death of at least 1 animal/group (Table 1). The data depicted in Fig. 3 reveal that only in the cases of taxol and daunomycin, known for their cytotoxicity to bone marrow (19, 20), does the MTD in MDR-transgenic mice exceed by far that in normal control animals. All of the MDR-transgenic mice survived 24 mg/kg taxol or 14 mg/kg daunomycin, whereas normal control mice tolerated only 7 mg/kg or 8 mg/kg, respectively. There was no difference in MTD between the MDR-transgenic and control mice for vinblastine, vincristine, doxorubicin, or actinomycin D (Fig. 3), and the MTDs for normal C57Bl/6 × SJL F1 mice correspond to those reported for other mouse strains (21, 22). We have previously shown that the MDR transgene is mainly expressed in bone marrow cells (17). These results imply that the MTD of chemotherapeutic agents in this novel animal model reflects their relative toxicity to bone marrow as opposed to other organ systems in vivo (Fig. 3). Thus, vinblastine, vincristine, doxorubicin, and actinomycin D must have a limiting toxicity in mice which is not due to bone marrow suppression, whereas killing by taxol and daunomycin can be prevented by protecting bone marrow with the MDR1 gene. As shown in Table 2, only the toxicity due to taxol proved to be sufficiently bone marrow specific for the MTD to reduce the WBC significantly in the MDR-transgenic mice. With all other drugs, the MTD did not overcome the inherent MDR1 activity in bone marrow (Table 2), whereas in normal mice the WBC fell by >50% at the MTD for these animals (data not shown).

Determination of Minimum Effective Dose and Toxic Effects of Chemosensitizers. The reversal of chemoresistance, termed chemosensitization, is of major clinical importance for research on drug resistance. Previous studies have indicated that the clear-cut difference between MDR-protected bone marrow in our transgenic model and unprotected normal bone marrow enables a reliable identification of chemosensitizers that reverse MDR in vivo (15, 16). We next wished to determine the potency of chemosensitization by measuring the minimum effective dose of chemosensitizing agent. In the experiments illustrated in Fig. 4A, groups of mice received 7 mg/kg taxol and different amounts of R-verapamil. R-verapamil is an optical isomer of verapamil with reduced calcium channel blocking activity and less cardiovascular effect than racemic verapamil, which is still able to circumvent MDR in vitro (23, 24) and in vivo (15, 16). Since much higher doses of R-verapamil than racemic verapamil can be tolerated, clinical trials with this substance have been initiated. Our data indicate that a dose as low as 0.01 mg/kg R-verapamil induces a detectable reduction of the WBC after taxol treatment. This effect increases in a dose-dependent manner, and a dose of 0.5 mg/kg R-verapamil decreases the WBC by nearly 50% (Fig. 4A). Although it is difficult to extrapolate doses from mouse to human, the area of highest sensitivity in this transgenic system includes the dose range of R-verapamil used in clinical trials (25).

We also examined whether a combination of a chemosensitizer and cytotoxic drug could produce toxic side effects in this model. To do so, we determined the MTD (Table 2) of chemotherapeutic agents in conjunction with 5 mg/kg (~10 μM) R-verapamil. In our bone marrow preparations all normal cells were present. Myeloid and erythropoietic forms were abundant, whereas lymphatic and monocytic cells were present in fewer numbers. Occasionally, megakaryocytes were seen. The distribution and maturation of erythropoiesis and granulopoiesis were normal. There was no numerical enhancement of blast cells. BMC, bone marrow cells.
verapamil. Table 2 shows that chemosensitization under these conditions necessitates a reduction of the maximum applicable dose of most of the anticancer drugs by approximately 20%. Only in the case of taxol, which has predominantly bone marrow-toxic activity, does the circumvention of the MDR protection make a dose reduction by 45% necessary. This indicates that the reversal of resistance against a highly organ-specific drug such as taxol may be associated with more toxicity than that against drugs with a broader tissue spectrum such as Vinca alkaloids, etc. In addition, when we plotted these MTDs of drugs combined with 5 mg/kg R-verapamil against WBC, the drop in WBC depended on the chemotherapeutic drug (Fig. 4B). In the case of doxorubicin, daunomycin, and taxol, combined therapy with R-verapamil reduced the WBC to 22, 39, or 38% of original values, respectively, whereas the WBC was only decreased to 59, 54, or 63% when vinblastine, vincristine, or actinomycin D was applied. Resistance to the epipodophyllotoxin etoposide, which was used at 20 mg/kg in this experiment, was only moderately reversed. Since we used the same chemosensitizer at a fixed dose in conjunction with various chemotherapeutic agents, only the inherent properties of the cytotoxic drugs are responsible for the differences in WBC reduction. Hence, the underlying bone marrow toxicity of a chemotherapeutic agent is responsible for the effect once a chemosensitizer overcomes protection afforded by the MDR1 gene. These results indicate that resistance to anthracyclines such as doxorubicin and daunomycin, and to taxol, which are all highly bone marrow suppressive, is more readily reversed in this model than resistance to Vinca alkaloids such as vinblastine and vincristine, and the antibiotic actinomycin D, which are intrinsically less bone marrow toxic (26).

Pathological Effects of Chemotherapy Combined with Chemosensitizers. To determine the organ toxicity associated with combined chemotherapy and chemosensitization, groups of mice were treated with the MTD (Table 2) of vinblastine, doxorubicin, or daunomycin plus 5 mg/kg R-verapamil. On day 7, the mice were sacrificed, and a necropsy was performed. Clinically, none of the animals were in apparent distress, but in those animals which had received vinblastine or doxorubicin, but not daunomycin, a reduction in body weight of 5 to 17% was noted. This was presumably due to reduced food and fluid intake, since no obvious diarrhea occurred. At autopsy, the vinblastine- and doxorubicin-treated animals exhibited macroscopic signs of intestinal inflammation and occasional hemorrhages. In addition, vinblastine-treated mice showed enlargement of the liver, which was soft and pale. In daunomycin-treated mice, the spleens were enlarged and had focal areas of hemorrhage. No other gross pathological findings were detected.

Examples of the histopathological specimens are shown in Figs. 5 and 6. Microscopically, the small intestine of vinblastine-treated mice was characterized by generally moderate acute inflammation with occasionally severe exacerbation of the inflammatory reaction, moderate epithelial hyperplasia, and mild hemorrhage (Fig. 5A). In doxorubicin-treated mice, intestinal lesions included severe epithelial hyperplasia, moderate active chronic inflammation, and a few areas of focal necrosis (Fig. 5B). The intestinal toxicity of daunomycin-treated mice was much less pronounced (Fig. 5C) than in vinblastine- or doxorubicin-treated mice and consisted mainly of mild inflammation

Table 2 Effects of the maximum tolerated dose of several cytotoxic drugs on the WBC of MDR-transgenic mice with or without chemosensitization with R-verapamil

<table>
<thead>
<tr>
<th>Chemotherapeutic drug</th>
<th>Chemotherapeutic drug alone (mg/kg)</th>
<th>WBC (%)</th>
<th>+ 5 mg/kg R-verapamil (mg/kg)</th>
<th>WBC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>24</td>
<td>26</td>
<td>14</td>
<td>38</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>14</td>
<td>95</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>15</td>
<td>100</td>
<td>12</td>
<td>59</td>
</tr>
<tr>
<td>Vincristine</td>
<td>7</td>
<td>100</td>
<td>6</td>
<td>54</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>27</td>
<td>100</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>1.4</td>
<td>100</td>
<td>1.2</td>
<td>63</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of taxol chemotherapy on WBC in mice. A, normal mice. B, MDR-transgenic mice. C, MDR-transgenic mice. D, MDR-transgenic mice. E, 24 mg/kg taxol. F, 18 mg/kg taxol. D, MDR-transgenic mice. F, 24 mg/kg taxol. Experiments were conducted as explained in "Materials and Methods" and in Table 1.

Fig. 3. Determination of the maximum tolerated dose of chemotherapeutic agents in MDR-transgenic or in normal control mice. Experiments were conducted as explained in "Materials and Methods" and in Table 1. TAX, taxol; DAU, daunomycin; VBL, vinblastine; VCR, vincristine; DOX, doxorubicin; Act D, actinomycin D.

Fig. 4. Effects of taxol chemotherapy on WBC in mice. A, normal mice. B, MDR-transgenic mice. C, MDR-transgenic mice. D, 18 mg/kg; O, 20 mg/kg taxol. D, MDR-transgenic mice. 22 mg/kg; 8, 24 mg/kg taxol. Experiments were conducted as explained in "Materials and Methods" and in Table 1.
mild hyperplasia. Fig. 5D shows the normal histology of the small intestine of control mice, which received R-verapamil but not chemotherapy.

The spleens of daunomycin-treated mice displayed severe extramedullary hematopoiesis, and erythroidopoietic and granulocytic precursors and megakaryocytes were seen (Fig. 6A). In vinblastine-treated mice, liver alterations were noted that consisted of severe vacuolation, moderate cellular hyperplasia, and mild degeneration of connective tissues (Fig. 6B). All of the changes described in Figs. 5 and 6 are known to be associated with chemotherapy with vinblastine, doxorubicin, or daunomycin. They indicate that i.p. application of drugs in mice under these conditions may result in toxicity similar to that found with i.v. administration in humans. Thus, systemic absorption rather than local effects are more likely to cause these lesions. Moreover, chemosensitization did not change the pattern of organ toxicity. It therefore seems unlikely that inhibition of P-glycoprotein in normal tissues would have consequences other than a potential enhancement of anticipated effects, which are specific for particular cytotoxic drugs.

**DISCUSSION**

The MDR-transgenic mice have human MDR1 RNA in their bone marrow at levels comparable to those seen in the in vitro selected MDR cell line KB-8-5 (17). This level of expression corresponds to levels detected in many drug-resistant human cancers (6). Our data demonstrate that this MDR1 gene expression is sufficient to render resistance as high as 10-fold in vivo (Fig. 2). These results reinforce our previous conclusion that human cancers which express comparable levels of MDR1 RNA will be resistant to many natural-product chemotherapeutic drugs in vivo.

It currently appears difficult to develop new natural-product chemotherapeutic agents that will overcome the protection of many human tumors resulting from MDR1 gene expression without causing unacceptable toxic side effects due to the large amounts needed. Thus, an innovative approach that combines classical chemotherapy with chemosensitization seems more promising in theory, if toxicity associated with this treatment modality does not compromise clinical applicability. Currently available chemosensitizing agents, such as racemic verapamil, have potent pharmacological effects which limit their usefulness in patients. Most clinical studies using the prototype chemosensitizer verapamil have been uninformative, because the cardiovascular activities of verapamil have limited the plasma levels that can be achieved in humans (27, 28), and low plasma levels have only resulted in transient responses (29, 30). Only in the case of multiple myeloma have more favorable success rates been reported, which were attributed to an unusual susceptibility of these cancer cells to verapamil-enhanced chemotherapy (13). However, in all of these studies toxicities related to the chemosensitizer proved to be salient and thus to be the limiting factor. The situation is now changing, because new chemosensitizers such as R-verapamil, which have reduced cardiovascular side effects, have been introduced or will soon be available. It therefore appears likely that toxicities inherent to the chemotherapeutic agents will become more prominent, and studies addressing this question seem warranted. The MDR-transgenic mice described in this report provide a model system in which to test both the efficacy of new chemosensitizers and their toxicity when combined with chemotherapeutic agents.

One possible obstacle to the reversal of MDR in vivo is that expression of the MDR1 gene is not unique to neoplastic tissue (31, 32) and that chemosensitization may also sensitize nonmalignant cells to the toxicity of chemotherapy. It has been previously reported that verapamil significantly exacerbates vincristine-related intestinal toxicity in tumor-xenograft-bearing mice (33). More recently, these authors have demonstrated that the route of administration has an impact on the intensity of the intestinal lesions (34). Bolus i.p. injection of vincristine produces more side effects than continuous i.v. infusion, when a steady plasma level of 10 µM verapamil is maintained. This is far above a clinically achievable concentration of verapamil (25).

Our toxicity studies used doses of chemosensitizers which were efficient at reversing drug resistance and were readily tolerated by the mouse. We used 5 mg/kg (~10 µM) R-verapamil for chemosensitization, which is well below the dose of 150 mg/kg which is tolerated by mice (15). Moreover, we used bolus injections rather than continuous infusion, because this regimen is more likely to provoke toxic complications (34), and it was our goal to evaluate such complications. In addition, chemotherapy is nowadays often given on an outpatient basis, and
CHEMOSENSITIZATION OF MDR-TRANSGENIC MICE

Fig. 5. Intestinal toxicity associated with chemotherapy and chemosensitization in mice. A, vinblastine chemotherapy + R-verapamil (5 mg/kg) chemosensitization. Specimens are characterized by moderate acute inflammation and moderate epithelial hyperplasia (arrows). B, doxorubicin chemotherapy + R-verapamil (5 mg/kg) chemosensitization. Examples show severe epithelial hyperplasia, moderate inflammation, and few areas of focal necrosis (arrows). C, daunomycin chemotherapy + R-verapamil (5 mg/kg) chemosensitization. The intestines mainly displayed mild alterations such as mild inflammation and mild hyperplasia (arrow). D, control mouse (no chemotherapy) + R-verapamil (5 mg/kg). Experiments were conducted as explained in "Materials and Methods" and in Table 1. Groups of mice were challenged with the maximum tolerated dose of the chemotherapeutic agents as detailed in Table 1 in conjunction with R-verapamil.

prolonged infusions may compromise this strategy. Under these conditions, we found that the MTD for the chemotherapeutic agents had to be reduced by approximately 20% for most drugs compared to chemotherapy alone (Tables 1 and 2 and Fig. 3). This drug reduction appears to be acceptable, because most drugs still produced a decrease of 44–78% in the WBC when combined with R-verapamil. Since a fall in WBC in this transgenic model is a reliable indicator of the reversal of MDR (15), a therapeutic net benefit for the patient should be achieved using this approach (Table 1 and Fig. 4B).

Since earlier studies showed that the MDR transgene is not expressed in the small intestine (17), intestinal toxicity associated with chemotherapy and chemosensitization (Fig. 5) cannot be attributed to inhibition of the human MDRI transgene. However, expression of endogenous mouse mdr sequences must be considered. In the mouse, three mdr genes have been identified, and tissue-specific expression has been reported (35). In intestinal tissue, mRNA levels of mdr3 (also known as mdr1a) are quite high, and it was previously demonstrated that there was increased accumulation and retention of vincristine in the intestinal tissue of mice receiving verapamil infusions (33). The presence of such a verapamil-sensitive efflux mechanism in small intestine suggests that the mdr1a gene product mediates drug efflux in the mouse. Nevertheless, our data, from an aggressive approach to administering the MTD of chemotherapeutic agents as a bolus injection combined with 5 mg/kg of R-verapamil, indicate that the pathological changes observed as toxic side effects (Figs. 5 and 6) are tolerable.

The chemosensitization studies have also allowed a determination of the underlying organ or tumor toxicities of the chemotherapeutic agents. These studies, in which the bone marrow of the MDR-transgenic animals is protected from the toxicity of natural-product chemotherapeutic agents, make it possible to determine what limiting (fatal) toxicity is for each of the drugs. A striking result of our studies is that the MDRI-transgenic mice are protected from death by daunomycin and taxol, but not by vincristine, vinblastine, doxorubicin, or actinomycin D (Fig. 3). These results imply that, in mice, non-marrow-mediated toxicity is responsible for death associated with high doses of these latter drugs.

Our experiments involving chemotherapy and chemosensitization also demonstrate that the reversal of MDR did not change the organ-related pattern of the toxic lesions (Figs. 5 and 6). Hence, if a more organ- or tumor-specific effect in such
Arrows, severe vacuolation and moderate cellular hyperplasia. Experiments were conducted as explained in "Materials and Methods" and in Fig. 5.

vinblastine chemotherapy + R-verapamil chemosensitization: effects on liver.

chemosensitization: effects on spleen. Arrows, severe extramedullary hematopoiesis in MDR-transgenic mice. A, daunomycin chemotherapy + R-verapamil will be important. We have previously reported that in human effect on peripheral WBC, drugs like anthracyclines and taxol, trials should carefully consider the selection of a potent and to be active against certain cancers. Therefore, future clinical These data support the idea that certain drugs are more likely therapeutic agents, which should be active against the tumor type examined in the absence of drug resistance mechanisms. To design such studies, the MDR-transgenic mouse model will be a useful tool. It provides a reliable system of assessing whether chemosensitizers possess the appropriate bioactivity and pharmacokinetic properties to justify clinical trials (15), and it is most sensitive to chemosensitizers at concentrations within the clinically achievable dose range of these agents (Fig. 4A).

Finally, essential information on new chemotherapeutic agents may be acquired using this model. Since the function of the MDR transgene is restricted to bone marrow protection, new drugs with high bone marrow specificity may be administered in much higher concentrations to reveal activity in other organs, which otherwise would have been missed. New chemotherapeutic agents which are not affected by the multidrug transporter will reduce WBC in the MDR-transgenic mice, and thus these animals may serve as an in vivo screen for such agents. Since bone marrow suppression is the dose-limiting factor for many kinds of chemotherapy, the clear-cut difference between MDR-protected bone marrow and normal bone marrow (Fig. 3) also means that the MDR-transgenic mice can be used to screen for bone marrow toxicity when chemotherapeutic agents are evaluated.

Fig. 6. Spleen and liver toxicity associated with chemotherapy and chemosensitization in MDR-transgenic mice. A, daunomycin chemotherapy + R-verapamil chemosensitization: effects on spleen. Arrows, severe extramedullary hematopoiesis and erythropoietic and granulocytic precursors and megakaryocytes. B, vinblastine chemotherapy + R-verapamil chemosensitization: effects on liver. Arrows, severe vacuolation and moderate cellular hyperplasia. Experiments were conducted as explained in "Materials and Methods" and in Fig. 5.

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REFERENCES

15. Mickisch, G. H., Merlino, G. T., Galski, H., Gottesman, M. M., and Pastan, A clinical study is desired, the choice of chemotherapeutic agent will be important. We have previously reported that in human renal cell carcinoma cells vinblastine resistance is much more readily reversed by R-verapamil than is anthracycline resistance (16, 24). Vinblastine is considered to be the single most effective drug against renal cell carcinoma (36). In our MDR-transgenic model, where bone marrow toxicity is a prerequisite for an effect on peripheral WBC, drugs like anthracyclines and taxol, known for their bone marrow suppression (19, 20), lowered the WBC more than the other chemotherapeutic agents (Fig. 4B). These data support the idea that certain drugs are more likely to be active against certain cancers. Therefore, future clinical trials should carefully consider the selection of a potent and less toxic chemosensitizer as well as the choice of chemothera-
CHEMOSENSITIZATION OF MDR-TRANSGENIC MICE


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