Retroviral Transfer of the Human MDR1 Gene Confers Resistance to Bisantrene-specific Hematotoxicity

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ABSTRACT

In this work, we demonstrate a protective effect conferred by the human multidrug resistance gene (MDR1) to populations of the murine hematopoietic system against the toxic effects of bisantrene, a novel intercalating cytotoxic agent under investigation as an anticancer agent. In vitro, MDR1-expressing cell lines are highly cross-resistant to bisantrene, and low levels of P-glycoprotein (the MDR1 gene product cell surface protein) confer resistance to the drug. MDR1-positive mice were generated after transplantation of bone marrow cells (BMC) transduced in vitro with a MDR1 retrovirus. Control mice were transplanted with BMC transduced with the neomycin resistance gene. Administration of a single i.v. dose of 50 mg/kg of bisantrene resulted in a decrease of the total WBC count of approximately 40%. In contrast, a decrease of the total WBC count of only 17% was observed in mice transplanted with MDR1-transduced BMC. Immunofluorescence studies with cell lineage-specific monoclonal antibodies showed that bisantrene was specifically toxic for B lymphocytes and macrophages. Double-staining with MRK16 (a monoclonal antibody specific for P-glycoprotein) demonstrated that a single dose of bisantrene increased the relative number of MDR1-transduced positive cells, macrophages, and (to some extent) granulocytes when compared to the number found in MDR1-untreated mice or the bisantrene-treated neomycin-transduced control mice. These results provide in vivo evidence that bisantrene is a hematotoxic drug capable of selecting for MDR1-transduced hematopoietic cells. Bisantrene might be useful for gene therapy as an in vivo selective agent for cells transduced with MDR1 vectors that also coexpress therapeutic genes of interest.

INTRODUCTION

The MDR1 gene encodes the Mr 170,000 ATP-dependent P-gp that is overexpressed in multidrug-resistant cell lines and acts as a multidrug transporter that actively pumps drugs out of cells. Bisantrene is a new anthracycline derivative that has shown cytotoxic activity in experimental in vitro and in vivo models against solid tumors and hematological neoplasias (2, 3). The mechanism by which it exerts this cytotoxic effect is DNA intercalation (4). Bisantrene is being tested in clinical trials with variable success, with a majority of cancers showing no response to treatment (2, 3, 5–11). In tissue culture and transgenic animal models, it has been demonstrated that P-gp plays a major role in resistance to bisantrene-related agents such as doxorubicin or anthracycline (12). A recent report indicates that high-level resistance to bisantrene results from the expression of P-gp (13). These data suggest that multidrug resistance resulting from the expression of the human MDR1 gene may play a major role in the mechanisms of cancer refractoriness to bisantrene observed in these trials. Higher therapeutic doses that could overcome this mechanism of resistance have not been tried due to the dose-limiting toxicity of bisantrene, principally myelosuppression (14), phlebitis (15), and anaphylactoid-type response (16).

Using retroviral vectors to transfer the MDR1 gene into BMC, our group and others have developed a mouse model in which BMC express the human MDR1 gene product. BMC transduced with MDR1 retroviral vectors selectively survive in vivo treatment with Taxol (17–19). Because bisantrene seemed to be an even better P-gp substrate than Taxol in vitro, it was our intention to test bisantrene in vivo to find out if the expression of MDR1 in BMC would result in protection against the toxic effect of the drug. We wished to see which cells would be the most affected by toxic doses of bisantrene and if bisantrene treatment of mice could enrich for MDR1-transduced BMC.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. NIH3T3 (the drug-sensitive parental cell line used in this study) is a continuous line of contact-inhibited cells derived from NIH Swiss mouse embryo cultures (a gift from C. Scher, Harvard University, Boston, MA). The previously characterized multidrug-resistant cell lines NIH3T3-MDR-G185 and NIH3T3-MDR-V185 (20) are populations of NIH3T3 cells transfected with the wild-type pHaMDR1/A (G185; Ref. 21) and mutant pHaMDR1/A (V185; Ref. 22) MDR1 retroviral vectors by the calcium phosphate coprecipitation method. The transfectants were initially selected in 60 ng/ml colchicine. The wild-type NIH-MDR-G185 was maintained in the presence of 60 ng/ml colchicine, whereas the mutant NIH-MDR-V185 (which confers greater relative resistance to colchicine) was exposed to increasing concentrations of the drug and was maintained in 1 μg/ml colchicine. Both transfected populations of cells displayed approximately the same amount of P-gp on their surfaces, as determined by fluorescence-activated cell sorting analysis and comparison to the parental negative population of NIH3T3 cells (23). The parental NIH3T3 cells and the transfectants were
grown in monolayer at 37°C in 5% CO₂, using DMEM supplemented with 10% fetal bovine serum (Whitaker BioProducts), 5 mM glutamine, 50 mM/L penicillin, and 50 mM/L streptomycin (Life Technologies, Inc.).

Drugs. Bisantrene hydrochloride was obtained from Dr. L. Greenberger (Wyeth-Ayerst Research, Pearl River, NY). Taxol was obtained from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD). Daunorubicin and colchicine were purchased from Sigma Chemical Co. DMSO is a gold-label Aldrich Chemical Co. product that was used as drug solvent for in vitro studies.

Colony-forming Assay for Bisantrene Resistance In Vitro. The dose-response curves of the NIH3T3 cell line and its drug-resistant transfectants NIH3T3-MDR-G185 and NIH3T3-MDR-V185 were determined by plating 300 exponentially growing cells per 60-mm dish in the absence of drug (24, 25). After a 16-h incubation at 37°C, the appropriate concentrations of stock solutions of bisantrene or Taxol dissolved in DMSO (<0.5% of total volume/dish) were added to each cell line. After incubating the cells at 37°C for 8–10 days, colonies were stained with 0.5% methylene blue in 50% ethanol and counted with a Manosat colony counter. The LD₅₀ for each cell line was determined by the concentration of drug that reduced the cloning efficiency of the transfectant to 50% of the control without drug. Relative resistance was determined by dividing the LD₅₀ of the resistant cell line by the LD₅₀ of the parental cell line.

Viral Producer Cell Lines. A high-titer, helper-free, ecotropic producer clone from the GP+E86 packaging cell line (provided by Dr. A. Bank, Columbia University, New York, NY; Ref. 26) generating a retroviral vector containing the MDR1 cDNA with a Harvey murine sarcoma virus long terminal repeat (pHaMDR1, a plasmid containing the MDR1 gene under control of the long terminal repeat promoter sequences of the Harvey murine sarcoma virus) was used (22). A Harvey murine leukemia vector containing the bacterial neomycin resistance (Neo-r) gene produced in GP+E86 was used as a control vector (pHaNeo; Ref. 26). The viral producer cell lines were cultured in DMEM supplemented with 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine (Life Technologies, Inc., Greenbelt, MD), and 10% calf serum (Colorado Serum Co., Denver, CO). The MDR1 producer cell line was cultured in 60 ng/ml colchicine, and the neomycin producer cell line was cultured in 650 μg/ml G418 (Sigma Chemical Co.). Twenty-four h before the experiment, the cells were trypsinized, and 1 × 10⁶ cells were seeded in 10 ml of drug-free medium.

Transduction of BMC and BMT. BMC were harvested from 6–12-week-old C57Bl/6 mice purchased from Frederick Research Laboratories (Frederick, MD) 48 h after they had received 150 mg/kg of 5-fluorouracil. BMC were counted, and 3 × 10⁶ cells were resuspended with 7–10 ml of cell-free viral supernatant in a T-75 flask. Cultures were incubated for 3–4 days, during which time they were subjected to a total of 6–8 rounds of transduction with 7–10 ml cell-free viral supernatant from either GP+E86MDR or GP+E86Neo producer cells. The growth factors (100 ng/ml of mouse interleukin 3 and 200 ng/ml of human interleukin 6; Collaborative Science, Inc.) and 2.5 ng/ml of rat stem cell factor (Amgen, San Diego, CA) were added before each round of viral infection. Before BMT, BMC cultures were collected and counted in a hemocytometer, using trypan blue to determine cell viability. The BMT procedure was based on a protocol described previously (27). C57Bl/6 mice were reconstituted with 1–3 × 10⁶ BMC 6–8 h after receiving a lethal irradiation dose of 1.1 Gy. A group of mice, designated as MDR mice, was transplanted with pHaMDR1-transduced BMC. As negative controls, designated Neo mice were transplanted with pHaNeo-transduced BMC. Nonreconstituted irradiated control mice were included in all experiments and were no longer alive 9–12 days postirradiation.

Preparation and Administration of Bisantrene. Bisantrene hydrochloride was dissolved in 12.5% DMSO and 87.5% of 0.9% saline solution (bisantrene stock solution of 10 mg/ml). At 25–30 days after BMT, mice were injected i.v. via tail vein with bisantrene at different doses (ranging from 10 mg/kg to 150 mg/kg) or the equivalent volume of the carrier alone, or were not injected at all.

WBC and Differential Staining Obtained by Flow Cytometry Analysis. Eye bleeds were obtained before bisantrene administration and 3, 7, and 21 days postinjection. WBC counts were performed on a hemocytometer after the RBC were lysed using a RBC lysing buffer solution (Becton Dickinson, San Jose, CA). Analyses of stained cells were performed on a FACSsort (Becton Dickinson). Differential staining was performed by incubating blood cells for 30 min at 4°C with 5 μg/ml phycocyanin (orange-red fluorescence)-labeled monoclonal antibodies specific for immature and mature granulocytes (Gr-1; Ref. 28), immature and mature B cells (CD45R/B220; Ref. 29), immature and mature macrophages (Mac-1; Ref. 30), and pan-T lymphocytes (Thy-1.2; Ref. 31), all purchased from PharMingen (San Diego, CA). The cells were washed twice and then analyzed by measuring fluorescence intensity levels registered as histograms plotted against the X axis displaying the relative channel number on a logarithmic scale, with the relative cell number displayed on a linear axis. For detection of P-gp, we performed a double-staining procedure using 10 μg/ml MRK16 (a P-gp-specific monoclonal antibody provided by Dr. Ono, Hoechst, Kawagoe City, Japan; Ref. 32) conjugated in our laboratory with FITC (green fluorescence). The double staining was performed by coincubating the cells with lineage-specific monoclonal antibodies and MRK16-FITC antibody. Cells that stained positively with the respective lineage-specific antibody were gated out, and their MRK16 staining levels were plotted against the X axis displaying the fluorescence level on a linear scale, with the relative cell number on the Y axis.

Statistical Analyses. Statistical analyses were performed using Student’s t test for comparison of means. Ps were obtained by comparing the differences between the means of cell counts at day 0 with the means of cell counts at days 3 and 7.

RESULTS

MDR Cell Lines Are Highly Cross-Resistant to Bisantrene. Resistance to anticancer drugs was assayed in a clonogenic cell-killing assay. As expected, both drug-resistant NIH3T3 transfectants G185 (wild-type P-gp) and V185 (mutant-type P-gp) exhibited the usual MDR1 phenotype characterized by cross-resistance to four unrelated antitumor agents (Table 1) including colchicine, the selecting agent; bisantrene and
daunorubicin, the anthracyclines; and Taxol, the natural product drug. Compared to colchicine, the G185 cells exhibited a somewhat enhanced resistance to Taxol and daunorubicin. More significant was the approximately 11-fold greater resistance demonstrated against bisantrene as compared to colchicine. The mutant V185 cells showed preferential relative resistance to colchicine (10-fold > G185). In addition, the mutant V185 cells showed a 2-fold decrease in relative resistance to Taxol when compared to the relative resistance of G185 cells and little difference in daunorubicin resistance. Relative resistance was higher to bisantrene than to any of the other drugs for both wild-type and mutant transfecants, with no major difference in bisantrene resistance seen for mutant or wild-type. In this assay, bisantrene was the most cytotoxic drug tested with the lowest LD_{50}.

**Bisantrene Induces Leukopenia in a Dose-dependent Manner.** Bisantrene induced leukopenia in Neo mice in a dose-dependent manner, with WBC count nadirs at day 3 postinjection. Seven days after injection, the counts increased slightly but were still less than normal values. Results are displayed as bar graphs in Fig. 1, representing percentages of WBC counts in all groups of mice at days 3 and 7, compared to their WBC counts at day 0. The counts within the untreated and carrier-treated mice remained relatively stable during the experiment. In the untreated group (n = 12), WBC counts varied from 96.5% (P = 0.2) at day 3 to 100.7% (P = 0.9) at day 7. No statistically significant variation of WBC was observed in the group of mice treated with the carrier alone (n = 8) at days 3 and 7, with WBC counts of 110.8% (P = 0.119) and 122.8% (P = 0.542), respectively. When 10 mg/kg bisantrene were administered (n = 5), WBC counts dropped to 74.9% (P = 0.05) at day 3 and returned to 84.8% (P = 0.3) at day 7 postinjection. With administration of 20 mg/kg (n = 5), 50 mg/kg (n = 16), 75 mg/kg (n = 11), or 100 mg/kg (n = 9) bisantrene, the WBC counts fell to 44.3, 42.3, 33.5, and 29.6% at day 3 after i.v. injection, respectively (P < 0.05 in all groups). At day 7, the WBC counts were 84.9% (P = 0.3), 58.3% (P < 0.05), 53.2% (P < 0.05), and 46.9% (P < 0.05) compared to the WBC counts before drug administration. Bisantrene given at a dose of 150 mg/kg (n = 5) induced the WBC counts to fall to 12.7% of normal at day 3 postinjection (P < 0.05) and led to death in 60% of the mice by day 7 postinjection (3 of 5 mice died at day 4), whereas counts in the surviving mice (n = 2) were 13.3% lower than their day 0 counts (P = 0.01). When tested 3 weeks after bisantrene administration, the counts were back to baseline values in all surviving mice (data not shown).

**MDR Mice Are Resistant to Bisantrene-induced Leukopenia.** The dose of 50 mg/kg seemed to reproducibly reduce the WBC of control Neo mice without causing death in any of the 16 mice tested with this dose. We administered that dose to test the effect of bisantrene in MDR mice. Table 2A displays the mean ± SD WBC counts obtained before bisantrene administration and at days 3 and 7 postinjection in all groups (untreated, treated with carrier alone, and treated with bisantrene) for the MDR mice and the Neo mice described in Fig. 1. Table 2B shows the Ps comparing the means of each group at day 0 with its respective means at days 3 and 7 after treatment, if any. The WBC counts in the Neo and untreated mice remained stable throughout the experiment. The carrier alone did not significantly affect the WBC in any of the groups. Bisantrene had a significant toxic effect on leukocytes in the Neo mice, whose WBC fell to 3.8 × 10^6/ml at day 3 postinjection, which was a decrease of 42.3% from the day 0 value (9.0 × 10^6/ml; P = 0.001). At day 7 after bisantrene injection, the WBC increased slightly to 5.2 × 10^6, or 58.3% of the day 0 counts (P = 0.001). In contrast, the MDR mice showed a small (but not statistically significant) drop in WBCs compared to day 0 counts: 84.5% at day 3 (P = 0.100) and 86.9% at day 7 (P = 0.541).

**B Cells and Macrophages Are Targets for Bisantrene Toxicity and Are Protected in MDR Mice.** After administration of 50 mg/kg bisantrene, we were able to calculate the number of B cells, T cells, granulocytes, and macrophages for each mouse by staining with lineage-specific monoclonal antibodies. Fig. 2 shows the mean WBC for each cell type (expressed as millions per ml) obtained from each group of mice at day 0 and day 3 of the experiment. These results were obtained after analysis of five untreated, five carrier-treated, and six bisantrene-treated Neo mice and three untreated, three carrier-treated, and six bisantrene-treated MDR mice. Fig. 2A shows that the number of B cells in the group of untreated Neo mice increased from 3.2 × 10^6/ml at day 0 to 4.8 × 10^6/ml at day 3. However, this increase was not significant (P = 0.525). The B-cell number in the untreated MDR mice remained stable from day 0 to day 3 (P = 0.696). None of the Neo or MDR mice treated with carrier alone showed any significant change in the number of B cells from day 0 to day 3 (P = 0.656 for Neo mice and P = 0.125 for MDR mice). Bisantrene induced a fall in the number of B cells in the Neo mice from 2.3 × 10^6/ml to 1.13 × 10^6/ml, which was probably significant (P = 0.051). In contrast, B-cell counts in the MDR mice treated with the same dose of bisantrene seemed to increase between day 0 and day 3 (1.8 × 10^6/ml and 2.56 × 10^6/ml, respectively). However, this change seemed not to be statistically significant (P = 0.70). The mean number of T cells and granulocytes for each group of mice is shown in Fig. 2B and C, respectively. In none of the groups did T cells or granulocyte counts change significantly (Ps ranged from 0.258 to 0.824 for the T cells and from 0.07 to 0.819 for the granulocytes). As shown in Fig. 2D, the macrophages seemed to be sensitive to bisantrene toxicity. Bisantrene injected in Neo mice induced a probably significant decrease in macrophage counts from 1.77 × 10^6/ml at day 0 to 0.7 × 10^6/ml at

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**Table 1** Relative resistance of multidrug-resistant NIH3T3 transfectant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Colchicine</th>
<th>Daunorubicin</th>
<th>Taxol</th>
<th>Bisantrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH3T3</td>
<td>100 (19)</td>
<td>1 (12)</td>
<td>1 (34)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>NIH-MDR-G185</td>
<td>10</td>
<td>13</td>
<td>57</td>
<td>112</td>
</tr>
<tr>
<td>NIH-MDR-V185</td>
<td>102</td>
<td>16</td>
<td>25</td>
<td>141</td>
</tr>
</tbody>
</table>

* Values represent the mean of three different cloning assays for each of the four cytotoxic drugs. The resistant cell lines are clones of NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters.

* Numbers in parentheses show the LD_{50} (ng/ml) of the parental NIH3T3 cells for each drug. Relative resistance is expressed as the LD_{50} of the resistant cell line divided by the LD_{50} of the parental drug-sensitive cell line.
The MDR1 Gene Confers Resistance to Bisantrene in Mouse BMC

976 The MDR1 gene confers resistance to bisantrene in mouse BMC. Bisantrene treatment selects for MDR1-expressing B-cell and macrophage populations. Bisantrene was found to increase the percentage of B cells and macrophages in the blood of MDR mice compared to Neo mice. The increase in B cell population was statistically significant (P < 0.001), while the increase in macrophage population was not statistically significant (P = 0.07). The MDR mice macrophage counts stayed stable after bisantrene administration, with 2 x 10^9/ml at day 0 and 2.5 x 10^9/ml at day 3 (P = 0.420). No significant difference in macrophage counts appeared between day 0 and day 3 in any of the untreated mice (P = 0.5 in Neo mice and P = 0.326 in MDR mice) or in the mice treated with carrier alone (P = 0.295 in Neo mice and P = 0.616 in MDR mice). We observed large variations of cell counts in the eight bisantrene-treated MDR mice. These variations may have contributed to the large P values for the counts, showing preferential resistance of B cells and macrophages in that group of mice.

Bisantrene Treatment Selects for MDR1-expressing B-Cell and Macrophage Populations. To assess if the demonstrated resistance to bisantrene in the MDR mice would result in a selective increase in specific cell lineages expressing P-gp, we used anti-P-gp and lineage-specific antibodies to perform double-staining studies on MDR mice that were treated with the carrier alone (n = 3) and on MDR (n = 6) and Neo (n = 6) mice that were treated with a single dose of 50 mg/kg bisantrene. Mice were evaluated 7 days after treatment. After gating the cell lineages that stained positively with the lineage-specific antibodies, we assessed the fluorescence of these cells stained with MRK16-FITC antibody. Shown in Fig. 3, top panels, are representative examples of blood cells stained with the cell lineage-specific monoclonal antibodies [Fig. 3A, CD45R/B220 (B lymphocytes); Fig. 3B, Thy-1.2 (T lymphocytes); Fig. 3C, Gr-1 (granulocytes); and Fig. 3D, Mac-1 (macrophages)]. The positive cells for each cell type were gated, and their respective fluorescence for MRK16-FITC is shown on the bottom panel as overlays of typical representative histograms of a bisantrene-treated Neo mouse, a carrier-treated MDR mouse, and two bisantrene-treated MDR mice (Fig. 3E, B cells; Fig. 3F, T cells; Fig. 3G, granulocytes; and Fig. 3H, macrophages). Staining was considered positive if a positive shift of the histogram (expressed as an increase of MCF) was observed in comparison to the MCF of the Neo mouse histogram. In our hands, flow cytometry analysis was sufficiently sensitive to specifically detect populations that represent at least 1% of the total, and we

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**Table 2** Absolute WBC counts in different groups of mice, untreated or treated with carrier alone or 50 mg/kg bisantrene

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 3 postinjection</th>
<th>Day 7 postinjection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo mice untreated</td>
<td>9.1 ± 1.2 (n = 12)</td>
<td>8.7 ± 1.8 (n = 12)</td>
<td>9.1 ± 1.7 (n = 12)</td>
</tr>
<tr>
<td>Neo mice + carrier</td>
<td>8.1 ± 2.2 (n = 8)</td>
<td>8.9 ± 1.3 (n = 8)</td>
<td>9.9 ± 1.2 (n = 8)</td>
</tr>
<tr>
<td>Neo mice + bisantrene</td>
<td>9.0 ± 1.1 (n = 16)</td>
<td>3.8 ± 1.2 (n = 16)</td>
<td>5.2 ± 1.2 (n = 16)</td>
</tr>
<tr>
<td>MDR mice untreated</td>
<td>7.9 ± 2.3 (n = 5)</td>
<td>7.3 ± 2.2 (n = 5)</td>
<td>7.7 ± 1.1 (n = 5)</td>
</tr>
<tr>
<td>MDR mice + carrier</td>
<td>7.9 ± 1.6 (n = 3)</td>
<td>7.3 ± 2.4 (n = 3)</td>
<td>7.8 ± 1.3 (n = 3)</td>
</tr>
<tr>
<td>MDR mice + bisantrene</td>
<td>8.4 ± 3.1 (n = 8)</td>
<td>7.1 ± 2.2 (n = 8)</td>
<td>7.3 ± 2.7 (n = 8)</td>
</tr>
</tbody>
</table>

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*Panel A displays the mean number of WBCs in millions per ml ± SD for each group of mice at days 0, 3, and 7.

*Panel B shows the Ps for the difference of the mean between the indicated groups at days 3 and 7 and the mean of each group at day 0.

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Fig. 1 Dose-dependent effect of bisantrene on Neo mice. Bar graph displays the percentage of WBCs obtained at day 3 and day 7 after treatment with different doses of carrier solvent alone (DMSO-saline) or bisantrene at day 0.
found that comparison of the MCF represents a simple and accurate way for assessing the positively stained samples when they are compared to their negative controls. Fig. 3E shows that the B cells of the untreated MDR mouse stained positively with MRK16-FITC (MCF, 180) when compared to the Neo mouse staining (MCF: 135). The B cells in the bisantrene-treated MDR mice demonstrated an even higher shift of fluorescence level. The T cells did not stain positively with MRK16-FITC in any of the MDR mice, whether treated or not, when compared to the Neo mouse histogram shown in Fig. 3F. Fig. 3G shows histograms of granulocytes stained with MRK16-FITC. It seemed that MCFs for the Neo mouse and the MDR untreated mouse were similar at 160. However, bisantrene treatment seemed to have increased the MCF of the MDR mice to 175 and 195, indicating that more of their granulocytes expressed P-gp. No such effect was seen in bisantrene-treated Neo mice. The number of MDR-positive macrophages clearly increased after bisantrene treatment of the MDR mice when compared to the bisantrene-treated Neo mouse or the untreated MDR mouse that was negative (Fig. 3H). The MCF increased from approximately 120 in the bisantrene-treated Neo mouse to 155 and 160 in the two bisantrene-treated MDR mice.

**DISCUSSION**

Several recent reports have appeared describing transgenic and mouse BMT transplantation models that demonstrate that MDR1 cDNA can protect BMC against cytotoxic P-gp substrates such as daunorubicin and Taxol (12, 17–19, 33). This work offers evidence that bisantrene seems to be among the best known cytotoxic substrates for P-gp because low levels of P-gp produce high levels of resistance to bisantrene and MDR cell lines are relatively more resistant in vitro to bisantrene than to any other drug tested. This work also presents direct evidence that MDR1 can mediate resistance to bisantrene in vivo, suggesting that this drug may prove to be a useful agent for selection of populations of BMC rendered MDR1-positive for the purpose of genetic therapy.

Zhang et al. (13), using multidrug-resistant human epidermoid and melanoma cells, showed that compared to vinblastine, colchicine, and Taxol (among others), bisantrene is a very powerful cytotoxic drug that is an excellent substrate for P-gp, because it seemed that even low level expression of P-gp was sufficient to confer resistance to bisantrene. Our current data using NIH3T3 cells transfected with wild-type or mutant MDR1 cDNA confirm this finding. Moreover, it is interesting that contrary to what is seen with Taxol, vinblastine, or colchicine, mutation of the MDR1 gene does not substantially affect the substrate specificity for bisantrene.

In this study, we show how a significant decrease of WBC counts was registered in all of the control mice treated with as little as a single dose of 10 mg/kg administered i.v. Doses of bisantrene ranging from 10 mg/kg to 150 mg/kg induced leukopenia in a dose-dependent manner. At doses of 150 mg/kg, bisantrene caused severe leukopenia, leading to death in 60% of the control mice. The wide dose range that induced leukopenia but not death means that the use of bisantrene as a selective agent for MDR1-transduced cells has a wide margin of safety. Recovery from bisantrene-induced leukopenia is rapid; the WBCs in all of the control mice began to recover by day 7 and were back to normal values 3 weeks after injection. Bisantrene
Fig. 3 Detection of MDR1-(P-gp)-positive cells 7 days after bisantrene administration using MRK16-FITC antibody. A-D: histograms obtained after staining of peripheral blood cells with CD45R/B220-PE (mature and immature B lymphocytes), Thy-1.2-PE (pan-T lymphocytes), Gr-1-PE (mature and immature granulocytes), and Mac-1-PE (mature and immature macrophages), respectively. The histograms are plotted against the X axis on a logarithmic scale showing the orange-red fluorescence intensity (FL2) and the Y axis representing the relative cell number. R, the gating of the respective cell population stained. E-H: the gated B lymphocytes, T lymphocytes, granulocytes, and macrophages, respectively, displayed as histograms plotted against the X axis on a linear scale showing the fluorescence intensity of cells stained with MRK16-FITC (FL1:MRK16-FITC) and the Y axis representing the relative cell number. Solid line, a bisantrene-treated Neo mouse; small dotted line, an untreated MDR mouse; large dotted and dashed/dotted lines, two different MDR mice treated with bisantrene, respectively.

at 50 mg/kg induced a significant fall of WBC counts in the control mice (42.3%; $P = 0.001$) and seemed to be a good MDR1 substrate because little or no toxicity to BMC was observed in the transplanted MDR mice (84.5%; $P = 0.1$).

It seems that bisantrene at a dose of 50 mg/kg is specifically toxic for populations of B lymphocytes and macrophages. These results correlated with the finding that treatment of mice with bisantrene enriched for B-cell and macrophage lineages expressing P-gp, as detected with anti-P-gp monoclonal antibodies. An intriguing result was observed with the granulocytes. Although no decrease in their numbers was found in the bisantrene-treated group of mice (Fig. 2C), we found an increased percentage of P-gp-expressing cells in the MDR-positive mice after treatment with bisantrene. This finding suggests that bisantrene might be slightly toxic for granulocytes; however, due to the rapid turnover of these cells, this effect is not detectable by counting methods. This is in contrast to the flow cytometry techniques that are more sensitive and allow the detection of the selected MDR1-positive cells.

The specific toxic effect bisantrene seems to have on B cells and macrophages is not a unique phenomenon because several other drugs are known to affect specific cells at different stages of their maturation. 5-Fluorouracil will stop differentiation of progenitor cells (34). Zidovudine, among other effects, will cause anemia by decreasing the bone marrow-derived, erythropoietin-responsive progenitor cells (35), and the FK506 immunosuppressive effect is caused by lack of development of activated T lymphocytes (36). We do not know at which level of cell differentiation bisantrene acts. However, the fact that the cell lineage-specific antibodies used are not cross-reacting with other cell populations (such as progenitor cells), in addition to the fact that cell counts came back relatively rapidly, suggest that mature cells are targeted. Other drugs that induce leukopenia (such as Taxol or daunomycin) have been used in vivo to achieve selection of MDR1-positive cells and (contrary to bisantrene) have seemed to be especially toxic to granulocytes (19, 37). A direct side-by-side comparison of different drugs with bisantrene will be needed to determine which is best for in vivo selection of MDR1-expressing BMC. In addition, dosage studies should be done to investigate bisantrene toxicity for progenitor cells, granulocytes, or other types of cells. These
studies could also help us learn what level of MDR1 expression is needed to protect these cells.

We do not suggest that the use of bisantrene for selection of MDR1-positive cells in clinical situations will be advantageous in comparison to other P-gp-mediated drugs such as Taxol and the anthracyclines. Although not fully characterized, it seems that the toxicity of bisantrene may extend outside of the hematopoietic system, and for this reason, the drug is not currently useful in clinical settings. In addition, several clinical trials have been done in which bisantrene showed variable success as an anticancer drug (2, 3, 5–11). One reason for refractoriness of cancer to bisantrene might be that the trials were done either on patients with cancers already expressing P-gp intrinsically or on patients treated previously with other cancer drugs that might have selected for P-gp-positive cancer cells (38). Assuming that problems with nonhematological toxicity of bisantrene can be circumvented, it might be possible to use mutant MDR1 vectors to render bone marrow resistant to bisantrene while simultaneously reversing the resistance of wild-type MDR1 expressed on cancers (23). In addition, because of the specific toxic effect of bisantrene on B lymphocytes and macrophages, bisantrene might be considered useful as a specific selective agent for cells expressing both the MDR1 gene and a nonselectable gene of therapeutic relevance.

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