Multiple Mechanisms Confer Drug Resistance to Mitoxantrone in the Human 8226 Myeloma Cell Line

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ABSTRACT

Selection for in vitro drug resistance can result in a complex phenotype with more than one mechanism of resistance emerging concurrently or sequentially. We examined emerging mechanisms of drug resistance during selection with mitoxantrone in the human myeloma cell line 8226. A novel transport mechanism appeared early in the selection process that was associated with a 10-fold resistance to mitoxantrone in the 8226/MR4 cell line. The reduction in intracellular drug concentration was ATP-dependent and ouabain-insensitive. The 8226/MR4 cell line was 34-fold cross-resistant to the fluorescent aza-anthrapyrazole BBR 3390. The resistance to BBR 3390 coincided with a 50% reduction in intracellular drug concentration. Confocal microscopy using BBR 3390 revealed a 64% decrease in the nuclear:cytoplasmic ratio in the drug-resistant cell line. The reduction in intracellular drug concentration of both mitoxantrone and BBR 3390 was reversed by a novel chemosensitizing agent, fumitremorgin C. In contrast, fumitremorgin C had no effect on resistance to mitoxantrone or BBR 3390 in the P-glycoprotein-positive 8226/DOX6 cell line. Increasing the degree of resistance to mitoxantrone in the 8226 cell line from 10 to 37 times (8226/MR20) did not further reduce the intracellular drug concentration. However, the 8226/MR20 cell line exhibited 88 and 70% reductions in topoisomerase II \( \beta \) and \( \alpha \) expression, respectively, compared with the parental drug-sensitive cell line. This decrease in topoisomerase expression and activity was not observed in the low-level drug-resistant, 8226/MR4 cell line. These data demonstrate that low-level mitoxantrone resistance is due to the presence of a novel, energy-dependent drug efflux pump similar to P-glycoprotein and multidrug resistance-associated protein. Reversal of resistance by blocking drug efflux with fumitremorgin C should allow for functional analysis of this novel transporter in cancer cell lines or clinical tumor samples. Increased resistance to mitoxantrone may result from reduced intracellular drug accumulation, altered nuclear/cytoplasmic drug distribution, and alterations in topoisomerase II activity.

INTRODUCTION

Multidrug resistance is a major obstacle to successful cancer treatment. Given the poor therapeutic index of chemotherapeutic agents, even low levels of drug resistance may diminish therapeutic benefit. Multiple resistance mechanisms may occur during selection by chemotherapeutic agents in vitro. However, whether these mechanisms of drug resistance are selected for concurrently or consecutively is unknown.

In this paper, we examine emerging resistance mechanisms during selection with mitoxantrone, a clinically effective anthracenedione. Mitoxantrone, like doxorubicin, is a DNA-reactive compound that intercalates between bp of DNA and inhibits topoisomerase II (1–3). This ATP-dependent enzyme cuts both strands of DNA and changes the DNA linking number by two. Many inhibitors of topoisomerase II stabilize this normally transiently bound DNA-protein complex and form what is referred to as the cleavable complex (4). Both doxorubicin and mitoxantrone induce the formation of stable cleavable complexes.

Three distinct drug-resistant mechanisms are identified in cell lines selected with mitoxantrone: (a) mitoxantrone seems to select for a novel resistance phenotype associated with decreased drug accumulation not associated with either MDR1 or MRP3 expression. We first reported this novel phenotype in a WiDr human colon cancer cell line (5). We, and others, observed a similar phenotype in the human breast cancer cell line MCF7 (6–8); (b) mitoxantrone selects for alterations in topoisomerase II \( \alpha \) and \( \beta \) in HL-60 cells, a human leukemia cell line (9). In the mitoxantrone-selected HL-60 cell line, the appearance of a \( M_r \) 160,000 isoform of topoisomerase II that localizes predominantly to the cytoplasm of HL-60 cells also is evident (10); and (c) Pgp overexpression is documented in a K562 cell line selected with mitoxantrone (7).

We reported that neither Pgp nor MRp contribute to drug resistance in the human myeloma cell line 8226, selected with mitoxantrone (7). In this study, we further characterize the resistant phenotype in this cell line. Our evidence shows that, early in the selection process, drug resistance is at least partially mediated by an ATP-dependent, ouabain-insensitive drug transport mechanism. The drug-resistant phenotype could be reversed by a novel chemosensitizer, fumitremorgin C. This effect is specific to the mitoxantrone-selected cell lines, inasmuch as fumitremorgin C did not overcome resistance in the Pgp-positive 8226/DOX6 cell line. With further mitoxantrone drug selection and rising levels of resistance, alterations in topoisomerase II levels and activity were observed. Thus, higher levels of drug resistance to mitoxantrone are conferred by several drug-resistant mechanisms, including reduced drug accumulation and altered topoisomerase II.

MATERIALS AND METHODS

Cell Culture. The RPMI 8226 human myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD), and grown as a suspension in RPMI 1640, supplemented with: 5% FBS; 1% (v/v) penicillin; (100 units/ml); streptomycin (100 µg/ml); and 1% (v/v) \( \beta \)-glutamine (all from Life Technologies, Inc. (Grand Island, NY). Cells were maintained at 37° in 5% \( \text{CO}_2 \)/95% air atmosphere and were passaged once every 6 days. The 8226/DOX6 cell line was maintained as described previously (11).

Drugs. Mitoxantrone and fumitremorgin C were kindly provided by Dr. Lee Greenberger of Wyeth-Ayerst (Pearl River, NY); the aza-anthrapyrazole, BBR 3390, was obtained from Boehringer Mannheim Italia (Monza, Italy); doxorubicin was from Adria Laboratories (Columbus, OH); and ansamycin (m-AMSA) came from Ben Venue Laboratories, Inc. (Bedford, OH). Etoposide and methotrexate were obtained from Bristol Myers Squibb Co. (Evansville, IN). Dexamethasone, verapamil, and daunorubicin were obtained from Sigma Chemical Co. (St. Louis, MO); Vincristine was obtained from Eli Lilly

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3 The abbreviations used are: MRP, multidrug resistance-associated protein; MR, multidrug resistant; Pgp, P-glycoprotein; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; FBS, fetal bovine serum; kDNA, kinetoplast DNA.
MULTIPLE MECHANISMS CONFER DRUG RESISTANCE TO MITOXANTRONE

Laboratories (Indianapolis, IN); melphalan from Burroughs Wellcome (Research Triangle Park, NC); and cytarbamine (ara-C) from Upjohn (Kalamazoo, MI). [14C]Mitoxantrone (specific activity, 8.1 mCi/mmol; 18.2 μCi/mg) was obtained from Research Triangle Institute (Research Triangle Park, NC).

Selection of Mitoxantrone-resistant Cells. Two mitoxantrone-resistant cell lines were selected by continuous exposure to mitoxantrone. The 8226/ MR4 cell line was established by exposing 8226 parental cells (8226/DS) to 5 × 10^{-8} m mitoxantrone. The drug concentration was gradually increased to 4 × 10^{-7} m mitoxantrone. The 8226/MR20 cell line was established by exposing the 8226/MR4 cell line to increasing concentrations of mitoxantrone for 18 weeks. The final selection pressure for the 8226/MR20 cell line was 3 × 10^{-7} m mitoxantrone. Cells were grown in drug-free medium for 1 week before performing any experiments.

In Vitro Cytotoxicity Assay. Cytotoxicity was determined using a modified MTT dye assay (12). Cells were plated into 96-well microtiter plates at 1 × 10^4 cells/well (8226/MR and 8226/DOX6) or 8 × 10^3 cells/well (8226/S) in 0.2 ml of medium, in replicates of 8. After a 96-h incubation at 37°C, 50 μl of MTT dye (2 mg/ml) were added to each well, and the cells were incubated for an additional 4 h. Plates were centrifuged at 500 g for 10 min, 100 μl of DMSO was added to each well, and the absorbance was read at 570 nm using a microplate reader.

Drug Accumulation of [14C]Mitoxantrone and BBR 3390. Cellular accumulation of [14C]mitoxantrone was determined after a 1-hr exposure of cells (2 × 10^6 cells/ml, 1 ml/15-ml conical tube) to 2.5 μM [14C]mitoxantrone at 37°C (specific activity, 8.1 mCi/mmol). After incubation at 37°C, cells were washed twice with ice-cold PBS, and 1 × 10^6 cells were added to the scintillation vials and digested overnight with 100 μl of 1N HCl. The [14C]mitoxantrone was determined by liquid scintillation counting.

Intracellular drug accumulation of the highly fluorescent aza-anthrapyrazole BBR 3390 was measured by flow cytometry. One ml of this cell suspension (10^6 cells/ml) was transferred to 15-ml conical tubes. BBR 3390 (5 μM final concentration) was added to appropriate tubes, and samples were incubated for 1 h at 37°C. Cells were washed twice with cold PBS, centrifuged, and resuspended in 1 ml of ice-cold PBS, and analyzed immediately on a FACScan flow cytometer (Becton Dickinson) with an excitation wavelength of 488 nm. Emission was measured at 525 nm. Cells not exposed to BBR 3390 were used as a control for autofluorescence.

Experiments performed with the reversal agent fumitremorgin C were performed as described above, except fumitremorgin C was added 15 min before the addition of either 1 μM or 5 μM BBR 3390.

Drug Efflux. Approximately equal intracellular concentrations of BBR 3390 were obtained for each cell line by exposing 8226/DS, 8226/MR4 and 8226/MR20 to 2.0, 5.7, and 6.4 μM, respectively, for 1 h. After 1 h, cells were resuspended in drug-free media for the indicated times (Fig. 3). One ml aliquots containing 1 × 10^6 cells were washed twice in cold PBS and analyzed by FACS as described previously. Each time point is the average of three samples. The efflux experiment investigating the role of the chemosensitizer fumitremorgin C was performed as described above with the following exceptions: (a) the BBR 3390 concentration was 1 μM for each condition; and (b) 10 μM fumitremorgin C was added to appropriate vials during the accumulation and/or efflux as indicated in Figs. 3 and 6.

ATP Depletion Assay. ATP was depleted by a 2-h treatment incubation in glucose-free RPMI 1640 supplemented with 2% FBS and 10 μM NaF. Cells were subsequently treated with [14C]mitoxantrone, and drug accumulation was measured as described previously. Briefly, cells were washed in PBS, and 500 μl of trichloroacetic acid were added to lyse cells and precipitate proteins. Precipitated protein was solubilized with 0.1% SDS in 5 μl NaOH and samples were frozen at −80°C until assayed. ATP levels were determined photometrically using a luciferin-luciferase bioluminescent assay kit (Sigma Chemical Co.).

Confocal Microscopy. Confocal microscopy was used to determine intracellular drug distribution patterns. Briefly, coverslips were treated with 4 μg of neutralized Celltrack (Collaborative Biomedical Research, Bedford, MA). Approximately 1 × 10^6 cells in serum-free RPMI 1640 were pipetted directly onto the coverslip. Cells were allowed to adhere for 20 min, and the media was replaced with RPMI containing 5% FBS. Ten μM fumitremorgin C was added to appropriate samples for 15 min before the addition of 2 μM BBR 3390. Cells were incubated with BBR 3390 for 1 h, washed in PBS, and then analyzed for drug distribution by confocal microscopy. Confocal microscopy was performed using a Zeiss confocal scanning microscope (LSM 510) with an argon laser (488 nm). Fig. 7 is representative of three independent experiments. For each experimental condition, 20 individual cells were analyzed, and the mean pixel density of the nucleus and the cytosol were determined. The mean pixel density of the background was subtracted from all of the values before calculating the nuclear:cytoplasmic ratio.

Topoisomerase II Assays. Nuclear extracts were prepared from log phase cells as described by Sullivan et al. (13) with the following modifications: (a) to minimize proteolysis, all of the procedures were performed at 4°C, and the protease inhibitor Pefabloc (1 mM; Boehringer Mannheim, Indianapolis, IN) was added to buffers A-F; and (b) antipain, aprotinin, leupeptin, and pepstatin A (each at 20 μg/ml) were added to buffers B-F. Cells (3–4 × 10^6) were initially washed twice with PBS and then were spun down and resuspended in 15 ml of Buffer A (0.15 μM NaCl and 10 mM KH2PO4). Cells were washed a second time in Buffer A and then were resuspended in 10 ml of Buffer B (5 mM KH2PO4, 2 mM MgCl2, 4 mM DTT, and 0.1 mM Na3EDTA) and allowed to swell for 30 min on ice. Cells were then dounce-homogenized for 10 strokes, and released nuclei were collected at 2500 × g for 15 min. Nuclei were resuspended in 4 ml of Buffer C (Buffer B + 0.25 mM sucrose) and layered over 1.2 ml of Buffer D (Buffer B + 0.6 M sucrose). This sucrose gradient was centrifuged in a swinging bucket rotor for 20 min at 2000 × g. The nuclear pellet was resuspended in 300 μl of Buffer B (5 mM KH2PO4, 4 mM DTT, and 1 mM Na3EDTA), and the total volume was measured. An equal volume of Buffer F [40 mM Tris (pH 7.5), 2 mM NaCl, and 4 mM DTT] was added, and the solution was incubated at 37°C. After adjusting to 10% glycerol, the solution was centrifuged at 100,000 × g for 1 h, and the supernatant was divided into aliquots and stored at −70°C. Protein concentrations were determined with the Bio-Rad (Bio-Rad, Hercules, CA) protein assay kit according to the manufacturer’s instructions.

For immunoblotting, 50 μg of fresh nuclear extract from the cell lines were separated on a 7% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The blot was probed with a polyclonal antibody from Dr. M. Danks (St. Jude Hospital, Memphis, TN) that recognizes both M2 and 180,000 forms of topoisomerase II (14, 15). After the primary incubation, the blot was washed and probed with [125I]-labeled goat anti-rabbit IgG (specific activity, 6.20 μCi/μg; NEN, Boston, MA). The membrane was washed and dried, and autoradiography was performed. The autoradiograms were quantified by the Imagequant software program (Molecular Dynamics, Sunnyvale, CA).

Catalytic activity was measured as the decatenation of networks of kDNA isolated from C. fasciculata (13). Nuclear extract was added to an E. coli containing 0.5 μg kDNA, 30 μg of nuclear extract protein, and topoisomerase II reaction buffer [50 mM Tris (pH 7.5), 85 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.5 mM Na3EDTA, 30 μg/ml BSA, and 1 mM ATP]. The samples were incubated for 30 min at 30°C, and the reaction was terminated by the addition of 5 μl of 0.5% bromphenol blue, 2% SDS, and 50% glycerol. The samples were then electrophoresed (1% agarose gel) for 2.5 h at 75 v, and DNA was visualized using ethidium bromide. Agarose gel electrophoresis of released minicircles was used to determine topoisomerase II activity.

RESULTS

Selection and Characterization of the Mitoxantrone-resistant Cell Lines. The 8226/MR4 cells were approximately 10-fold resistant to mitoxantrone and were cross-resistant to etoposide, the anthracines, and the aza-anthrapyrazole BBR 3390 (see Table 1). The 8226/MR20 cell line was approximately 37-fold resistant to mitoxantrone, 15-fold resistant to etoposide, and 77-fold resistant to the aza-anthrapyrazole BBR 3390. Similar to the doxorubicin-resistant Pgp-expressing cells 8226DOX6, mitoxantrone-resistant cell lines were not cross-resistant to antimetabolites or alkylating agents. However, unlike the doxorubicin-selected Pgp-expressing cells, the 8226/MR cell lines are cross-resistant to the topoisomerase I inhibitor.
8226/MR20 cell lines, respectively. The decrease in the intracellular BBR 3390 concentration was 50 and 60% in the 8226/MR4 and MR20 cell line compared with the MR4 cell line. The reduction in decrease in drug accumulation was observed in the more resistant 8226/MR4 and 8226/MR20 cell lines, respectively. Thus, no further reduction in mitoxantrone accumulation was 40 and 36% in the concentrations of mitoxantrone and BBR 3390 were decreased. The 8226/MR20 drug-resistant cell lines (Figs. 1 and 2), intracellular determined in the resistant cell lines. In both the 8226/MR4 and accumulation, the intracellular drug concentrations of two drugs was

Results from cytotoxicity assays

Table 1

A MTT assay was used to determine the IC_{50} values in the drug-sensitive and drug-resistant cell lines. The degree of cross-resistance in 8226 cells was related to a decrease in drug
cross-resistance value is in parentheses. Three or more experiments were performed for each drug in each cell line.

Table 1

<table>
<thead>
<tr>
<th>Agent and class</th>
<th>8226/S</th>
<th>8226/MR4</th>
<th>8226/MR20</th>
<th>8226/DOX6</th>
</tr>
</thead>
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<tr>
<td>Topo^a II inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>9.7 x 10^-8</td>
<td>1.05 x 10^-6 (10.8)</td>
<td>3.6 x 10^-6 (37.0)</td>
<td>6.0 x 10^-7 (6.2)</td>
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<td>Etoposide</td>
<td>6.35 x 10^-8</td>
<td>3.79 x 10^-7 (6.0)</td>
<td>9.72 x 10^-7 (15.3)</td>
<td>3.97 x 10^-7 (6.3)</td>
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<td>m-AMSA</td>
<td>4.71 x 10^-6</td>
<td>6.36 x 10^-6 (1.4)</td>
<td>6.22 x 10^-6 (1.3)</td>
<td>5.42 x 10^-6 (1.2)</td>
</tr>
<tr>
<td>Aza-anthrapyrazoles</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BBR 3390</td>
<td>2.6 x 10^-9</td>
<td>8.8 x 10^-8 (33.8)</td>
<td>2 x 10^-7 (77.3)</td>
<td>5.6 x 10^-8 (21.5)</td>
</tr>
<tr>
<td>Topo I inhibitor</td>
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<tr>
<td>SN-38</td>
<td>1.08 x 10^-8</td>
<td>5.0 x 10^-8 (4.8)</td>
<td>1.27 x 10^-7 (12.2)</td>
<td>1 x 10^-8 (1.0)</td>
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<td>Anthracyclines</td>
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<tr>
<td>Doxorubicin</td>
<td>8.08 x 10^-9</td>
<td>2.32 x 10^-8 (2.9)</td>
<td>4.49 x 10^-8 (5.6)</td>
<td>1.72 x 10^-7 (21.3)</td>
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<tr>
<td>Daunorubicin</td>
<td>8.05 x 10^-9</td>
<td>3.23 x 10^-8 (4.0)</td>
<td>4.52 x 10^-8 (6.5)</td>
<td>8.30 x 10^-8 (10.3)</td>
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<td>Vinca alkaloids</td>
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<td></td>
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<tr>
<td>Vincristine</td>
<td>3.37 x 10^-8</td>
<td>3.45 x 10^-8 (1.0)</td>
<td>3.85 x 10^-8 (1.1)</td>
<td>1.30 x 10^-6 (38.6)</td>
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<td>Alkylating agents</td>
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<td>Melphalan</td>
<td>3.98 x 10^-6</td>
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<td>Antimetabolites</td>
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<td>Methotrexate</td>
<td>1.88 x 10^-8</td>
<td>1.92 x 10^-8 (1.0)</td>
<td>2.14 x 10^-8 (1.1)</td>
<td>2.39 x 10^-8 (1.3)</td>
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<td>ARA-C</td>
<td>3.72 x 10^-6</td>
<td>2.18 x 10^-6 (0.6)</td>
<td>1.57 x 10^-6 (0.4)</td>
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<td>Glucocorticoids</td>
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<td>Dexamethasone</td>
<td>2.6 x 10^-6</td>
<td>3.12 x 10^-6 (1.2)</td>
<td>2.07 x 10^-6 (0.8)</td>
<td>9.2 x 10^-7 (0.002)</td>
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</table>

^a Topo, topoisomerase.

Fig. 1. One-hour drug accumulation assay. Cells were treated with 2.5 μM [14C]mitoxantrone for 1 h, washed twice in cold PBS, and lysed; and radioactivity was determined as described in “Materials and Methods.” The data points are the mean of four samples ± SD. Relative to the 8226/S cell line, a 40, 36, and 46% reduction in drug accumulation was observed in the 8226/MR4, 8226/MR20, and 8226/DOX6 cell lines, respectively.

SN-38 and are not cross-resistant to the Vinca alkaloids. Furthermore, unlike the 8226/DOX6 cell line, no enhanced sensitivity was observed for glucocorticoids in the mitoxantrone-resistant cell lines.

Drug Accumulation and Efflux. To determine whether mitoxantrone resistance in 8226 cells was related to a decrease in drug accumulation, the intracellular drug concentrations of two drugs was determined in the resistant cell lines. In both the 8226/MR4 and 8226/MR20 drug-resistant cell lines (Figs. 1 and 2), intracellular concentrations of mitoxantrone and BBR 3390 were decreased. The reduction in mitoxantrone accumulation was 40 and 36% in the 8226/MR4 and 8226/MR20 cell lines, respectively. Thus, no further decrease in drug accumulation was observed in the more resistant MR20 cell line compared with the MR4 cell line. The reduction in BBR 3390 concentration was 50 and 60% in the 8226/MR4 and 8226/MR20 cell lines, respectively. The decrease in the intracellular concentration of BBR 3390 corresponded with a higher degree of resistance of BBR 3390 compared with mitoxantrone in the mitoxantrone-selected cell lines.

To determine whether the decrease in total drug accumulation was associated with an increase in drug efflux, equal intracellular concentrations of BBR 3390 were obtained in both drug-sensitive and resistant cells before the cells were placed in drug-free media. As shown in Fig. 3, BBR 3390 was rapidly effluxed out of the drug-resistant cells according to a biphasic model, whereas drug efflux in the 8226/S cells is best depicted as a single phase indicative of passive diffusion. The maximum rate of drug efflux in the resistant cells occurred within the first 15 min; only 30% of the original drug concentration remained in the cells at that time. In contrast, the parental drug-sensitive cells contained 82% of the drug 15 min after drug treatment.

To determine whether differences in drug accumulation were related to an ionic gradient generated by Na^+ K^+ ATPase, cells were
pretreated for 15 min with varying concentrations of ouabain, an inhibitor of the Na\(^+\)K\(^+\) ATPase, and then incubated with 2.5 \(\mu\)M \([^{14}\text{C}]\)mitoxantrone. As shown in Fig. 4, poisoning of the Na\(^+\)K\(^+\) ATPase with ouabain did not increase intracellular drug concentration in either the Pgp-positive 8226/DOX6 cell line or the 8226/MR20 cell line, which indicates that the reduction of intracellular mitoxantrone is due to the direct efflux of the compound.

To determine whether the decrease in drug accumulation was energy-dependent, ATP pools were depleted by over 97% with 10 mM NaN\(_3\) in glucose-free media. As shown in Table 2, this resulted in a significant increase (as determined by Student’s \(t\) test, \(P < 0.05\)) in intracellular \([^{14}\text{C}]\)mitoxantrone concentration in the 8226/MR20 cells; we did not observe a similar significant increase in the intracellular mitoxantrone concentration in the parental 8226/S cells. These results strongly suggest that the reduced intracellular concentration of drug in the mitoxantrone-resistant cells is due to the presence of an energy-dependent drug efflux pump similar to Pgp and MRP.

**Reversal of Mitoxantrone Resistance.** Nontoxic doses of the novel chemosensitizer agent fumitremorgin C reversed the degree of mitoxantrone resistance from 8- to 1.8-fold in the 8226/MR4 cell line. Fumitremorgin C also reversed mitoxantrone resistance in the 8226/MR20 phenotype from approximately 16-fold resistant to 2.4-fold. This demonstrates that fumitremorgin C can reverse drug resistance associated with both of the mitoxantrone-resistant cell lines. Fumitremorgin C produced similar results reversing the resistance that was associated with BBR 3390 in the mitoxantrone-selected cell lines (Table 3). In contrast, fumitremorgin C did not reverse resistance to mitoxantrone or BBR 3390 in the Pgp-positive 8226/DOX6 cell line.

Drug accumulation assays determined whether the reversal of the drug-resistant phenotype was associated with an increase in intracellular drug accumulation. As shown in Fig. 5, fumitremorgin C increased the intracellular concentration of BBR 3390 in the mitoxantrone-selected drug-resistant cells. Fumitremorgin C also significantly (\(P < 0.05\)) increased the intracellular drug concentration of the parental 8226/S cell line. This increase in intracellular drug concentration may account for the increase in sensitivity of BBR 3390 and mitoxantrone in 8226/S cells. Fumitremorgin C had no effect on the 8226/DOX6 cells, a result consistent with the in vitro cytotoxicity data.

Drug efflux experiments also determined that fumitremorgin C inhibits the efflux of BBR 3390 in resistant cells (see Fig. 6). Furthermore, fumitremorgin C must be present during the accumulation and efflux phases of the experiment in order for the compound to be effective.

**Table 2 Effect of ATP depletion on intracellular mitoxantrone accumulation**

To determine whether the reduction in intracellular drug concentrations was ATP-dependent, cells were depleted of ATP pools by treatment with 10 mM NaN\(_3\) in media devoid of glucose (\(\sim\)Glu) and assayed for drug accumulation as described in “Materials and Methods.” Each condition is the mean of six independent samples. Depleting ATP pools significantly (\(P < 0.05\)) increased the intracellular concentration of \([^{14}\text{C}]\)mitoxantrone in the drug-resistant 8226/MR20 cell line. Depletion of ATP in the parental 8226/S cell line had no effect on the intracellular concentration of mitoxantrone.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Condition</th>
<th>(\mu)M ([^{14}\text{C}])mitoxantrone</th>
<th>ATP value ng/(\mu)g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226/S</td>
<td>RPMI</td>
<td>1788.7 ± 93</td>
<td>126</td>
</tr>
<tr>
<td>8226/S</td>
<td>+NaN(_3)−/−Glu</td>
<td>1899.8 ± 253</td>
<td>23</td>
</tr>
<tr>
<td>MR20</td>
<td>RPMI</td>
<td>1366.7 ± 98.0</td>
<td>178</td>
</tr>
<tr>
<td>MR20</td>
<td>+NaN(_3)−/−Glu</td>
<td>1671.6 ± 132</td>
<td>0.41</td>
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</tbody>
</table>

**Table 3 Reversal of drug-resistance by fumitremorgin C**

An MTT assay was performed with varying concentrations of mitoxantrone or BBR 3390 in the presence or absence of 5 \(\mu\)M fumitremorgin C. The values represent IC\(_{50}\) calculations, and the degree of resistance for each condition is reported in parentheses. For samples containing fumitremorgin C, the degree of resistance was calculated using the IC\(_{50}\) for 8226/S treated with fumitremorgin C as the reference point. A nontoxic dose of fumitremorgin C significantly reduced the degree of resistance to mitoxantrone and BBR 3390 in the 8226/MR4 and 8226/MR20 cell lines. In contrast, fumitremorgin C did not decrease the sensitivity of mitoxantrone or BBR 3390 in the Pgp-positive 8226/DOX6 cell line.

<table>
<thead>
<tr>
<th>Drug</th>
<th>8226/S</th>
<th>8226/DOX6</th>
<th>8226/MR4</th>
<th>8226/MR20</th>
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<tbody>
<tr>
<td>Mitoxantrone</td>
<td>4.2 × 10(^{-8})</td>
<td>2.0 × 10(^{-7}) (4.8)</td>
<td>3.3 × 10(^{-7}) (7.9)</td>
<td>6.7 × 10(^{-7}) (16)</td>
</tr>
<tr>
<td>Mitoxantrone + 5 (\mu)M fumitremorgin C</td>
<td>1.1 × 10(^{-8})</td>
<td>3.7 × 10(^{-7}) (33.6)</td>
<td>2.0 × 10(^{-7}) (1.8)</td>
<td>2.6 × 10(^{-7}) (2.4)</td>
</tr>
<tr>
<td>BBR 3390</td>
<td>3.2 × 10(^{-9})</td>
<td>1.3 × 10(^{-8}) (9)</td>
<td>1.0 × 10(^{-7}) (31)</td>
<td>4.4 × 10(^{-7}) (138)</td>
</tr>
<tr>
<td>BBR 3390 + 5 (\mu)M fumitremorgin C</td>
<td>1.5 × 10(^{-9})</td>
<td>1.3 × 10(^{-8}) (9)</td>
<td>1.1 × 10(^{-7}) (7.3)</td>
<td>1.5 × 10(^{-7}) (8.5)</td>
</tr>
</tbody>
</table>
Confocal Microscopy. As shown in Table 1, the mitoxantrone-resistant cell lines are cross-resistant to BBR 3390. Because BBR 3390 is more fluorescent than mitoxantrone, we examined the intracellular drug distribution pattern in cell lines using this compound. In the drug sensitive parental cell line we observed primarily a nuclear distribution of BBR 3390 (Fig. 7, A and B). In contrast, in the 8226/MR20 cell line the most intense staining was localized to punctated clusters of drug in the cytoplasm (Fig. 7C). The change in staining pattern corresponded to a significant (P < 0.05) 64% decrease in the nuclear/cytoplasmic ratio of BBR 3390 relative to the drug sensitive parental cell line. Preincubation of the 8226/MR20 cell line with fumitremorgin C significantly (P < 0.05) increased the nuclear/cytoplasmic ratio by 54% with redistribution of the drug from the cytoplasm to the nucleus (Fig. 7D).

Topoisomerase II Expression and Activity. Mitoxantrone resistance is associated with alterations in topoisomerase II activity or expression (9). To determine whether decreased expression of topoisomerase II occurred in drug-resistant cells, we performed a Western blot analysis using an antibody that recognizes both topoisomerase II isoforms (9). Each condition is the mean of four samples ± SD. Fumitremorgin C increased the intracellular concentration of BBR 3390 by 18, 31, and 49% in the 8226/S, 8226/MR4, and 8226/MR20 cell lines, respectively, but was significantly less effective in increasing the drug concentration in the Pgp-positive 8226/DOX6 cell line.

DISCUSSION

It has become evident that in vitro selection of drug-resistant cells results in a multimodal drug-resistant phenotype. Historically, drug-resistant cell lines are characterized after prolonged exposure to increasing concentrations of drug. Because of the narrow range of efficacy for cytotoxic drugs, a 2-fold increase in drug resistance could theoretically be sufficient to develop clinical drug resistance. Thus, it is critical to identify mechanisms that confer low levels of drug resistance. Slapak et al. (16–18) characterized sequential selection with doxorubicin in a murine erythroleukemia (PC4) and a human myeloid leukemia cell (U937). They demonstrated that Pgp expression in these cell lines was a late event and, furthermore, in the U937 cell line, occurred after overexpression of MRP. These reports also indicated that drug resistance may be associated with either a redistribution of drug or a decreased intracellular drug concentration. Thus, alterations in drug distribution and/or transport unrelated to the expression of MDR1/Pgp, may account for low levels of drug resistance.

In this study, we examined the mechanism of resistance for the human myeloma cell lines selected for increasing levels of resistance to mitoxantrone. Although mitoxantrone and doxorubicin are both considered to be topoisomerase II inhibitors, selection with mitoxantrone results in a different drug-resistant phenotype than doxorubicin selection. In 1988, Dalton et al. (5) reported that selection with mitoxantrone in the human colon WiDr cell line results in a phenotype characterized as containing less intracellular drug without overexpression of Pgp. This novel putative drug transport phenotype was repeated with selection of mitoxantrone in the breast cancer cell line MCF7 (6). Thus, in three different human cell lines (WiDr, MCF7, and 8226), mitoxantrone selects for resistance associated with reduced intracellular drug accumulation not caused by the overexpression of MDR1 or MRP (5–7). This study further characterizes the drug-transport mechanism and demonstrates its association with low levels of drug resistance.

This novel drug transporter is ATP-dependent and independent of the Na+ K+ ATPase. Taken together, these data suggest that the drug is not cotransported but is effluxed out of the cell by a direct ATP-dependent transport mechanism. Others (19) have shown that mitoxantrone-selected cell lines are cross-resistant to topotecan and that the resistance to topotecan was not due to changes in topoisomerase I but rather to transport of the drug. In this report, we show that the mitoxantrone-resistant cell line is cross-resistant to the active metabolite SN-38 of the topoisomerase I inhibitor irinotecan. Furthermore, we demonstrate that the aza-anthrapyrazole, BBR 3390, is a substrate and is effluxed out of the mitoxantrone-resistant cell line. Thus, similar to Pgp, the mitoxantrone-selected putative transporter can recognize, and confer resistance to, diverse chemical structures.
Using BBR 3390, a compound with a high degree of intrinsic fluorescence, we analyzed intracellular drug distribution in mitoxantrone-selected cell lines. We observed by confocal microscopy that drug-resistant cells contained more cytoplasmic staining compared with the nuclear staining of sensitive cells. Furthermore, in the 8226/MR20 cell line, fumitremorgin C reversed the reduction in the nuclear:cytoplasmic ratio of drug. This suggests that alterations in drug distribution may contribute to drug resistance in mitoxantrone-selected cell lines. Dietel et al. (20) have also shown cytoplasmic vesicles containing mitoxantrone in their mitoxantrone-selected gastric carcinoma cell line. Future studies are needed to discern the exact cytoplasmic component that is associated with drug accumulation.

Several reports of drug-resistant cell lines show alterations in drug distribution (17, 21, 22). We previously described (23) one mechanism of altered intracellular drug distribution in a Pgp-positive 8226 cell line selected with doxorubicin and verapamil. We demonstrated that this cell line redistributed Pgp from the plasma membrane to the cytosol, and that the redistribution of Pgp coincided with the redistribution of doxorubicin to the cytoplasm. Like the mitoxantrone-selected cell lines, several reports associated low levels of drug resistance with alterations in intracellular drug distribution. This provides further evidence that cytoplasmic sequestration of drug may be an early mechanism of drug resistance (22, 24).

As the levels of resistance to mitoxantrone increased from 10-fold to 37-fold, no further alterations in total drug accumulation were observed. This differs from 8226 cells selected for doxorubicin resistance, in which we observed increased levels of resistance correlated directly with Pgp levels and increased drug efflux (11). This suggests that higher levels of resistance in mitoxantrone-selected 8226 cells are mediated by a nontransport mechanism of drug resistance. One of the targets of mitoxantrone is topoisomerase II. Cell lines that contain altered topoisomerase II are also resistant to mitoxantrone (25, 26). Several different mechanisms may result in diminished topoisomerase II activity. These mechanisms include a decrease in expression of topoisomerase II (27), point mutations (28), and alterations in the cellular localization of topoisomerase II α (10, 29–32). Changes in subcellular distribution of topoisomerase II α have been attributed to large deletions or truncations in the COOH-terminal region of the enzyme. Specifically, Harker et al. (10, 33) reported that HL-60 cells selected with mitoxantrone had a $M_r$ 160,000 isoform of topoisomer-
ase II that was predominantly localized to the cytoplasm. We did not observe the M₆₀,000 isofrom of topoisomerase II in nuclear extracts from the mitoxantrone drug-resistant cells. However, in the more drug-resistant 8226/MR20 cell line, we observed an 88 and 70% reduction in expression of topoisomerase II β and α, respectively. The decrease in expression of topoisomerase II coincided with diminished enzymatic activity as measured by the ability of nuclear extracts to convert kDNA to minicircles. Alterations of topoisomerase II activity were found only in the higher-level drug-resistant cell line 8226/MR20. Friche et al. (34) observed a similar paradigm on selection with the anthracycline daunorubicin in Ehrlich ascites tumor cells. In this drug-resistant cell line, they observed overexpression of Pgp as well as reduced expression of topoisomerase II. In our mitoxantrone-resistant cell lines, reduced drug accumulation associated with a novel ATP-dependent transporter is observed at low levels of resistance, whereas alterations in topoisomerase II seem to be associated with higher levels of resistance. Further studies are required to determine whether genetic alterations such as mutations explain the reduction in topoisomerase II levels and activity.

Additional evidence supporting the existence of non-Pgp mechanisms of drug resistance have been observed in the clinic. For example, a phase I/II clinical trial by List et al. (35) used cyclosporine in combination with daunorubicin in patients with acute myelogenous leukemia. They found that Pgp-positive leukemic blasts were eliminated and patients relapsed with a non-Pgp mechanism of drug resistance. This observation provides the clinical rationale to develop reversing agents that target novel drug-resistance mechanisms. Fumitremorgin C may be an important chemosensitizer to reverse resistance caused by the expression of the novel drug transporter associated with mitoxantrone selection. In addition, this agent in combination with the aza-anthrapyrazole BBR 3390 may allow for the development of a functional assay for this novel transporter that would facilitate its detection in cancer cell lines or clinical tumor samples. In summary, our data indicate that resistance to mitoxantrone is due to multiple mechanisms including drug efflux associated with a novel drug transport mechanism, alterations in intracellular drug distribution, and reduced topoisomerase II activity. In the 8226 cell line, reduction in intracellular drug accumulation seems to be the predominant mechanism associated with low levels of drug resistance, and alterations in topoisomerase II occur primarily at higher levels. We are currently conducting studies to identify the novel mechanism of drug transport associated with mitoxantrone selection. 

Note Added in Proof

Since acceptance of this article, we now know that the 8226/MR cell lines overexpress Breast Cancer Resistance Protein mRNA (Atypical multidrug resistance: Breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. Ross et al., J. Natl. Cancer Inst., in press 1999).

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