Reduced Intracellular Drug Accumulation in the Absence of P-Glycoprotein (mdr1) Overexpression in Mitoxantrone-resistant Human MCF-7 Breast Cancer Cells

Masayuki Nakagawa, Erasmus Schneider, Katharine H. Dixon, Julie Horton, Kristin Kelley, Charles Morrow, and Kenneth H. Cowan

ABSTRACT

A mitoxantrone-resistant human MCF-7 breast cancer subline (MCF/MX) which is approximately 4000-fold resistant to mitoxantrone was isolated by serial passage of the parental wild-type MCF-7 cells (MCF/WT) in stepwise increasing concentrations of drug. MCF/MX cells were also approximately 10-fold cross-resistant to doxorubicin and etoposide but were not cross-resistant to vinblastine. Intracellular accumulation of radiolabeled mitoxantrone was markedly reduced in MCF/MX cells relative to that in the drug-sensitive MCF/WT cells. This decrease in intracellular drug accumulation into MCF/MX cells was associated with enhanced drug efflux, which was reversed when cells were incubated in the presence of sodium azide and 2,4-dinitrophenol, suggesting an energy-dependent process. Incubation of MCF/MX cells with verapamil did not affect either the accumulation of mitoxantrone or the level of resistance in these cells. Furthermore, RNase protection and Western blot analyses failed to detect the expression of the mdr1 RNA or P-glycoprotein, a drug efflux pump known to be associated with the development of multidrug resistance in vitro. However, a polyclonal antibody directed against a synthetic peptide corresponding to the putative ATP binding domain of P-glycoprotein reacted with two (M, 42,000 and 85,000) membrane proteins from MCF/MX cells which were not found in MCF/WT. Functional assays and Western blot analysis for topoisomerase II revealed no differences in topoisomerase II activity or protein levels in MCF/MX cells. Thus, resistance in this cell line is apparently associated with enhanced drug efflux involving a pathway distinct from the mdr1-encoded multidrug transporter P-glycoprotein.

INTRODUCTION

The emergence of drug-resistant tumor cells remains a major problem in cancer chemotherapy. Numerous previous studies have shown that cells selected in vitro for resistance to antineoplastic agents have obtained the MDR phenotype in the absence of detectable expression of P-glycoprotein. The anthracenedione mitoxantrone is an antineoplastic agent which, like anthracyclines, binds DNA (7). Clinical trials with mitoxantrone have indicated that this agent has activity in the treatment of acute leukemia, malignant lymphoma, breast cancer, and ovarian cancer (8-10). Results of phase II clinical trials have indicated that this drug is well tolerated, with fewer side effects than anthracyclines such as doxorubicin, including a reduced incidence of cardiotoxicity (11). While the mechanism of cytotoxicity of this drug is not clear, preclinical studies have shown that it intercalates DNA and induces both protein-associated and non-protein-associated DNA strand breaks (7). Previous studies have indicated that multidrug-resistant cells overexpressing P-glycoprotein displayed some cross-resistance to mitoxantrone (12). On the other hand, cells selected for primary resistance to mitoxantrone have been isolated in vitro which display cross-resistance to other agents associated with the multidrug-resistant phenotype in the absence of detectable increases in mdr1/P-glycoprotein expression (13-16).

In the present study, we have isolated a mitoxantrone-resistant human breast cancer cell line (MCF/MX) which was approximately 4000-fold resistant to the selecting agent and displayed low levels (10-fold) of cross-resistance to doxorubicin and VP-16. Resistance in MCF/MX cells was associated with decreased drug accumulation, although no expression of mdr1/P-glycoprotein was detected. However, these multidrug-resistant cells were found to contain two membrane proteins which cross-reacted with antibodies directed against a portion of the putative ATP binding domain of P-glycoprotein (17).

MATERIALS AND METHODS

Materials. [14C]Mitoxantrone (66 mCi/mmole) was kindly provided by the American Cyanamid Co. (Lederle Laboratory, Pearl River, NY). [methyl-3H]Thymidine (100 Ci/mmole), [alpha-32P]dATP (120 Ci/mmole), and [alpha-32P]dCTP (800Ci/mmole) were obtained from Amersham (Arlington Heights, IL). Mitoxantrone, VP-16, cisplatinum, and m-AMSA were obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). Vinblastine was obtained from Sigma (St. Louis, MO), doxorubicin was obtained from Adria Laboratories (Columbus, OH), and melphalan was obtained from Burroughs Wellcome (Research Triangle Park, NC). Drugs were prepared by dissolving in distilled water, dimethyl sulfoxide, or 0.5x HCI. C219 monoclonal antibody directed against P-glycoprotein was obtained from Centocor (Malvern, PA), and rabbit antisera ASP-14 (17) was kindly provided by Dr. M. S. Center (Kansas State University, Manhattan, KS). Rabbit DNA topoisomerase II antibody was kindly provided by Dr. L. F. Liu (The Johns Hopkins University School of Medicine, Baltimore, MD). Kinetoplast DNA was obtained from Topogen (Columbus, OH).

Cell Lines. A mitoxantrone-resistant subline (MCF/MX) was established from the human MCF-7 breast cancer cell line (MCF/WT) by continuously exposing cells to gradually increasing concentrations of mitoxantrone. MCF/WT cells were initially placed in 10 nm mitoxantrone and passed in 2-fold increasing concentrations of mitoxantrone whenever cells had grown to confluency. After 9 months in culture the MCF/MX cells were able to grow uninhibited in 250 nm mitoxantrone and were thereafter passaged in that concentration of
The doxorubicin-resistant MCF-7 subline (MCF/Adr) was isolated by continuous culture in IMEM containing 10% fetal calf serum and incubated at 37°C. MCF/WT, MCF/MX, and MCF/Adr cells were grown in IMEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum and various concentrations of anticancer agents, and the cells were incubated for 7 days. The medium was then replaced, and the colonies were stained for 20 min with 5 mM methylene blue in methanol. Colonies containing more than 50 colonies were counted using a Dissecting colony counter (model 820; Dynatech Laboratories, Inc., Chantilly, VA). To examine the effects of verapamil on mitoxantrone resistance, MCF/MX cells were incubated with 10 μM of verapamil with various concentrations of mitoxantrone as described above, and colonies were counted after 7 days of incubation.

Drug Accumulation and Drug Efflux. Exponentially growing cells (4 x 10^5 cells/dish) were plated and incubated at 37°C for 24 h. Blank dishes were also prepared simultaneously using the same medium. The medium was replaced with serum-free IMEM, and cells were incubated with 1 μM [*C]mitoxantrone for the indicated time. Cells were then washed three times with ice-cold PBS and lysed with PBS containing 1% Triton X-100, and the radioactivity was counted in a liquid scintillation counter. In order to examine the effect of verapamil on drug accumulation in both cell lines, MCF/WT and MCF/MX cells were incubated with 1 μM [*C]mitoxantrone for 4 h in the presence or absence of 10 μM verapamil, and drug accumulation was determined. For efflux studies, cells (4 x 10^5 cells/dish) were preloaded with 1 μM [*C]mitoxantrone at 37°C for 4 h and then rapidly chilled on ice and washed with ice-cold PBS. After radioactive mitoxantrone was removed, cells were incubated in fresh medium at 37°C for the indicated time and harvested by gentle scraping. The radioactivity remaining in cells was determined as described above. In order to examine the effects of ATP depletion on drug efflux, cells were first incubated with [*C]mitoxantrone at 37°C for 4 h in serum-free IMEM. The cells were then placed on ice, the medium removed, and the cell monolayers were washed twice in ice-cold glucose-free HBSS. The cells were then incubated at various times, and the radioactivity remaining at each time point was determined. Protein concentration was determined by the micro BCA (Bicinchoninic acid) method (19).

RNase Protection Assay. Total cellular RNA was hybridized with 32P-labeled antisense mdr1 or GSTα RNA probes, digested with RNAses A and T1, and analyzed by polyacrylamide gel electrophoresis as described previously (20). A 283-nt mdr1 RNA probe was transcribed with SP6 RNA polymerase from the 5' mdr1 complementary DNA (pADR2) subcloned in pGEM3 (Promega, Madison, WI), which had been linearized with Styl (21). This probe protects a 227-nt fragment of the 512-nt riboprobe generated from the complementary DNA (pADR2) subcloned in pGEM3 (Promega, Madison, WI), which had been linearized with EcoRl. The 512-nt riboprobe was transcribed with T7 RNA polymerase from a pBluescript SKII plasmid (Stratagene, La Jolla, CA) containing 465 nt of the 5' end of the GSTα complementary DNA (22), which had been linearized with EcoRI. The 512-nt riboprobe generated protected 465 nt of the 5' portion of GSTα mRNA.

Western Blot Analysis. Plasma membranes and nuclear extracts were prepared from MCF/WT, MCF/MX, or MCF/Adr cells (10^6) as previously described by Naito et al. (23). Whole cell extracts were obtained by lysis of cells in 2x SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris/HCl, pH 6.8; 6% SDS; 10% glycerol; 10 mM 2-mercaptoethanol). Crude plasma membrane or whole cell extracts (30 or 50 μg protein) or nuclear extracts (25 or 50 μg protein) were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes in 25 mM Tris-HCl (pH 8.3) containing 90 mM glycine and 20% methanol for 2 h at 150 mA. The nitrocellulose membranes were incubated with either monoclonal antibody C219 (3 μg/ml), rabbit anti-topoisomerase II polyclonal antibody (1:2000) (supplied by L. Liu) for 30 min at room temperature, or ASP-14 polyclonal antibody (1:200) (supplied by M. Center) at room temperature for 12 h in 100 mM Tris-HCl (pH 7.5) containing 1% Tween-20 and 100 mM NaCl. The filters were then rinsed several times with 100 mM Tris-HCl (pH 7.5) containing 1% Tween-20 and 100 mM NaCl and treated with biotinylated secondary antibodies at room temperature for 30 min, followed by development according to the manufacturer's specifications (Vectorstain ABC-AP kit; Vector Laboratories, Burlingame, CA).

DNA Topoisomerase II Assays. Nuclear extracts were prepared from MCF/WT and MCF/MX cells. Following trypsinization, cells (10^6) were washed once in ice-cold HBSS and resuspended in 0.25 ml lysis buffer (20 mM Tris-HCl, pH 7.2; 25 mM KCl; 5 mM MgCl2; 1 mM EGTA; 250 mM sucrose; 0.5% Nonidet P-40) and kept on ice for 10 min. After 2 min of centrifugation in a microfuge, nuclei were resuspended in 0.25 ml nuclei buffer (20 mM Tris-HCl, pH 7.2; 400 mM NaCl; 20 mM EDTA; 20 mM 2-mercaptoethanol), and nuclear protein was extracted for 30 min on ice, followed by 15 min of centrifugation in a microfuge. The supernatant was made 50% in glycerol, diluted to equal protein concentrations of approximately 1 mg/ml, and kept at -20°C.

The formation of cleavable topoisomerase II DNA complexes was measured using K+SDS precipitation assays. Nuclear extracts (approximately 0.2 μg protein) from MCF/WT and MCF/MX cells were incubated with linearized pBR322, 3'-end-labeled with [32P]dATP, as previously described (24). Decatenation and P4 unknotting assays were performed using 2-fold serial dilutions of nuclear extracts incubated with 0.1 μg kinetoplast DNA or 0.25 μg P4 DNA for 30 min at 37°C, followed by 30 min of proteinase K digestion, using the same reaction conditions as previously described for P4 unknotting (24). Reaction products were analyzed on a 1% agarose gel in TBE (Tris-Borate-EDTA) buffer.

RESULTS

Drug Sensitivity of MCF/MX Cells. The relative resistance of MCF/MX cells to various anticancer agents was compared to that of MCF/WT cells using colony formation assays. As shown in Table 1, MCF/MX cells are 3900-fold resistant to mitoxantrone and display low levels of cross-resistance to doxorubicin (11.4-fold), VP-16 (10.6-fold), melphalan (2.4-fold), and m-AMSA (2.2-fold). Although reproducible, the low levels of cross-resistance to m-AMSA and melphalan are probably not significant, thus suggesting that the mechanism(s) for mitoxantrone resistance are specific for this drug. MCF/MX cells are not cross-resistant to vinblastine or cisplatin. This pattern of multidrug resistance in MCF/MX cells is distinctly different from the multidrug resistance phenotype in mdr1/P-glycoprotein-overexpressing cells, such as the doxorubicin-resistant MCF-7 cells (MCF/Adr) (18, 21), which is associated with resistance to Vinca alkaloids, anthracyclines, and epipodophyllotoxins.

Table 1 Sensitivity of MCF/WT and MCF/MX to various anticancer agents

<table>
<thead>
<tr>
<th>Drugs</th>
<th>50% inhibition concentration for MCF/WT (μM)</th>
<th>Relative resistance</th>
</tr>
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<tbody>
<tr>
<td>Mitoxantrone</td>
<td>0.158 ± 0.0025a</td>
<td>3932</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>15.5 ± 0.50</td>
<td>11.4</td>
</tr>
<tr>
<td>VP-16</td>
<td>166 ± 8.7</td>
<td>10.6</td>
</tr>
<tr>
<td>Melphalan</td>
<td>2400 ± 100</td>
<td>2.4</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>36.5 ± 0.50</td>
<td>2.2</td>
</tr>
<tr>
<td>CDDP b</td>
<td>750 ± 26</td>
<td>1.1</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.50 ± 0.04</td>
<td>1.0</td>
</tr>
</tbody>
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a 50% inhibition concentration values were determined by colony formation assay. Values are means ± SD of triplicate determinations.
b cis-diamine dichloroplatinum(II) (cisplatin).
Drug Accumulation and Efflux in MCF/WT and MCF/MX Cells. In order to examine whether altered membrane permeability is involved in the mechanism of mitoxantrone resistance in MCF/MX cells, the uptake and efflux of radiolabeled mitoxantrone was compared in MCF/WT and MCF/MX cells. As shown in Fig. 1A, there was a 5-fold reduced accumulation of \([^{14}C]\)mitoxantrone in MCF/MX cells relative to that in MCF/WT. The efflux of drug from MCF/WT and MCF/MX cells preincubated for 4 h with \([^{14}C]\)mitoxantrone was also compared (Fig. 1B). Following the removal of radiolabeled drug from the medium, there was a more rapid loss of intracellular radioactivity from MCF/MX than from MCF/WT, with more than 60% of radioactive mitoxantrone effluxed from MCF/MX cells after 120 min. In contrast, more than 95% of the radiolabeled drug remained in MCF/WT cells incubated under the same conditions. Thus, resistance in MCF/MX cells was associated with enhanced drug efflux and/or decreased intracellular drug binding.

Effect of Verapamil on MCF/WT and MCF/MX Cells. Numerous previous studies have shown that the development of multidrug resistance in human cells in vitro is frequently associated with overexpression of the putative energy-dependent drug efflux pump \(mdr1/P\)-glycoprotein (1-3, 18, 21). Several compounds including verapamil have been shown to competitively inhibit the binding of anticancer drugs to \(P\)-glycoprotein (25). Moreover, verapamil inhibits the efflux of anticancer drugs from multidrug-resistant cells, resulting in the reversal of drug resistance. We therefore examined the effect of verapamil on mitoxantrone accumulation and sensitivity in MCF/MX

Fig. 1. Drug accumulation (A) and efflux (B) in MCF/WT and MCF/MX cells. A, exponentially growing MCF/WT (■) and MCF/MX (•) cells (4 × 10^5 cells/dish) incubated with 1 μCi \([^{14}C]\)mitoxantrone at 37°C for the indicated time. Cells were then washed and lysed with PBS containing 1% triton X-100, and the radioactivity was determined by liquid scintillation counting. The values represent the mean ± SD obtained from triplicate determinations. B, following incubation of MCF/WT (■) and MCF/MX (•) cells with 1 μCi \([^{14}C]\)mitoxantrone for 4 h, the medium was replaced with drug-free medium and the cells were incubated at 37°C for the indicated time. Cells were harvested at the indicated times and the radioactivity remaining in the cells was determined by liquid scintillation counting. The values represent the mean ± SD obtained from triplicate determinations.
cells. Incubation of MCF/MX cells with 10 \( \mu M \) verapamil, a concentration which by itself results in a <10% decrease in colony formation of these cells, did not have any effect on \([^{14}C]\)mitoxantrone accumulation in either MCF/MX or MCF/WT cells (Fig. 2A). Furthermore, incubation of MCF/MX cells in verapamil did not alter the sensitivity of these cells to mitoxantrone (Fig. 2B). Thus, MCF/MX cells lack some of the characteristics normally associated with P-glycoprotein (mdr1)-mediated multidrug resistance.

**mdr1** (P-glycoprotein) Expression in MCF/WT and MCF/MX. RNase protection and Western blot analyses were performed to directly assess the level of mdr1 RNA and P-glycoprotein in MCF/WT and MCF/MX cells. As has been previously shown (18), mdr1 RNA is readily detected in multidrug-resistant MCF/Adr cells (Fig. 3A). In contrast, mdr1 RNA was not detected by RNase protection assay in either MCF/WT or MCF/MX cells. Similarly, Western blot analysis failed to detect the Mr 180,000 P-glycoprotein in membranes of either MCF/WT or MCF/MX cells (Fig. 3B). Thus, the diminished intracellular drug accumulation observed in MCF/MX cells was not associated with overexpression of the mdr1 gene.

**Effect of ATP Synthesis Inhibition on Drug Efflux.** In order to examine the influence of ATP depletion on the enhanced drug efflux in MCF/MX cells, cells were first preloaded with \([^{14}C]\)mitoxantrone, and the efflux of radiolabeled drug from preloaded cells was then examined during subsequent incubation in complete medium containing glucose, glucose-free medium containing 10 mM sodium azide, or glucose-free medium containing 2,4-dinitrophenol. As shown in Fig. 4, 35% of the radioactivity remained in MCF/MX cells following incubation for 2 h in drug-free medium. In contrast, only 60% of the radioactive drug remained in preloaded cells incubated for 2 h in glucose-free medium containing sodium azide, and 70% of the drug remained in cells incubated in glucose-free medium containing both sodium azide and 2,4-dinitrophenol. Thus, the enhanced elimination of mitoxantrone from MCF/MX cells was sensitive, at least in part, to conditions that deplete intracellular ATP levels.

**Western Blot Analysis with ASP-14.** Two laboratories have independently isolated doxorubicin-resistant HL-60 cell lines that display a multidrug resistance phenotype in the absence of detectable mdr1/P-glycoprotein expression (17, 25, 26, 27). Using a polyclonal antibody (ASP-14) directed against an oligopeptide sequence located within the putative ATP binding domain of P-glycoprotein, Marquardt and co-workers (17) have detected membrane proteins in doxorubicin-resistant HL-60 cells that differ in size from mammalian P-glycoprotein and that do not cross-react with other antibodies directed against P-glycoprotein.

Shown in Fig. 5 are the results of a Western blot analysis in which membrane proteins from MCF/WT, MCF/MX, and MCF/Adr cells were reacted with the polyclonal ASP-14 antibody. As expected from the previous results showing cross-reactivity of this antibody with P-glycoprotein (17), this antibody recognized the Mr 180,000 P-glycoprotein present in MCF/Adr cells. While there was no cross-reactivity of this antibody with high-molecular-weight (Mr 160,000-180,000) membrane proteins in either MCF/WT or MCF/MX cells, this antibody did cross-react with two lower-molecular-weight membrane proteins of Mr 42,000 (Fig. 5A) and Mr 85,000 (Fig. 5B) in MCF/MX cells that were not detected in MCF/WT cells.

**Topoisomerase II Levels in MCF/WT and MCF/MX Cells.** Mitoxantrone, like doxorubicin, binds to DNA and interacts with topoisomerase II (7, 28). Previous studies have shown that resistance to doxorubicin and VP-16 may be associated with alterations in topoisomerase II activity (29-32). Since

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The rest of the text continues with detailed experiments and figures regarding ATP depletion, drug efflux, and topoisomerase II levels in MCF/WT and MCF/MX cells.
MITOXANTRONE-RESISTANT BREAST CANCER CELLS

Fig. 5. Western blot analysis with ASP-14 in MCF/WT, MCF/MX, and MCF/Adr cells. Plasma membranes were prepared from each cell line, separated by electrophoresis, and transferred to a nitrocellulose membrane as described in "Materials and Methods." The membrane was incubated with rabbit antiserum ASP-14 (1:200) at room temperature for 12 h, followed by incubation with biotinylated goat anti-rabbit IgG. Arrows A, B, and C mark M, 42,000 and a M, 85,000 protein in MCF/MX cells and the M, 170,000 P-glycoprotein in MCF/Adr cells, respectively.

MCF/MX cells are slightly cross-resistant to VP-16 and doxorubicin, topoisomerase II levels in whole cell and nuclear extracts were determined by Western blot analysis using a polyclonal antibody directed against topoisomerase II. As shown in Fig. 6, there was no apparent difference in total immunoreactive topoisomerase II protein levels between MCF/WT and MCF/MX cells or in the relative levels of the two isoforms of topoisomerase II (the two bands in Fig. 6).

Similarly, no differences were found in catalytic strand passing activity between MCF/WT and MCF/MX cells when assayed by ATP-dependent decatenation of kinetoplast DNA (Fig. 7) or P4 unknotting (data not shown). Furthermore, nuclear extracts from both MCF/WT and MCF/MX cells were equally sensitive to the stimulation of cleavable complex formation by mitoxantrone (Fig. 8). These results indicated that there were no detectable quantitative or qualitative differences in topoisomerase II in the MCF/MX cells relative to the parental cell line.

DISCUSSION

Cells selected in vitro for resistance to natural product anticancer drugs frequently develop broad-spectrum cross-resistance to structurally dissimilar agents, a phenomenon referred to as multidrug resistance. Classical multidrug resistance is associated with simultaneous resistance to anthracyclines, epipodophyllotoxins, and Vinca alkaloids and is associated with enhanced drug efflux and mdr1/P-glycoprotein overexpression (1-3, 18, 21). Other cell lines have been isolated which displayed broad-spectrum resistance to anticancer drugs without detectable expression of P-glycoprotein. In some instances alterations in topoisomerase II activity have been identified in cell lines with broad-spectrum resistance (29-35). In other multidrug-resistant cells, alterations in intracellular drug accumulation, binding, or sequestration have been noted in the absence of P-glycoprotein expression (26, 27).

While our mitoxantrone-resistant human MCF-7 breast cancer cells (MCF/MX) were approximately 4000-fold resistant to the selecting agent, they also displayed low levels (2- to 11-fold) of cross-resistance to doxorubicin, VP-16, m-AMSA, and melphalan. Resistance in MCF/MX cells was associated with decreased drug accumulation and enhanced drug efflux and/or reduced intracellular drug binding. However, the defect in drug accumulation was not reversed following incubation of MCF/MX cells with verapamil. In contrast, a decrease in drug efflux was observed under conditions that deplete intracellular ATP levels. This suggests that the defect in MCF/MX cells

Fig. 6. Western blot analysis of DNA topoisomerase II in MCT/WT and MCF/MX cells. Aliquots of whole cell extracts (A) containing 50 μg protein or nuclear extracts (B) containing 5 μg protein were prepared from each cell line, electrophoresed on a 7.5% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit antibody against DNA topoisomerase II followed by biotinylated goat anti-rabbit IgG.

![Western blot analysis of DNA topoisomerase II in MCT/WT and MCF/MX cells.](cancerres.aacrjournals.org)
mitoxantrone-resistant breast cancer cells

Involves an energy-dependent process similar to that observed in P-glycoprotein-expressing multidrug-resistant cells. However, the lack of cross-resistance of MCF/MX cells to Vinca alkaloids and the inability of verapamil to influence the defect in intracellular drug accumulation and reverse drug resistance clearly distinguish this cell line from P-glycoprotein-overexpressing multidrug-resistant cells. Furthermore, no detectable mdrl RNA or P-glycoprotein was found in MCF/MX cells.

Other human cell lines have been selected for primary resistance to mitoxantrone, including colon cancer cells (13), gastric cancer cells (14), HL-60 leukemia cells (15), and human breast cancer cells (16). These other mitoxantrone-resistant cells, like the MCF/MX cells described in this report, displayed low levels of cross-resistance to doxorubicin and no detectable expression of P-glycoprotein. In human colon, gastric, and breast cancer cell lines previously described, mitoxantrone resistance was associated with an apparent decrease in intracellular drug accumulation (13, 14, 16). In contrast, mitoxantrone-resistant HL-60 cells underwent no apparent alteration in drug accumulation (15) but did display higher levels of relative cross-resistance to agents that interact with topoisomerase II, including VP-16, VM-26, and m-AMSA. Studies by Harker et al. (33) indicated that mitoxantrone resistance in HL-60 cells was associated with reduced levels of topoisomerase II catalytic activity. Furthermore, relative to parental HL-60 cells, mitoxantrone-resistant HL-60 cells exhibited a marked decrease in the level of the Mf 180,000 form of topoisomerase II and an increase in a Mf 160,000 protein immunoreactive with topoisomerase II antibodies (33). However, in our MCF/MX cells resistance does not appear to be associated with any apparent quantitative or qualitative change in topoisomerase II activity or with the relative amounts of the Mf 180,000 and 170,000 topoisomerase II species.

Taylor et al. (16) have described the isolation of a mitoxantrone-resistant subline from MCF-7 human breast cancer cells. Resistance in that subline was also associated with a decrease in intracellular drug accumulation and enhanced efflux. However, while the MCF/MX cells described in this report displayed little cross-resistance to vincristine, cisplatinum, or m-AMSA, the mitoxantrone-resistant MCF-7 subline described by Taylor et al. (16) displayed higher levels of relative cross-resistance to vincristine, vinblastine, cisplatinum, and m-

AMSA, suggesting some differences in the mechanisms of resistance in these two cell lines.

Previous studies have indicated that increased levels of the drug-metabolizing enzyme glutathione S-transferase may play a role in the development of anticancer drug resistance. Increased levels of different isozymes of glutathione S-transferases have been found in cell lines selected for antineoplastic drug resistance. Increased levels of the human α class isozyme of GST (GSTα) were reported in MCF-7 cells selected for primary resistance to doxorubicin (36) and mitoxantrone resistance (37). Also, the relative insensitivity of the human colon cancer cell line Caco-2 to Adriamycin and mitoxantrone has been linked to higher levels of GSTα and γ (38). In addition, several anticancer agents, including 1-phenylalanine mustard and mitoxantrone, have been shown to be metabolized by GSTs (39, 40). We have examined the level of cytosolic GST in MCF/MX cells and found no difference in GST levels compared to that in MCF/WT cells (data not shown). Furthermore, there was no detectable expression of GSTα RNA in the MCF/MX cells (data not shown). Therefore, we believe that multidrug resistance in our MCF/MX cells is not associated with altered drug metabolism.

As noted previously, doxorubicin-resistant HL-60 cell lines have been isolated which display broad-spectrum resistance and decreased drug accumulation in the absence of P-glycoprotein overexpression (17, 26, 27). One doxorubicin-resistant HL-60 cell line was noted to have an increase in a high-molecular-weight membrane phosphoprotein as well as increased binding of an ATP photoaffinity analogue to a Mf 190,000 membrane protein (17). Furthermore, using a polyclonal antibody (ASP-14) directed against an oligopeptide generated from the putative ATP binding domain of the mdrl encoded P-glycoprotein, Center and co-workers (17, 27) identified a high-molecular-weight (Mf 190,000) membrane protein in two independently derived doxorubicin-resistant HL-60 cell lines and another, lower-molecular-weight (Mf 50,000) protein in one of the resistant cell lines that were not detected in drug-sensitive HL-60 cells. In the present study, we have shown that this polyclonal antibody ASP-14 also reacts with Mf 42,000 and 85,000 membrane proteins found in MCF/MX that are not found in MCF/WT cells. The identities of the Mf 42,000 and 85,000 proteins and their possible roles in resistance in the MCF/MX cell line are not known. On the basis of sequence homology with other mammalian genes (6) as well as genes from bacteria (1-3), yeast (4), and plasmodium (5), it has been proposed that the mammalian mdrl genes belong to a superfamily of membrane transport genes. The region with greatest homology within this family of membrane transport proteins involves the region encoded by the putative ATP binding domain of the human mdrl gene. Since the antibody ASP-14 is directed against an oligopeptide sequence contained within the putative ATP binding domain of P-glycoprotein, it is possible that the membrane proteins overexpressed in MCF/MX may also represent members of this superfamily. Additional studies are under way to identify some of the ASP-14 cross-reactive membrane proteins found in MCF/MX cells as well as their possible roles in drug resistance.

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