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 analysis 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 (Mr, 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 anthracyclines, Vinca alkaloids, or epipodophyllotoxins frequently develop cross-resistance to the other classes of agents. This broad-spectrum resistance to natural products has been referred to as MDR. MDR is frequently associated with the overexpression of a membrane protein, P-glycoprotein, which is thought to act as an energy-dependent drug efflux pump encoded in human cells by the mdr1 gene (1-3). Sequence analysis of the mammalian P-glycoprotein has shown that it contains regions with homology to other proteins presumably involved in membrane transport, including the STE6 gene of yeast (4), a gene associated with chloroquine resistance in plasmodium (5), and the gene associated with cystic fibrosis (6). The region of greatest homology with other membrane proteins occurs within the putative ATP binding domain of P-glycoprotein.

The anthracyclene 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/mmol) was kindly provided by the American Cyanamid Co. (Lederle Laboratory, Pearl River, NY). [methyl-3H]Thymidine (100 Ci/mmol), [3H]-3'-deoxyATP (120 Ci/mmol), and [32P]dCTP (800Ci/mmol) 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 0.5w HCl. C219 monoclonal antibody directed against P-glycoprotein was obtained from Centocor (Malvern, PA), and rabbit antisera to 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 passaged in 2-fold increasing concentrations of mitoxantrone whenever cells had grown to confluence. 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 described previously (18). MCF/WT, MCF/MX, and MCF/Adr cells were grown in IMEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum and 50 μg/ml gentamicin sulfate (Gibco). Cells were grown in drug-free IMEM containing 10% fetal calf serum for two passages prior to their use in any studies.

Colony Formation Assay. Cytotoxicity was assessed in MCF/WT and MCF/MX by colony-forming assay. MCF/WT (300 cells/ml) and MCF/MX (1500 cells/ml) were plated in triplicate in 12-well Linbro dishes in IMEM containing 10% fetal calf serum and incubated at 37°C for 24 h. The medium was replaced with IMEM containing 10% fetal calf serum and various concentrations of anticancer agents, and the cells were incubated for 7 days. The medium was then removed, and the colonies were stained for 20 min with 5 mM methylene blue in methanol. Colonies containing more than 50 cells were counted using a Artrek colony counter (model 800; 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 × 10⁵ 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 [¹⁴C]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 [¹⁴C]mitoxantrone for 4 h in the presence or absence of 10 μM verapamil, and drug accumulation was determined.

For efflux studies, cells (4 × 10⁵ cells/dish) were preloaded with 1 μM [¹⁴C]mitoxantrone at 37°C for 4 h and then rapidly chilled on ice and washed twice 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, and 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 1 μM [¹⁴C]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 37°C in glucose-free HBSS containing 10 mM sodium azide in the presence or absence of 1 mM 2,4-dinitrophenol. Cells were harvested 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 mdrl RNA probes, digested with RNAses A and T₁, and analyzed by polyacrylamide gel electrophoresis as described previously (20). A 283-nt mdrl RNA probe was transcribed with SP6 RNA polymerase from the 5' mdrl complementary DNA (pADR2) subcloned in pGEM3 (Promega, Madison, WI), which had been linearized with Styl (21). This probe protects a 227-nt fragment of mdrl mRNA corresponding to complementary DNA nt +956 to 1182 (2). The GST* riboprobe was transcribed with T7 RNA polymerase from a pBluescript SKII plasmid (Stratagene, San Diego, CA) containing 465 nt of the 5' end of the GST* complementary DNA (22), which had been linearized with EcoRI. The 512-nt riboprobe generated protected 465 nt of the 5' portion of GST* mRNA.

Western Blot Analysis. Plasma membranes and nuclear extracts were prepared from MCF/WT, MCF/MX, or MCF/Adr cells (10⁶) 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 (Vectastain 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 (one 135-mm dish) were washed twice with ice-cold PBS, resuspended in 0.25 ml lysis buffer (20 mM Tris-HCl, pH 7.2; 25 mM KC1; 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; 2 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 pH322, 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 mAMSA 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 cisplatinum. This pattern of multidrug resistance in MCF/MX cells is distinctly different from the multidrug resistance phenotype in mdrl/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>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>0.158 ± 0.0025 *</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>CDPD *</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>
</table>

*50% inhibition concentration values were determined by colony formation assay. Values are means ± SD of triplicate determinations.

** cis-diammine 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 \([^{14}\text{C}]\)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 \([^{14}\text{C}]\)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 \(\text{mdr1/P-glycoprotein}\) (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...
cells. Incubation of MCF/MX cells with 10 μM verapamil, a concentration which by itself results in a <10% decrease in colony formation of these cells, did not have any effect on [14C]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 (mdrl)-mediated multidrug resistance.

mdrl (P-glycoprotein) Expression in MCF/WT and MCF/MX. RNase protection and Western blot analyses were performed to directly assess the level of mdrl RNA and P-glycoprotein in MCF/WT and MCF/MX cells. As has been previously shown (18), mdrl RNA is readily detected in multidrug-resistant MCF/Adr cells (Fig. 3A). In contrast, mdrl 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 mdrl 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 [14C]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 10 mM sodium azide and 1 mM 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, 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 mdrl/P-glycoprotein expression (17, 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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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 at 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 unknotted (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 mdrl/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

A. Whole cell extracts

B. Nuclear extracts

Fig. 6. Western blot analysis of DNA topoisomerase II in MCF/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.

Fig. 7. Decatenation assay for topoisomerase II with nuclear extracts from MCF/WT and MCF/MX cells. Nuclear extracts (1 mg/ml protein each) from MCF/WT and MCF/MX cells were serially 2-fold diluted and assayed for ATP-dependent decatenation activity.
cross-resistance to vincristine, vinblastine, cisplatinum, and m-
crease in a MT 160,000 protein immunoreactive with topoi-
cell lines previously described, mitoxantrone resistance was as-
of P-glycoprotein. In human colon, gastric, and breast cancer
cancer cells (16). These other mitoxantrone-resistant cells, like
cancer cells (14), HL-60 leukemia cells (15), and human breast
expressing 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 resis-
tance 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 accu-
mulation (13, 14, 16). In contrast, mitoxantrone-resistant
HL-60 cells underwent no apparent alteration in drug accumu-
tion (15) but did display higher levels of relative cross-resis-
tance to agents that interact with topoisomerase II, including
VP-16, VM-26, and m-AMSA. Studies by Harker et al. (33)
demonstrated that mitoxantrone resistance in HL-60 cells was
associated with reduced levels of topoisomerase II catalytic activity.
Furthermore, relative to parental HL-60 cells, mitoxantron-
ne-resistant HL-60 cells exhibited a marked decrease in the
level of the M, 180,000 form of topoisomerase II and an in-
crease in a M, 160,000 protein immunoreactive with topoi-
somerase 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 M, 180,000 and 170,000
topoisomerase II species.

Taylor et al. (16) have described the isolation of a mitox-
trone-resistant subline from MCF-7 human breast cancer cells.
Resistance in that subline was also associated with a de-
crease in intracellular drug accumulation and enhanced efflux.
However, while the MCF/MX cells described in this report
displayed little cross-resistance to vinblastine, cisplatinum, or
m-AMSA, the mitoxantrone-resistant MCF-7 subline de-
scribed 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 re-
stance 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 resis-
tance. Increased levels of the human alpha class isozyme of GST
(GSTa) were reported in MCF-7 cells selected for primary re-
stance 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 GSTa and GST (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 GSTa RNA in the MCF/MX cells
(data not shown). Therefore, we believe that multidrug resis-
tance 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
altered 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 Mr 190,000 membrane
protein (17). Furthermore, using a polyclonal antibody (ASP-
14) directed against an oligopeptide generated from the puta-
tive ATP binding domain of the mdrl encoded P-glycoprotein,
Center and co-workers (17, 27) identified a high-molecular-
weight (M, 190,000) membrane protein in two independently
derived doxorubicin-resistant HL-60 cell lines and another,
lower-molecular-weight (M, 50,000) protein in one of the re-
sistant 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 M, 42,000 and 85,000 mem-
brane proteins found in MCF/MX that are not found in
MCF/WT cells. The identities of the M, 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 en-
coded by the putative ATP binding domain of the human mdrl
gene. Since the antibody ASP-14 is directed against an oli-
gopeptide 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 mem-

mbrane proteins found in MCF/MX cells as well as their possible roles in drug
resistance.

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the preparation of the manuscript.
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