Cytogenetic and Phenotypic Analysis of a Human Colon Carcinoma Cell Line Resistant to Mitoxantrone

William S. Dalton, Anne E. Cress, David S. Alberts, and Jeffrey M. Trent

ABSTRACT

A human colon carcinoma cell line selected for a 21-fold resistance to mitoxantrone was cross-resistant to the anthracycline, doxorubicin, but not to the anthracene, bisantrene. A 2-fold resistance was observed with vinblastine, another drug associated with multidrug resistance. Net intracellular mitoxantrone and doxorubicin accumulation were decreased at 1 h for all dose levels in the resistant cell line compared to the sensitive cell line. Although the resistant cells were more resistant to mitoxantrone than doxorubicin, the net accumulation of mitoxantrone was only 19% less than the sensitive cell line; whereas doxorubicin accumulation was decreased by 49%. No significant difference between the sensitive and resistant cell lines was observed in the initial accumulation of mitoxantrone; however, the efflux of mitoxantrone was increased in the resistant cell line. Verapamil did not overcome the resistance to mitoxantrone and did not increase the net accumulation of drug. No alterations in the electrophoretic mobility of membrane proteins were observed. Using immunoblotting techniques, the resistant cell line did not express P-glycoprotein which is frequently observed for cells resistant to anthracycline antibiotics. Cytogenetic analysis showed a putative homogeneously staining region on the short arm of chromosome 7 in the resistant cell line. The limited cross-resistant phenotype, lack of verapamil reversal, nondetection of P-glycoprotein, and cytogenetic evidence of gene amplification suggest the involvement of a novel drug-resistant gene associated with resistance to mitoxantrone.

INTRODUCTION

The development of drug resistance is a major obstacle in the treatment of cancer. In order to successfully overcome this problem, the origin of resistant cells and the mechanism by which drug resistance is mediated must be determined. Although the mechanisms for drug resistance are diverse, there are unifying factors which are beginning to emerge (1, 2). Primary among these is the hypothesis that drug-resistant neoplastic stem cells arise from genetic mutations and that these genetic changes produce a unique phenotype which is inherited (3–5). Tissue culture systems offer a method of studying the genetic alterations and mechanisms of drug resistance in human tumor cells. In this study, we have characterized a human colon carcinoma cell line made resistant to the anthrancenedione derivative, mitoxantrone or 1,4-dihydroxy-5,8-bis[2-{(2-hydroxyethyl)amino}ethyl]amino]-9,10-anthracenedione dihydrochloride (6).

Recently, several reports have associated the overexpression of a glycoprotein with a molecular weight of 170,000, termed the P-glycoprotein, with the development of resistance to many natural products including anthracyclines and Vinca alkaloids (7, 8). The mechanism of resistance appears to be due to a decreased drug accumulation secondary to enhanced efflux of drug which is energy dependent (7). The role of P-glycoprotein is uncertain; however, recent evidence suggests that it may directly bind the drug and act as an efflux pump (9, 10). An alternative explanation likens the mammalian P-glycoprotein to the bacterial transport protein, hemolysin B, which transports the toxin α-hemolysin (11). In this setting, drugs would bind a carrier protein and the drug-protein complex would be actively transported from the cell. This hypothesis is attractive in that it explains how one mechanism might confer resistance to agents which lack any structural or mechanistic similarities. While decreased drug accumulation is perhaps the most well-documented mechanism for multidrug resistance, other possibilities include: (a) enhanced detoxification of drugs or drug products by metabolic conversion (12); (b) altered intracellular drug distribution or binding which would decrease the amount of drug at the target (13); (c) altered DNA damage by changes in enzymes such as topoisoasemerase II (14); or (d) enhanced DNA repair. In reality, a combination of these mechanisms may play a role in chemotherapeutic drug resistance as these mechanisms are likely not mutually exclusive.

Mitoxantrone is similar to the anthracycline, doxorubicin in that both compounds possess dihydroxyquinones (Fig. 1) and, as a result, often have been compared with respect to antitumor activity and toxicities. In all tumor models tested, the activity of mitoxantrone was comparable or superior to that of doxorubicin, however, its cardiotoxicity was much less, making it an attractive agent for clinical trials (15, 16). Clinical studies have shown mitoxantrone to be clinically useful in the treatment of breast cancer, leukemias, and non-Hodgkin’s lymphomas (17–19). The studies reported here describe cytogenetic characteristics, patterns of drug resistance, analysis of membrane proteins, and drug accumulation characteristics of a mitoxantrone resistant human colon carcinoma cell line.

MATERIALS AND METHODS

Drugs. The sources of drugs were as follows: [3H]mitoxantrone (s.a., 15 μCi/nmol) and bisantrene (9,10-anthracenedecarboxaldehyde-bis(4,5-dihydro-1H-imidazol-2-yl)hydrazone dihydrochloride), American Cyanamid Co., Lederle Laboratories, Pearl River, NY; [14C]doxorubicin, (s.a., 21 μCi/mmol) vinblastine, Eli Lilly and Co., Indianapolis, IN; methotrexate, National Cancer Inst., Bethesda, MD; and 4-hydroperoxycyclophosphamide from Dr. Robert Struck of the Southern Research Institute, Birmingham, AL. Verapamil HCl was obtained from Knoll Pharmaceuticals (Whippany, NJ). Drugs were prepared by dissolving in methanol or 0.9% saline with dilutions made by adding conditioned medium. The final concentration of methanol was 0.01% when exposed to cells and was included in controls.

Cell Lines. The WiDr human colon carcinoma cell lines (WiDr-sensitive, WiDr/S and WiDr/resistant, WiDr/R) were kindly provided by Dr. R. Wallace of American Cyanamid Co., Lederle Laboratories, Pearl River, NY. The mitoxantrone-resistant subline, WiDr/R, was established by continuously exposing cells to gradually increasing concentrations of mitoxantrone as described previously (6). Cultures were grown as a monolayer in Eagle’s minimum essential medium (Grand Island Biological Co.) supplemented by 10% fetal bovine serum (Hyclone Co.), 2% L-glutamine (Grand Island Biological Co.).
formed as previously described (21). The results presented conform to calculated from linear transformation of the dose response curves.

Blastine, methotrexate, or 4-hydroperoxycyclosphamide for 1 h at 37°C. At the end of 1 h the cells were washed and plated in triplicate in 35-mm petri dishes at 20,000 cells per plate. Plates were then incubated at 37°C. In some cases, verapamil 5 μg/ml was added to the media and incubated with 1 x 10^6 cells grown as a monolayer at 37°C. At the end of the 1-h incubation period, the media with drug was diluted by incubating drug with 1 x 10^6 cells grown as a monolayer at 37°C. The multidrug resistant human myeloma cell line was used as a control body, (C219; kindly provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario, Canada), according to the method described by Kartner et al. (26). This method is capable of detecting the overexpression of P-glycoprotein associated with multidrug resistance (25, 26). The multidrug resistant human myeloma cell line was used as a control for the detection of P-glycoprotein (27).

Determination of Intracellular Drug Accumulation. Net intracellular accumulation of [3H]mitoxantrone or [14C]doxorubicin was measured by incubating drug with 1 x 10^6 cells grown as a monolayer at 37°C. The concentration of drug was determined by adding liquid scintillation fluor and counting in replicates of three for each cell group following the 1-h drug accumulation to assure equivalent cell numbers. To determine if verapamil increased intracellular accumulation of mitoxantrone, 5 μg/ml of verapamil was added to the media containing mitoxantrone and cells were incubated with this mixture for 1 h.

Initial [3H]mitoxantrone intracellular accumulation over the first 60 s was determined by using rapid dilution techniques. Cells were grown at 37°C as a monolayer in glass scintillation vials with 1 x 10^6 cells per vial. Media containing [3H]mitoxantrone was added to the vials and rapidly diluted with 10 volumes of 4°C PBS at appropriate time intervals (0-60 s). The zero time value was determined by keeping cells, drug, and media at 4°C to estimate nonspecific binding of drug. Following removal of drug and media the cells were rinsed once with cold PBS. One molar NaOH was used to solubilize cells and subsequently neutralized with HCl. Radioactivity was determined by liquid scintigraphy.

Efflux of mitoxantrone was determined by incubating WiDr/S and WiDr/R cells with [3H]mitoxantrone for 1 h at 37°C. PBS was added after 1 h and the drug with media was replaced by drug-free media. At appropriate time intervals following placement in drug-free media, cells were solubilized and radioactivity determined as above. Terminal phases of the elimination curves were compared by linear regression analysis (28).

HPLC analysis was performed in sensitive and resistant cells to evaluate for changes in metabolism (29). The HPLC apparatus consisted of a model 660 solvent programmer, two model 6000 A solvent delivery systems, and a Waters Associates model C18-μ Bondapak reversed-phase column, used for all analyses. Mitoxantrone was eluted isocratically at ambient temperature with acetonitrile/0.2 mM ammonium

Fig. 1. Structures of doxorubicin, a naturally occurring anthracycline antibiotic, and two anthracene derivatives, mitoxantrone and bisantrene.

Co.) and 1.0% (v/v) penicillin/streptomycin (10,000 units/ml; Grand Island Biological Co.). Cell lines were cultured at 37°C in 95% humidity, 5% CO₂, and 20% O₂. Resistance to mitoxantrone had previously been demonstrated to be stable for more than 42 cell generations following removal of the drug from culture media (6); therefore, cells were maintained in drug-free media for at least 1 week prior to study.

Chemosensitivity Assay. Techniques for preparing cells for drug exposure by plating cells in soft agar have been reported previously (20). Exponentially growing cells were exposed to varying concentrations of anticancer drugs (mitoxantrone, doxorubicin, bisantrene, vincristine, methotrexate, or 4-hydroperoxycyclosphamide) for 1 h at 37°C. In some cases, verapamil 5 μg/ml was added to the media and cytotoxic drug to determine if verapamil reversed drug resistance. At the end of 1 h the cells were washed and plated in triplicate in 35-mm petri dishes at 20,000 cells per plate. Plates were then incubated at 37°C for 10 days. Colonies greater than 60 μm were counted by inverted microscopy or by computerized image analyzer (Omincon FAS II; Bausch and Lomb, Inc., Rochester, NY). The concentration of drug which produced a 50% inhibition of cloning efficiency (IC₅₀) was calculated from linear transformation of the dose response curves.

Chromosomal Analysis. Chromosome banding analysis was performed as previously described (21). The results presented conform to ISCN recommendations (22).

Protein Analysis. Cellular protein was labelled for 18 h with 5 μCi/ml of [35S]methionine (New England Nuclear Corp.) in McCoy's 5A media (GIBCO, Inc.) containing 10% of the normal concentration of methionine. The cells were fractionated and the plasma membrane isolation performed using a modification of the method of Brunette and Till (23). After radioactive labeling, cells were rinsed and harvested with PBS, pH 7.2. The cells are resuspended at a concentration of 2.0 x 10⁴ cells/ml in a hypotonic buffer containing 10 mM Tris-HCl, 1 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, and are allowed to swell at 25°C for 15 min, followed by 5 min on ice. The swollen cells are fractionated by Dounce homogenation (Kontes, Inc.) with a type B pestle and viewed microscopically to ensure a maximum yield of nuclei. The cytoplasmic fraction is further fractionated by use of the Dextran-polyethylene glycol two-phase systems. A representative portion of the whole cell, cytoplasmic, nuclear, and membrane fractions was utilized for gel electrophoresis.

Samples were prepared for electrophoretic analysis by denaturing the proteins in SDS sample buffer containing 2% SDS, 5% mercaptoethanol, 50 mM Tris-HCl, pH 7.2, 3% sucrose and 0.01% bromophenol blue. The samples were boiled for 10 min and centrifuged (900 x g/3 min) prior to application to the gel. SDS-polyacrylamide gel (7%) electrophoresis was performed according to Laemmli's procedure (24). Equal amounts of protein were applied to each gel lane.

Immunoblot Analysis for P-Glycoprotein. After preparing plasma membranes according to the method of Riordan and Ling (25), immunoblot analysis was performed with an [125I]-labeled monoclonal antibody, (C219; kindly provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario, Canada), according to the method described by Kartner et al. (26). This method is capable of detecting the overexpression of P-glycoprotein associated with multidrug resistance (25, 26). The multidrug resistant human myeloma cell line was used as a control for the detection of P-glycoprotein (27).

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The abbreviations used are: PBS, phosphate buffered saline, 137 mM NaCl, 2.65 mM KCl, 7.66 mM Na₂HPO₄, and 133 mM KH₂PO₄; HPLC, high-performance liquid chromatography; IC₅₀, concentration resulting in 50% inhibition of colony formation; SDS, sodium dodecyl sulfate; HSR, homogeneously staining region.

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acetate, pH 4.0 (22:78) as solvent at a flow rate of 1.5 ml/min. Mitoxantrone was detected at 658 nm.

RESULTS

In Vitro Chemosensitivity Testing. Mitoxantrone survival curves for the sensitive WiDr/R cells were 21-fold resistant to mitoxantrone when compared to the parent line WiDr/S (Fig. 2). In addition, an 8-fold increase in resistance to the anthracycline, doxorubicin was observed for the WiDr/R subline. The relative patterns of WiDr/S and WiDr/R to these and several other commonly used chemotherapeutic agents is shown in Table 1. This pattern is expressed as a ratio of the IC50 concentration of each drug for the resistant subline versus the parent sensitive cell line. In addition to the relatively marked cross-resistance to doxorubicin, there was a slight (2-fold) cross-resistance to the Vinca alkaloid, vinblastine. No cross-resistance was observed for the anthracycline, bisantene; the antimetabolite, methotrexate; or for the active metabolite of cyclophosphamide, 4-hydroperoxycyclophosphamide. Verapamil at 5 μg/ml did not enhance the cytotoxicity of mitoxantrone in either cell line when IC50 values were compared.

Cytogenetic Analysis. G- and Q-banding analysis was performed on WiDr/S cells and revealed numerous numeric and structural chromosome alterations (Fig. 3, A–C). The WiDr/S line demonstrated a modal chromosome number of 69 (range, 64–72) with six unidentifiable marker chromosomes. In addition to these marker chromosomes, several other clonal structural alterations could be identified including an isochromosome of 3p [(3p)], a Robertsonian translocation involving chromosome 13[(13;13)(p11;q11)], and the simple deletion of the short arm of chromosome 7 [del(7)(p15)]. The karotype of the WiDr/R subline was markedly different than the sensitive, parental line. Although the modal chromosome number was similar (68), the range of chromosomes per cell was significantly greater in the resistant subline (range, 49 > 4N). Also, although the six marker chromosome present in WiDr/S were also present in WiDr/R cells, several specific chromosome changes accompanied the acquisition of drug resistance (Fig. 3C). Those changes unique to WiDr/R included the presence of five additional marker chromosomes, the simple deletion of the short arm of chromosome 1 [del(1)(p21)], and a translocation between chromosomes 1 and 13 [(1;13)(p13;q11)]. In addition to these alterations, there were two aberrations of chromosome 7p which were unique to the resistant line. The first was a region of homogeneously staining present at band 7p15[7pHSR], and second a pericentric inversion of this 7pHSR marker (Fig. 3C).

Drug Accumulation Studies. As a possible mechanism of resistance, [³H]mitoxantrone and [¹⁴C]doxorubicin accumulation were compared for the sensitive and resistant cell lines. Table 2 demonstrates the net accumulation of mitoxantrone and doxorubicin at 1 h. The amount of intracellular drug in both the sensitive and resistant cell lines was directly related to the extracellular concentration range without evidence of plateau over a 10-fold concentration range. For mitoxantrone, the resistant cells accumulated approximately 19% less drug in 1 h compared to the sensitive cells for all concentrations. The differences in intracellular accumulation was greater for doxorubicin; the resistant cells accumulated 51% less drug in 1 h compared to the sensitive cells for all concentrations. There is therefore a discordance between the degree of resistance (21-fold for mitoxantrone and 8-fold for doxorubicin) and differences in drug accumulation for these two drugs in the sensitive and resistant cells.

Verapamil has been shown to enhance the cytotoxicity of Vinca alkaloids and anthracyclines in various multidrug resistant cell lines by increasing intracellular concentration of cytotoxic drugs (9, 30, 31). It can be seen in Fig. 4, however, that verapamil had no effect on intracellular mitoxantrone concentration at 1 h. This finding is consistent with the observation that verapamil did not enhance the cytotoxicity of mitoxantrone in the resistant cell line.

We evaluated the possibility that decreased influx might account for the decrease in net accumulation of mitoxantrone in the resistant cell lines. Fig. 5 demonstrates, however, that the amount of intracellular drug during the first 60 s of mitoxantrone exposure was not different for the sensitive and resistant cells. The rate of initial drug accumulation was linear over the 60-s exposure, and the amount of intracellular drug was not different at any of the time points measured in the sensitive and resistant cells. Comparison of slopes by linear regression analysis also demonstrated no statistical difference (28).

In contrast, the rate of efflux of mitoxantrone from resistant cells appeared to be greater compared to the sensitive cells. Fig. 6 compares [³H]mitoxantrone retention at various time points for sensitive and resistant cells in drug-free media. Following an initial rapid loss of intracellular mitoxantrone in both cell lines, the sensitive cell line retained 67% of the original mitoxantrone concentration; whereas, the resistant cells retain only 43% at 1 h. Comparison of the terminal slopes (from 10 to 60 min) also showed a significantly higher rate of drug loss for the resistant cells compared to the sensitive cells (P < 0.01). Thus, it would appear that the difference in net accumulation between sensitive and resistant cells is due to differences in drug efflux.

HPLC analysis of mitoxantrone and metabolites for both cell lines confirmed the difference in net accumulation at one hour (data not shown). Also, there were no additional HPLC peaks observed for WiDr/R which would have suggested altered mitoxantrone metabolism in the resistant cell line.
Fig. 3. G-Banded chromosomes from the WiDr/S (A and B) and WiDr/R (C) cell lines. A, representative karotype of the sensitive parental cell line WiDr/S. Clonal chromosomal abnormalities included six unidentifiable marker chromosomes (M1-M6); B, an additional clonal alteration (M5) is documented which was not found in the cell presented in A; C, chromosomal alterations unique to the resistant subline (M7-M11), as well as structural chromosomal alterations of chromosome 1 [del(1)(p21) and t(1;13) (p13;q11)]. The presence of two versions of homogeneously staining region of the short arm of chromosome 7 (7pHSR & inv7pHSR) was also a unique feature of the drug-resistant subline (see text).
**ANALYSIS OF A CELL LINE RESISTANT TO MITOXANTRONE**

### Table 2. Accumulation of drug in sensitive (WiDr/S) and resistant cells (WiDr/R) at 1 h

<table>
<thead>
<tr>
<th>Drug concentration (µM)</th>
<th>WiDr/S (cpm ± SD)</th>
<th>WiDr/R (cpm ± SD)</th>
<th>WiDr/R:WiDr/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone 0.1</td>
<td>8,179 ± 184</td>
<td>6,466 ± 184</td>
<td>0.79</td>
</tr>
<tr>
<td>0.5</td>
<td>43,318 ± 1,015</td>
<td>35,501 ± 1,267</td>
<td>0.82</td>
</tr>
<tr>
<td>1.0</td>
<td>89,689 ± 8,688</td>
<td>74,101 ± 6,042</td>
<td>0.82</td>
</tr>
<tr>
<td>Doxorubicin 0.1</td>
<td>607 ± 45</td>
<td>332 ± 12</td>
<td>0.54</td>
</tr>
<tr>
<td>0.5</td>
<td>2,484 ± 143</td>
<td>1,192 ± 76</td>
<td>0.48</td>
</tr>
<tr>
<td>1.0</td>
<td>4,557 ± 54</td>
<td>2,113 ± 117</td>
<td>0.46</td>
</tr>
</tbody>
</table>

**Fig. 4.** Intracellular accumulation in WiDr/S and WiDr/R cells after a 1-h exposure to 0.1 µM [3H]mitoxantrone with or without the addition of verapamil (5 µg/ml). Verapamil did not enhance mitoxantrone accumulation in the resistant cell line. Bar, mean of four replicates with one standard deviation noted.

**Fig. 5.** Initial accumulation of [3H]mitoxantrone during the first 60 s of drug exposure in WiDr/S (○) and WiDr/R (●) cells. Intracellular drug concentration was not different at any time point and the slopes were not significantly different by linear regression analysis. Points, mean of four replications with one standard deviation noted.

**Fig. 6.** Efflux of [3H]mitoxantrone from WiDr/S (○) and WiDr/R (●). Cells were exposed to [3H]mitoxantrone 0.1 µM for 1 h at 37°C, washed with PBS, and then resuspended in drug-free medium at 37°C. Points, mean of six replicates with one standard deviation noted. Drug efflux was significantly greater in the WiDr/S cell line when terminal slopes (10 to 60 min) were compared by linear regression analysis (P < 0.05).

**Fig. 7.** SDS-polyacrylamide gel electrophoresis (7.0%) of cellular proteins. The WiDr sensitive (WiDr/S) and resistant (WiDr/R) cells were grown in the presence of [35S]methionine and fractionated to yield cytoplasmic (lanes B and F), nuclear (lanes D and H) and plasma membranes (lanes C and G) components. Proteins from unfractionated cells are represented in lanes A and E. Identical protein amounts were applied to each gel lane. No difference in membrane proteins were identified by this method.

**DISCUSSION**

Several cell lines which have been selected for resistance to one drug often exhibit a cross-resistance to a wide range of drugs which are unrelated either structurally or mechanistically (2, 7). For example, variant mammalian cell lines which have been selected for resistance to anthracyclines frequently develop a high level of cross-resistance to Vinca alkaloids (2, 7, 27). This type of multidrug resistance has been associated with the overexpression of a particular integral membrane protein termed P-glycoprotein alternatively called mdrl (7, 8). The function of this protein is thought to be related to decreased net drug accumulation secondary to enhanced drug efflux (2, 7). Unlike the multidrug resistant cell lines, the WiDr/R cell line does not appear to have increased amounts of this integral membrane protein compared to the sensitive cell line WiDr/S. Using a second murine monoclonal antibody against P-glycoprotein, JSB-1 (32), also failed to detect an overexpression in the WiDr/R cell line (data not shown).

**Protein Analysis in Sensitive and Resistant Cells.** As a first step in characterizing the WiDr sensitive and resistant cell lines, we analyzed the qualitative protein profiles in cell fractions enriched for cytoplasmic, nuclear or membrane proteins. Fig. 7 illustrates the protein profiles observed in both the WiDr/S parent line and the WiDr/R-resistant line. The major proteins which appear unique to the membrane fractions have apparent molecular weights of 200,000; 180,000; 145,000–125,000; and 72,000–70,000. The molecular weight classes of the major membrane proteins are not significantly different when the sensitive and resistant cell lines are compared.

Immunoblot analysis of WiDr-sensitive and -resistant cells using the C-219 monoclonal antibody is shown in Fig. 8. This antibody is capable of recognizing a conserved portion of the P-glycoprotein and is species independent (26). In the multiple drug-resistant cell line, 8226/Dox40, P-glycoprotein is overexpressed compared to the sensitive cell line 8226/S. In contrast, the WiDr/R cell line does not appear to have increased amounts of this integral membrane protein compared to the sensitive cell line WiDr/S. Using a second murine monoclonal antibody against P-glycoprotein, JSB-1 (32), also failed to detect an overexpression in the WiDr/R cell line (data not shown).
The cell remains to be determined. Although there was a significant reduction of mitoxantrone in the WiDr/R cell line, it appears insufficient to explain the over 20-fold resistance seen in the resistant cell line. This is especially true in light of the fact that the difference in doxorubicin uptake was over twice that seen for mitoxantrone (Table 2) and yet the degree of resistance for doxorubicin was only 8-fold compared to 21-fold for mitoxantrone (Table 1).

The fact that this mitoxantrone-resistant cell line is resistant to the anthracycline, doxorubicin, but not to the anthracene derivative, bisantrene, poses interesting questions regarding the structure-activity relationships and mechanisms of action for these related drugs. All three drugs have features which are considered to be essential for DNA intercalation; that is, all three compounds are planar, electron-rich chromophores. However, in comparing the molecular pharmacology of the anthracene compounds, mitoxantrone and bisantrene, Bowden et al., described major differences in the way these compounds interact with cellular DNA which might explain the lack of cross-resistance to bisantrene (34). Bisantrene binds DNA in a manner typical of classical intercalating drugs by causing protein-associated DNA breaks and changes in DNA supercoiling. Mitoxantrone, on the other hand, does not act as a classical intercalating drug and induces both protein-associated and non-protein-associated DNA strand breaks. Other investigators have suggested that Adriamycin and related antitumor drugs induce cytotoxicity via DNA damage mediated by mammalian DNA topoisomerase II (14). A difference in the DNA-protein complex induced by mitoxantrone or bisantrene might explain the lack of cross-resistance observed between these two drugs.

A second notable difference between doxorubicin, mitoxantrone, and bisantrene is that both mitoxantrone and doxorubicin possess quinone groups (see Fig. 1) which are capable of reduction and autoxidation to produce oxygen radicals; bisantrene, on the other hand, lacks this structural characteristic and should not be capable of oxygen radical formation. The generation of doxorubicin-free radicals has been demonstrated to produce DNA strand breaks (35). It is possible therefore that a mechanism to reduce free radicals and their associated DNA damage might account for the cross-resistance observed for mitoxantrone and doxorubicin which is lacking for the more classical intercalating agent, bisantrene. Candidates for such a mechanism might include a variety of detoxifying enzymes such as glutathione-S-transferase or more specifically a quinone-specific enzyme such as DT-diaphorase (36, 37). Studies to measure the activity of these enzymes in the WiDr/S and WiDr/R cell lines are in progress.

The results of the cytogenetic analysis suggest that the acquisition of mitoxantrone resistance has been accompanied by several chromosomal alterations. Of particular interest was the finding of cytological evidence for gene amplification associated with the resistant phenotype. Specifically, the finding of a putative homogeneously staining region in drug resistant (but not sensitive) cells may be analogous to other systems where drug resistance is mediated by amplification of specific gene products (38). Studies are now underway to confirm the presence of an amplified DNA domain in resistant cells using the technique of gel renaturation (39).

Drug accumulation studies for the sensitive and resistant cell lines demonstrate that the WiDr/R cells accumulate less mitoxantrone and doxorubicin at 1 h than the WiDr/S cells. This decrease in net accumulation appears to be due to enhanced efflux and not due to altered influx. Whether this enhanced efflux is energy dependent and related to a membrane protein or whether it is related to altered subcellular distribution and drug binding which would allow the drug to more readily exit the cell remains to be determined. Although there was a significant decrease in net accumulation due to enhanced efflux, it appears insufficient to explain the over 20-fold resistance seen in the resistant cell line. This is especially true in light of the fact that the difference in doxorubicin uptake was over twice that seen for mitoxantrone (Table 2) and yet the degree of resistance for doxorubicin was only 8-fold compared to 21-fold for mitoxantrone (Table 1).

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This cell line appears of particular interest for further study for several reasons. First, in contrast to cell lines resistant to anthracyclines such as doxorubicin, the WiDr/R line displays a limited multidrug-resistant phenotype, and does not significantly overexpress P-glycoprotein. Secondly, the net accumulation data indicate that mechanisms other than just drug accumulation may be playing a major role. Thirdly, the cyto-
genetic evidence suggests that amplification of a novel drug-resistant gene mediating the resistant phenotype has occurred. Recently, similar reports have described multidrug resistance to natural products in various human cell lines which do not over express P-glycoprotein (40, 41). Whether these cell lines share a common mechanism of resistance to drugs or whether these mechanisms are unique to each individual cell line or drug remains to be determined.

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