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Increased DT-diaphorase Expression and Cross-Resistance to Mitomycin C in a Series of Cisplatin-Resistant Human Ovarian Cancer Cell Lines

Peter J. O'Dwyer, Raymond P. Perez, Kang-Shen Yao, Andrew K. Godwin and Thomas C. Hamilton*

FOX CHASE CANCER CENTER, PHILADELPHIA, PA 19111, U.S.A.

ABSTRACT. In a series of ovarian carcinoma cell lines selected *in vitro* for resistance to cisplatin by continuous exposure to increasing drug concentrations, the level of resistance is proportional to the expression of γ-glutamylcysteine synthetase (γ-GCS). To determine if other detoxicating genes are coordinately expressed, we measured the activity of DT-diaphorase and cytochrome P450 reductase. The specific activity of DT-diaphorase, but not that of cytochrome P450 reductase, increased with increasing resistance to cisplatin. Steady-state mRNA levels for DT-diaphorase correlated with enzyme activity and hence with cisplatin resistance. Since the activity of DT-diaphorase has been associated with sensitivity to quinones, we studied the cytotoxicity of mitomycin C under oxic conditions. Unexpectedly, resistance to mitomycin C increased proportionally with that to cisplatin ($r = 0.997$). Pretreatment with buthionine sulfoximine, which inhibits glutathione (GSH) synthesis, failed to sensitize either the sensitive or the resistant lines to mitomycin C. Thus, the basis for collateral resistance to mitomycin C in the cisplatin-resistant lines under oxic conditions is unrelated to overproduction of GSH. Under hypoxia, the toxicity of mitomycin C to the most sensitive (A2780) cell line was unchanged. However, the most resistant (C200) line was 2-fold more resistant to mitomycin C under hypoxic conditions. The coordinate overexpression of DT-diaphorase and γ-GCS in the resistant cell lines is thus associated with hypoxic cell resistance, and supports the involvement of shared mechanisms of gene regulation in the observed resistant phenotype. BIOCHEM PHARMACOL 52;1:21–27, 1996.

KEY WORDS. ovarian cancer; mitomycin C; glutathione; DT-diaphorase; gene expression; cisplatin resistance

Although many anticancer drugs have substantial efficacy, they are often not curative as a result of the development of tumor cell resistance. The activity of cisplatin in ovarian cancer provides an example. In this disease, the complete response rate after aggressive cisplatin-based chemotherapy is 60–80%. However, a majority of these patients ultimately develop recurrent disease that is drug refractory. The development of cell lines with acquired resistance to selected active agents such as cisplatin has been one approach used to study the important clinical problem of drug resistance. In the case of ovarian cancer, we have developed a series of stably resistant cell lines by selection *in vitro* to increasing concentrations of cisplatin [1, 2]. The IC_{50} values of cisplatin required for cytotoxicity range from 0.19 to 205 μM, a 1000-fold difference in sensitivity [3]. Resistance to cisplatin in these cell lines is mediated by multiple mechanisms including but perhaps not limited to: decrease in cell-associated drug, increase in the potential for GSH†-mediated drug inactivation, increased DNA repair, and alteration in the DNA lesions formed [4, 5]. It is also noteworthy that these cell lines show moderate to substantial cross-resistance to diverse drugs including carboplatin, melphalan, doxorubicin, etoposide, mitoxantrone, and paclitaxel. Furthermore, the broad cross-resistance to natural products is not related to expression of GP-170, the product of the multidrug resistance 1 gene or to multidrug resistance-associated protein [6].

In this model of cisplatin resistance, one of the more striking changes observed is a 50-fold increase in cellular GSH. We found this change to be mediated by increased expression of steady-state mRNA levels for γ-GCS and γ-GT [7]. We also found recently induction of GSH and γ-GCS in HT29 cells exposed to hypoxia [8] or to the chemopreventive dithiolethione oltipraz.‡ Hypoxia is associated with resistance to cytotoxic drugs, and overexpres-

* Corresponding author: Peter J. O'Dwyer, M.D., Kimmel Cancer Institute, Thomas Jefferson University, 233 South 10th St., Suite 502, Philadelphia, PA 19107. Tel. (215) 503-3155; FAX (215) 503-3047.

† *Abbreviations:* GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; γ-GT, γ-glutamyl transpeptidase; FBS, fetal bovine serum; SRB, Sulforhodamine B; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; BSO, buthionine sulfoximine; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

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sion of detoxicating enzymes is a plausible mechanism of resistance [8]. In this stress-response setting, the overexpression of other enzymes associated with detoxication is also found, including DT-diaphorase and cytochrome P450 reductase [8, 9]. These changes in detoxication gene expression appear to be a consequence of alterations in the levels of expression of the genes encoding several ubiquitous transcription factors [10–12]. Additionally, we have begun to investigate the mechanism of increased transcription of γ-GCS in cisplatin resistance. One interesting difference is that selection of cells for cisplatin resistance yields cell lines with constitutively increased expression of GSH and γ-GCS, whereas that induced by hypoxia is transient.

Our combined studies suggest some commonality between the mechanism of transcriptional regulation by hypoxia and chronic cisplatin exposure. There is some overlap in the response elements present in the promoters of the individual genes, and in the altered expression of transcription factors that bind these elements. The possible similarity in gene regulation in these two models prompted an investigation of whether there was constitutive induction of the classical stress-response enzymes DT-diaphorase and cytochrome P450 reductase in cisplatin-resistant cells. Furthermore, since both of these enzymes participate in the bioreductive activation of the clinically important alkylating agent mitomycin C [9], it was of interest to determine the sensitivity of the cisplatin-resistant cells to this drug under oxic and hypoxic conditions. We found that the cisplatin-resistant cells were cross-resistant to mitomycin C, and that only the resistant lines showed additional resistance under hypoxia.

MATERIALS AND METHODS

Drugs, Chemicals

Commercially available sources of cisplatin (Bristol–Myers Squibb, Wallingford, CT) and mitomycin C (Bristol Laboratories, Evansville, IN) were reconstituted according to manufacturer's directions. All other reagents were purchased from Sigma (St. Louis, MO), except where noted.

Cells

The selection of this series of cisplatin-resistant ovarian cancer cell lines from the parent cell line A2780 has been described in detail [2]. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS (GIBCO, Grand Island, NY), 100 μg/mL streptomycin, 100 U/mL penicillin, 0.3 mg/mL glutamine, and 0.3 U/mL insulin. Cells were grown at 37°, in a humidified atmosphere of 5% CO_2.

SRB Assay

The SRB assay was performed as described by Skehan *et al.* [13], with minor modifications. Briefly, 5×10^3 cells in 0.2 mL medium were plated in each well (0.16 cm^2) of 96-well plates and allowed to attach for several hours. Drug was added to the wells to produce the desired final concentrations, and plates were incubated at 37° for 3 days. Cells were then fixed by gentle addition of 50 μL of cold (4°) 50% trichloroacetic acid to each well, followed by incubation at 4° for 1 hr. Plates were washed with deionized water five times and allowed to air dry. Cells were then stained by addition of 50 μL SRB solution [0.4% SRB (w/v) in 1% acetic acid (v/v)] to wells for 10 min. Following staining, plates were quickly washed five times with 1% acetic acid to remove unbound dye, and allowed to air dry. Bound dye was solubilized with 10 mmol/L Tris base (pH 10.5) prior to reading plates. Plates were read at 595 nm on a Bio-Rad microplate reader.

MTT Assay and Hypoxic Cell Treatment

The MTT assay involves the conversion of tetrazolium salt to colored formazan by cells serving as an indirect measurement of cell proliferation [14]. The cells were plated in flat-bottom NUNC microtiter plates at a cell density of 1500 cells/well with 8 replicates for each drug concentration. Microtiter plates were harvested when controls reached 80–90% confluency in approximately 5 days.

A2780/C200 cells were trypsinized from a monolayer and suspended in Spinner's minimal essential medium (Ca^{2+} and Mg^{2+} free) supplemented with 5% FBS. Cell suspensions at 10^5 cells/mL in 8-mL aliquots were transferred to glass incubation chambers and slowly stirred by a magnetic bar in a waterbath maintained at 37°. The glass chambers contained inlet and outlet ports for gassing with either humidified 95% ultrapure nitrogen with 5% CO_2 or CO_2 balanced 95% air. The gas flow rate through each chamber was 1 L/min. After 30 min of pre-gassing, a small amount of mitomycin C was added to a final concentration as required. Aliquots of the cell suspension (0.6 mL) containing various drug concentrations were removed by 4 hr post gas treatment. Cells were washed immediately with Dulbecco's PBS and plated for MTT assay.

Data Analysis

The IC_{50} values for cisplatin and mitomycin C in individual cell lines were determined for each experiment by median effects analysis, using commercially available software [15]. Data used for median effects analysis fit the regression lines used for the analysis with correlation coefficients ≥ 0.95. Data in tables and figures represent the means (±SD) of three individual experiments, unless otherwise indicated. Cytotoxicity (IC_{50}) data for each assay were analyzed by Spearman rank correlation.

Biochemical Assays

GSH. Cells ($2–5 \times 10^6$) grown to ~60% confluence were sonicated in 1 mL of ice-cold PBS, and centrifuged at 10,000 *g* for 10 min at 4°; the cytoplasmic supernatant was

deproteinized, and the protein-free filtrate assayed by a modification of the method of Griffith [16], in which the rate of formation of the GSH conjugate of 5,5′-dithiobis(2-nibrobenzoic acid) was determined at 412 nm, and results expressed as GSH content per 10^6 cells. GSH concentrations were determined by reference to a standard curve.

DT-DIAPHORASE. This enzyme was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm [17]. Dicumarol was used as a negative control. Results were normalized to protein by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

NADPH CYTOCHROME P450 REDUCTASE. This enzyme was measured by a spectrophotometric assay in which the rate of reduction of cytochrome *c* was monitored at 550 nm in the presence or absence of NADPH [18].

GSH TRANSFERASE. Activity was measured spectrophotometrically as the rate of GSH conjugation of 1-chloro-2,4-dinitrobenzene [19].

Protein was measured by a modification of the Bradford assay (Bio-Rad) using a standard curve of bovine serum albumin.

RNA Isolation and Northern Analysis

The total RNA was isolated from cells as described by Chomczynski and Sacchi [20]. Northern blot analysis was performed with 15 μg of total RNA after electrophoresis through 1% agarose gel in 1x HEPES buffer and 2.2 M formaldehyde, and capillary transfer to a Nylon transfer membrane (MSI Magna NT). Filters were prehybridized in 50% (v/v) formamide, 5x SSPE, 2x Denhart's reagent, and 0.1% SDS, at 42° for 4–6 hr (1x Denhart's reagent is 0.02% polyvinylpyrolidione, 0.02% Ficoll, 0.02% bovine serum albumin; 1x SSPE is 180 mM NaCl, 10 mM sodium phosphate, 1 mM disodium EDTA, pH 8). An 880-bp *Pst* I fragment from the coding region of rat DTD gene cDNA [21] was labeled by the multiprimer method (Amersham) to >1 × 10^9 cpm/μg, denatured, and added directly to the prehybridization mixture. Hybridization was continued overnight for 16–24 hr at 42°. The optimal washing conditions were as follows: 20 min at room temperature in 1x SSC, 0.1% SDS, 20 min at 55° in 0.2x SSC, 0.1% SDS, and 20 min at 55° in 0.1x SSC, 0.1% SDS. Autoradiography was carried out at −80° for 4–7 days. The blot was subsequently stripped in boiling water and reprobed with β-actin, and the relative labeling was determined by scanning densitometry.

RESULTS

Activity of Detoxication Enzymes in Cisplatin-Resistant Ovarian Cancer Cells

It was shown previously in this series of cisplatin-resistant ovarian cancer cell lines that the content of GSH varies by 50-fold [6]. The relationship between GSH content and resistance to cisplatin (as expressed by the IC_{50} was linear ($r = 0.942$). The increased cell content of GSH is associated with proportionally elevated steady-state mRNA content of γ-GCS and γ-GT [6]. There is no increase in the activity of GSH transferase [5]. To determine if the changes in resistant cells extended to other detoxication enzymes, the activities of DT-diaphorase and NADPH cytochrome P450 reductase were determined. Resistant cells had markedly elevated activity of DT-diaphorase, but not of cytochrome P450 reductase (Table 1). The activity of DT-diaphorase was proportionally related to cisplatin resistance ($r = 0.727$).

DT-diaphorase Steady-State mRNA Content of Cisplatin-Resistant Lines

To determine the basis for the increase in the specific activity of DT-diaphorase in these cell lines, we investigated the content of mRNA encoding DT-diaphorase by northern analysis (Fig. 1). A proportional increase in DT-diaphorase mRNA levels was observed with increasing re-

TABLE 1. GSH content and activity of detoxication enzymes in human ovarian cancer cell lines with acquired resistance to cisplatin

Cell line	IC_{50} of cisplatin* (μM)	GSH (nmol/10^6 cells)	GSH (nmol/min/mg protein)	DT-diaphorase (nmol/min/mg protein)	Cytochrome P450 reductase (nmol/min/mg protein)
A2780	0.19	1.75	1.30	166	4.27
CP8				152 (0.9)†	
CP20				214 (1.3)	
CP70	5.5	22.6 (13)	1.22	420 (2.5)	1.33
C30	54	26.0 (15)	1.13	536 (3.2)	2.95
C50	100	37.8 (22)	0.97	694 (4.2)	1.69
C80	115	52.4 (30)	1.14	1044 (6.3)	4.57
C100	125	31.6 (18)	0.93	1304 (7.9)	3.24
C200	205	83.2 (48)	1.10	823 (5.0)	4.10

* Data are from cytotoxicity studies using the MTT assay [4].

† Values in parentheses represent activity relative to A2780.

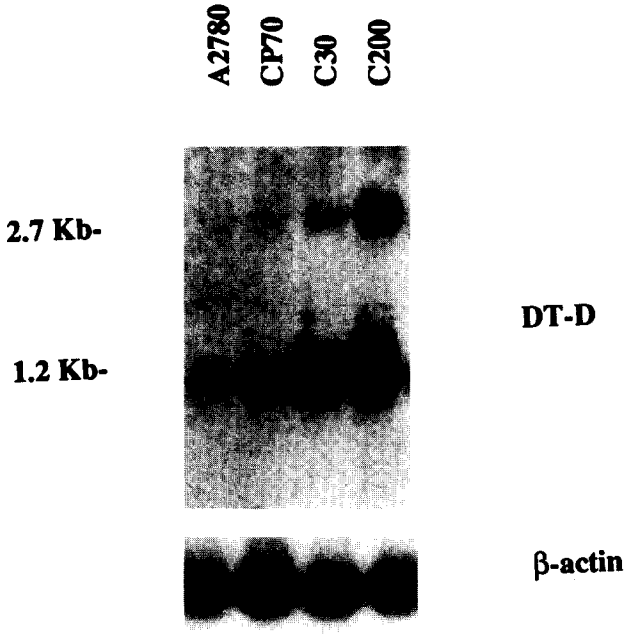

FIG. 1. Northern analysis of total RNA from cisplatin-resistant ovarian cancer cell lines probed for expression of DT-diaphorase. Total RNA (15 μg/lane) from each cell line was separated in a 1% agarose gel, blotted, and probed with an 880 bp fragment from the DT-diaphorase coding region.

sistance to cisplatin. This result shows the close concordance in the RNA expression of DT-diaphorase and γ-GCS [6].

Cross-Resistance to Mitomycin C in the Presence of Primary Cisplatin Resistance

The substantial cross-resistance to diverse drugs characteristic of these cisplatin-resistant cells, coupled with their increased DT-diaphorase activity, prompted an investigation of their sensitivity to mitomycin C. Using the SRB assay, there was substantial cross-resistance to mitomycin C (Fig. 2). The IC_{50} for cisplatin and that for mitomycin C were closely correlated ($r = 0.997$). A plot of the IC_{50} values versus DT-diaphorase activity was linear (Fig. 3, $r = 0.925$).

Effects of BSO-Mediated GSH Depletion on Mitomycin C Sensitivity

To test the possibility that the elevated GSH content might be the sole underlying basis for cross-resistance to mitomycin C in these cell lines, the moderately cross-resistant CP70 cells and the highly resistant C200 cells were pretreated for 24 hr with BSO, a specific inhibitor of γ-GCS. The A2780 and CP70 cell lines were pretreated with 30 μM BSO. We have shown previously that BSO treatment at these concentrations for 24 hr depletes GSH to <10% of control in these cells (unpublished data). In A2780 and CP70, this treatment has also been shown to sensitize cells to the cytotoxicity of cisplatin (unpublished data). Such treatment, however, failed to affect mitomycin C sensitivity in either cell line, indicating that elevated GSH content is not a major determinant of resistance to mitomycin C in these lines (Fig. 4).

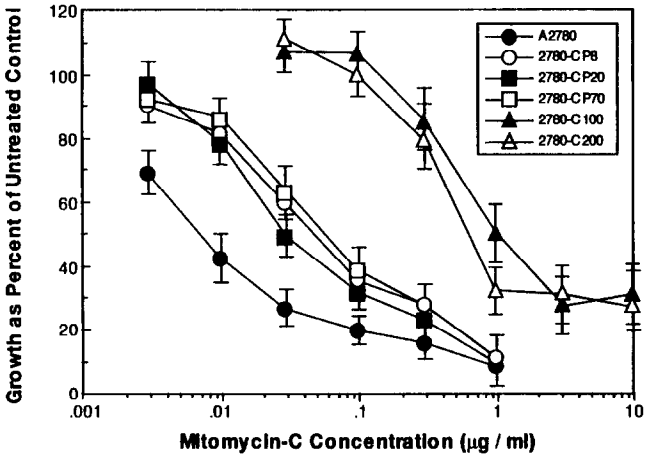

FIG. 2. Survival curves (measured by SRB absorbance at 595 nm) for cisplatin-resistant ovarian cancer cell lines treated with a 72-hr continuous exposure to mitomycin C in 96-well plates. Shown are representative curves from over five individual experiments; values are means ± SD.

Effect of Hypoxia on Mitomycin C Sensitivity

The activity of mitomycin C as a bioreductive drug derives from its greater activity against certain cell lines under hypoxic conditions. The basis for this observation has been attributed to greater radical formation under these conditions. Both DT-diaphorase and cytochrome P450 reductase have been proposed as responsible for this hypoxic effect. However, elevated sensitivity to mitomycin C in hypoxia is not a uniform phenomenon, and the role of DT-diaphorase in the activation of mitomycin C is disputed. The most

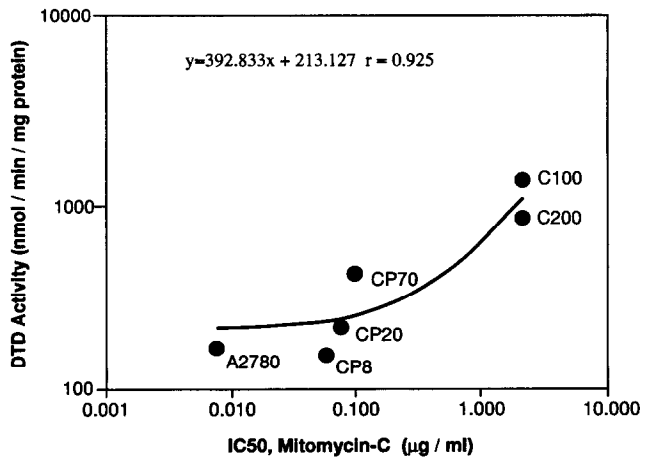

FIG. 3. Plot of sensitivity of cisplatin-resistant ovarian cancer cell lines to mitomycin C as expressed by their IC_{50} values in the SRB B assay versus their measured activity of DT-diaphorase (DTD).

sensitive (A2780) and most resistant (C200) cell lines were treated during a 4-hr exposure to both mitomycin C and hypoxia. In A2780 cells, no change in the survival curve was observed: hypoxic cells were neither more sensitive nor more resistant to the drug (Fig. 5A). In C200 cells on the other hand, marked resistance to mitomycin C (2-fold increase in IC_{50}) was observed under hypoxia (Fig. 5B).

DISCUSSION

The cell lines described in this paper were selected for primary cisplatin resistance by stepwise exposure to the drug *in vitro* [2]. We have demonstrated previously that elevated GSH content is a likely contributor to resistance based on the relationship between the GSH content of the cells and cisplatin sensitivity [7]. This increase in GSH appears to be the result of increased expression of mRNA for γ-GCS and γ-GT [7]. Gene amplification has been excluded as a basis for the increased expression, and we have shown that for γ-GCS the mechanism by which steady-state mRNA levels are elevated is by increased rate of transcription with no change in mRNA stability [7]. This finding supports the involvement of *cis-* or *trans*-regulatory elements. To investigate this possibility, we have cloned the promoter of γ-GCS [22]. Sequence analysis revealed motifs characteristic of several well-known transcription factor binding elements including AP-1, NF-κB, XRE, AP-2, EpRE, CAAT and TATA box elements. Some of these elements are common to those present in the promoter for DT-diaphorase. Of especial interest was the finding of AP-1 and NF-κB motifs in both promoters since we and others have produced substantial evidence that transcription factor binding to these elements may be pivotal to the stress-related induction of DT-diaphorase [10–12, 23]. Coupled with our previous findings that γ-GCS is also induced by hypoxia [8], this suggests that there may be at least one common pathway by which both genes are induced. The data presented here demonstrating the constitutive up-regulation of DT-diaphorase in our model of cisplatin resistance adds support to this hypothesis. It should be noted, however, that there is discordance between the expression of at least two other stress response genes, GSH transferase and cytochrome P450 reductase, in the two models [6, 7]. The basis for differences in the regulation of these genes in the resistant lines may provide insights into a transcriptional basis for acquired drug resistance.

The cross-resistance of these cisplatin-resistant cells to mitomycin C suggests that multiple mechanisms of detoxication may have been induced. High levels of expression of this enzyme are associated with increased sensitivity of some tumors to mitomycins [24, 25]. In aerobic cells, two-electron reduction of mitomycin C by DT-diaphorase generates a cytotoxic intermediate in some but not all tumor models [26]. In these cisplatin-resistant and mitomycin C cross-resistant human ovarian cancer cell lines, the opposite pattern was observed (Fig. 3). Despite recent data that show the involvement of GSH in resistance to mitomycin C in colon cancer cell lines [27], we were unable to sensitize cells to mitomycin C by GSH depletion using conditions that sensitize these cells to cisplatin [28]. It thus seems likely that a mechanism other than increased GSH content is responsible for the mitomycin C resistance in these cell lines and that this mechanism more than compensates for the increased activation of the drug, which may be accomplished by the elevated DT-diaphorase. Candidates might include variation in the activity of xanthine oxidase/xanthine dehydrogenase, superoxide dismutase, or other bioreductive enzymes, or increased DNA repair capacity. Such variation in the metabolism and DNA interactions of mitomycin C alone may not account for the observed differences in susceptibility to cytotoxicity. We have noted previously that C200 cells undergo cell death at a Pt-DNA adduct content higher than that of the sensitive cells [5]. Therefore, differences in the susceptibility to cell death may also have a role.

With a shorter exposure to mitomycin C, striking differences are evident between the most sensitive and the most resistant cell lines in their response to treatment under hypoxia. As with many other cell lines, A2780 is as sensitive under either oxic or hypoxic conditions [29]. However, in contrast to some cell lines with high DT-diaphorase expression [30], the hypoxic C200 cells were more resistant to mitomycin C. This finding implies that at least in this cell line DT-diaphorase does not contribute materially to the activation of mitomycin C to an active cytotoxic species. It also suggests that any effect of hypoxia that might favor the bioreductive activation of mitomycin C is outweighed by effects favoring protection. We recently demonstrated the coordinate induction of multiple protective mechanisms in colon cancer cells exposed to hypoxia [8]. The association between pleiotropic resistance to several cytotoxics and additional resistance under hypoxic condi-

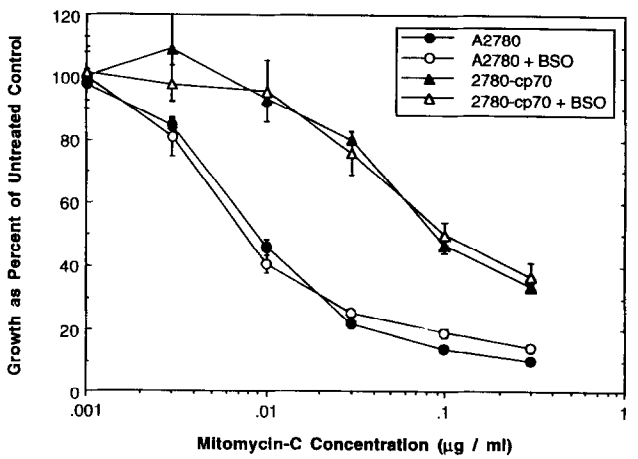

FIG. 4. Survival curves (measured by SRB absorbance at 595 nm) for the sensitive (A2780) and resistant (CP70) lines treated with a 72-hr exposure to mitomycin C with or without BSO (as indicated). Shown are representative curves from three experiments; values are means ± SD.

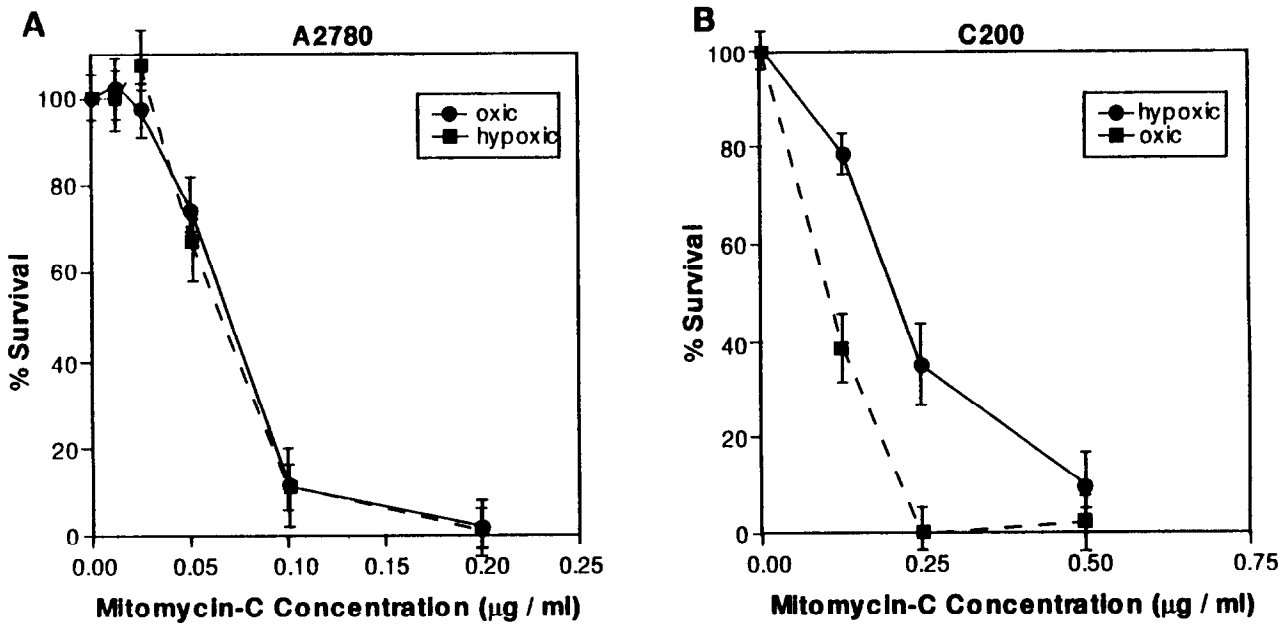

FIG. 5. Survival of the (A) sensitive (A2780) and (B) resistant (C200) cell lines exposed to mitomycin C for 4 hr in suspension culture under oxic and hypoxic conditions, and quantitated using the MTT assay. Values are means ± range of two separate experiments.

tions in the C200 cells provides a model to explore the relationship further.

In conclusion, the coordinate overexpression of more than one detoxication enzyme in these multidrug-resistant ovarian cancer cell lines is consistent with findings in MCF-7 human breast cancer cells selected for resistance to doxorubicin [31]. The resistant MCF-7 cells express multiple genes potentially involved in resistance, including elevated expression of the P-glycoprotein and increased activity of GSH transferases. This finding in MCF-7 has been interpreted to reflect the multiplicity of mechanisms by which doxorubicin may produce cytotoxicity. DT-diaphorase, however, has no known role in the expression of cisplatin toxicity. Therefore, our results support the possibility of a "molecular switch" that may control the expression of several detoxication enzymes, some involved in primary resistance and others in cross-resistance. Identification of such a control point would provide a novel potential target for the therapy of resistant tumors.

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