Potentiation of Doxorubicin Cytotoxicity by Buthionine Sulfoximine in Multidrug-resistant Human Breast Tumor Cells

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ABSTRACT
Resistance to antineoplastic drugs is a major problem in the clinical management of cancer. Previous studies have demonstrated that the cytotoxicity of certain anticancer drugs is increased by lowering the glutathione (GSH) levels with buthionine sulfoximine (BSO), a specific inhibitor of L-glutamylcysteine synthetase. In this study we report that the resistant human breast tumor cell line MCF-7/ADR is 7-fold more resistant than its sensitive parental cell line, MCF-7 WT. These MCF-7/ADR8 cells were approximately 250-fold resistant to doxorubicin when compared with the sensitive cells (MCF-7/WT), and this resistance was stable in drug-free medium for at least 52 wk. Previous studies from our laboratory (23, 24) have shown that resistance in MCF-7/ADR cells is associated with decreased 'OH formation in the presence of doxorubicin compared to drug-sensitive cells, implying that formation of oxygen radicals from doxorubicin may be partially responsible for its cytotoxic effect in the tumor cells (23-25). Furthermore, this differential 'OH formation in MCF-7 WT and MCF-7/ADR cell lines was due neither to a significant decrease in the activity in activating flavin-dependent enzymes, nor as a result from a decreased accumulation of the drug in the cells, since the lysed cells also showed the same differential 'OH formation. Furthermore, the differential 'OH formation in the resistant cells was not due to increased levels of SOD, catalase, or GST. This decreased 'OH formation in the resistant cell line, however, appeared to be related to the enhanced activities of detoxifying enzymes like GSHp and GST in the MCF-7/ADR cell line (24). GSH is a nonprotein thiol present in almost all mammalian cells in substantial concentrations (0.5 to 10.0 mM), and because of these high concentrations, GSH can be very important in modulating the action of commonly used antitumor agents by direct chemical reaction with either the drug or reactive species formed as a result of drug activation. In addition, GSH is an important substrate for both GSHp and GST. Selenium-containing GSHp uses GSH to reduce H2O2 and various organic peroxides to water and the corresponding organic alcohols, respectively. Thus, depletion of cellular GSH pools by chemical agents can be used to indirectly assess the role of glutathione and GSHp in preventing doxorubicin cytotoxicity.

INTRODUCTION
The anthracycline antitumor drug, doxorubicin, has been proven to be one of the most effective drugs in the treatment of breast cancer. However, repeated courses of doxorubicin therapy frequently result in a decreased therapeutic response accompanied by the emergence of an acquired drug-resistant cell population, ultimately limiting the usefulness of doxorubicin. Although biochemical effects of doxorubicin are well studied, there is no consensus as to how this drug kills cancer cells. Currently, three aspects of doxorubicin biochemistry are frequently considered as a basis for its action; DNA intercalation (1, 2); membrane binding (3, 4); and reductive activation (5-9). In addition, doxorubicin has also been shown to bind to a range of transition metal ions (10-15) and to alter topoisomerase II activity (16, 17). It is clear from these studies that a wide range of potentially lethal biochemical events might be operative in tumor cell killing by doxorubicin in different cell lines.

The acquired resistance to antitumor drugs is frequently the major cause of therapeutic failures in cancer patients. The methods by which cells become resistant to drugs are also complex, and a number of specific mechanisms have been discovered (18-20). The molecular mechanism(s) responsible for the primary as well as the acquired pleiotropic cross-resistance in human breast cancer cells remains to be established.

In order to study the mechanisms associated with the drug resistance in breast tumors, MCF-7, a well characterized (21, 22) cell line, was chosen which was selectively made resistant to doxorubicin by stepwise increases in drug concentrations (10^-4 M to 10^-3 M). These MCF-7/ADR8 cells were approximately 250-fold resistant to doxorubicin when compared with the sensitive cells (MCF-7/WT), and this resistance was stable in drug-free medium for at least 52 wk. Previous studies from our laboratory (23, 24) have shown that resistance in MCF-7/ADR cells is associated with decreased 'OH formation in the presence of doxorubicin compared to drug-sensitive cells, implying that formation of oxygen radicals from doxorubicin may be partially responsible for its cytotoxic effect in the tumor cells (23-25).

MATERIALS AND METHODS
Doxorubicin hydrochloride (NSC 123127) was a gift of the Drug Development Branch of the National Cancer Institute, Bethesda, MD. Buthionine sulfoximine was obtained from Sigma Chemical Co. (St. Louis, MO). DMPO was obtained from Aldrich Co. (Milwaukee, WI) and was purified by passing over activated charcoal before use.

1The abbreviations used are: ADR8, doxorubicin-resistant cells; WT, sensitive cells; GSH, reduced glutathione; GSHp, glutathione peroxidase; GST, glutathione transferase; BSO, buthionine sulfoximine; SOD, superoxide dismutase; DMPO, 5,5-dimethylpyrroline-N-oxide; ESR, electron spin resonance; PBS, phosphate-buffered saline.

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Cell Lines

Human breast cancer cells, MCF-7/ADR<sup>R</sup>, resistant to doxorubicin as described earlier (22), were grown in improved modified essential medium (Zinc Option) supplemented with 2 m L-glutamine, 2 mg/l of L-proline, gentamycin (50 µg/ml), and 5% fetal bovine serum (Grand Island Biological Co., New York, NY) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in air.

Assay for GSH

The total reduced GSH levels in breast cancer cells were measured according to Ellman’s method (26). Briefly, the MCF-7/ADR<sup>R</sup> cells were trypsinized and washed twice in nitrogen-bubbled phosphate-buffered saline. Cells were resuspended at 10<sup>6</sup> cells/ml in nitrogen-bubbled 0.1 M sodium phosphate buffer (pH 7.4) containing 1.0 mM EDTA. Cells were lysed by diluting 1:1 with 4% sulfosalicylic acid with rapid mixing, and the samples were centrifuged at 10,000 x g for 10 min in a refrigerated centrifuge. Aliquots of 1 ml of resulting supernatants were added to 2 ml of Ellman’s reagent (8 mg/100 ml of dinitro-5-thiobenzoic acid in 0.1 M sodium phosphate buffer, pH 8.0), and the absorbance was recorded at 412 nm after 5 min. GSH is expressed as nmol per mg of protein. Protein determinations were done by the method of Lowry et al. (27).

Drug Sensitivity with or without Prior GSH Depletion

Growth Inhibition Assay. Cells (50,000) were seeded in monolayer in 6-well tissue culture clusters in triplicate (Costar), and 24 h later replicate cultures received a medium change with or without 50 µM BSO. Preliminary studies of growth rate indicated that this dose of BSO was not very toxic to the cells (10 to 15% cell kill). After an additional 24 h, cells in both treated groups were exposed to various concentrations of doxorubicin prepared in medium, with or without BSO. After 24 h the medium was changed to remove both the drug and BSO. Cells were allowed to grow for 4 to 5 days (0.5 to 1.0 x 10<sup>6</sup> cells) and then trypsinized and counted in a ZM Coulter Counter (Coulter Electronics, England).

Clonogenic Assay. Cells were treated as described above, trypsinized, resuspended, and seeded in 6-well tissue culture plates at a density of 500 cells per well in 3 ml of medium. Plates were incubated at 37°C for 10 to 12 days. Colonies were fixed with methanol, washed, and stained with 0.16% méthylène blue, and colonies of more than 10 cells were counted.

ESR Studies. For the detection of free radical intermediates, MCF-7/ADR<sup>R</sup> cells were grown in log phase and harvested by trypsinization. Cells were washed twice with ice-cold PBS (pH 7.4) and resuspended in PBS at a cell density of 2.5 × 10<sup>6</sup> cells/ml. A typical incubation mixture for the trapping of OH contained: 2.5 × 10<sup>6</sup> cells; 50 mM DMPO; 1 mM NADPH; and doxorubicin (up to 200 µM) in a total volume of 1 ml. The reaction mixtures were incubated in air at room temperature for 5 min before ESR spectra were recorded on an ER 220-D IBM-Bruker spectrometer (9.5 GHz) equipped with a TM cavity and operating at a field modulation of 100 kHz. The effects of inhibitors, SOD and catalase, on OH formation were examined by preincubating them with the cells for 30 min on ice before adding the drug or DMPO.

Drug Uptake. [14C]Daunorubicin, a closely related analogue of doxorubicin, was used as a model to determine the cellular uptake of doxorubicin (22). The intracellular drug accumulation study was carried out with MCF-7/ADR<sup>R</sup> cells (with or without BSO treatment) grown in the log phase as previously described (28). Briefly, the cells were incubated with 10 µM drug for various time periods, ranging from 0 to 90 min, and washed 3 times with ice-cold PBS. Proteins were digested with 1 N NaOH and then neutralized with 1 N HCl. The radioactivity in the samples was counted in a Packard (Tri-Carb 2000, CA) liquid scintillation analyzer, and appropriate quenching corrections were made. Accumulated daunorubicin is expressed as nmol of the drug per mg of protein.

RESULTS

Effects of BSO on the Cellular GSH Level. Experiments were performed with MCF-7/ADR<sup>R</sup> cells to select a dose and exposure time of BSO that produced an optimum rate of GSH depletion with minimal toxicity. Table 1 shows the extent of GSH depletion following exposure with 25 and 50 µM BSO for 24 and 48 h. An 80 to 90% depletion of GSH was seen in the cells treated with 50 µM BSO for 24 h. The absolute value of GSH in untreated MCF-7/ADR<sup>R</sup> cells was found to be 9.6 ± 0.6 nmol/10<sup>6</sup> cells (Table 1). The effect of BSO was not specific for the drug-resistant cell line; BSO also depleted GSH levels in parental MCF-7 WT to a similar extent (data not presented).

To determine the time dependence of GSH depletion following the BSO (50 µM) treatment, GSH assays were done at different time points (12, 24, 36, and 48 h). MCF-7/ADR<sup>R</sup> cells exhibited maximum depletion of GSH at 24 to 48 h (Fig. 1). The time point of 48 h was selected for the subsequent series of experiments. Cell viability was checked by trypan blue exclusion dye test at every time point, and BSO concentration and viability was greater than 90%.

The cellular GSH levels were also monitored in MCF-7/ADR<sup>R</sup> cells exposed to doxorubicin alone, and no difference from controls was found. It was also observed that the combination of doxorubicin and BSO did not further deplete GSH below the levels measured in the BSO-treated groups (Table 1).

Effect of BSO-mediated GSH Depletion on Doxorubicin Toxicity. The cytotoxicity of doxorubicin was significantly enhanced by BSO. The average results of 3 separate growth inhibition experiments in which MCF-7/ADR<sup>R</sup> cells were pre-treated with 50 µM BSO are shown in Fig. 2A, and a dose modification factor of 5 to 7 was found. Similarly, the clonogenic assay carried out with doxorubicin MCF-7/ADR<sup>R</sup> cells also showed an enhanced level of doxorubicin cytotoxicity.

![Fig. 1. Time course of GSH depletion caused by BSO exposure in MCF-7/ADR<sup>R</sup> cells.](image-url)
MODULATION OF DOXORUBICIN RESISTANCE BY BSO

Fig. 2. Augmentation of doxorubicin cytotoxicity by BSO-mediated GSH depletion in MCF-7/ADR® cells determined by growth inhibition (A) and clonogenic assay (B). Cells were treated with different concentrations of doxorubicin in the presence (•) or absence (□) of 50 μM BSO and were allowed to grow for 5 days for growth inhibition or 12 days for clonogenic assay, respectively.

Fig. 3. Uptake of daunorubicin in BSO-treated (●) or control (□) MCF-7/ADR® cells. Cells growing in log phase and pretreated with 50 μM BSO for 48 h were exposed to [14C]daunorubicin for the indicated times, after which cells were washed with cold PBS, and drug-derived radioactivity was counted.

following BSO pretreatment. The results presented in Fig. 2B show a dose modification factor of 4 to 6. In the present study, we did not observe any significant toxicity associated with the BSO treatment (10 to 15% cell kill), even after prolonged exposure (48 h), although there are reports that extended GSH depletion by BSO produced a delayed cell cycle progression and cell death in murine mammary carcinoma (29). This BSO-induced toxicity may be cell type dependent.

Drug Uptake in BSO-treated Cells. The uptake of daunorubicin in BSO-treated or -untreated cells was studied in order to verify that the enhanced toxicity seen in the BSO-treated MCF-7/ADR® cells was not due to an increased drug accumulation. Results presented in Fig. 3 indicate that there was no difference in either the rate or the net amount of drug taken up at 90 min by controls and BSO-treated cells.

Free Radical Formation in BSO-treated Cells. In order to quantitate the relative doxorubicin-mediated 'OH formation by ESR in BSO-treated and -untreated cells, a spin trapping technique was used. The incubation of doxorubicin (200 μM) with MCF-7/ADR® cells that were pretreated with 50 μM BSO for 24 or 48 h in the presence of 1 mM NADPH and DMPO (a spin trap) resulted in the formation of a spin adduct consisting of a quartet (1:2:2:1) with the following splitting constants αr = αt = 14.9 G (Fig. 4). This represents a typical DMPO-hydroxyl radical adduct (30) formed as a result of trapping of 'OH by DMPO. In contrast to control cells, at least 2-fold more of the DMPO-hydroxy radical adduct was detected in cells pretreated with 50 μM BSO for 48 h. Addition of either SOD or catalase significantly inhibited DMPO-hydroxyl radical adduct formation (Fig. 4), indicating that both O₂⁻ and H₂O₂ were involved in 'OH formation. In contrast, when GSH or cysteine (1 to 2 mM) was added to control or WT cells (intact or lysed) but not treated with BSO, only a very small decrease (less than 20%) in the amplitude of the DMPO-hydroxyl radical signal was observed (data not shown). This would indicate that decreased 'OH formation in MCF-7/ADR® cells was not due
to a direct chemical reaction of GSH with free radicals and H$_2$O$_2$ but required the presence of both GSH and GSHPx.

**DISCUSSION**

Free radical formation by one electron enzymatic reduction of doxorubicin followed by oxidation-reduction cycling of the drug semiquinone radical in the presence of molecular oxygen is implied as the principal mechanism in the drug-induced host toxicity (10, 24, 25). The toxic products generated in this process are superoxide, hydrogen peroxide, and \( \text{OH} \), which in turn react with cellular constituents like DNA, enzymes, and membrane lipids, thereby causing cell death. Doxorubicin has also been shown to generate oxygen radicals in human breast tumor cells (24, 25, 31), and in a recent study, we have shown that doxorubicin induced significantly more (4-fold) \( \text{OH} \) formation in WT cells than MCF-7/ADR$_R$ cells. This decrease in \( \text{OH} \) formation in MCF-7/ADR$_R$ cells was found to be directly related to the enhanced defenses against oxygen radical toxicity, as these cells have a significantly elevated activity of selenium-containing GSHPx (12-fold) and, thereby, are protected from cytotoxic effects of doxorubicin-generated oxygen radicals (24). This enzyme has an absolute requirement for GSH as the reducing cofactor.

GSH is a major thiol-containing compound which regulates intracellular free radical concentrations (32). The recent findings (33, 34) that certain human tumor cells contain high levels of GSH have also led to a suggestion that GSH is an important factor capable of limiting the therapeutic efficacy of conventional cancer treatment. Olson *et al.* (35) have reported modulation of doxorubicin toxicity in rodent by N-acetylcysteine and diethylmaleate which, respectively, increase and decrease GSH levels in various tissues like heart, liver, and erythrocytes. Treatment of mice *in vivo* with a concentration of 4 mmol/kg of BSO also decreased GSH levels in most tissues (36, 37). It has also been reported that the cytotoxicity of doxorubicin to EHP3 carcinoma cells (38) and to A2780 ovarian cancer cells (39) is enhanced following depletion of GSH levels by 80 to 90% by BSO treatment. Similarly, treating isolated hepatocytes with 1,3-bis(2-chloroethyl)-1-nitrosourea, an inhibitor of glutathione reductase, depleted GSH by 70% and concomitantly potentiated the toxicity of doxorubicin (40). In the absence of adequate levels of cystolic GSH, the GSHPx would no longer be able to adequately scavenge H$_2$O$_2$ or other peroxides resulting from the metabolic activation of doxorubicin. Alternatively, thiols may also facilitate the repair of oxidative damage to DNA and other target enzymes caused by oxygen radicals (41).

These findings prompted us to examine whether the GSH depletion could diminish or overcome the doxorubicin resistance expressed by MCF-7/ADR$_R$ cells. The present investigation was designed in an effort to investigate this aspect of doxorubicin toxicity. Our results presented here clearly show the involvement of GSH and GSHPx in modulating the free radical levels and the cytotoxicity of doxorubicin.

The depletion of GSH in human breast tumor cells was slow and dose dependent. Maximum depletion of GSH was achieved with BSO concentrations between 25 and 50 \( \mu \)M. The potency of BSO is very well illustrated by GSH depletion to 20% of the control level at 24 h with a dose of 50 \( \mu \)M. It has been reported previously in a cell-free study with purified \( \gamma \)-glutamylcysteine synthetase, a key enzyme for GSH synthesis, that as low as 1 \( \mu \)M dose of BSO inhibited the enzyme by 52%, and complete inhibition was obtained with 20 \( \mu \)M BSO (42). The difference between the degree of inhibition and the concentration of BSO required for complete enzyme inhibition and maximum depletion of cellular GSH in this study may be due to inadequate BSO transport, or the presence of an access barrier to BSO for the enzyme, or just simply by compartmentalization of cellular GSH in subcellular fractions (43). There is good evidence that GSH levels in mitochondria and in the nucleus are not easily depleted by BSO (43, 44). The potent inhibition of GSH synthesis in MCF-7/ADR$_R$ cells was clearly seen with micromolar concentrations of BSO, and depletion of GSH levels by 80% probably represents nearly total depletion of cytosolic GSH. It is possible under these conditions, therefore, that cytosolic GSHPx is rendered incapable of effectively scavenging cytosolic H$_2$O$_2$ generated by the oxidation-reduction cycling of doxorubicin. The activity of GSHPx in BSO-treated and -untreated cells, however, was not significantly altered compared to untreated cells.\(^3\) Furthermore, doxorubicin exposure (10 \( \mu \)M) likewise did not inhibit GSHPx activity.

BSO treatment of MCF-7/ADR$_R$ cells did not damage the cell membranes and impair transport processes as there was no difference in the rate or net cellular accumulation of [\(^{14}\)C]-daunorubicin even after 90 min. This comparison of doxorubicin with daunorubicin is valid, since daunorubicin shows differential cytotoxicity towards MCF-7 WT and MCF-7/ADR$_R$ cells similar to doxorubicin. This suggests that the enhanced toxicity of doxorubicin in GSH-depleted cells was not due to increased intracellular drug accumulation.

Since the toxicity of doxorubicin is directly related to its reductive activation to a semiquinone radical and subsequent production of toxic oxygen radicals in breast tumor cells (24, 25), we also quantitated free radical formation in BSO-treated MCF-7/ADR$_R$ cells and compared it to \( \text{OH} \) produced by MCF-7/ADR$_R$ cells. Our studies show a 2-fold increase in DMPO-hydroxyl radical adduct formation in BSO-treated cells upon exposure to doxorubicin. Preincubation of BSO-mediated GSH-depleted cells with superoxide dismutase or catalase significantly inhibited \( \text{OH} \) formation, indicating that doxorubicin-stimulated \( \text{OH} \) formation in these cells was superoxide anion radical and hydrogen peroxide dependent. Since there was no appreciable decrease in the formation of DMPO-hydroxyl radical adducts when GSH or cysteine was added exogenously, suppressed formation of \( \text{OH} \) in MCF-7/ADR$_R$ cells was not due to chemical scavenging by thiols, but was most likely the result of enhanced detoxification of hydrogen peroxide by increased activities of GSHPx.

In conclusion, we have shown that, by lowering the GSH levels through BSO (50 \( \mu \)M) treatment, the toxicity of doxorubicin in multidrug-resistant breast tumor cells can be increased. It is interesting to note that the concentration of BSO used in our experiments is 100 times less than what is achieved in the mouse with a dose of 4 mmol/kg and where the plasma concentration has been estimated to be 5 to 6 mM (36, 37). The enhanced toxicity was not due to an increase in the drug accumulation, but resulted from diminished cellular capacity to reduce peroxides or hydroperoxides via glutathione peroxidase-linked reactions, as indicated by an enhanced \( \text{OH} \) formation in BSO-pretreated cells. The present study further confirms the previous report (24) that oxygen radicals, formed from doxorubicin, are cytotoxic to human breast tumor cells. It is therefore possible that the clinical use of BSO in combination with doxorubicin might provide a means of overcoming drug resistance in human tumors.

\(^3\) Unpublished observation.
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