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INTRACELLULAR GLUTATHIONE AS A DETERMINANT OF RESPONSIVENESS TO ANTITUMOR DRUGS

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Abstract—The effect of glutathione depletion on cytotoxicity of the anthracycline daunorubicin, and of a copper : bis-thiosemicarbazone chelate, was examined in the P388 murine leukemia and its anthracycline-resistant subline, P388/ADR. Depletion of intracellular glutathione was accomplished through exposure to buthionine sulfoximine, a specific inhibitor of glutathione synthesis. Cytotoxicity of daunorubicin was not altered by glutathione depletion, while responsiveness to the bis-thiosemicarbazone chelate was thereby enhanced.

The effectiveness of a number of antitumor agents is limited by the emergence of resistant cell populations. In P388/ADR, an anthracycline-resistant subline of the P388 murine leukemia, resistance is associated with an energy-dependent exodus process [1–8], although outward transport of the drug accounts for only part of the observed 30-fold difference in sensitivity to daunorubicin (DNR†) in P388/ADR [9]. It is clear that other resistance mechanisms must account for this difference in drug responsiveness.

Anthracyclines can be enzymatically activated to free radical semiquinones that either react directly with biological targets, such as DNA and RNA, or generate cytotoxic oxygen-dependent free radicals such as superoxide anion or hydroxyl radical [10, 11]. Anthracycline resistance could therefore result from decreased activity of enzymes responsible for semiquinone formation [12], or from an increase in intracellular glutathione concentration resulting in enhanced detoxification of drug-induced free radicals [13–16]. Glutathione (GSH) is a thiol containing tripeptide which is present in millimolar concentrations in mammalian cells. Important functions associated with GSH include protection of essential thiol groups from oxidation, detoxification of metabolites of physiological and xenobiotic origin via formation of mercapturic acids, and scavenging of cytotoxic free radicals [17]. BSO is an irreversible inhibitor of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme in GSH synthesis [18]. Recent studies have shown that depletion of glutathione via inhibition of GSH synthesis with BSO promotes anthracycline toxicity in human ovarian tumor cell lines [14–16]. In L1210 mouse lymphoma cells, BSO concentrations as high as 10 mM do not inhibit cell growth and proliferation [19]. We measured the BSO-induced depletion of intracellular glutathione levels in P388 and P388/ADR cells and the resulting effect on anthracycline responsiveness.

As a positive control, we also examined the effect of BSO on the cytotoxicity of a bis-thiosemicarbazone, B2844 [20], in the P388 and P388/ADR cell lines. The bis-thiosemicarbazones chelate extracellular copper ions and transfer the ions into the cell where interactions between nucleophilic moieties and Cu^{2+} result in inhibition of DNA synthesis and other crucial cellular processes [21, 22]. The intracellular GSH concentration should, therefore, be an important determinant of the toxicity of the thiosemicarbazone.

MATERIALS AND METHODS

Cell lines. The P388 murine leukemia and its anthracycline resistant subline, P388/ADR [5], were maintained in suspension culture using Fischer's media (GIBCO) supplemented with 10% horse serum, 15 μM 2-mercaptoethanol and antibiotics (Gentamycin, 10 μg/ml). Two P388 cell lines were obtained, one from Dr. T. Corbett (Michigan Cancer Foundation, P388-1) and one from Dr. R. Johnson (Smith Kline & French, P388-2). The P388/ADR cell line was obtained from Dr. Corbett.

Drugs. DNR and ADR were obtained from the Division of Cancer Treatment, NCI, NIH. DL-BSO was purchased from the Chemical Dynamics Corp., South Plainfield, NJ. Reduced glutathione (GSH), NADPH, dithionitrobenzoic acid (DTNB) and glutathione reductase were purchased from the Sigma Chemical Co., St. Louis, MO. The bis-thiosemicarbazone, B2844, was synthesized as described in Ref. 20.

Cytoxicity studies. P388 and P388/ADR cells were incubated for 24 hr in growth medium containing specified levels of BSO. Cells were then diluted to approximately 8×10^4 cells/ml and incubated for 24 hr in medium containing DNR or B2844. B2844

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† Abbreviations: DNR, daunorubicin; ADR, adriamycin; GSH, glutathione; and BSO, DL-buthionine sulfoximine.

Table 1. Glutathione levels and drug sensitivity in P388 cell lines

Cell line	Glutathione (nmoles/10^6 cells)	IC_{50} Values (μM)	
		DNR	B2844
P388-1	9.5	0.08	0.018
P388-2	18.3	0.07	0.022
P388/ADR	14.0	2.8	0.014

GSH levels were determined as described in Materials and Methods. The IC_{50} values were determined from growth curves. The IC_{50} level represents the amount of drug required to inhibit cell growth by 50%. Data represent the average of two or three experiments which differed by less than ±5%.

was used in combination with an equimolar concentration of $CuSO_4$. Control cultures were not treated with BSO. Growth curves of treated versus control cultures were compared. Counting and sizing of cells were done as described previously [23].

Glutathione assay. Cells were collected by centrifugation and lysed with distilled water. Sulfosalicylic acid was added to a final concentration of 3%. After centrifugation (5000 *g*, 20 min) to remove the protein precipitate, samples were degassed with nitrogen to prevent oxidation and stored at −80° for assay within 1 week. Glutathione was analyzed by the recycling assay using dithionitrobenzoic acid and glutathione reductase as described by Tietze [24] and modified by Griffith [25]. Values are expressed as the total glutathione level, the sum of the reduced and oxidized forms of the tripeptide (GSH + GSSG).

RESULTS

Relationship between intracellular GSH and anthracycline responsiveness. GSH levels were determined in two different P388 cell lines, and in one anthracycline-resistant subline designated P388/ADR (Table 1). While the two P388 lines were equally responsive to DNR, their GSH content varied by a factor of two. A comparison with P388/ADR showed no relationship between intracellular GSH concentration and drug responsiveness.

Depletion of glutathione by BSO. Experiments to characterize the dose-dependent decrease in total glutathione by BSO were carried out by incubating cells with various levels of this drug for 24 hr and then assaying for glutathione as described above. Figure 1 shows the resulting dose–response curves. Complete inhibition of glutathione synthesis occurred at a 100 μM concentration of BSO in P388 and at 1 mM in P388/ADR. Growth was not affected by the presence of 100 μM–1 mM BSO in any cell line during a 1-week observation period.

GSH turnover was examined by incubating P388 and P388/ADR cells with an inhibitory dose of BSO as determined from the dose–response curves. GSH was measured at 0, 1, 2, 5, 8, 24, and 48 hr (Fig. 2). GSH depletion followed the same time course in the P388 and P388/ADR cell lines, although the level of BSO required to completely inhibit GSH synthesis was ten times greater in P388/ADR than in either P388 cell line.

Cytotoxicity after BSO treatment. Depletion of GSH by BSO had no effect on the cytotoxicity of

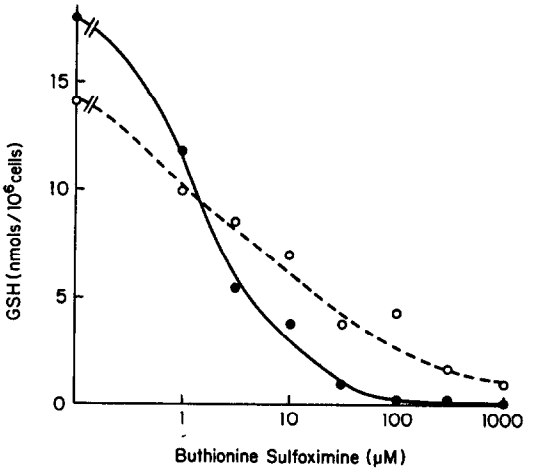

Fig. 1. Depletion of GSH by BSO. Cells were grown in the presence of specified levels of BSO and assayed for GSH after 24 hr (P388, ●—●; P388/ADR, ○—○). Data represent the average of two experiments which differed by less than ±10%.

DNR in either cell line (Table 2). This result suggests that the level of GSH is not a determinant of anthracycline responsiveness in P388 or P388/ADR. In contrast, sensitivity to the bis-thiosemicarbazone chelate was enhanced 2-fold by 10 μM BSO and 3.4-

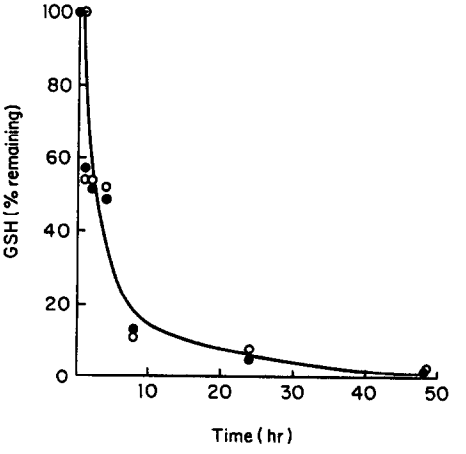

Fig. 2. Time course of GSH depletion by BSO. P388 cells were incubated with 100 μM BSO (●) and P388/ADR cells with 1 mM BSO (○) for indicated times and then assayed for GSH content. Data represent the average of two experiments which differed by less than ±10%. The initial glutathione level was 15.1 nmoles/10^6 cells in P388 and 14.5 nmoles/10^6 cells in P388/ADR.

Table 2. Effect of glutathione depletion with BSO on DNR sensitivity

Treatment	P388-1		P388/ADR	
	Glutathione (nmoles/10^2 cells)	IC_{50} (μM)	Glutathione (nmoles/10^6 cells)	IC_{50} (μM)
Control	10.0	0.08	14.0	3
100 μM BSO	1.0	0.08	3.5	3
1 mM BSO			1.2	3

GSH levels were determined after 24 hr of treatment with BSO. The IC_{50} level was determined from subsequent growth curves with or without BSO. The IC_{50} level is the amount of drug required to inhibit cell growth 50% when compared to DNR-free control. Data represent the average of two experiments which differed by less than ±5%.

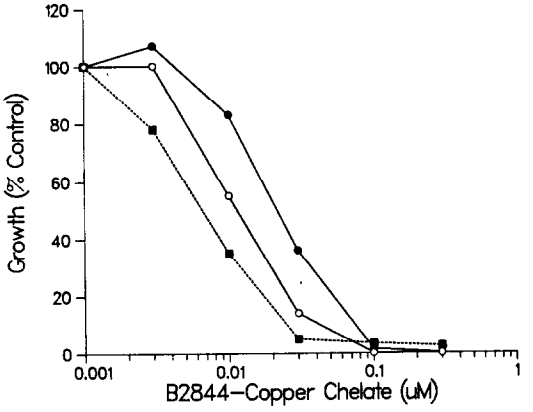

Fig. 3. Growth inhibition of P388 cells by B2844 copper chelate after GSH depletion. Cells were treated with BSO for 24 hr and then grown in the presence of the B2844 copper chelate + BSO (10 μM, O——O; 100 μM, ■——■). Control cultures (●——●) received no BSO exposure. Data are expressed as growth of cells (% control) compared to cells grown in the absence of the B2844 chelate. Data represent the average of three experiments which differed by less than ±10%.

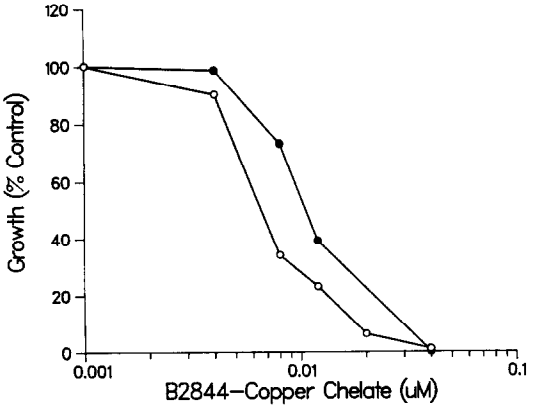

Fig. 4. Growth inhibition of P388/ADR cells by B2844 copper chelate after GSH depletion with BSO. Cells were treated with 1 mM BSO (O——O) for 24 hr and then grown in the presence of the B2844 copper chelate and BSO. Control cultures (●——●) received no BSO exposure. Data are expressed as growth of cells (% control) compared to cells grown in the absence of the B2844 chelate. Data represent the average of two experiments which differed by less than ±10%.

fold by 100 μM BSO in P388 (Fig. 3), and cytotoxicity was increased 1.4-fold in P388/ADR cells line by 1 mM BSO (Fig. 4).

DISCUSSION

In several tumor cell lines, GSH depletion enhances ADR responsiveness [14–16]. We examined intracellular GSH levels in two P388 murine leukemia cell lines obtained from different sources. These P388 lines presumably arose from a single carcinogen-induced mutation, but their properties have diverged during maintenance in different laboratories. While others have reported the finding of a correlation between anthracycline responsiveness and GSH levels [14–16], the present data do not bear out such a relationship. Moreover, depletion of intracellular GSH in both P388 and P388/ADR cell lines did not affect DNR responsiveness.

GSH depletion by BSO has been shown to enhance sensitivity to oxidative cytolysis in a P388 cell line [26]. Yet, P388/ADR cells are more sensitive to ionizing radiation than P388, suggesting that the ability to detoxify reactive oxygen metabolites may not be a determinant of anthracycline resistance [27]. Hamilton *et al.* [15] studied the human ovarian tumor cell line, A2780, and reported that cells responsive to ADR are further sensitized by glutathione depletion. In contrast, ADR responsiveness in the adriamycin-resistant 2780^{AD} cells is enhanced to a much lesser degree by BSO compared to the drug-responsive A2780 line. These results imply that GSH may be a determinant of ADR toxicity in A2780 and in 2780^{AD}, but not in the P388 and P388/ADR cell lines. Brodie and Reed [28] reported that, in addition to inhibiting GSH synthesis, BSO also inhibits the uptake of cysteine by human lung carcinoma cells in culture. Since, in our experiments, addition of BSO had no effect on DNR toxicity, any concurrent inhibition of cysteine uptake apparently does not alter anthracycline toxicity.

We also examined the role of intracellular GSH as a factor in the cytotoxicity of a bis-thiosemicarbazone, B2844. This drug shuttles copper ions into the cell, resulting in inhibition of thiol-requiring enzyme systems. Sensitivity to the bis-thiosemicarbazone was increased by depletion of glutathione (Figs. 3 and 4) in both P388 and P388/ADR. It is likely that GSH acts to protect critical sulfhydryl groups from inactivation by intracellular Cu^{2+}. It has been suggested that some tumor cells have glutathione levels near the minimum level required for survival, while normal cells have an excess [29]. If this is the case, GSH and/or cysteine depletion by BSO may sensitize tumor cells to the cytotoxic effects of Cu^{2+} without promoting toxicity toward normal host cells.

We conclude that, in P388 and P388/ADR, the intracellular GSH concentration can be manipulated so as to play a role in responsiveness to the copper chelate of B2844, but not to the anthracyclines.

It should be noted that there is an inherent difference in sensitivity to BSO in the P388 versus the P388/ADR cell lines. Maximal inhibition of glutathione synthesis required a 10-fold excess of BSO in the resistant cell line. Despite this difference, turnover of glutathione followed the same time course in both cell lines (Fig. 2). This suggests that the difference in sensitivity to BSO is due to an increased level of γ-glutamylcysteinyl synthetase in P388/ADR. Alternatively, the difference may be due to impaired uptake or enhanced exodus of BSO in P388/ADR since this cell line exhibits a broad-spectrum outward transport system which recognizes such diverse structures as ADR, vincristine, actinomycin and *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulforamide (*m*-AMSA) [1–8].

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