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MOLECULAR PHARMACOLOGY, 49:1012–1020 (1996).

Buthionine Sulfoximine Induction of γ-L-Glutamyl-L-cysteine Synthetase Gene Expression, Kinetics of Glutathione Depletion and Resynthesis, and Modulation of Carmustine-Induced DNA-DNA Cross-linking and Cytotoxicity in Human Glioma Cells

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Received October 13, 1995; Accepted March 7, 1996

SUMMARY

Glutathione (GSH) depletion by buthioninine sulfoximine (BSO) is being explored clinically as a means of enhancing the efficacy of cancer chemotherapy. We investigated the kinetics of GSH depletion and altered γ-L-glutamyl-L-cysteine synthetase (γ-GC-S) gene expression in two human malignant glioma cell lines, HBT5 and HBT28, and examined how these relate to GSH resynthesis and changes in DNA interstrand cross-link induction and cytotoxicity of 1,3-bis(2-chloroethyl)-nitrosourea (BCNU). GSH content was 54 and 126 nmol/mg/protein in HBT 5 and HBT 28, respectively, and after a 24-hr exposure to 100 μM BSO was decreased by 95% in HBT 5 and 91% in HBT 28. Basal γ-GC-S enzyme activity in HBT 28 was twice that in HBT 5, and steady state γ-GC-S gene transcripts were 2.6-fold higher in HBT 28 than in HBT 5, with no apparent amplification or rearrangement of the gene in either cell line. BSO exposure (100 μM) for 24 hr increased γ-GC-S gene transcripts by 1.7-fold in HBT 5 and 2.8-fold in HBT 28. After BSO removal, the rate of GSH resynthesis in HBT 28 was twice that in HBT 5. Continuous BSO exposure increased the level of BCNU-induced DNA interstrand cross-links, and cytotoxicity was significantly higher in cells exposed continuously to BSO than in cells with only a 24-hr BSO preexposure. This increase was, however, greater in HBT 28 than in HBT 5. These findings indicate significant heterogeneity in the effects of BSO on γ-GC-S gene expression and in the ability of BSO to sensitize tumors and cell lines to BCNU. The data also suggest that by preventing GSH resynthesis, a greater level of cytotoxicity is achieved with continuous BSO exposure than with BSO preexposure alone.

A major mechanism of cellular protection against endogenous and exogenous electrophiles, free radical-producing compounds and alkylating agents involves *S*-conjugation with the tripeptide GSH. The resultant GS-X conjugates are often less reactive than the parent compounds or their active intermediates (1–7), and recent data indicate that some GS-X conjugates can be effluxed from cells via the multiple drug resistance-associated protein MRP (8–12). GSH has also been shown to react with and to quench DNA monoadduct precursors of potentially lethal DNA cross-links generated by cisplatin (13) and bifunctionally alkylating nitrosoureas (14) and to be involved in the repair of cellular DNA damage (15–17). Consequently, much interest has been focused on the depletion of cellular GSH as a means of enhancing the response of tumor cells to alkylating agents and radiation and thereby improving the efficacy of cancer therapy (2, 5, 6, 18–29).

Cellular GSH depletion can be achieved through treatment of cells with thiol-reactive compounds such as *N*-ethyl maleimide and diethyl maleate. GSH depletion by these methods are, however, short lived, and the *S*-conjugates of the depleting compounds, if not efficiently effluxed from cells, could have other biological effects unrelated to GSH depletion. An alternate method of cellular GSH depletion is the inhibition of *de novo* GSH biosynthesis with BSO, a potent and specific inhibitor of γ-GC-S (30, 31). Intracellularly, BSO is rapidly phosphorylated and in the phosphorylated form inhibits γ-GC-S and blocks the first and rate-limiting step of GSH biosynthesis, that of formation of the dipeptide, γ-L-glutamyl-L-cysteine (31). The actual depletion of cellular GSH, however, is much slower and occurs over several hours. Despite the known action of BSO as an inhibitor of γ-GC-S and its use in a large number of studies to deplete cellular GSH, the

This work was supported by Grants CA55835 and CA55261 from the National Cancer Institute and a grant from the Kleberg Foundation.

ABBREVIATIONS: GSH, γ-L-glutamyl-l-cysteinylglycine (glutathione); BCNU, 1,3-bis(2-chloroethyl)-nitrosourea (carmustine); BSO, dl-*S*-(*n*-butyl)-homocysteine-*DR*-sulfoximine; γ-GC-S, γ-l-glutamyl-l-cysteinylglycine synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; GSH-S, γ-l-glutamyl-l-cysteinylglycine-synthetase.

underlying biochemical factors and the kinetics of BSO-mediated GSH depletion and subsequent GSH resynthesis after BSO removal have not been well characterized. Consequently, in GSH depletion studies, investigators have used a wide range of BSO concentrations (μM to mM) and exposure times (4–48 hr) in attempts to achieve the GSH-depleted state in cells.

Previous studies from our laboratory (32) have shown that human glial tumors differ significantly in their GSH content and that this, in part, contributes to their differential alkylator sensitivity. In the current study, we have used two human malignant glioma cell lines that differ by >2-fold in their GSH content to investigate the interrelationship of tumor GSH content, γ-GC-S gene expression, and the kinetics of BSO-mediated GSH depletion. The effect of BSO treatment on γ-GC-S gene expression and how this affects the rate of GSH replenishment in cells after BSO removal were investigated. Finally, we examined the effects of both preexposure and continuous BSO exposure on the level of DNA interstrand cross-links and cytotoxicity induced in the tumor cells by BCNU, an agent used in first-line brain tumor chemotherapy. These studies should contribute to a better understanding of the biochemical and molecular factors involved in the regulation of the GSH status in tumor cells and to the rational exploitation of BSO-induced GSH depletion for enhanced therapeutic response to alkylating agents.

Materials and Methods

Cell lines and reagents. The cell lines, HBT 5 (anaplastic astrocytoma) and HBT 28 (glioblastoma multiforme), were established in our laboratory from fresh tumor biopsies, as described previously (33). Their astrocytic nature was confirmed through positive immunoreactivity for glial fibrillary acidic protein. The cells were routinely maintained in DMEM supplemented with 15% fetal calf serum. Cells used in these studies had undergone ≤20 *in vitro* passages since establishment in culture. All chemicals and biochemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture media, fetal calf serum, and other related reagents were purchased from either Flow Laboratories or GIBCO. [^{35}S]L-methionine and [α-^{32}P]dCTP were obtained from New England Nuclear.

cDNA probes. The 222-base-pair γ-GC-S cDNA probe covered the region +393–614 of the human liver γ-GC-S heavy subunit cDNA (34) and was amplified through reverse transcription-polymerase chain reaction with RNA from HBT 28 cells. The amplification primers for this cDNA were 5′-CTGCTGTCCCAGGGCTCGCC-3′ (forward) and 5′-GTTGGATGAGTCAGTTTTACT-3′ (reverse) and were synthesized using the phosphoradimite method on an automated two-column DNA synthesizer (Applied Biosystems, Forster City, CA). A 1.1-kb cDNA and a 2.0-kb human β-actin cDNA, both from Clontech (Palo Alto, CA), were used as control probes in the Northern and Southern analyses, respectively. The cDNA were ^{32}P-labeled through random priming and purified on Bio-Spin 6 columns (Bio-Rad, Richmond, CA).

Southern and Northern hybridizations. These was performed as described previously (35). Briefly, for Southern blotting, genomic DNA was isolated from tumor cells through the use of standard techniques (35). Then, 10 μg DNA was *Eco*RI digested and electrophoresed in 0.75% agarose. After denaturation, neutralization, and capillary transfer onto nylon, the membranes were prehybridized and hybridized overnight with a ^{32}P-labeled γ-GC-S probe, and after washing, the membranes were autoradiographed on Kodak XAR-5 X-ray film and photographed. The γ-GC-S probe was then stripped off by heating the membrane at 65° in 5× standard saline citrate (5× = 0.75 M NaCl, 0.075 M sodium citrate, pH 7.0) containing 50% formamide and 1% sodium dodecyl sulfate and rehybridized with a ^{32}P-labeled β-actin probe.

Northern blotting was performed with total RNA extracted from tumor cells according to the acid guanidinium thiocyanate/phenol/chloroform method (36). Then, 10 μg of RNA was loaded per lane and subjected to electrophoresis in a 1.3% agarose-formaldehyde gel. The fractionated RNA was capillary transferred to nylon membranes and probed with the ^{32}P-labeled γ-GC-S cDNA probe as described above for the Southern hybridizations. After autoradiography and photodocumentation, the membranes were stripped of the γ-GC-S probe and rehybridized with a ^{32}P-labeled GAPDH probe.

Ion-exchange HPLC for cellular glutathione. Cellular GSH was assayed through ion-exchange HPLC, as described previously (32). Briefly, to 250 μl of cell-free supernatants of tumor cells prepared in 10% $HClO_4$ we added γ-L-glutamyl-L-glutamic acid (internal standard), 10-phenanthroline, and iodoacetic acid to achieve 10 μM, 2.5 mM, and 10 mM, respectively. The mixture was neutralized with $KHCO_3$ and incubated at room temperature in the dark for 2 hr to complete *S*-carboxymethyl derivatization. 1-Fluoro-2,4-dinitro-benzene was then added to achieve 0.5%, and the mixture was incubated overnight at room temperature. After centrifugation at 12,000 × *g* for 10 min, the supernatant was diluted with 80% methanol and used for HPLC on a 3-aminopropyl-spherosorb ion exchange column, with a methanol/H_2O-acetate binary gradient. Detection was at 365 nm. Protein content was determined in the cell pellets according to the method of Lowry (37), and cellular GSH content was expressed in nmol/mg protein.

Assays of cellular γ-GC-S and GSH-S activities. Tumor cells in exponential phase growth were homogenized in 50 mM Tris·HCl, pH 8, containing 1 mM phenymethylsulfonyl fluoride. After centrifugation at 30,000 × *g* for 20 min at 4°, supernatants were recovered and used to determine the activities of the two enzymes.

γ-GC-S was assayed essentially as described previously (38, 39). Briefly, to a 1-ml reaction mixture consisting of 0.1 M Tris·HCl buffer, pH 8, 150 mM KCl, 5 mM ATP, 2 mM phosphoenol pyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM Na_2EDTA, 0.2 mM NADH, and 17 mg of lactate dehydrogenase, we added 12.5, 25, and 50 μg cell extract protein. After mixing, the decrease in absorbance at 340 nm was followed for 2 min in a Beckman DU65 spectrophotometer. γ-GC-S activities were computed using the rates of enzyme-catalyzed reactions that had been normalized by subtracting the rates of control reactions in which cell extracts had been replaced with extraction buffers. γ-GC-S activity was defined based on the amount (μmol) of L-γ-glutamyl-L-α-aminobutyrate formed/min/mg protein under the experimental conditions.

GSH-S was assayed using a previously described method (40), which is based on the ability of the enzyme to catalyze the conjugation of L-γ-glutamyl-L-α-aminobutyric acid with L-glycine. A reaction mixture (0.9 ml) consisted of 100 mM Tris·HCl buffer, pH 8.2, 50 mM KCl, 5 mM L-γ-glutamyl-L-α-aminobutyrate, 10 mM ATP, 5 mM L-glycine, 20 mM $MgCl_2$, and 2 mM Na_2EDTA to which 12.5–50 mg cell extract protein were added. The mixture was incubated in a water bath at 37° for 30 min, and the reactions terminated with the addition of 20 μl of 10% sulfosalicylic acid. Control reactions were set up as above but contained cell extraction buffer instead of cell extract protein. Then, 0.1 ml of a 250 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM phosphoenol pyruvate, 0.2 mM NADH, 1 unit pyruvate kinase, 40 mM $MgCl_2$, and 50 mM KCl was added, followed by 1 unit of lactate dehydrogenase. The amount of ADP formed under these conditions is proportional to the activity of GSH-S. The reaction was monitored as the decrease in absorbance at 340 nm over 20 min. After subtracting the values of controls obtained with cell extraction buffer instead of cell extracts, we used the reaction rates to compute GSH-S activity. The activity of GSH-S was defined as the amount (μmol) of L-γ-glutamyl-L-α-aminobutyrylglycine formed/min/mg of protein under the reaction conditions.

Purification of γ-GC-S through ATP-affinity chromatography. γ-GC-S was purified from exponentially growing HBT 28 and HBT 5 cells through ATP-agarose affinity chromatography as described previously (42, 43). Briefly, cell pellets were homogenized in phosphate-buffered saline containing 5 mM 2-mercaptoethanol and centrifuged at 30,000 × *g* for 20 min (4°), and the supernatants were treated with ammonium sulfate at 65% saturation. The supernatants then were dialyzed against 50 mM Tris·HCl, pH 7.4, containing 5 mM $MgCl_2$ and retreated with ammonium sulfate at 20% saturation. The precipitates were redissolved, dialyzed, and chromatographed on a DE-52 cellulose column over a linear 0–0.2 M NaCl gradient. Active γ-GC-S fractions were pooled, concentrated through a YM-10 membrane (Amicon, Beverly, MA), dialyzed, and then subjected to ATP-agarose affinity chromatography as we described previously (42). Active fractions were pooled and concentrated over an Amicon YM10 membrane. γ-GC-S activity was assayed as described earlier.

Inhibition of γ-GC-S by BSO. These experiments were performed to determine whether differential inhibition of γ-GC-S in the two cell lines could account for the differences in BSO-mediated GSH depletion and/or repletion in HBT 5 and HBT 28. Stock BSO solutions in 50 mM Tris·HCl, pH 8, were added to 0.5 unit of γ-GC-S from each cell line to achieve final concentrations of 50 and 100 μM BSO. Controls were similarly set up with 50 mM Tris·HCl without BSO. The reaction mixtures were incubated at 25° in a water bath for 30 min, after which 50-μl aliquots were removed and assayed for γ-GC-S activity, as described above. Residual γ-GC-S activities relative to non-BSO controls were computed and plotted against BSO concentrations.

Effect of GSH depletion on cellular protein synthesis and cell survival. To investigate the effects of GSH depletion on cellular protein synthesis, exponentially growing tumor cells were plated at 5×10^4 cells/ml, in replicates of six per data point, onto a 96-well microtiter plate. After 24 hr, BSO was added to achieve a final concentration of 100 μM, and after an additional 24 hr, the cells were rinsed and refed with L-methionine-free DMEM, containing 100 μM BSO, for 2 hr, and then [^{35}S]L-methionine was added to each well to achieve 10 μCi/ml. After a 2-hr incubation at 37°, the cells were trypsinized, transferred to glass fiber filters, protein precipitated, and washed with cold 5% trichloroacetic acid. The filters were dried, and the incorporated radioactivity was quantified through β scintillation counting. The effect of the BSO treatment on protein synthesis was determined relative to control non-BSO-treated cultures using the following equation: Protein synthesis inhibition (%) = 100 (cpm of BSO-treated cells/cpm of control cells).

The capillary clonogenic cell assay (41) was used to determine the effect of GSH depletion on tumor cell survival. Exponentially growing cultures of each cell line were treated with BSO to achieve final concentrations of 0–200 μM. After 24 hr, the cells were trypsinized, washed, and used to set up cell-cloning mixtures containing 2.5×10^4 cells/ml and 0.2% agarose in enriched cloning medium (41). Then, 50 μl of the mixture was introduced in triplicate into sterile glass capillary tubes, the agarose allowed to solidify on a cold surface, and the tubes were incubated at 37° for 2 weeks. Colonies were counted, and surviving fractions were computed and plotted against BSO concentrations, as described previously (41).

Effect of GSH depletion on BCNU sensitivity. HBT 5 and HBT 28 cells in exponential growth were treated with 100 μM BSO, and after 24 hr, control and BSO-treated cultures were trypsinized, and the cells were used to set up clonogenic assays containing 50 μM BCNU. In the experiments to determine the effects of continuous GSH depletion on BCNU sensitivity, additional BSO was added to the cloning mixture to achieve a final concentration of 100 μM. Controls contained (a) additional BSO without BCNU and (b) BCNU without additional BSO. Next, 50 μl of each cloning mixture was drawn into triplicate capillary tubes and incubated at 37° in a humidified atmosphere. After a 2-week incubation, colonies were counted, surviving fractions were computed, and the degree of potentiation of BCNU sensitivity was expressed relative to controls.

Effect of GSH depletion on BCNU-induced DNA interstrand cross-links. This was determined using the assay for total genomic DNA interstrand cross-links that we described previously (41). To cultures of HBT 5 and HBT 28 cells, we added BSO stock solution to achieve a final concentration of 100 μM BSO. After 24 hr, the cultures were rinsed twice with prewarmed (37°) Hanks' balanced salt solution and refed with fresh culture medium containing (a) no BSO and (b) 100 μM BSO. A freshly prepared 100× stock BCNU solution was then added to the cultures to a final concentration of 50 μM. Controls received no BCNU. After an additional 24 hr, the cells were harvested, and high molecular weight DNA was extracted and dissolved in a buffer (pH 7.2) of 0.1 M Tris·HCl containing 2 mM EDTA. To 100-μl aliquots (triplicates) containing 10 μg DNA, we added 0.9 ml of a 20 mM potassium phosphate buffer, pH 11.75, containing 2 mM EDTA. One set of tubes was heated at 100° for 10 min and cooled to 15° in a water bath for 15 min. Another set of tubes was similarly prepared and maintained at 15° without prior heating. Then, 1.5 ml of a 2 μg/ml ethidium bromide solution was added to each tube, and the fluorescence was measured with an LS 50 variable wavelength spectrofluorometer (Perkin Elmer, Norwalk, CT) at an excitation wavelength of 305 nm and an emission wavelength of 585 nm. Background fluorescence was corrected with a solution of 1 μg/ml ethidium bromide in the assay buffer. Assuming a Poisson distribution of cross-links in a constant DNA size, the number of cross-links per DNA molecule, N, is given by: $N = -\ln X$, where X is the fractional change in DNA-ethidium bromide fluorescence after the denaturation/renaturation cycle. It is a measure of the recovery in DNA double-helical structure, a function of the number of cross-links in the DNA. A DNA cross-link index (CLI) was computed as $CLI = \ln[X_o/X_d]/[-\ln X_o]$, where X_o and X_d are the fractional fluorescence changes of DNA from controls and BCNU-treated cells, respectively.

Kinetics of cellular GSH depletion and resynthesis. Triplicate tumor cell cultures in late exponential growth were refed with culture medium containing 100 μM BSO. At 3, 6, 9, 12, and 24 hr, a set of BSO-treated and control cultures with no BSO treatment were trypsinized, washed twice, and extracted in 10% $HClO_4$. GSH was assayed by HPLC, as described earlier. Residual GSH, both absolute and as a percentage of control GSH content, was plotted against BSO exposure time. The rate of GSH depletion was computed by linear regression analysis and expressed as nanomoles per minute per milligram.

To determine the kinetics of GSH resynthesis, cells were treated with 100 μM BSO for 24 hr and rinsed twice with fresh prewarmed (37°) culture medium and refed with DMEM containing 10% fetal calf serum. After 3, 6, 12, and 24 hr, the cells were harvested, extracted in 10% $HClO_4$, and assayed for GSH. The GSH content was plotted against time, and the rate of GSH resynthesis was computed by linear regression as for the depletion studies.

Effects of BSO treatment on γ-GC-S gene transcripts. Total RNA was extracted from tumor cells that had been treated with 100 μM BSO for 24 hr and from control cells without BSO treatment. After electrophoresis and transfer onto nylon membranes, the RNA was hybridized with the ^{32}P-labeled γ-GC-S cDNA probe, as described earlier. After autoradiography, the filters were stripped of the γ-GC-S probe and rehybridized with the GAPDH cDNA probe. Bands were quantified through densitometry, and the levels of γ-GC-S gene transcripts were determined as the ratios of the areas of the γ-GC-S band intensities relative to GAPDH band intensities.

Statistical analysis. All data points were determined in triplicate and expressed as mean ± 1 SD. Significance of differences between the cell lines in measured parameters, e.g., GSH content, or in the effect of specific modality, e.g., BSO treatment, were determined with the Student's t test ($p = 0.05$).

Results

GSH content and activities of γ-GC-S and GSH-S. The two glioma cell lines, HBT 5 and HBT 28, differed significantly in their basal GSH content and in their basal γ-GC-S activities. GSH content/mg protein was 54 ± 7 nmol in HBT 5 and 126 ± 16 nmol in HBT 28, a 2.3-fold difference. γ-GC-S enzyme activity was 86 ± 11 and 176 ± 21 μmol/mg/min, respectively, in HBT 5 and HBT 28. In contrast to γ-GC-S, the activities of GSH-S in HBT 5 and HBT 28 were similar at 5.2 ± 0.7 and 4.9 + 1.1 μmol/mg/min, respectively.

Purification and BSO inhibition of γ-GC-S. After the final step of ATP-agarose affinity chromatography, an 80-fold purification of γ-GC-S from the crude homogenate of HBT 28 cells and 74-fold from HBT 5 was achieved, with an overall yield of 32% and 20%, respectively. The results of the inhibition studies (Fig. 1) showed that in both cell lines, γ-GC-S was inhibited by BSO to the same degree. After 30 min, residual γ-GC-S activity in incubates containing 100 μM BSO was 2% and 7%, respectively, for HBT 5 and HBT 28.

BSO dose-response effects on cellular GSH content, protein synthesis, and survival. The relationship between BSO concentration and GSH depletion in HBT 5 and HBT 28 is summarized in Fig. 2a. After a 24-hr exposure, GSH content decreased dose-dependently with increasing BSO concentration, and at 50 μM BSO was 5.6% and 9.3% of control values, respectively, for HBT 5 and HBT 28. BSO concentrations of >50 μM had no significant additional effect on GSH levels in either cell line after 24 hr. Fig. 2, b and c, show that even at 200 μM, BSO had relatively little effect on protein synthesis or cell survival in both HBT 5 and HBT 28 cell lines. Clonogenic surviving fractions at 200 μM BSO were 0.93 for HBT 5 and and 0.87 for HBT 28.

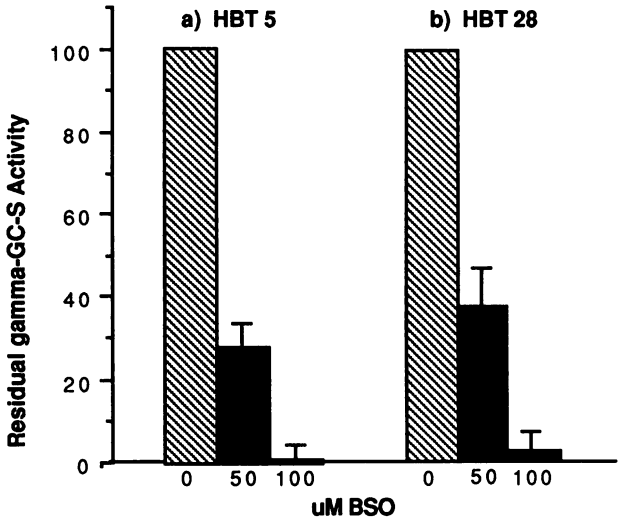

Fig. 1. Inhibition of γ-GC-S by BSO. The enzyme was purified through ATP-agarose affinity chromatography and incubated with BSO for 30 min before determination of γ-GC-S activity as described in the text. Residual activity was computed relative to controls without BSO. Reactions were in triplicate, and results are expressed as mean + 1 SD.

Southern and Northern analyses and BSO effects on γ-GC-S gene transcripts. The results of these studies are shown in Figs. 3 and 4. Fig. 3 shows the Southern blots for the γ-GC-S and the reference β-actin genes in HBT 5 and HBT 28 cells. As is evident, no apparent amplification or

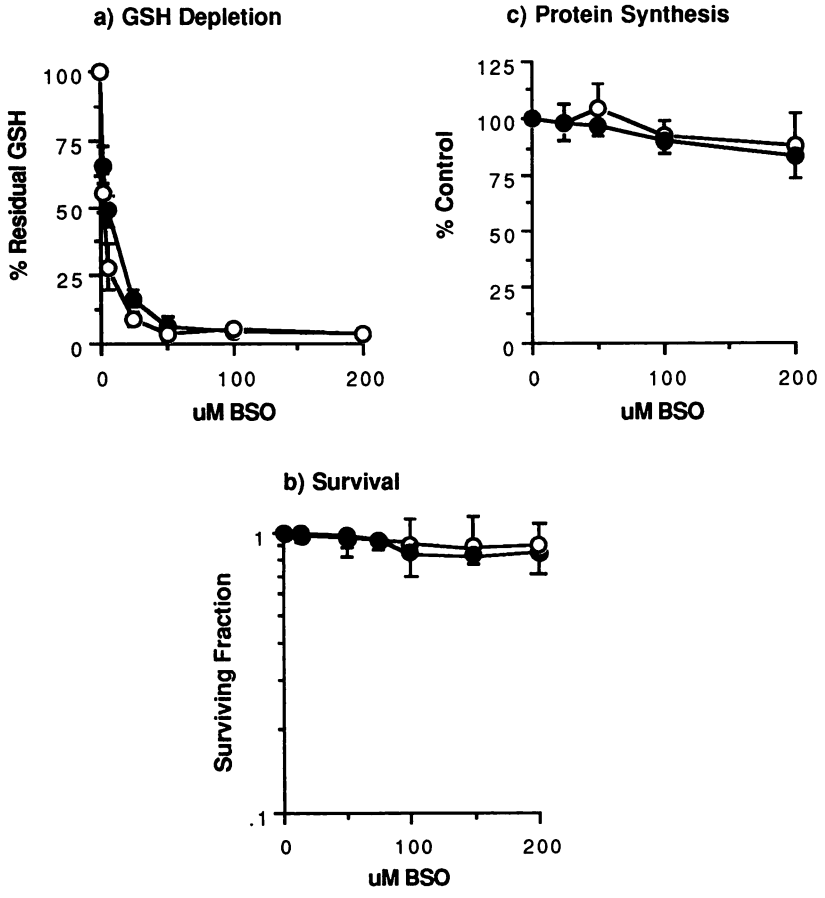

Fig. 2. Relationship between BSO concentration and GSH depletion (a), *de novo* protein synthesis (b), and clonogenic survival (c) in HBT 5 (○) and HBT 28 (●) human glioma cell lines. All end points were assayed in triplicate and expressed as mean + 1 SD.

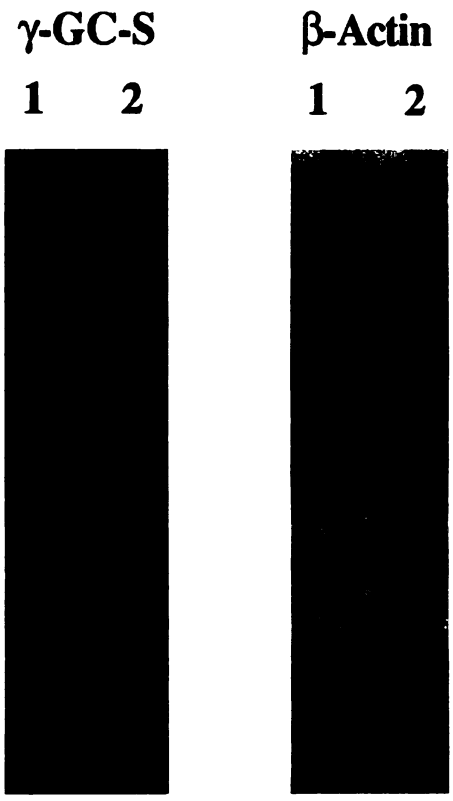

Fig. 3. Southern analysis of *Eco*RI digests of genomic DNA from HBT 5 and HBT 28 cells showing no apparent amplification or rearrangement of the γ-GC-S gene in both cell lines. *Lane 1*, HBT 5; *lane 2*, HBT 28.

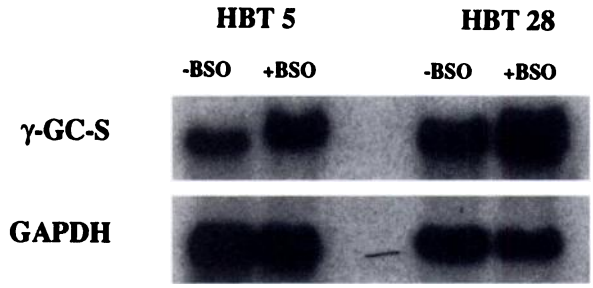

Fig. 4. Northern analysis of γ-GC-S gene transcript levels in HBT 5 and HBT 28 cell lines showing the effect of a 24 hr treatment with 100 μM BSO on γ-GC-S mRNA levels. Controls received no BSO treatment.

rearrangement of the γ-GC-S gene was observed in the two cell lines. The Northern analyses showed that in both cell lines, the same RNA species, ~4.0 kb, hybridized to the γ-GC-S probe. Relative to the level of GAPDH gene expression, basal level of γ-GC-S gene transcripts was 2.6-fold higher in HBT 28 than in HBT 5 and correlates well with the 2.3-fold higher GSH content in HBT 28. After a 24-hr exposure to 100 μM BSO treatment, γ-GC-S gene transcripts were increased by 1.7-fold in HBT 5 and 2.8-fold in HBT 28 (Fig. 4).

Kinetics of GSH depletion and resynthesis after BSO exposure. In both HBT 5 and HBT 28, GSH content decreased linearly with increasing duration of BSO exposure (Fig. 5a). Despite the >2-fold difference in GSH content and the 2-fold difference in γ-GC-S activity between the two cell lines, their rates of GSH depletion were essentially the same, 0.33 nmol/mg/min for HBT 5 and 0.28 nmol/mg/min for HBT 28. The kinetics of GSH resynthesis after BSO removal, were, however, significantly different between the cell lines (Fig. 5b and Table 1). The rate of GSH repletion in HBT 28 cells was 0.57 nmol/mg/hr compared with 0.26 nmol/mg/hr in HBT 5 cells, a 2.2-fold difference. At 24 hr after BSO removal, the GSH content of HBT 28 cells had increased to 110% of controls, whereas HBT 5 had recovered only ~50% of its control GSH level. This rapid rate of GSH repletion in HBT 28 cells after BSO removal correlates with the higher level of induction of γ-GC-S gene transcripts by BSO in this cell line.

Effects of GSH depletion on BCNU-induced DNA cross-links. These results are summarized in Table 1. In controls without BSO treatment, the cross-link index after exposure to 50 μM BCNU was 0.36 for HBT 5 and 0.32 for HBT 28. When cells were preexposed to 100 μM BSO before BCNU, the cross-link index increased to 0.39 in HBT 5 and 0.48 in HBT 28, respectively. Under conditions of continuous BSO exposure, i.e., cells were exposed to BSO for 24 hr, treated with BCNU, and incubated with additional BSO for a further 24 hr, the cross-link indices were increased to 0.43 in HBT 5 and 0.72 in HBT 28. This represents a 1.7-fold higher level of BCNU-induced cross-links in HBT 28 compared with HBT 5 under conditions of continuous BSO exposure. In contrast, only a 1.2-fold difference in cross-linking was observed between the cell lines after a 24-hr BSO preexposure. Relative to cells without BSO treatment, BCNU-induced cross-links were enhanced by 1.1-fold (HBT 5) and 1.5-fold (HBT 28) when cells were preexposed to BSO and by 1.2-fold (HBT 5) and 2.25-fold (HBT 28) in cells exposed to BSO before and after BCNU treatment.

Effect of GSH depletion on BCNU sensitivity. The histograms in Fig. 6, a and b, show the effects of BSO exposure on BCNU sensitivity. ID_{50} values for BCNU in the absence of BSO were 51 μM and 68 μM, respectively, for HBT 5 and HBT 28. In HBT 5, the effect of BSO on BCNU sensitivity was moderate. Clonogenic surviving fraction of control HBT 5 cells (no BSO exposure) treated with 50 μM BCNU was 0.62 compared with 0.54 for cells preexposed to 100 μM BSO for 24 hr and 0.47 in cells exposed continuously to BSO, i.e., before and after BCNU treatment. The enhancement of BCNU sensitivity by BSO was higher in HBT 28 than in HBT 5 cells. Surviving fractions of BCNU-treated HBT 28 cells was 0.58 without BSO exposure, 0.38 in cells with a 24-hr BSO preexposure, and 0.22 in cells with a continuous BSO exposure. Relative to non-BSO-treated cells, BCNU sensitivity was increased by 1.25-fold in HBT 5 cells exposed to BSO for 24 hr and by 1.45-fold in cells exposed to BSO continuously. In HBT 28 cells, the enhancement of BCNU sensitivity was 1.39-fold (24-hr BSO) and 2.7-fold (continuous BSO).

Discussion

In vitro and *in vivo* studies (19–29) and clinical trials in humans (43, 44) have shown GSH depletion with BSO to be a potentially useful strategy with which to biochemically enhance the efficacy of cancer chemotherapy. A better understanding of the cellular and molecular factors involved in the achievement and maintenance of the GSH-depleted state after BSO treatment in tumor cells should contribute to further successful use of this strategy. We used two human malignant glioma cell lines with a 2.5-fold difference in basal GSH content to investigate some of these factors. We exam-

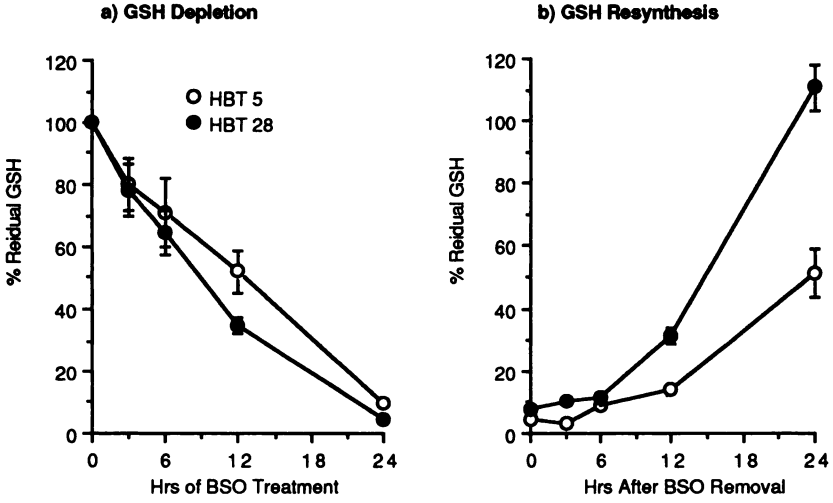

Fig. 5. Time course of GSH depletion in human glioma cells treated with 100 μM BSO (a), and GSH repletion in cells after removal of BSO (b). GSH was quantified through reverse-phase HPLC, as described in Materials and Methods.

	HBT 5	HBT 28	HBT 28/HBT 5
Rate of GSH depletion (nmoles/mg protein/hr)	0.33 ± 0.03	0.28 ± 0.04	0.85
Rate of GSH resynthesis (nmoles/mg protein/hr)	0.26 ± 0.05	0.57 ± 0.07	2.19

TABLE 1

Effect of BSO treatment on DNA interstrand cross-links in HBT 5 and HBT 28 human malignant glioma cells

Tumor cells were exposed to BSO for 24 hr, treated with 50 μM BCNU, and then incubated with and without additional BSO for 24 hr to allow cross-links to form. Controls received no BSO before BCNU treatment.

	DNA cross-link index after exposure to 50 μM BCNU		
	HBT 5	HBT 28	HBT 28/HBT 5
Control (no BSO)	0.36 ± 0.05	0.32 ± 0.03	0.89
24-hr exposure to 100 μM BSO	0.39 ± 0.03	0.48 ± 0.06	1.23
Continuous exposure to 100 μM BSO	0.43 ± 0.07	0.72 ± 0.11	1.67

ined the kinetics of BSO-mediated depletion of GSH and its resynthesis after BSO removal and how these relate to BSO treatment-mediated alterations in γ-GC-S gene expression and affect tumor cell sensitivity to BCNU, a first-line drug in the treatment of malignant brain tumors. We observed a strong positive correlation between cellular GSH content and both γ-GC-S enzyme activity and gene expression, similar to previous observations in multiple myeloma cells (45).

Despite the significant difference in GSH content between the HBT 5 and HBT 28 cell lines, treatment with 100 μM BSO for 24 hr resulted in similar levels of GSH depletion of 91% and 95%, respectively, of controls. The residual GSH in the cells could not be depleted even after exposure of the cells to 200 μM BSO for 24 hr. These observations are consistent with previous *in vitro* and *in vivo* reports of a BSO-resistant cellular GSH pool. The nature of this residual GSH pool is still not completely clear but is likely to represent compartmentalized GSH that is not readily utilized during the duration of BSO exposure. A unique mitochondrial GSH pool involved in the maintenance of intramitochondrial redox status and in protection against peroxides, free radicals, and other DNA-damaging agents has been demonstrated in many cell types (46, 47). This mitochondrial GSH is readily depleted by agents that directly react with GSH but is resistant to depletion by BSO, and, as such, may represent the major contribution to the residual GSH pool observed in cells exposed to BSO. In addition to this mitochondrial GSH pool, other studies (48, 49) have suggested that a nuclear GSH pool exists in cells that is not readily depleted by BSO. The existence of a unique nuclear GSH pool, however, remains controversial as the conjugate between GSH and monochlorobimane, the fluorophore used to characterize nuclear GSH in these studies, is capable of diffusion from the cytoplasm to the nucleus (50). Nevertheless, compartmentalized GSH, whether mitochondrial or nuclear, if not readily depletable by BSO, will have significant implications for strategies aimed at enhancing cellular response to DNA-damaging agents because these GSH pools will be most readily available for quenching of DNA cross-link precursors (13, 14) and for repair of DNA damage (15–17) in these important cellular organelles. The lack of significant inhibition of both protein synthesis and cell survival in the glioma cells at BSO concentrations at which significant decrease in GSH content were observed are in agreement with previous reports (12, 51) and may suggest that the GSH contents of critical organelles such as the nucleus and the mitochondria are still relatively intact.

Steady state γ-GC-S gene transcript levels were significantly higher in HBT 28 than in HBT 5 cells, without any apparent amplification or rearrangement of the γ-GC-S gene in either cell line. The higher transcript levels in HBT 28 are, therefore, most likely due to a higher transcriptional activity of the γ-GC-S gene and/or to increased γ-GC-S mRNA stability in this cell line. The association between high γ-GC-S transcript levels with high cellular GSH content observed in

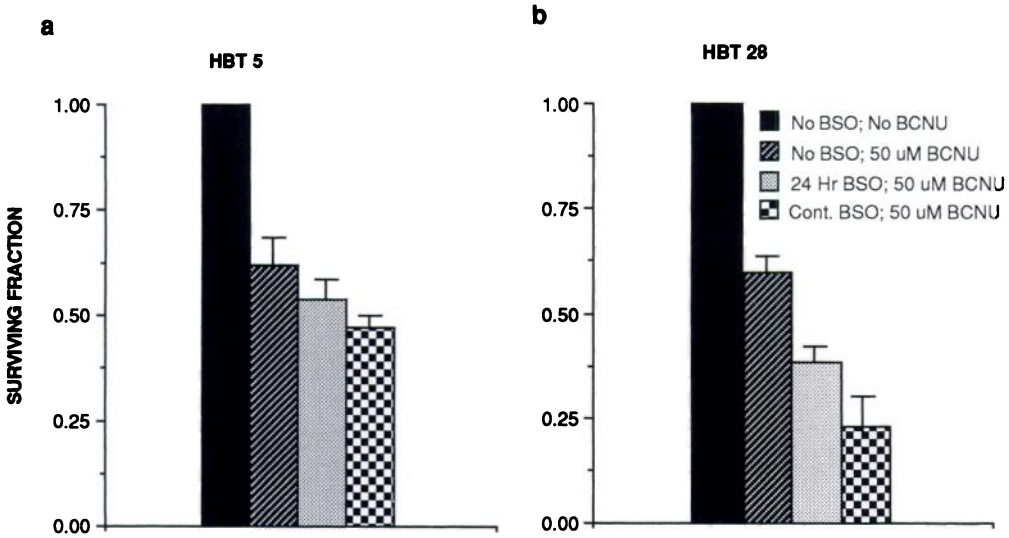

Fig. 6. Modulation of BCNU sensitivity by BSO in HBT 5 and HBT 28 cells. Cells were treated with 50 μM BCNU after a 24-hr BSO preexposure and then cloned in the capillary assay with and without additional BSO exposure. Cells without BSO preexposure and without BCNU treatment were used as controls.

this study may not occur to the same degree in all tumors or cell types because other factors, such as the recently reported MRP-mediated efflux of GSH (12), could also contribute to intracellular GSH status. In both cell lines, the γ-GC-S gene transcript size detected by Northern blotting was ~4.0 kb, similar to that previously observed in human kidney (34). In contrast, in ovarian carcinoma (51) and multiple myeloma (45) cell lines, this 4-kb transcript and a smaller 3.5-kb transcript were observed. The latter has been suggested to represent alternatively spliced γ-GC-S transcripts (34).

The rate of cellular GSH repletion in HBT 28 cells after removal of BSO was twice that in HBT 5 cells, although γ-GC-S purified from both cell lines was inhibited to similar levels by BSO. The differential rates of GSH replenishment after BSO removal can thus be attributed to differences in *de novo* γ-GC-S synthesis. This is supported by the observation that after exposure to BSO, the level of γ-GC-S gene transcripts in HBT 28 increased to almost twice that in HBT 5, consistent with previous observations in a human ovarian carcinoma cell line (52) and in the white blood cells of patients receiving *in vivo* BSO (43). Although the molecular mechanisms by which BSO increases γ-GC-S gene transcript levels, as has been observed in this and in other studies, remain to be determined, it is likely that this would include activation of the γ-GC-S gene and/or stabilization of γ-GC-S mRNA. Recently (53), it has been shown that BSO increases the induction of the genes encoding γ-GC-S and dihydrothiol dehydrogenase by ethacrynic acid, its glutathione conjugate, and the GSH analogue γ-glutamyl-*S*-(octyl)cysteneiyl-glycine diethyl ester, T.199. Based on these and other observations, a model for the action of electrophilic inducers of γ-GC-S and other phase II detoxifying genes has been proposed (53) in which these agents act by altering the redox status of the cell, which in turn causes gene activation mediated through binding of *trans*-activating factors to antioxidant response elements present in these genes. In addition to the increased transcription of these genes, however, other evidence (54) obtained with human colon, ovarian, and prostatic carcinoma cell lines suggest that the action of these redox-modifying agents may also include stabilization of the mRNA of the affected genes, in particular, γ-GC-S and glutathione conjugate.

We observed a significant enhancement of BCNU-induced DNA interstrand cross-links in the cells of both cell lines exposed to BSO, both before and after BCNU treatment, compared with cells treated with BCNU, either without prior BSO exposure or with only a 24-hr BSO preexposure. The data indicate that because of the greater capacity of HBT 28 cells to replenish GSH, BSO exposure for 24 hr before BCNU produced only a modest increase in DNA cross-links (~1.5-fold) compared with a 2.5-fold increase in cross-links observed with continuous BSO exposure. In contrast, in HBT 5 with a lower GSH resynthesizing ability, little difference was observed in cross-link enhancement between a 24-hr BSO and continuous BSO. Overall, the increase in the level of BCNU-induced DNA cross-links and cytotoxicity after GSH depletion was higher in HBT 28 than in HBT 5 cells. It is possible that in the latter cell line, other mechanisms, including non-GSH-related ones, may play a more significant role in mediating the response of the cells to BCNU. In previous studies (55, 56), we showed that two classes of proteins critically involved in DNA cross-link repair, namely, DNA polymerases and DNA ligases, were functionally down-regulated in GSH-depleted glioma cells. Because the repair of DNA interstrand cross-links is a process that continues for several hours (42), the maintenance of a low intracellular GSH over a long period will enhance cytotoxicity by ensuring that these drug-induced DNA lesions are not readily repaired but accumulate to cause cytotoxicity. Although GSH depletion is a major mechanism by which BSO enhances cellular alkylator sensitivity, there is evidence to suggest that BSO may increase drug sensitivity, especially of nonalkylators, by other mechanisms. In a recent study (56), BSO-mediated increase in the sensitivity of MCF 7 breast carcinoma cells to etoposide seemed to result from an enhancement of intracellular etoposide concentration and protein binding.

The data presented in this study indicate that gliomas are heterogeneous in their response to BSO modulation and that the achievement and maintenance of the GSH-depleted state are the result of both the inhibition of γ-GC-S enzyme activ-

ity and the suppression, directly or indirectly, of γ-GC-S resynthesis by BSO. These conclusions are supported by the results of a phase I clinical trial (44) in which the level of GSH depletion in peripheral blood lymphocytes was shown to be highly dependent on BSO plasma pharmacokinetics. At the end of BSO infusions, plasma clearance of BSO was rapid, and γ-GC-S activities and GSH levels in PBLs also recovered rapidly, reaching near-baseline levels after 6–12 hr. This was accompanied by increased GSH content of peripheral blood lymphocytes. These *in vivo* observations are consistent with those in this study and together suggest that the use of protocols that ensure effective plasma BSO levels before, during, and several hours after administration of an alkylating agent are likely to result in higher therapeutic efficacy than strategies in which BSO is administered only before the alkylating agent.

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