Decreased DNA Interstrand Cross-Linking and Cytotoxicity Induced in Human Brain Tumor Cells by 1,3-Bis(2-chloroethyl)-1-nitrosourea after in Vitro Reaction with Glutathione

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ABSTRACT

Although both direct and glutathione S-transferase (GST)-catalyzed interactions between many electrophiles and GSH generally result in inactivation of the former, there are several reports of compounds whose electrophilic, alkylating, and cytotoxic activities are potentiated by GSH. This study investigates the effects of direct in vitro interaction between GSH and BCNU at physiological pH (7.2) and temperature (37°C) and how this affects the cytotoxic and DNA cross-linking activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in target human malignant brain tumor cells. The kinetics and dose-response relationship of this interaction were determined by measuring residual GSH and residual BCNU-cytotoxicity in a GSH/BCNU mixture over a 45-min period and at varying BCNU concentrations. The results demonstrate that reaction of BCNU with four times its molar concentration of GSH for 45 min significantly inactivates BCNU, as expressed by a 32% decrease in induction of cellular DNA cross-linking, a 21% increase in DNA synthesis, and a 15% increase in clonogenic survival of human brain tumor cells compared to incubates of BCNU alone. Equine liver (EL)-GST increased the inactivation of BCNU only slightly (insignificant at p = 0.05). These results suggest that, in contrast to agents such as the alkyl-N,N'-nitroso-N'-nitrosoguanidines which become more potent alkylators after reacting with GSH, the 2-chloroethylnitrosoureas (CENUs) undergo inactivation by GSH. We propose that such interactions between GSH and the CENUs may constitute an important aspect of CENU metabolism and provide a potential means by which brain tumor cells can circumvent CENU toxicity and exhibit resistance to this class of agents.

INTRODUCTION

GSH is present in mammalian cells in concentrations of up to 10 mM (1). Its strongly nucleophilic thiol group confers on the molecule the unique ability to react with a wide variety of agents including free radicals, reactive oxygen species, alkylating groups, and many other endogenous and exogenous compounds (2, 3). However, not all reactions of GSH and cytotoxic electrophilic compounds result in inactivation of the latter (4). Indeed, for some classes of alkylators, such as the alkyl-N'-nitro-N'-nitrosoguanidines (5), reaction with GSH has been shown to produce the opposite effect, i.e., increased alkylating activity with a corresponding increase in cytotoxicity and mutagenicity (6, 7). Potentially, interaction between intracellular GSH and CENUs, currently the most effective agents used in clinical brain tumor therapy (8), could play a significant role in modulating CENU cytotoxic action. Intracellularly, CENUs are hydrolyzed to yield organic isocyanates and bifunctionally alkylating chloroethyl carbonium ions (9). The latter are very reactive electrophiles capable of alkylating and cross-linking cellular macromolecules with nucleophilic centers, e.g., nucleic acids, proteins, and peptides. Considerable evidence (10-14) suggest that interstrand cross-linking of cellular DNA constitutes a major molecular cytotoxic lesion induced by CENUs in tumor cells. Consequently, intra- and extracellular reactions between a CENU and other molecules that result in decreased DNA cross-linking activity of the CENU will be expected to result in a concomitant decrease in CENU cytotoxicity. One such potential CENU inactivating reaction is that between GSH and a CENU.

The primary objective of this study, therefore, was to investigate the effect of direct in vitro interaction of GSH with BCNU, a model clinically active CENU, on cellular and molecular endpoints of BCNU-induced cytotoxicity in human brain tumor cells in vitro. We measured the clonogenic cell survival, the inhibition of cellular DNA-synthesis and the degree of cellular DNA interstrand cross-linking induced by BCNU alone and after in vitro incubation with GSH at 37°C and pH 7.2. We also examined the kinetics of the interaction by measuring residual GSH in GSH/BCNU incubates.

MATERIALS AND METHODS

Brain Tumor Cell Line. The UWR-2 cell line was established in our laboratories from a fresh tumor biopsy obtained from a patient with a pathologically confirmed glioblastoma multiforme. The specimen was obtained on a protocol approved by the Human Subjects Committee of the University of Washington School of Medicine. Cultures were initiated by treatment of the tumor specimen with an enzyme mixture of 0.4% collagenase, 0.2% neutral protease, and 0.02% DNase for 60 min, washing the cells twice with HBSS, and plating them in DMEM containing 20% FCS. After achieving confluency, the cell monolayers were trypsinized and subcultured into new flasks. The malignant and glial nature of the cells was confirmed by cytopathological examination of cultures grown on tissue culture grade microscope slides and stained both with hematoxylin & eosin, and with the avidin-biotin immunocytochemical technique for expression of glial fibrillary acidic protein. The cells used in these studies had undergone 16 in vitro passages and were shown to be free of mycoplasma contamination.

Drugs and Biochemicals. BCNU was kindly provided by the Developmental Therapeutics Program of the National Cancer Institute, Bethesda, MD, and was dissolved in minimal absolute ethanol. Final stock BCNU solutions were prepared by further dilution with HBSS. Final ethanol concentration in cultures was less than 0.001%. GSH, equine liver GST, and all other chemicals were obtained from Sigma, St. Louis, MO.

GSH Assay. GSH was determined based on the reaction of its thiol group with DTNB as previously described (15). To 1.5 ml of GSH solution in 1 x potassium phosphate buffer, pH 8.3, was added 0.1 ml of a 1.5 mg/ml DTNB solution in potassium phosphate buffer and the absorbance at 412 nm determined in a Beckmann DU-50 spectrophotometer. Standard curves were established using known GSH dilutions.

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4 The abbreviations used are: GSH, glutathione (γ-glutamyl-L-cysteinylglycine); CENU, 2-chloroethylnitrosoureas; BCNU, 1,3-bis(2-chloroethyl)-1-nitroso- urea; FCS, fetal calf serum; EL-GST, equine liver-glutathione S-transferase; HBSS, Hank's balanced salt solution; DTNB, 5,5-dithiobis(2-nitro)benzoic acid; DMEM, Dulbecco's minimal essential medium.

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BCNU Cytotoxicity Assay: Clonogenic Cell Survival. This was performed using the capillary tumor clonogenic cell assay as previously described (16). Briefly, late exponential phase cell cultures were trypanoylized, washed, and resuspended in fresh cloning medium. A cloning mixture (0.3 ml) was then made up containing 2.5 × 10⁶ brain tumor cells, test solution (BCNU alone, GSH alone, BCNU/GSH, or BCNU/GSH/GST), 0.2% agarose, and enriched CMRL 1066 medium (16). The mixture was vortexed and 30 μl drawn into each of triplicate sterile glass capillary tubes (9.75 cm long and 1.38 mm in diameter; Clay Adams, Parsippany, NJ). After gelling on a cold surface, the tubes were incubated in a humidified atmosphere at 37°C and 5% CO₂/air. Two weeks later, the cultures were flushed out of the capillary tubes onto glass slides and the colonies counted under phase contrast microscopy at 100× magnification. The surviving fraction (SF) at each drug treatment was determined, and survival curves were constructed by plotting the SFs against the drug concentrations or incubation times.

BCNU Cytotoxicity: Inhibition of DNA Synthesis. We used a previously described method (16) for measuring the incorporation of [³H]thymidine in brain tumor cells to quantitate DNA synthesis. Briefly, 200 μl of a cell suspension containing 2.5 × 10⁶ cells/ml in DMEM supplemented with 15% FCS were pipetted into each of replicate wells of 96-well microtiter plates. The cells were allowed to attach and grow for 48 h and then three sets of six wells were treated with 50 μl of stock incubates so as to achieve final concentrations of 100 μM BCNU, 400 μM GSH, 100 μM BCNU/400 μM GSH, or 100 μM BCNU/400 μM GSH/0.25 unit/ml GST. After 24 h, the cells were pulsed for 24 h with 10 μCi/ml of [³H]thymidine. The cells were then washed, treated with cold 5% trichloroacetic acid, washed, and the filters dried, and placed in scintillation liquid (Ready Safe, Beckman Instruments, Fullerton, CA). Incorporated radioactivity was counted in a Beckman LS1801 β-scintillation counter. The decrease in [³H]TdR incorporation in treated cells relative to untreated controls was computed as the inhibition of cellular DNA synthesis.

Measurement of DNA-Interstrand Cross-Linking. DNA-interstrand cross-linking was determined in these studies using the filter elution technique of Kohn et al. (17), as modified by Kuo et al. (18). UWR-2 cells were plated at 1.5 × 10⁶ cells/flask in T75 tissue culture flasks for 72 h, treated as required, and incubated at 37°C for a further 15 h. The cells were then trypsinized, washed twice with X-irradiated ice cold with 6 Gy, using a J. L. Shepherd Model 141.5-cm-137 unit. The irradiated cells were immediately centrifuged (precooled centrifuge) at 300 × g for 4°C for 10 min. The cell pellet was resuspended in cold phosphate buffered saline and 5 × 10⁶ cells layered on to a 47-mm, 0.2-μm-pore polycarbonate filter. After rinsing with ice-cold phosphate buffered saline, the cells were lysed in the dark for 60 min with a lysis solution, pH 12.2, containing 0.2% Sarkosyl, 2 mM NaCl, 0.04 M Na-EDTA and 0.5 mg/ml proteinase K. The DNA was rinsed and then eluted in the dark with a solution of 0.1 M tetrapropyl ammonium hydroxide in 0.02 M EDTA, pH 12.2, and fractions collected every 90 min for 18 h. DNA retained on the filter was removed by mincing and heating the filter for 30 min at 60°C in 5 ml of eluting solution. Remaining DNA on the filter holder and column was removed by rinsing with 5 ml eluting buffer. To each solution was added 1 ml of 30% KH₂PO₄ followed by 1 ml of a 1.5 μM Hoechst 33258 solution. The fractions were vortexed and the DNA measured fluorometrically (350 nm excitation and 460 nm emission) in a Perkin-Elmer LS 5 spectrophotometer. The data were plotted as the log of the fraction of DNA remaining on the filter versus elution time. A cross-link index (CLI) for each treatment was computed as described by Kohn et al. (17):

\[ \text{CLI} = \left( \frac{1 - R_d}{1 - R_i} \right) e^{R_i} - 1 \]

where \( R_d \) and \( R_i \) are the fractions of DNA remaining on the filter after 30 min elution for irradiated control cells and irradiated treated cells, respectively.

Kinetic and Dose-Response Studies. Five ml 400 μM GSH was incubated with 5 ml 400 μM BCNU at 37°C. Both solutions were made by diluting stock solutions with phosphate buffer, pH 7.2, and there was no precipitation of GSH or BCNU at this concentration. At various time points up to 45 min, 375-μl aliquots were removed, diluted to 1.5 ml with phosphate buffer, pH 8.3, and the residual GSH measured as previously described. To determine whether the reaction of GSH with BCNU is dose dependent, 1-ml aliquots of 400 μM GSH was incubated with 1 ml of phosphate buffer containing 0, 100, 200, 400, and 800 μM BCNU for 45 min under the conditions described above and the residual GSH determined.

Effect of BCNU-GSH Interaction on BCNU Cytotoxicity. Five ml triplicates of a mixture of BCNU (final concentration, 0–100 μM) and GSH (final concentration, 400 μM) was freshly prepared in phosphate buffer, pH 7.2, and incubated at 37°C in a water bath. To another set of similarly prepared tubes, 0.25 unit/ml EL-GST was added. Aliquots of 75 μl of the incubate were removed after 45 mins, and added to 225 μl of a cloning mixture containing UWR-2 brain tumor cells and the clonogenic cell survival determined as described earlier. Three control cultures were similarly set up and treated with 75 μl of 45-min incubates of: (a) BCNU alone; (b) GSH alone; and (c) GSH + EL-GST at the concentrations described above. Inhibition of cellular DNA synthesis by the incubates containing 100 μM BCNU and 400 μM GSH with and without EL-GST were determined using the method described earlier.

Effect of BCNU/GSH Interactions on DNA Cross-Link Induction. UWR-2 cells were plated at 1.5 × 10⁶/cells/T 75 flask in 7 ml DMEM containing 20% FCS for 72 h. BCNU/GSH incubates were prepared in HBSS at concentrations such that adding 100 μl to a final culture volume of 7 ml yielded concentrations of 150 μM BCNU and 600 μM GSH, respectively. After 45 min at 37°C, 1 ml of the incubate was aliquoted into triplicate near confluent UWR-2 cultures in T 75 flasks. After 2 h at 37°C each culture was rinsed twice, refed, and incubated at 37°C for a further 15 h, and the degree of cellular DNA cross-linking induced was determined as described earlier. Simultaneously, the degree of cross-linking induced in control cultures treated with 45-min incubates of BCNU alone was determined.

Statistical Analysis. All data points are expressed as the mean of triplicate measurements and one standard deviation. To determine the significance of the effects of GSH interaction with BCNU, the one-way analysis of variance (19) was used at a \( P = 0.05 \).

RESULTS

Kinetics and Dose-Response Relationship of BCNU-GSH Interaction. Fig. 1 shows the kinetics of depletion of GSH in a mixture of 400 μM GSH and 400 μM BCNU. The decrease in GSH concentration was linear for up to 45 min; the lowest value achieved was 53% of the original GSH concentration. Fig. 2 shows the effect of a 45-min incubation of GSH (final concentration, 200 μM) with increasing concentrations of BCNU (up to 400 μM). Under these experimental conditions, GSH depletion by BCNU was linearly dose dependent. Control experiments with GSH alone showed only a 5.6% decrease in the initial GSH concentration over 45 min.

![Fig. 1. Kinetics of in vitro depletion of GSH in incubates of equimolar (200 μM) concentrations of GSH and BCNU in K-phosphate buffer, pH 7.2, 37°C. Aliquots were removed at various times and the GSH content measured.](cancerres.aacjrournals.org)
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Fig. 2. Dose-response relationship of in vitro GSH depletion by BCNU. 400 µM GSH (200 µM final concentration) was reacted with equal volumes of increasing concentrations of BCNU (0–400 µM) for 45 min and the residual GSH in the mixture was measured.

Fig. 3. Alkaline elution profiles of UWR-2 human glioblastoma cells treated with 150 µM BCNU with and without prior interaction with 600 µM GSH, for 45 min at 37°C. The elution profiles of control cells treated with radiation (600 rads) alone, and without drug or radiation are also shown.

Effect of GSH/BCNU Interaction on DNA Interstrand Cross-linking. Fig. 3 shows the alkaline elution profiles of UWR-2 cells treated with 150 µM of BCNU preincubated with and without 600 µM GSH, and of control cells with and without X-irradiation. The results demonstrate that after a 45-min interaction, the amount of DNA interstrand cross-linking induced in the cells by the BCNU/GSH incubates was reduced by 32% relative to the BCNU controls. The actual cross-link indices computed for the various treatments are 0.28 for BCNU alone and 0.12 for the BCNU/GSH incubate.

Effect of GSH/BCNU Interaction on Cell Survival. Fig. 4 shows the effect on UWR-2 cells of 45-min incubates of increasing concentrations (up to 100 µM) of BCNU alone, with 400 µM GSH or with 400 µM GSH and 0.25 U/ml EL-GST. Over the whole range of BCNU concentrations (0–100 µM), there was a consistent increase in clonogenic survival of cells treated with the BCNU/GSH incubates over those treated with BCNU alone. A slight increase in survival was observed when EL-GST was added to the incubates of GSH and BCNU. This increase was not statistically significant at P = 0.05.

Effect of GSH/BCNU Interaction on Cellular DNA Synthesis. The effects of GSH/BCNU interaction on the ability of BCNU to inhibit cellular DNA synthesis in UWR-2 cells are summarized in Fig. 5. As in the clonogenic cell studies (Fig. 4), cellular DNA synthesis is decreased to a lesser degree (74% of control) in cells treated with 100 µM BCNU that had been incubated with 400 µM GSH for 45 min than in cells treated with incubates of BCNU alone (53% of control). Addition of 0.25 unit/ml EL-GST had only slight potentiating effect on the decrease in DNA synthesis inhibition by GSH/BCNU incubates.

DISCUSSION

One of the major biologically significant functions of GSH in mammalian cells is that of conjugation with and subsequent detoxification of a wide range of electrophiles (2, 3, 20). These reactions of GSH may be spontaneous and nonenzymatic or may be catalyzed enzymatically by a group of glutathione S-transferases (3, 21). Although most of the interactions between GSH and these compounds result in inactivation of the latter, there is evidence (4) that, for some compounds, interaction with GSH can have the opposite effect, namely activation. For example, GSH interacts with alkyl-N-nitro-N'-nitrosoguanin-
dines both at the iminocarbon and at the nitroso group to yield products with higher alkylating activity than the parent compounds (4-7). Thus, the extent of methylation of cellular DNA in Chinese hamster ovary cells treated with N-methyl-N'-nitro-N-nitrosoguanidine has been shown to increase proportionately with increases in cellular GSH content. Furthermore, it has been demonstrated that the mutagenic activities of N-methyl-N'-nitro-N-nitrosoguanidine and ENNG are significantly reduced in GSH- mutants relative to GSH+ mutants. Other mutagenic and cytostatic compounds reported to be activated by either GST-catalyzed or direct interaction with GSH include dihaloalkanes (22-24), vicinal dihalogen compounds (25), nercarcinostatin (26, 27), azathioprine (28, 29), and bleomycin (30).

The potential ability of GSH to activate some alkylating agents and inactivate others makes it important to determine for a given class of alkylators what effect direct or GST-catalyzed interaction with GSH has on the key cellular and molecular endpoints of action of the agents. This is particularly important if GSH is to be implicated in molecular mechanisms upon which cellular sensitivity and/or resistance to these agents are based. CENUs are clinically the most active agents used to treat human brain tumors and the electrophilic products of their intracellular hydrolysis are capable of reacting with GSH (31). Such CENU/GSH interaction may thus provide a potential means by which brain tumor cells can become CENU resistant. The present study was therefore designed to investigate the effect of direct in vitro interaction of GSH with BCNU on the action of BCNU against target cells of the UWR-2 human glioblastoma cell line. The cellular and molecular endpoints of BCNU action, namely clonogenic cell kill, inhibition of cellular DNA synthesis and induction of cellular DNA-interstrand cross-linking were measured as a function of prior interaction of BCNU with GSH, with and without equine liver GST. The reaction times used were within the in vitro half-life of BCNU under our experimental conditions (32). Our data demonstrate that both the bifunctional alkylating and cytotoxic activities of BCNU are decreased significantly after direct interaction of the drug with GSH.

The exact mechanisms underlying the inactivation of BCNU by GSH under these conditions are unclear. However, a possible explanation for the results is that adducts of GSH and BCNU that have little or no cross-linking and/or cytotoxic activity against the UWR-2 cell line are formed when GSH and BCNU are incubated together. Indeed, one such putative adduct, namely, S-(2-chloroethyl)glutathione, has been shown (33) not to induce relaxation in supercoiled plasmid pBR 322 DNA, suggesting that the adduct does not react readily with DNA. We also cannot rule out the possibility that adducts are formed that are potentially cytotoxic but are either too short lived or else are not taken up significantly by UWR-2 cells. An interesting finding in our studies was that the inactivation of BCNU was potentiated to only a relatively small, and statistically insignificant (P = 0.05), extent by the addition of equine liver GST to the GSH/BCNU mixtures. One likely reason for this could be that BCNU is a relatively poor substrate for the equine liver GST. It has been demonstrated previously that some GST-catalyzed GSH/BCNU reactions, e.g., the denitrosation of BCNU (34) are specific GST isozyme dependent. Based on our observations and reported studies on the chemistry and modes of action of BCNU, and known adducts that can result from CENU/GSH interaction, we have summarized in Fig. 6 possible reactions that can result in reduced bifunctional alkylating and cytotoxic activities of BCNU upon interaction with GSH.

We hypothesize further that based on our results, such reactions occurring intracellularly could provide the means by which tumor cells, could become resistant to the CENUs. Current studies in our laboratories are aimed at establishing the nature and quantity of the key adducts formed both in cell-free systems and intracellularly in human brain tumor cells.

We realize that the conditions of GSH/BCNU interaction employed in this study are far simpler than the complex conditions that exist intracellularly. Furthermore, we have observed, as has also been demonstrated by other investigators (35-41), that intracellularly, CENUs and/or their decomposition products can react with other cellular targets, including the enzymes of glutathione metabolism (35, 36, 41), DNA repair (37, 38), RNA metabolism (39), and chromatin (13, 36, 40). The effects of direct or GST-catalyzed GSH/BCNU interactions on cells are therefore best viewed as part of a complex interaction of BCNU with a variety of cellular targets, some of which are enzymes that regulate the metabolism of GSH itself. McConnell et al. (42), have reported that treatment of normal and L1210-bearing mice with BCNU resulted in significant decreases in hepatic GSH content suggesting that, indeed, the GSH/CENU interaction we observed in this study may also occur in vivo.

We recently (43, 44) showed that in addition to the possible interaction of GSH with BCNU as demonstrated in the present study, GSH is also capable of quenching chloroethylated DNA and attenuating DNA interstrand cross-link induction by BCNU. These observations imply that such interactions may constitute important potential mechanisms by which human brain tumor cells may become resistant to chloroethylnitrosoureas. The extent of resistance will presumably be dependent not only upon the intracellular GSH concentration and its biosynthetic turnover rate but also upon the intracellular levels of GST isoenzymes with high substrate specificity for either the CENUs or chloroethylated DNA. Future studies should further clarify this and better define the role of GSH and its metabolism in molecular mechanisms of human brain tumor CENU resistance and/or sensitivity.

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REFERENCES


* F. Ali-Osman et al., unpublished observation.
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