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CHARACTERIZATION OF THE INHIBITION OF GLUTATHIONE REDUCTASE AND THE RECOVERY OF ENZYME ACTIVITY IN EXPONENTIALLY GROWING MURINE LEUKEMIA (L1210) CELLS TREATED WITH 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA

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Abstract—The inactivation of the enzyme glutathione reductase by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was studied in exponentially growing murine leukemia cells. A 1-hr incubation with 1.6 ± 0.2 μM BCNU resulted in a 50% inhibition of glutathione reductase, while 10 μM BCNU caused total inhibition of the enzyme. The time required for 50% inhibition of glutathione reductase by 10 μM BCNU was 7 min. The recovery of glutathione reductase activity was studied by incubating cells with 10 μM BCNU for 30 min to inhibit all glutathione reductase activity, washing the cells free of drug, and continuing the incubation in fresh medium. Fifty percent of the activity returned within 12 hr. Glutathione reductase activity recovered normally when cell growth and DNA synthesis were inhibited in the cells, but it failed to recover when protein synthesis was inhibited. Therefore, the inactivation of glutathione reductase appears irreversible, and the recovery of enzymatic activity is dependent on the synthesis of new protein. Continuous incubation with 19.8 ± 0.4 μM BCNU resulted in a 50% inhibition of cell growth. A 1-hr incubation with 7.3 ± 0.8 μM BCNU resulted in a 50% loss of viability as measured by a soft agar clonogenic assay. These experiments quantify the inhibition of glutathione reductase by BCNU and the recovery of enzyme activity in the context of the toxic effects of the compound. A clinically useful inhibitor of glutathione reductase will require a wider difference between the concentrations required for enzyme inhibition and cytotoxicity than BCNU provides.

Glutathione is the principal non-protein thiol in cells. Along with glutathione-dependent enzymes, it participates in many intracellular reactions involved in the detoxification of xenobiotics and of damaging chemical species produced by ionizing radiation or during normal cellular processes [1–3]. Glutathione also has a role in cellular functions such as maintenance of membrane integrity, polymerization of tubulin, synthesis of macromolecules, and amino acid transport [2, 4].

There is intense interest in the relationship of intracellular glutathione levels and the resistance of tumor cells to alkylating chemotherapeutic agents and radiation. The sensitivity of tumors to alkylating agents is related to the ratio of unbound to protein bound thiols [5], and cell lines induced to become resistant to alkylating agents *in vitro* have elevated levels of glutathione and detoxify alkylating agents more quickly than the sensitive cell lines from which they are derived [6, 8]. A mutant human fibroblast cell line with reduced glutathione content was more sensitive to radiation than a related control cell line [9]. There is increasing interest in the manipulation of glutathione levels to potentiate therapy and potentially to overcome resistance to these agents. Depletion of glutathione levels by BSO†, an inhibitor of the *de novo* synthesis of glutathione, increases the cytotoxicity of melphalan in both drug-sensitive and -resistant cell lines [10, 11], sensitizes cells to radiation [12], and increases the efficacy of melphalan in an *in vivo* nude mouse model [13].

There have also been many reports of the ability of BCNU to potentiate the activity of ionizing radiation [14–17]. However, while potentiation has been consistently observed in studies using cell culture, the results are variable in studies using animal models and in clinical trials [14–17].

One potential mechanism for therapeutic potentiation by BCNU is the inhibition of glutathione reductase as originally observed by Frisher and Ahmad [18]. Glutathione reductase is an enzyme critical for maintaining the proper proportions of GSH and GSSG in cells [2]. This is extremely important for the effective detoxification of radiation-induced radicals and hydrogen peroxide [1]. The isocyanates derived from the breakdown of BCNU have been shown to inactivate purified yeast glutathione reductase, enzyme from lysed murine leukemia cells, and enzyme in isolated hepatocytes

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† Abbreviations: BSO, buthionine sulfoximine; GSH, reduced glutathione; GSSG, oxidized glutathione; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; and 4-OH-BCyNU, 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea.

[19, 20]. In this study we expand on these previous studies by investigating the inhibition of glutathione reductase and the recovery of enzyme activity in growing cells treated with BCNU. The results are presented within the context of the cellular toxicity of BCNU and explain the variability observed in therapeutic potentiation by BCNU.

MATERIALS AND METHODS

Materials. BCNU was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). BCNU was dissolved in 95% ethanol and stored at −20°.

Cell culture. Murine leukemia (L1210) cells were grown in RPMI 1640 medium containing 15% fetal bovine serum, 2 mM glutamine, and 50 μg/ml gentamycin, at 37° under 5% CO_2.

Growth inhibition. One milliliter of exponentially growing L1210 cells in complete medium (approximately 1.5×10^5 cells/ml) was added to 10 μl of various concentrations of BCNU in 24 well plates (Falcon). The ethanol-solubilized BCNU was diluted 10-fold in water just prior to addition. The final ethanol concentration was 0.1%. Initial cell density and cell density after 24 hr were measured by a Coulter counter. Growth is reported as fraction of control growth which is defined as the number of cell doublings of treated cells divided by the number of cell doublings of control cells.

Cytotoxicity. Cell viability was determined by a soft agar clonogenic assay. Following drug exposure for 1 hr, cells were washed free of drug and 100–10,000 cells were placed in cloning medium consisting of RPMI 1640 medium, 20% fetal bovine serum, 2 mM glutamine, 50 μg/ml gentamycine, and 0.15% agar. The cells were dispersed in a tube and allowed to grow for 10 days at which time the percent viable cells was determined as the number of colonies in treated tubes divided by the number of colonies in control tubes. The cloning efficiency was 65–75%.

Preparation of cell lysates. L1210 cells (50 ml) at a density of $6–8 \times 10^5$ cells/ml were incubated with the compounds of interest for the appropriate times, harvested by centrifugation for 10 min at 400 *g*, washed with phosphate-buffered saline, collected, and resuspended in 300 μl of buffer, 0.12 M sodium phosphate, 0.5 mM EDTA, pH 7.2. The cell suspension was freeze-thawed three times, and the enzyme containing supernatant fraction was separated from cell debris by centrifugation for 10 min at 30,000 *g*. The protein content of the cell lysate was determined by the method of Bradford [21].

Assay for glutathione reductase activity. The assay determines the activity of glutathione reductase by measuring the decrease in absorbance at 339 nm caused by the utilization of NADPH by the enzyme [22]. The reaction mixture consisted of 2.60 ml of 0.12 M sodium phosphate, 100 μl of 15 mM EDTA, 100 μl of 65 mM oxidized glutathione, and 100 μl of a lysate preparation. After mixing and waiting 5 min, 100 μl of a 4.5 mM NADPH solution was added, and the absorbance at 339 nm was monitored by a Beckman DU-50 spectrophotometer programmed for automatic data acquisition every 15 sec for 5 min.

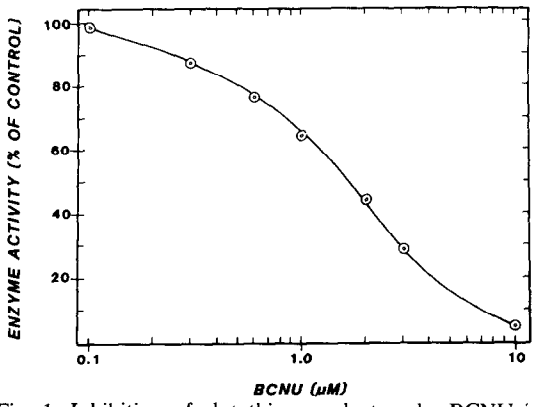

Fig. 1. Inhibition of glutathione reductase by BCNU in L1210 cells. Cells were incubated with increasing concentrations of BCNU for 1 hr. Cell lysates were prepared, and enzyme activity was analyzed as described in Materials and Methods. The experiment was conducted two additional times with the same results. The activity of glutathione reductase in lysate from untreated cells was 53 ± 6 nmol NADPH oxidized·(mg protein)$^{-1}$·min^{-1}.

Enzyme activity was determined as the best fit computed line through the acquired data on a per milligram of protein in the lysate basis.

RESULTS

The inhibition of glutathione reductase in L1210 cells was dependent on the concentration of BCNU and the time of exposure. Exponentially growing L1210 cells (50 ml) were exposed to various concentrations of BCNU for 1 hr, a lysate was prepared, and glutathione reductase activity was measured. Glutathione reductase activity was inhibited 50% by 1.6 ± 0.2 μM BCNU and totally inhibited by 10 μM BCNU (Fig. 1). Cells lost 50% of their glutathione

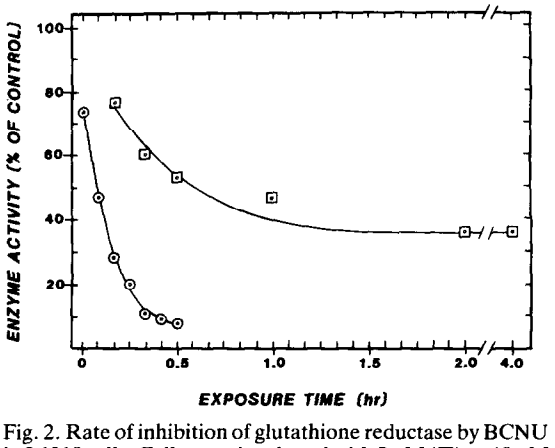

Fig. 2. Rate of inhibition of glutathione reductase by BCNU in L1210 cells. Cells were incubated with 2 μM (□) or 10 μM (○) BCNU for increasing times. Cell lysates were prepared, and enzyme activity was analyzed as described in Materials and Methods. The activity of glutathione reductase in lysate from untreated cells 53 ± 6 nmol NADPH oxidized·(mg protein)$^{-1}$·min^{-1}. The experiment was conducted two additional times with the same results.

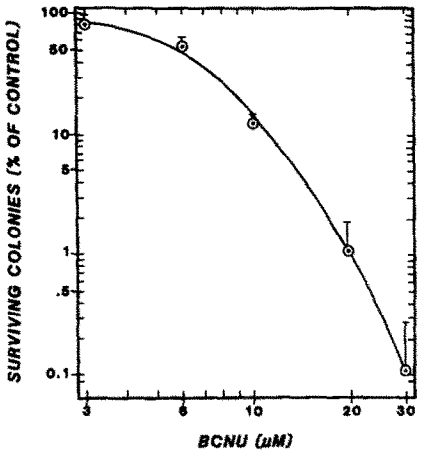

Fig. 3. Viability of L1210 cells exposed to BCNU. Cells were exposed for 1 hr to various concentrations of BCNU and viability was determined by soft agar colony formation as described in Materials and Methods. The cloning efficiency of untreated L1210 cells was 75%. Each point is the mean ± SD of four determinations. The experiment was conducted two additional times with the same results.

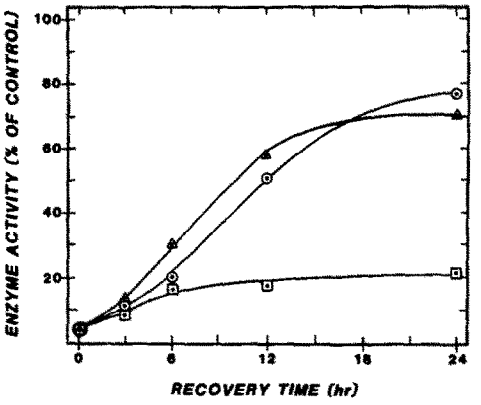

Fig. 4. Recovery of glutathione reductase activity in L1210 cells after inhibition by BCNU. Cells were incubated with 10 μM BCNU for 30 min to inhibit glutathione reductase, washed free of drug, and incubated in fresh medium containing no additions (△), 150 μM hydroxyurea to inhibit DNA synthesis (○), or 5 μM sparsomycin to inhibit protein synthesis (□). Cell lysates were prepared, and glutathione reductase activity was analyzed at various times. The activity of glutathione reductase in lysate from untreated cells was 53 ± 6 nmol NADPH oxidized·(mg protein)$^{-1}$·min^{-1}. The experiment was repeated once with the same results.

reductase activity within 7 min of addition of 10 μM BCNU (Fig. 2). Cells exposed to 2 μM BCNU lost enzymatic activity over a 1.5-hr period, after which no additional inhibition was observed (Fig. 2).

Exponentially growing L1210 cells were treated with various concentrations of BCNU for 24 hr and cell growth was determined. Cell growth was inhibited to 50% of normal by 19.8 ± 0.4 μM BCNU (data not shown). The viability of cells exposed to various concentrations of BCNU for 1 hr was determined by a soft agar clonogenic assay. Cell viability was reduced to 50% of control by 7.3 ± 0.8 μM BCNU (Fig. 3), a concentration which had minimal effect on cell growth during the first 24 hr after incubation.

The ability of cells to regain their glutathione reductase activity was studied. Cells were incubated with 10 μM BCNU for 30 min to inhibit glutathione reductase activity, washed free of drug, and incubated in fresh medium. The cells grew 80% relative to untreated cells during the following 24 hr. Fifty percent of the glutathione reductase activity returned within 12 hr and 70% returned within 24 hr (Fig. 4. Judged by the cell growth rate, 47% of the cells present at 12 hr and 74% of the cells present at 24 hr had not been present during the original incubation with BCNU. Likewise, 40% of the cellular protein present at 12 hr had not been present during the original incubation with BCNU. Therefore, the recovery of activity was related to cell growth and/or the synthesis of new protein. To distinguish between these two possibilities, cells were incubated with BCNU, washed as previously described and then incubated either in the presence of 150 μM hydroxyurea to inhibit cell growth via DNA synthesis inhibition or in the presence of 5 μM sparsomycin to inhibit protein synthesis. Cell growth over 24 hr was 20% of control with hydroxyurea and 10% of control with sparsomycin. Sparsomycin inhibited the incorporation of [^{3}H]leucine into macromolecules by 70% within 3 hr and by 85% within 5 hr. Hydroxyurea had no effect on the incorporation of [^{3}H]leucine within 12 hr, but did cause a 50% reduction at 24 hr. Hydroxyurea-treated and sparsomycin-treated cells were 95 and 80% viable as measured by the exclusion of trypan blue at 24 hr. Cells treated with BCNU + hydroxyurea recovered their glutathione reductase activity similarly to that of BCNU-treated cells when measured on a per milligram of protein basis. On a per cell basis, BCNU + hydroxyurea-treated cells had twice the glutathione reductase activity of BCNU-treated cells and 1.5 times the activity of untreated cells. The glutathione reductase activity of cells treated with BCNU + sparsomycin recovered slowly for approximately 6 hr and then had no additional increase. This correlates with the degree of protein synthesis inhibition caused by sparsomycin. Therefore, the recovery of glutathione reductase activity is dependent on the synthesis of new protein rather than on cell growth or the synthesis of DNA.

DISCUSSION

The results presented in this paper demonstrate that BCNU is a potent inhibitor of glutathione reductase in exponentially growing murine leukemia (L1210) cells. These results extend previous reports that BCNU inhibits purified yeast glutathione reductase, enzyme from lysed cells, and enzyme in isolated erythrocytes and hepatocytes [18–20]. The 1.6 and 10 μM concentrations of BCNU required for 50% inhibition and total inhibition of the enzyme, respectively, indicate that the compound is much more potent than the 0.197 mM concentration used to inhibit the enzyme in concentrated solutions and cell lysates might indicate [19].

Covalent modification of glutathione reductase by

BCNU-generated isocyanates has been proposed as the mechanism of enzyme inactivation [19]. In agreement with the irreversible nature of this type of inactivation, the synthesis of new protein was required for cells to regain active enzyme. However, neither cell growth nor DNA synthesis was required to regain active enzyme. Therefore, it is not a new population of cells which contains the newly synthesized active enzyme but rather all surviving cells. Cell growth rate does not appear to determine the ability to regain active enzyme.

This report compares the concentration of BCNU that effectively inhibits glutathione reductase to that which can be tolerated by cells. In the L1210 cell line, which is sensitive to the alkylating effects of nitrosoureas, concentrations of BCNU that inhibited glutathione reductase also resulted in substantial toxicity. BCNU might inhibit glutathione reductase at non-toxic concentrations in cells which are less sensitive to the alkylating effects of nitrosoureas.

There has been continued interest in the manipulation of the glutathione status of cells to potentiate the activity of alkylating agents and radiation and to overcome resistance to these agents. Presently, the most studied mode of manipulation of glutathione status is the inhibition of the *de novo* synthesis of glutathione by BSO. However, there have also been many reports of the ability of BCNU to potentiate the activity of ionizing radiation [14–17]. Therefore, we are studying the suitability of the inhibition of glutathione reductase as an alternative or to augment the inhibition of *de novo* glutathione biosynthesis. The potentiation of ionizing radiation by BCNU has been consistently observed in studies using cell culture, but results are inconsistent in studies using animal models and in clinical trials [14–17]. Our results may explain these variable findings. BCNU is already toxic to cells via alkylation at concentrations that inhibit glutathione reductase or DNA repair [23], two possible mechanisms of potentiation. Therefore, in many cases tolerable amounts of BCNU administered *in vivo* may not initiate these additional biochemical effects. Although synergy can be observed at lethal concentrations in cell culture, morbidity makes the use of these concentrations useless for the evaluation of synergy in animals.

We conclude that BCNU is not a suitable compound for potentiation of therapy. A compound which inhibits glutathione reductase at a concentration achievable *in vivo* and below that which is toxic may be an effective potentiator *in vivo*. Tew *et al.* [24] studied the pure carbomoylating nitrosourea 4-OH-BCyNU in the Walker 256 rat carcinoma cell line. Glutathione reductase is inhibited by approximately 50% at 50 μM 4-OH-BCyNU, while 100 μM 4-OH-BCyNU caused 25% cell death within 6 hr as measured by trypan blue exclusion [24]. We have similar results in our system showing that a 1-hr incubation with 4-OH-BCyNU inhibited glutathione reductase by 50% at 80 ± 11 μM and reduced viability by 50%, as measured by soft agar cloning, at 27 ± 4 μM.* Therefore, the pure carbamoylating nitrosourea 4-OH-BCyNU is not a particularly potent inhibitor of glutathione reductase, nor does it effectively inhibit glutathione reductase at non-toxic concentrations. The development of a compound which fits the criteria for potency and being non-toxic is in progress.

* M. B. Cohen, unpublished observation.

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