Glutathione Depletion Greatly Reduces Neocarzinostatin Cytotoxicity in Chinese Hamster V79 Cells*

William G. DeGraff, Angelo Russo, and James B. Mitchell

From the Radiation Biology Section, Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

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The role of the intracellular thiol glutathione in the reductive activation of neocarzinostatin was investigated in Chinese hamster V79 cells. The cells were pretreated with agents that either lower (buthionine sulfoximine or diethyl maleate) or elevate (oxothiazolidine carboxylate) intracellular glutathione levels. These cells were then exposed to 1-5 µg/ml neocarzinostatin for 1 h and assayed for survival. Depletion of glutathione to levels at or below the limit of detection resulted in a marked reduction in neocarzinostatin cytotoxicity, while increasing glutathione levels to 250% of control values had little or no effect on neocarzinostatin toxicity. High performance liquid chromatography analysis of cysteine in untreated and glutathione-depleted cells showed cysteine levels lower than 0.2 µM, indicating that cysteine does not play a major role in the reductive activation of neocarzinostatin in untreated or glutathione-depleted cells. When intracellular cysteine levels were artificially elevated by oxothiazolidine carboxylate treatment of glutathione-depleted cells, neocarzinostatin toxicity was about two-thirds that seen in cells with normal glutathione levels. In cell-free systems, others have shown that reducing agents such as 2-mercaptoethanol are necessary for the activation of neocarzinostatin to a species that will cleave DNA. In this study, we have identified glutathione as the major cellular reducing agent for the activation of neocarzinostatin in a mammalian cell line.

NCS* is a polypeptide antibiotic that has shown some antitumor activity in clinical trials (1, 2) and which continues to be the subject of clinical investigation (3, 4). NCS contains a nonprotein chromophore (5), which is responsible for the biological activities of the parent compound (6). NCS induces single-strand breaks in DNA by damaging the deoxyribose moiety of a nucleotide (7). These breaks occur preferentially at thymine residues and occasionally at adenine residues (8); the base is released intact from the DNA molecule (9, 10), leaving a gap bounded on the 3' side by a phosphate group and on the 5' side by the deoxyribose cleavage moiety attached to a phosphate group (7, 11). The reaction of high concentrations (100 µg/ml) of the NCS chromophore with DNA occurs in cell-free systems in the absence of any reducing agent, but sulphydryl group donors such as 2-mercaptoethanol stimulate the DNA strand scission activity at least 1000-fold (12). DNA strand scission has been shown to be oxygen-dependent (13). Recently, it has been shown that 2 mol eq of sulphydryl and 1 mol eq of O₂ are consumed for each mole equivalent of NCS chromophore reacting with DNA (14). GSH is usually the major nonprotein thiol in the mammalian cell (15) and is a potential source of sulphydryl groups for the bioactivation of NCS in vivo or in cell culture. Based on this fact alone, one might expect that depletion of the cellular GSH pool would lower the toxicity of NCS, since there would be fewer sulphydryl groups available to react with the NCS chromophore. On the other hand, GSH is important in protecting cells from free radical damage (15), and there is evidence that free radical production is important in the activity of NCS (16, 17). Favaudon (18) has proposed that oxygen is necessary in the thiol-mediated activation of the NCS chromophore because in the absence of oxygen, a third thiol group would react with the methylene radical produced at the C-5' position of deoxyribose by activated NCS, terminating the radical, and preventing DNA damage. Thus, it is theoretically possible that depletion of cellular GSH could actually increase NCS toxicity.

It is possible to deplete GSH to levels of less than 5% of control values in V79 cells by exposing the cells to BSO, a specific inhibitor of γ-glutamylcysteine synthetase (19). GSH levels can also be depleted by treatment of the cells with DEM (20), an agent that binds GSH in a reaction catalyzed by GSH S-transferase (21, 22). Treatment of V79 cells with OTZ increases intracellular GSH levels to 150-300% of control values (23). Using these three compounds, we have investigated the effect of reduced and elevated GSH levels on the cytotoxicity of NCS in V79 cells.

Another thiol compound that could have a role in the bioactivation of NCS is cysteine. To investigate this possibility, high levels of intracellular cysteine were induced, without a concurrent increase in GSH levels, by simultaneous BSO and OTZ treatment. Cysteine levels were monitored by HPLC in untreated, BSO-treated, and OTZ/OTZ-treated cells. Glutathione depletion greatly reduces neocarzinostatin cytotoxicity in Chinese Hamster V79 Cells.
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RESULTS

Pretreatment with BSO or DEM decreased total GSH levels to less than 5% of control values. OTZ pretreatment increased GSH levels to 250-260% of control values. Combined BSO and OTZ treatment also reduced total GSH to less than 5% of control values. All GSH measurements were made at the time NCS was added. In one experiment, GSH determinations were made after the 1-h NCS exposure (at 5 μg/ml) and showed no change from pre-NCS exposure levels. NCS treatment (5 μg/ml for 1 h) caused no detectable change in total GSH. Control levels of GSH for these experiments were from 2 to 5 mM, as measured by the GSH reductase procedure (26). Treated samples were compared to control samples.

GSH depletion by either BSO or DEM reduced the toxicity of NCS by a factor of 1000 or greater at 5 μg/ml (Figs. 1 and 2), indicating that GSH plays a major role in the activation of NCS in mammalian cells. Another way to compare these data is to calculate D0 values, the amount of NCS needed to reduce the surviving fraction to 0.37 along the linear portion of the curve (27) for the survival curves. The average D0 for NCS in these experiments was 0.57 μg, while in BSO-treated cells, the D0 for NCS was 6 μg, and in DEM-treated cells, it was 10 μg. To rule out that a reaction between NCS and BSO in the medium could be causing these results, GSH depletion by BSO was done as described above, but the BSO was removed before NCS exposure. GSH levels remained low for several hours following the removal of BSO from V79 cells (data not shown). These data are also shown in Fig. 1 and are nearly identical to the data from experiments where BSO was left on during NCS exposure.

Increasing intracellular cysteine levels in the absence of GSH resulted in intermediate NCS toxicity (Fig. 3). HPLC analysis of untreated and BSO-treated cells showed no detectable cysteine (Fig. 4). The limit of detection for cysteine in this assay was 50 pmol, which is equivalent to 0.2 μM when 50 x 10^6 cells are assayed. Cysteine and GSH standards for HPLC analysis were prepared by placing micromolar concent-
The method may better simulate experimental conditions found in mammalian cells, bacteria, fungi, or yeast by using millimolar concentrations, yet this variation in concentrations of the compounds directly into the monobromobimane exposure results from a sulfhydryl-independent NCS-induced DNA cleavage. Alternately, reducing agents other than GSH may activate NCS. The HPLC analysis in the present study indicated that the concentration of cysteine in untreated and BSO-treated cells is less than 0.2 μM (see Fig. 4 and "Results"). The concentrations of NCS used in these experiments were 0.1-0.5 μM. Since 2 mol of sulfhydryl groups is needed to activate each mole of NCS (14), normal intracellular levels of cysteine could, at most, only activate a small portion of the NCS molecules.

It is possible that there is some GSH left in the cell after depletion by BSO or DEM, but at a concentration too low to be detected by the assay used. The GSH assay used in these studies can detect a minimum of 1-5 ng of GSH (24), which corresponds to 0.8-4 μM when 4 x 10^6 cells are assayed (one 100-mm Petri dish seeded with 10^6 cells 24 h prior to starting the experiment, with a doubling time of 10-12 h). Thus, it is possible that concentrations of GSH that are still 10-fold higher than the concentration of NCS could remain in the cell after BSO or DEM treatment. This concentration could be sufficient to activate a small portion of the NCS molecules.

The nearly identical results obtained with two different thiol depleters and the fact that BSO need not be present at the same time as NCS for reduction of NCS toxicity to occur indicate that the observed reduction in NCS toxicity is due to thiol depletion, and not to inactivation of NCS in the medium by reaction with the thiol-depleting compounds. Taken together, these results indicate the GSH is the major thiol used in the reductive activation of NCS in V79 cells and argue against a significant role for cysteine, when cysteine is present at normal levels.

GSH concentrations in untreated cells in these experiments were 2-5 mM, nearly 10,000-fold higher than the NCS concentrations used. This explains the lack of potentiation of NCS toxicity by pretreatment with OTZ. Even though 2 mol of sulfhydryl groups is required for each mole of NCS activated (12), there is still a considerable excess of GSH in an untreated cell, and raising the GSH concentration with OTZ would not be expected to increase the toxicity of NCS. Without the GSH biosynthetic pathway being blocked by BSO, OTZ treatment would also not be expected to significantly increase intracellular levels of cysteine.

After combined BSO and OTZ treatment, higher levels of cysteine and very low levels of GSH are present in the cell, resulting in intermediate NCS toxicity (see Fig. 3). The D_50 for NCS for BSO/OTZ-treated cells was 0.9 μg, higher than the 0.57-μg D_50 for NCS in untreated cells, indicating that even at chemically induced high levels, cysteine does not activate NCS to the same extent as does GSH.

BSO is currently being considered as an adjuvant in cancer radiation and chemotherapy. If this were done with a drug like NCS, which relies on intracellular GSH for activation, it would be counterproductive, unless some way is found of lowering GSH levels in normal tissues to a greater extent than in the tumor tissue. Our work suggests that before in a marked reduction in NCS cytotoxicity, as evidenced by a 10-fold increase in the D_50 (a 10-fold increase in the slope of the survival curve).

Beerman et al. (12) have reported that NCS will cleave DNA in a cell-free system without the addition of a sulfhydryl compound, but such cleavage occurs slowly and at high NCS concentrations (100 μg/ml), compared to the same reaction when stimulated by 2-mercaptoethanol. They reported the stimulation by 2-mercaptoethanol to be at least 1000-fold. Thus, it is possible that the remaining toxic activity of NCS in GSH-depleted V79 cells (40-50% survival at 5 μg of NCS/ml) results from a sulfhydryl-independent NCS-induced DNA cleavage. Alternately, reducing agents other than GSH may activate NCS. The HPLC analysis in the present study indicated that the concentration of cysteine in untreated and BSO-treated cells is less than 0.2 μM (see Fig. 4 and "Results"). The concentrations of NCS used in these experiments were 0.1-0.5 μM. Since 2 mol of sulfhydryl groups is needed to activate each mole of NCS (14), normal intracellular levels of cysteine could, at most, only activate a small portion of the NCS molecules.

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combining BSO with chemotherapeutic agents, one should understand the biochemistry of the agents. In addition, our work suggests that the potential of selective biochemical modulation of malignant versus normal cells might provide some therapeutic advantages.

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