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The effects of O^6-benzylguanine and hypoxia on the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea in nitrosourea-resistant SF-763 cells

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Abstract. O^6-Alkylguanine-DNA alkyltransferase (AGT) activity is associated with resistance of brain tumor cell lines to the cytotoxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). SF-763 cells exhibit high AGT activity and are resistant to BCNU. In this study, we compared the effects of the AGT inhibitor O^6-benzylguanine (BG) on the cytotoxicity of BCNU in oxic and hypoxic SF-763 cells; we also measured AGT activity, ornithine decarboxylase (ODC) activity, and polyamine levels to determine if there was any correlation with cell survival as determined by colony-forming efficiency assay. Exponentially growing monolayer cells were pretreated with 10 μM BG for 2 h under oxic or hypoxic (95% nitrogen/5% CO_2) conditions and then exposed to graded concentrations of BCNU for 1 h. BG significantly lowered AGT activity but had no cytotoxic effect in oxic or hypoxic cells; hypoxia alone was not cytotoxic. The cytotoxicity of BCNU was 4 times higher in BG-treated hypoxic cells than in oxic cells treated with BCNU alone; the BCNU doses required for a 1-log cell kill were 75 and 300 μM, respectively. ODC activity was lowered by hypoxia alone but was not significantly affected by BG in either hypoxic or oxic cells. Polyamine levels were not significantly affected by hypoxia or BG. These results indicate that pretreatment with BG dramatically lowers AGT activity and increases the cytotoxicity of BCNU in both oxic and hypoxic SF-763 cells. The mechanism of this enhanced cytotoxicity is apparently unrelated to ODC activity or polyamine levels.

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Abbreviations: CENU, chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; ODC, ornithine decarboxylase; CMEM, complete minimal essential medium; PE, plating efficiency

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Introduction

Chloroethylnitrosoureas (CENUs) such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) are widely used to treat solid tumors, including brain tumors [31, 55]. These agents exert their cytotoxic effect by alkylation at the O^6 position of guanine in DNA, which leads to the formation of interstrand cross-links [2, 14]. The O^6-alkylguanine adduct in DNA is repaired by O^6-alkylguanine-DNA alkyltransferase (AGT), which transfers the alkyl group stoichiometrically to an internal cysteine residue of the enzyme to form S-alkylcysteine and in the process becomes inactivated [5, 6, 28, 36, 59]. The extent of DNA repair depends on the availability of AGT, which varies in different types of cells and tissues [37, 59]. AGT activity in human tumor cells correlates well with the cytotoxicity of CENUs [4, 15]. Cells with high levels of AGT activity are mort resistant to alkylating agents than cells with little or no AGT activity [3, 51].

One approach used to overcome resistance to CENUs is to deplete or inactivate AGT by pretreatment with methylating agents, such as streptozotocin [17, 19]. The clinical usefulness of these agents may be limited by their mutagenic and carcinogenic properties [45]. O^6-Methylguanine has also been used for this purpose [8, 24], but even when given at high doses for long periods, it does not inactivate AGT completely [10]. Recently it has been shown that O^6-benzylguanine (BG) inactivates AGT irreversibly at low, nontoxic doses and sensitizes oxic cells to CENUs [10, 12].

Most solid tumors contain hypoxic cells [7, 33, 34], which are more resistant to ionizing radiation than oxic cells [18, 20]. Hypoxic cells may be blocked partially or completely in their progression through the cell cycle and are distal to tumor vasculature; these cells may therefore be refractory to some chemotherapeutic agents [25, 48, 52]. Hypoxia can also induce gene amplification that results in resistance to some anticancer drugs [42]. Hypoxic cells are nonetheless susceptible to certain chemotherapeutic agents, such as mitomycin C, a bioreductive alkylating agent [41, 53]. Similarly, BCNU is more toxic to most hypoxic, as

compared with oxic, brain-tumor cell lines [46]. The mechanism of this enhanced cytotoxicity under hypoxic conditions is not known, but in some cell lines hypoxia depletes polyamine levels, which may increase sensitivity to BCNU [47].

Polyamines are small, highly charged cations that are important in a variety of fundamental intracellular processes [38], including the modulation of nucleic acid and protein biochemistry in eukaryotic cells [40]. DNA synthesis is preceded by increased polyamine synthesis, and rapidly dividing tissues typically have higher polyamine levels than slowly dividing or nondividing tissues [40, 49]. The biosynthesis of polyamines in mammalian cells is primarily initiated by the decarboxylation of ornithine to form putrescine; this step is governed by the highly regulated ornithine decarboxylase (ODC) [35]. Inhibition of ODC activity depletes intracellular polyamine levels and thus inhibits cell growth [26]. Pretreatment of brain-tumor cell lines with α-difluoromethylornithine, an ODC inhibitor, lowers putrescine and spermidine levels and increases the cytotoxicity of subsequent treatment with BCNU [21, 50].

In this study, we evaluated the effects of pretreatment with BG on the cytotoxicity of BCNU in oxic and hypoxic SF-763 human brain-tumor cells, which exhibit high AGT activity and are resistant to BCNU. The goal was to determine if BG increases sensitivity to BCNU under both oxic and hypoxic conditions and to determine if its cytotoxicity correlates with AGT, ODC, or polyamine levels.

Materials and methods

Drugs. BCNU was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.) and stored at –20° C. BG, synthesized as previously described [10], was a gift from Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.).

Cell culture. SF-763 was derived from a human recurrent glioblastoma; this cell line has a doubling time of approximately 28 h. The cells were grown as monolayers in complete minimal essential medium (CMEM) consisting of Eagle's minimal essential medium supplemented with 10% fetal calf serum and nonessential amino acids in a humidified atmosphere of 5% CO_2-95% air at 37° C.

Hypoxic culture. Cells were grown for 24 h in glass flasks (Bellco, Model 1903, Vineland, N. J.) fitted with sterile silicone stoppers. The cells were made hypoxic by gassing the flasks with a continuous flow of 95% N_2 and 5% CO_2 for 2 h at a flow rate of 75 ml/min at 37° C; pH was maintained at 7.4 throughout the experiment as described previously [46]. At the end of the hypoxic period, the flasks were placed on ice, the caps were removed, and the cells either were trypsinized for cell survival and polyamine assays or were scraped from the flasks for ODC and AGT assays.

Drug treatment. A stock solution of BG (100 m*M*) was made in dimethylsulfoxide and diluted to 1 m*M* with CMEM. Oxic and hypoxic cells were exposed to BG at a final concentration of 10 μ*M* for 2 h at 37° C and then to graded doses of BCNU for 1 h. BCNU in ethanol (final concentration, <1%) was injected into the medium through the silicone stoppers without breaking the hypoxia, and the cultures were incubated at 37° C under a 95% N_2 and 5% CO_2 atmosphere for 1 h and harvested for the cell survival assay. All drug solutions were made fresh before each experiment.

Cell survival assay. Cell survival was measured with an assay of colony-forming efficiency. Volumes (1 ml) of appropriately diluted single-cell suspensions containing known numbers of cells were added to plastic wells in a 6-well plate (Costar, Cambridge, Ma.) containing 2×10^4 heavily irradiated (52.7 Gy) feeder cells in 4 ml of CMEM and incubated for 12 days. The cultures were stained with crystal violet (0.125% in methanol), and colonies containing >50 cells were counted. The plating efficiency (PE) was calculated as the number of colonies per dish divided by the number of cells originally seeded. The surviving fraction was calculated as the PE of treated cells divided by the PE of untreated control cells.

AGT assay. BG-treated and control oxic and hypoxic cells (3×10^7) were scraped from flasks, rinsed twice with cold phosphate-buffered saline (PBS), and centrifuged. PBS was aspirated from the pellet; the tubes containing cell pellets were inverted and residual PBS was drained from the pellets and removed with a cotton swab. The pellets were stored at –70° C. Crude extracts from cells were prepared as described previously [13, 39]. AGT activity was measured as the removal of O^6-[^{3}H]-methylguanine from a [^{3}H]-methylated DNA substrate (21.5 Ci/nmol). The extracts were incubated with the substrate at 37° C for 30 min. DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 *M* and then hydrolyzed in 0.1 *M* HCl at 70° C for 30 min. The modified bases were separated by reverse-phase, high-performance liquid chromatography with 0.05 *M* ammonium formate (pH 4.5) containing 6.5% methanol [9]. Protein was determined by the method of Bradford [1]; the results were expressed as femtomoles of O^6-methylguanine released from the substrate per milligram of protein.

Polyamine measurement. Cells ($0.5-1 \times 10^6$) were collected from each sample, washed with isotonic phosphate buffer (pH 7.4), sonicated in 250 μl of 8% sulfosalicylic acid, dansylated, and analyzed for polyamine content by high-performance liquid chromatography [23].

ODC assay. The ODC assay was based on a CO_2-trapping method [44]. Cells ($1-3 \times 10^6$) were scraped from tissue-culture flasks, washed twice with cold PBS, and centrifuged. After the pellets had been resuspended in 600 μl of buffer containing 50 m*M* sodium-potassium phosphate buffer (pH 7.2), 1 m*M* dithiothreitol, 0.1 m*M* ethylenediamine tetraacetic acid (EDTA), and 0.1 m*M* pyridoxal 5-phosphate, they were sonicated and centrifuged. ODC activity was determined from the amount of $^{14}CO_2$ released from L-[1-^{14}C]-ornithine (specific activity, 50 mCi/nmol; Amersham, Arlington Heights, Ill.). The enzyme assay was carried out in triplicate with 180 μl of supernatant. The reaction was initiated by adding 20 μl of substrate L-ornithine (0.25 μCi/assay) and sufficient unlabeled ornithine to achieve a final concentration of 0.25 m*M*. The tubes were closed with rubber stoppers with inserted plastic center wells (Kontes, Vineland, N. J.) containing 200 μl of ethanolamine: ethoxythanol (2:1, v/v), and incubated at 37° C for 1 h. The reaction was stopped by adding 500 μl of 2 *M* citric acid. The next day, the wells were cut and dropped directly into glass scintillation vials containing 10 ml of scintillation fluid (Betamax + 15% ethanol, ICN Radiochemicals, Irvine, Calif.). The radioactivity was counted in a scintillation counter (Model LS 3801, Beckman, Palo Alto, Calif.). In control experiments, the cell extracts were replaced by 50 μl of bovine serum albumin at a concentration of 1 mg/ml. The protein content was determined with a protein assay kit (Bio-Rad, Richmond, Calif.). Absorbance at 595 nm was measured at room temperature in a Perkin-Elmer (Norwalk, Ct.) spectrophotometer; bovine serum albumin solution of known concentration was used as a standard.

Results

Cell survival

Hypoxia alone did not kill cells; the PEs of both oxic and hypoxic cells were 60%–70%. BCNU was more toxic to hypoxic than to oxic cells (Fig. 1). The BCNU concentra-

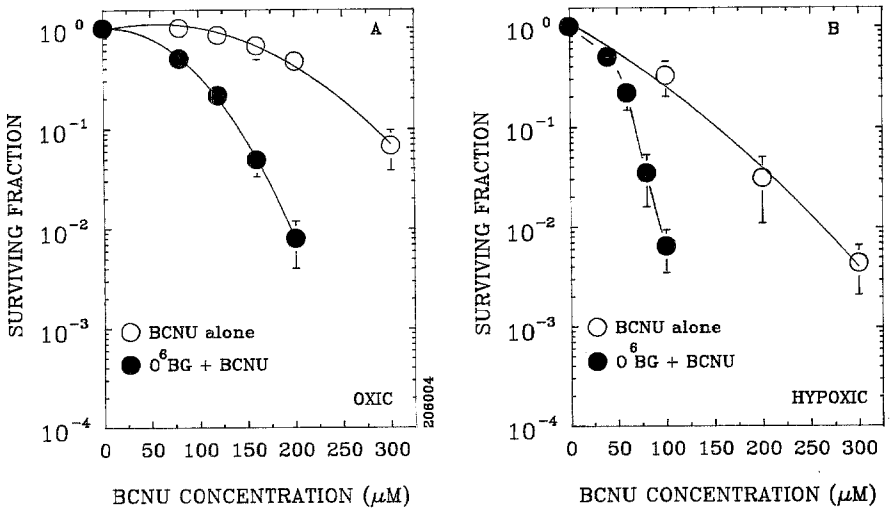

Fig. 1. Survival of SF-763 cells treated for 1 h with graded concentrations of BCNU alone (○) or with 10 μ*M* O^6-benzylguanine (BG) for 2 h (●) under oxic (*left*) and hypoxic (*right*) conditions. Each point represents the mean number of colonies from six wells; bars represent standard errors

Table 1. AGT activity in oxic and hypoxic SF-763 cells

Treatment	AGT activity (fmol/mg protein)
Oxic	84 (101, 67)
Oxic+BG	8 (12, 4)
Hypoxic	61 (70, 52)
Hypoxic+BG	13 (19, 7)

Control cells and cells that were pretreated with 10 μ*M* BG for 2 h were assayed for AGT. The mean activities of duplicate measurements are shown; the individual values are shown in parentheses

Table 2. ODC activityin oxic and hypoxic SF-763 cells

Group	ODC (dpm/mg protein/min)
Oxic cells:	
Control	264±39
BG-treated	224±42
Hypoxic cells:	
Control	10.3±3.1
BG-treated	13.6±0.2

Control cells and cells that were pretreated with 10 μ*M* BG for 2 h were assayed. Values are mean ± SD of three to five independent measurements

tion required to achieve a 1-log cell kill in hypoxic cells was about half that needed in oxic cells. Pretreatment with 10 μ*M* BG increased the cytotoxicity of BCNU in both oxic (Fig. 1, left) and hypoxic cells (Fig. 1, right). In both cases, the dose-enhancement ratio at a 1-log cell kill was about 2. Thus, the BCNU concentration required to achieve a 90% cell kill was about 4 times higher in oxic cells treated with BCNU alone than in hypoxic cells pretreated with BG (300 vs 75 μ*M*). BG alone was not cytotoxic to oxic or hypoxic cells.

AGT activity

There was minimal difference in AGT activity between oxic and hypoxic cells. Pretreatment with 10 μ*M* BG for 2 h lowered AGT activity similarly in both oxic and hypoxic cells (Table 1).

ODC activity and polyamine levels

ODC activity was much lower in hypoxic cells than in oxic cells (Table 2), but there were no significant differences in polyamine levels between oxic and hypoxic cells (data not shown). BG did not affect ODC activity or polyamine levels under either oxic or hypoxic conditions.

Discussion

The results of this study show that BG depletes AGT activityand increases the cytotoxicity of BCNU in oxic and hypoxic SF-763 human brain-tumor cells but does not affect ODC activity or polyamine levels. It is generally agreed that BCNU exerts its cytotoxic effect by inducing interstrand DNA cross-links, which occur by intramolecular rearrangement of the initially formed monoadducts. Evidence suggests that the initial O^6-chloroethylguanine adduct rearranges to forn O^6-N^1-ethanoguanine in DNA, which reacts with the complementary strand of DNA to form 1-(3-deoxycytidyl)-2-(1-deoxyguanosinyl)ethane [2, 30, 56, 57]. AGT prevents the formation of DNA interstrand cross-links by removing alkyl groups from the O^6 position of guanine in DNA [27, 43]. Therefore, compounds that inactivate AGT may be useful in BCNU therapy. Recently it has been reported that BG at nontoxic doses depletes AGT activity in cultured cells, tissues, and tumors and thereby enhances the effectiveness of antitumor drugs, including CENUs [10–12, 16, 32].

We previously found that both 9L rat brain-tumor cells, which have low AGT activity, and BTRC-19 cells, which have high AGT activity and are resistant to BCNU, were sensitized to BCNU under hypoxic conditions [46]; however, AGT activities were similar in oxic and hypoxic BTRC-19 cells (unpublished data). In SF-763 human brain-tumor cells, which exhibit even higher AGT activity than BTRC-19 rat brain-tumor cells, hypoxia alone did not

alter AGT much. Thus, the enhanced cytotoxicity of BCNU in hypoxic cells does not appear to be related to AGT activity. However, BG at nontoxic doses depleted AGT activity in SF-763 cells under both oxic and hypoxic conditions (Table 1) and rendered them more sensitive to BCNU (Fig. 1). Therefore, BG appears to sensitize cells to BCNU by a mechanism different from that by which hypoxia alone increases the cytotoxicity of BCNU.

Another possible mechanism for the enhanced BCNU cytotoxicity produced by either hypoxia or BG would be a decrease in ODC activity. Depletion of putrescine and spermidine enhances the cytotoxicity of BCNU [21, 22, 50]. We previously observed lower ODC and polyamine levels in 9L rat brain-tumor cells and U-251 MG and SF-126 human brain-tumor cells under hypoxic conditions ([47], unpublished results). In the present study, however, there was no difference in putrescine, spermidine, or spermine levels between oxic and hypoxic SF-763 cells, even though ODC activity was markedly lower under hypoxic conditions (Table 2). These contrasting results may be due in part to differences in uptake, excretion, or degradation of polyamines [58]. Alterations of ODC activity in specific cells and tissues do not necessarily correlate directly with changes in putrescine levels [29, 54, 55]. We have no information on the effects of hypoxia on protein synthesis in SF-763 cells. However, preliminary studies in 9L rat and SF-126 human brain-tumor cells showed no difference in total protein synthesis as measured by incorporation of [^{35}S]-methionine between oxic and hypoxic cells [unpublished results). Thus, depletion of ODC activity under hypoxic conditions may be specific and not the result of an overall decrease in protein synthesis.

Our results suggest that the cytotoxicity produced by BCNU will be greater in hypoxic regions of a tumor after treatment with BG than in oxic regions of a tumor with or without pretreatment with BG. Pretreatment with BG did not affect polyamine levels, indicating that the mechanism by which BG enhances cytotoxicity is apparently not related to polyamines.

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