Effect of O\(^6\)-BenzyIguanine Analogues on Sensitivity of Human Tumor Cells to the Cytotoxic Effects of Alkylating Agents\(^1\)

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ABSTRACT

The effect of 0\(^6\)-benzyIguanine, O\(^6\)-(p-chlorobenzyI)guanine, and O\(^6\)-(p-methylbenzyI)guanine on the sensitivity of various human tumor cell lines to alkylating agents is evaluated. The sensitivity of human colon tumor cells, HT29, to the chloroethyIating agents, 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cycIohexyl-1-nitrosourea, 2-chloroethyl(methyIsulfonyl) methanesulfonate (clomeseIone), and chlorozotocin was increased by pretreatment for 2 h with 25 \(\mu\)M of each analogue. O\(^6\)-BenzyIguanine was slightly more effective as a sensitizer in HT29 cells than the p-chlorobenzyI and p-methylbenzyI analogues. However, all analogues sensitized SF767 glioma cells to the cytotoxic effects of 1-(2-chloroethyl)-3-cycIohexyl-1-nitrosourea, 1,3-bis(2-chloroethyl)-1-nitrosourea, and clomeseIone to the same degree. Both cell lines were sensitized to the methylation agents streptozotocin and 5-(3-methyl-1-triazeno)imidazol-4-carboxamide, the active intermediate of 5-(3,3-dimethyl-1-triazeno)imidazol-4-carboxamide, by pretreatment with 10 \(\mu\)M 0\(^6\)-benzyIguanine for 2 h. The number of Raji cells surviving 50 \(\mu\)M clomeseIone decreased 3-fold upon pretreatment for 2 h with 1 \(\mu\)M O\(^6\)-benzyIguanine. The degree of enhancement was dependent on the amount of alkyltransferase protein present in cell lines. For example, HT29 cells (alkyltransferase activity, 381 fmol/mg protein) exhibited a greater degree of enhancement when treated with O\(^6\)-benzyIguanine than SF767 (77 fmol/mg protein) and M19-MEL melanoma (36 fmol/mg protein) cells. There was no enhancement observed in mer\(^+\) cell lines, U251 (<2 fmol/mg protein), and BE (3 fmol/mg protein), or with alkylating agents which did not produce a cytotoxic lesion at the O\(^6\) position of guanine in DNA such as cisplatin or 4-hydroperoxycycIophosphamide. Our studies suggest that O\(^6\)-benzyIguanine analogues may have utility in mer\(^+\) tumors as an adjuvant to a variety of alkylating agents which produce a toxic lesion at the O\(^6\) position of guanine.

INTRODUCTION

Alkylating antitumor drugs produce their cytotoxic effect through a covalent reaction with cellular macromolecules (1–3). These drugs are used singly, in combination with other drugs, or in high dose protocols with bone marrow transplantation. Some alkylating antitumor agents require metabolic activation (procarbazine, DTIC\(^-\)), while others spontaneously form active alkylating species (BCNU, CCNU, streptozotocin) which react with DNA. DTIC, procarbazine, and streptozotocin methylate DNA, while BCNU, chlorozotocin, and clomeseIone chloroethylate DNA. In general, the dose-limiting toxicity of alkylating agents is bone marrow suppression, although animal studies with streptozotocin and chlorozotocin (nitrosourea derivatives of glucosamine) indicate that these agents do not exhibit this toxicity (1, 4, 5). However, the dose-limiting toxicity of chlorozotocin in humans appears to be hematopoietic suppression (6).

The therapeutic effectiveness of alkylating agents can be severely limited by drug resistance which may be intrinsic or may develop after several courses of therapy. There are a variety of mechanisms which can contribute significantly to cellular resistance including decreased drug uptake, increased cellular thiol level, enzymatic detoxification mechanisms, and repair of lesions generated by alkylatIon (7–9). Since DNA appears to be the most critical target for alkylatIon, enzymes responsible for removal of DNA lesions act to protect cells from the cytotoxic effects of alkylatIing agents (9, 10). The presence of a DNA repair protein termed O\(^6\)-alkylguanine-DNA alkyltransferase protects cells against the effects of alkylatIon at the O\(^6\) position of guanine (7, 11, 12). Most human cell lines can be divided into two groups depending on their alkyltransferase levels. Cells with little or no alkyltransferase activity (termed mer\(^-\)) are more sensitive to the cytotoxic effects of alkylating agents, and cells which express the protein (termed mer\(^+\)) exhibit resistance to these effects (7, 11–13).

Recently, we demonstrated that exposure to O\(^6\)-benzyIguanine can effectively deplete cells, tissues, and tumors of alkyltransferase activity and enhance the therapeutic effectiveness of certain antitumor drugs (14, 15). We have extended these studies and describe here the effect of treating a number of human tumor cell lines including glioma, colon, melanoma, and lymphoma cell lines with O\(^6\)-benzyIguanine, O\(^6\)-(p-methylbenzyI)guanine, or O\(^6\)-(p-chlorobenzyI)guanine prior to treatment with chemotherapeutic alkylatIing agents. The alkylating agents examined include compounds that exert their cytotoxic effect through reaction at the O\(^6\) and N7 position of DNA guanine residues, for example, chloroethylnitrosoureas and methylnitrosoureas, as well as alkylating agents that react mainly at the 7 position of guanine residues, such as cisplatin and 4-hydroperoxycycIophosphamide.

MATERIALS AND METHODS

Chemicals and Drugs. BCNU (NSC 409962), CCNU (NSC 79037), clomeseIone (NSC 338947), methyl-CCNU (NSC 95441), streptozotocin (NSC 85998), and chlorozotocin (NSC 178248) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 4-HydroperoxycycIophosphamide was a gift from Dr. M. Colvin, Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, MD. MTIC was a gift from Dr. Neil Gibson, Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA. O\(^6\)-BenzyIguanine, O\(^6\)-(p-chlorobenzyI)guanine, and 7-benzyIguanine were synthesized as described previously (14, 16). MTIC and cisplatin were purchased from Sigma Chemical Co., St. Louis, MO. Other biochemicals were purchased from Bio-Rad Laboratories, Richmond, CA.

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3 The abbreviations used are: DTIC, 5-(3,3-dimethyl-1-triazeno)imidazol-4-carboxamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; alkyltransferase, O\(^6\)-alkylguanine-DNA alkyltransferase; MTIC, 5-(3-methyl-1-triazeno)imidazol-4-carboxamide; BCNU, 1,3-bis((2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cycIohexyl-1-nitrosourea; cisplatin, cis-diammine dichloride.
Cell Culture. Both human colon carcinoma cell lines, HT29 and BE, were obtained from Dr. L. C. Erickson, Stritch School of Medicine, Loyola University, Maywood, IL, and were maintained as described previously (14). The human glioma cell line, SF767, was a gift from Dr. M. E. Berens, Barrow Neurological Institute, Phoenix, AZ. Another glioma cell line, U251, was obtained from Jan Ponten, Department of Pathology, University of Uppsala, Uppsala, Sweden. Glioma cell lines were maintained as described for SF767 cells (15). Raji lymphoma cells were obtained from Dr. F. Rapp, Pennsylvania State University, College of Medicine, Hershey, PA, and maintained as described before (17). M19-MEL and LO2 melanoma cells were received from the Tumor Repository of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Cell cultures were grown at 37°C in 5% CO2/95% humidified air.

HT29 cells plated at a density of 2.5 x 10⁶ cells/T150 flask were allowed to grow for 3 days prior to treatment with 10 μM O6-benzylguanine for 30 min. At various times after addition of fresh medium, cells were harvested and stored at -80°C until alkyltransferase activity measurements were performed. Raji lymphoma cells at a density of 5 x 10⁵ cells/ml were grown in T150 flasks for 2 days prior to exposure to increasing concentrations of O6-benzylguanine for 4 h. Cells were pelleted and assayed for alkyltransferase.

Cytotoxicity Assay. Cytotoxicity was evaluated using modifications of the MTT assay as described previously (18). Briefly, cells were plated at a density of 500 cells/well in 96-well plates and allowed to grow for 24 h. Two h prior to drug addition, O6-alkylguanine was added to give a final concentration of 10 or 25 μM O6-benzylguanine. O6-β-propylguanine. O6-β-methylbenzyl)guanine, or O6-β-chlorobenzylguanine. A series of drug dilutions were prepared such that the addition of 20 μl of drug solution to cells resulted in final concentrations between 0 and 2 μM. After 2 h, medium was replaced with fresh medium or medium containing the same concentration of O6-benzylguanine analogue for an additional 16 h, at which time all medium was replaced with fresh medium. Five days after drug treatment, 25 μl of a 5 mg/ml solution of MTT was added to each well, followed 2 h later by the addition of 100 μl lysis buffer, pH 4.7 (20% sodium dodecyl sulfate, 50% N,N-dimethylformamide, and 0.8% acetic acid) for an additional 22 h. A microplate reader set at 570 nm was used to determine the absorbance. Results were expressed as the fraction of the absorbance determined for cells treated with drug compared to those treated with vehicle.

Raji lymphoma cells at a density of 1.4 x 10⁶ cells/ml were preincubated for 2 h with 1 μM O6-benzylguanine and subsequently treated with concentrations of clomiphen or CCNU between 0 and 100 μM while remaining in the same medium. After 2 h at 37°C, cells were diluted 20-fold and allowed to grow for 4 days. Cytotoxicity was...
determined by inhibition of cell growth as determined on a Coulter Counter.

Assay of Alkyltransferase Activity. The assay for alkyltransferase activity was performed as previously described (14, 19). Alkyltransferase activity was measured as removal of $O^6$-$[^3]H$-methylguanine from a $[^3]H$-methylated DNA substrate (21.5 C/mmole). The extracts were incubated with the substrate at 37°C for 30 min. The 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 (20). Protein was determined by the method of Bradford (21), and the results are expressed as fmol of $O^6$-methylguanine released from the DNA substrate/mg of protein.

RESULTS

Exposure of HT29 cells to 10 $\mu$M $O^6$-benzylguanine for 30 min and replacement with fresh medium led to a complete and rapid loss of alkyltransferase activity which remained below 10% of pretreatment levels for up to 16 h (Fig. 1). Previous data indicated that $O^4$-(p-chlorobenzyl)guanine and $O^6$-(p-methylbenzyl)guanine were as potent as $O^6$-benzylguanine at depleting alkyltransferase levels in cells at this concentration, although the regeneration rate with these analogues has not been determined (14).

The effect of pretreatment of HT29 cells with 25 $\mu$M $O^6$-benzylguanine, $O^6$-(p-chlorobenzyl)guanine, or $O^6$-(p-methylbenzyl)guanine for 2 h resulted in an enhancement of the sensitivity of cells to chlorozotocin, CCNU, BCNU, or clomoxone (Fig. 2). All three $O^6$-benzylguanine analogues were effective at increasing the sensitivity of HT29 cells to the cytotoxic effects of chloroethylating agents. These data are in agreement with our previous report that the sensitivity of HT29 cells to CCNU and clomoxone was enhanced by pretreatment with 10 $\mu$M $O^6$-benzylguanine as measured by the colony-forming assay (14). Exposure to $O^6$-benzylguanine or the analogues alone showed no toxic effects at the doses used in these experiments.

The sensitivity of human glioma cell lines, SF767 and U251, human glioma cell lines, and a human colon tumor cell line, BE, to clomoxone was also examined. Cells were treated with either vehicle (control) or 25 $\mu$M $O^6$-benzylguanine for 2 h prior to treatment with increasing concentrations of clomoxone (Fig. 3). The apparent lack of increase in toxicity of clomoxone upon pretreatment of U251 and BE cell lines with $O^6$-benzylguanine is due to a low intrinsic alkyltransferase activity. The enhancement ratios for cells treated with $O^6$-benzylguanine, $O^6$-(p-chlorobenzyl)guanine, and $O^6$-(p-methylbenzyl)guanine prior to chlorozotocin, CCNU, BCNU, and clomoxone are shown in Table 1. The enhancement ratio is the ratio of the percentage of cell survival without $O^6$-alkylguanine derivative pretreatment divided by the percentage of cell survival with $O^6$-alkylguanine pretreatment at a specific dose of alkylating agent.

Although no toxic effects at the doses used in these experiments. Cells with little or no alkyltransferase activity (mer$^-$) are already more sensitive to the alkylating agent and show little or no effect when treated.

Table 1. Enhancement ratio of alkylating agents pretreated with $O^6$-benzylguanine analogues

<table>
<thead>
<tr>
<th>Alkylating agent</th>
<th>Cell line</th>
<th>$O^6$-BenzyLguanine</th>
<th>$O^6$-(p-Chloro)BenzyLguanine</th>
<th>$O^6$-(p-Methyl)BenzyLguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorozotocin (100 $\mu$M)</td>
<td>HT29</td>
<td>10.0</td>
<td>3.1</td>
<td>4.2</td>
</tr>
<tr>
<td>CCNU (25 $\mu$M)</td>
<td>HT29</td>
<td>6.9</td>
<td>3.7</td>
<td>2.7</td>
</tr>
<tr>
<td>BCNU (38 $\mu$M)</td>
<td>SF767</td>
<td>1.9</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Clomoxone (100 $\mu$M)</td>
<td>HT29</td>
<td>10</td>
<td>7.6</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>SF767</td>
<td>4.3</td>
<td>5.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of $O^6$-benzylguanine on the sensitivity of human tumor cell lines to clomoxone. SF767 (A), U251 (B), and BE (C) cells were exposed to fresh medium containing vehicle (0.08% ethanol: $\Box$) or medium containing 25 $\mu$M $O^6$-benzylguanine ($\bullet$) for 2 h prior to the addition of increasing concentrations of clomoxone for 2 h. After 6 days, MTT analysis was performed to determine cell density. Each point, mean (bar, ±SD) of three determinations.
Table 2 Ratio of enhancement of BCNU and clomophone on human colon, melanoma, and brain tumor cell lines

Each cell line was assayed for alkyltransferase activity as described in Materials and Methods. Cells were treated with 25 μM O6-benzylguanine for 2 h prior to a 2-h incubation with the chloroethylating agent and the percentage survival relative to control was determined. The enhancement ratio was determined at the dose indicated for BCNU and clomophone.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Alkyltransferase activity (fmol/mg)</th>
<th>Enhancement ratio</th>
<th>BCNU (38 μM)</th>
<th>Clomophone (50 μM)</th>
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</thead>
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<tr>
<td>HT29 (colon)</td>
<td>381</td>
<td></td>
<td>4.0</td>
<td>3.3</td>
</tr>
<tr>
<td>SF767 (brain)</td>
<td>77</td>
<td></td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>M19-MEL (melanoma)</td>
<td>36</td>
<td></td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>BE (colon)</td>
<td>3</td>
<td></td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>U251 (brain)</td>
<td>&lt;2</td>
<td></td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>LOX (melanoma)</td>
<td>&lt;2</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of O6-benzylguanine on alkyltransferase activity and on the sensitivity of Raji cells to clomophone. Alkyltransferase activity was assayed in Raji cells after exposure to increasing concentrations of O6-benzylguanine for 4 h (A). Cells were pretreated with medium (C) or 1 μM O6-benzylguanine (M) for 2 h prior to clomophone (B). After 4 days of growth, cell number was determined by a Coulter Counter. Each point, mean (bar, ±SD) of three determinations.

with O6-benzylguanine prior to the alkylating agent (Fig. 3; Table 2).

Raji, a human lymphoma cell line which grows in suspension, was assayed for alkyltransferase activity after being incubated with increasing concentrations of O6-benzylguanine for 4 h (Fig. 4A). There was a dose-dependent decrease in the alkyltransferase activity in Raji cells with complete depletion observed at a concentration of 0.5 μM. Exposure of cells to 1 μM O6-benzylguanine for 2 h prior to increasing concentrations of clomophone for 2 h led to a greater sensitivity of cells to clomophone (Fig. 4B). This effect was also observed for CCNU (data not shown).

The ability of O6-benzylguanine and analogues to sensitize cells to chemotherapeutic methylating agents was also determined. SF767 and HT29 cells were exposed to 10 μM O6-benzylguanine for 2 h, followed by concentrations of MTIC, the active intermediate of DTIC, between 0 and 400 μM (Fig. 5A). At 200 μM MTIC, the enhancement ratio was about 1.5-fold for both cell lines after treatment with O6-benzylguanine. A greater increase was observed by treating SF767 cells with O6-benzylguanine prior to streptozotocin, a methylnitrosourea derivative of glucosamine (Fig. 5B). The sensitivity of SF767 cells to the cytotoxic effects of streptozotocin was similarly increased with O6-(p-methylbenzyl)guanine as the pretreatment agent (data not shown). A greater sensitivity of HT29 cells to streptozotocin upon pretreatment with 25 μM O6-benzylguanine, O6-(p-methylbenzyl)guanine, or O6-(p-chlorobenzyl)guanine is shown in Fig. 5D.

The effect of O6-benzylguanine analogues on alkylating agents whose mechanism of cytotoxicity does not involve reaction at the O6 position of DNA guanine residues was also evaluated. The sensitivity of HT29 cells to two such agents, 4-hydroperoxycyclophosphamide, an analogue which is metabolized to the active intermediate of cyclophosphamide, and cisplatin was not enhanced by treatment with O6-benzylguanine (Fig. 6A and B). As expected, 7-benzylguanine which does not deplete cells of alkyltransferase activity (14), does not enhance the sensitivity of cells to BCNU (Fig. 6C), CCNU, or clomophone (data not shown).

DISCUSSION

Our results demonstrate that O6-benzylguanine, O6-(p-chlorobenzyl)guanine, and O6-(p-methylbenzyl)guanine can be used to enhance the cytotoxicity of chloroethylating and methylating agents which react at the O6 position of cellular DNA guanine residues. An analysis of several tumor cell lines including human brain, melanoma, lymphoma, and colon has illustrated a correlation between the extent of enhancement and the amount of alkyltransferase. Cell lines with high levels of alkyltransferase activity (mer+) exhibited a greater enhancement of cytotoxicity than cell lines with little or no alkyltransferase (mer-). This may be advantageous clinically if the tumor and corresponding tissues differ in their alkyltransferase activity. The dose-limiting toxicity of the chloroethylnitrosoureas is myelosuppression. However, since bone marrow cells have very low levels of alkyltransferase (22), O6-benzylguanine would not be expected to increase the bone marrow toxicity of these agents.

The presence of the alkyltransferase protects human tumor cells from the cytotoxic effects of chloroethylating agents such as chloroethylnitrosoureas and clomophones. The mechanism of protection involves the prevention of DNA interstrand cross-links by the chloroethylating agents or the removal of methyl groups from the O6 position of guanine formed by the methylating agents (7). Cross-links occur by intramolecular rearrangement of O6-chloroethylguanine residues to O6,N1-ethanoguanine residues in DNA, and these can react with the complementary strand of DNA to form 1-(3-deoxyxytidyl)-2-(1-deoxyguanosinyl)ethane (23–25). The alkyltransferase removes chloroethyl groups from the O6 position (26, 27) and/or it reacts with O6,N1-ethanoguanine (28) in DNA to form a cross-
Fig. 5. Enhancement of methylating agents by pretreatment with O*-benzylguanine in SF767 and HT29 cells. SF767 (A and B) and HT29 (C and D) cells were exposed to medium containing vehicle (0.08% ethanol; ○) or medium containing 10 μM O*-benzylguanine (■) for 2 h prior to the addition of increasing concentrations of MTIC (A and C) or streptozotocin (B). HT29 cells were exposed to vehicle (○), 25 μM O*-benzylguanine (♦), 25 μM O*-p-methylbenzylguanine (▵), or 25 μM O*-p-chlorobenzylguanine (■) for 2 h prior to the addition of increasing concentrations of streptozotocin (D). MTT assay was used to determine cell growth after 6 days. Each point, mean (bar, ±SD) of three determinations.

Fig. 6. Lack of enhancement of sensitivity to cisplatin or 4-hydroperoxycyclophosphamide (4-HC) by O*-benzylguanine and lack of enhancement to effects of BCNU by 7-benzylguanine. HT29 cells were treated with medium containing vehicle (0.08% ethanol; ○) or 10 μM O*-benzylguanine (■) for 2 h prior to the addition of increasing concentrations of 4-hydroperoxycyclophosphamide (A) or cisplatin (B). SF767 cells were treated with vehicle (○) or 25 μM 7-benzylguanine (■) for 2 h prior to increasing concentrations of BCNU for 2 h (C). Cell growth after 6 days was measured using the MTT assay. Each point, mean (bar, ±SD) of three determinations.

link between guanine and the repair protein. Disabling the alkyltransferase protein presumably increases the cytotoxic effects of the chloroethylating agents by allowing increased numbers of cross-links to form.

The observation that O*-benzylguanine and analogues enhance the cytotoxic effects of chemotherapeutic methylating agents is also encouraging, since these agents are used extensively in the treatment of melanomas and lymphomas (1, 29). The effectiveness of the directly acting methylating agents streptozotocin and MTIC was increased in both SF767 and HT29 cells. Other methylating agents which require metabolic activation to generate a methylating species, such as procarbazine, would be expected to exhibit enhanced activity. Administration of procarbazine to nude mice bearing human brain tumor xenografts resulted in greater growth delay if the tumors were of the mer" phenotype (30). On the other hand, O*-methylguanine is a mutagenic lesion as well as a toxic lesion (31). The repair protein acts as an important defense mechanism against malignant transformation from exposure to methylating agents (32). Thus, it is unlikely that depletion of the alkyltransferase will result in enhancement of the cytotoxicity without an enhancement of the mutagenicity of these agents. The persistence of procarcinogenic lesions in the DNA of patients may increase the likelihood of secondary tumor development.

Not surprising was the lack of enhancement of the alkylating agents, cisplatin or 4-hydroperoxycyclophosphamide. The mechanism of toxicity of these drugs involves alkylation at the
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