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Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents

(DNA repair/nitrosoureas/chemotherapy)

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ABSTRACT **O^6-Alkylguanine-DNA alkyltransferase was rapidly and irreversibly inactivated by exposure to O^6-benzylguanine or the *p*-chlorobenzyl and *p*-methylbenzyl analogues. This inactivation was much more rapid than with O^6-methylguanine: incubation with 2.5 μM O^6-benzylguanine led to more than a 90% loss of activity within 10 min, whereas 0.2 mM O^6-methylguanine for 60 min was required for the same reduction. O^6-Benzylguanine was highly effective in depleting the alkyltransferase activity of cultured human colon tumor (HT29) cells. Complete loss of activity was produced within 15 min after addition of O^6-benzylguanine to the culture medium and a maximal effect was obtained with 5 μM. In contrast, at least 100 μM O^6-methylguanine for 4 hr was needed to get a maximal effect, and this reduced the alkyltransferase by only 80%. Pretreatment of HT29 cells with 10 μM O^6-benzylguanine for 2 hr led to a dramatic increase in the cytotoxicity produced by the chemotherapeutic agents 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) or 2-chloroethyl(methylsulfonyl)methanesulfonate (Clomesone). Administration of O^6-benzylguanine to mice at a dose of 10 mg/kg reduced alkyltransferase levels by more than 95% in both liver and kidney. These results indicate that depletion of the alkyltransferase by O^6-benzylguanine may be used to investigate the role of the DNA repair protein in carcinogenesis and mutagenesis and that this treatment may be valuable to increase the chemotherapeutic effectiveness of chloroethylating agents.**

The formation of adducts at the O^6 position of guanine by alkylating agents is widely believed to have the potential to initiate carcinogenesis or mutagenesis (1–3). Alkylation at this position also appears to be involved in the cytotoxicity of chloroethylating agents (4, 5). O^6-Methylguanine in DNA is repaired by the action of a protein which brings about a stoichiometric and covalent transfer of the methyl group to a cysteine moiety within the amino acid sequence of the protein (5–8). Although the rate of reaction decreases with the size of the adduct, this protein will also act at a significant rate on longer alkyl groups such as ethyl and *n*-propyl and can, therefore, be described as O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63) (9–11). Upon reaction, the alkyltransferase is inactivated. Therefore, the extent of repair which occurs in the cell is dependent on the initial amount of alkyltransferase. Cell types and tissues vary in their level of this repair protein (7, 8). There is an inverse correlation between the presence of this repair protein and the sensitivity of cells to the actions of alkylating agents, but the extent to which other factors and other DNA adducts not repaired by the alkyltransferase also play an important role is not well understood.

One approach to this problem is to design experimental methods to modulate the alkyltransferase activity and examine the consequences of such modulation on carcinogenesis, mutagenesis, and cytotoxicity. Several groups have been able to express the bacterial alkyltransferase in mammalian cells (12–15), and recently transgenic mice expressing this protein have also been developed (16, 17). These systems can be used to study the role of the alkyltransferase, but they have the disadvantage that *Escherichia coli* alkyltransferases differ significantly from the mammalian protein, particularly in their ability to act on O^4-methylthymine (18) and the relative rate of reaction with larger adducts (8, 11). Also, the extent of increased alkyltransferase activity in the transgenic animals is not particularly large. Another approach is to study the effects of the absence of alkyltransferase activity. This has been done by comparing human cell lines which lack alkyltransferase (termed mer$^-$ or mex$^-$) with cells which express the protein (mer$^+$ or mex$^+$) (reviewed in refs. 7 and 8). However, even with cells which have been converted to the mer$^+$ phenotype by transfection of mammalian DNA (19–21), it is by no means clear that the expression of alkyltransferase is the only difference between them and the mer$^-$ parental cells.

Attempts have also been made to deplete the alkyltransferase activity by the use of inhibitors. Such inhibitors, if potent and specific, not only would be useful for studies of the importance of the alkyltransferase in protection from the dangerous effects of alkylating agents but also they have considerable potential in chemotherapy since it appears that the tumoricidal effects of chloroethylating agents such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) are greater in cells lacking alkyltransferase and can be enhanced by reduction in alkyltransferase activity (4, 5). Streptozotocin, a methylating agent, is currently in clinical trials for the enhancement of chloroethylnitrosourea therapy of tumors (22, 23), but its usefulness may be limited due to the mutagenic and carcinogenic properties of such agents (1). Up to the present, the only compound available for this purpose which is not itself an alkylating agent is O^6-methylguanine (24, 25). We and others have demonstrated previously that the amount of active alkyltransferase could be decreased by about 80% when cells were exposed to O^6-methylguanine (25,

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Abbreviations: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; +ve FAB MS, positive ion fast atom bombardment mass spectra.

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26). This resulted in an increase in cell responsiveness to chemotherapeutic chloroethylating agents (27–30). Specifically, O^6-methylguanine acts as a weak substrate for the alkyltransferase protein, thereby leaving less alkyltransferase available for the repair of cytotoxic lesions introduced by chloroethylating agents (25, 28, 30). This agent was also useful in reducing the alkyltransferase content in tissues and human colon tumor xenografts in athymic mice (31, 32). However, the clinical potential of O^6-methylguanine is limited due to the high doses of drug and long exposure periods required and to the fact that even under these conditions there is only a partial depletion in alkyltransferase activity.

Through consideration of the chemical literature on the influence of the structure on the rates of displacement reactions, which indicates that benzyl groups are more easily displaced in bimolecular displacement reactions than methyl groups (33–35), we anticipated that the alkyltransferase may have increased affinity for O^6-benzylguanine. To test this we examined the effectiveness of O^6-benzyl-, O^6-(*p*-chlorobenzyl)-, and O^6-(*p*-methylbenzyl)guanine as depleters of alkyltransferase activity in mammalian cells, cell-free extracts, and mammalian tissues. We report here that these agents are potent inactivators of the alkyltransferase and that they can be used in this capacity to markedly enhance the cytotoxicity of chloroethylating agents toward human colon tumor HT29 cells.

MATERIALS AND METHODS

Analytical Methods. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian XL 200 instrument interfaced to an Advanced (Palo Alto, CA) data system. Samples were dissolved in completely deuterated dimethyl sulfoxide with tetramethylsilane as internal standard. Positive ion (+ve) fast atom bombardment (FAB) mass spectra (MS) were obtained with a reversed geometry VG Micromass ZAB-2F spectrometer interfaced to a VG 2035 data system. A mixture of dithiothreitol and dithioerythritol (1:1) was used as FAB matrix.

Chemicals and Drugs. CCNU (NSC 79037) and 2-chloroethyl(methylsulfonyl)methanesulfonate (Clomesone; NSC 33847) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. [^{3}H]Methylnitrosourea (5.6 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. All other chemicals were purchased from Fisher, Calbiochem, Sigma, or Aldrich.

The preparation of oligodeoxynucleotides containing O^6-substituted guanines has been reported previously (36). O^6-Benzylguanine (37, 38), O^6-(*p*-chlorobenzyl)guanine, and O^6-(*p*-methylbenzyl)guanine were prepared by treating 2-amino-6-chloropurine (0.018 mol) with 2.2 equivalents of sodium benzyloxide, sodium 4-chlorobenzyloxide, or sodium 4-methylbenzyloxide in 30 g of benzyl, 4-chlorobenzyl, or 4-methylbenzyl alcohol, respectively, at 130°C for 6 hr. The resulting suspensions were cooled to room temperature, treated with 2.5 ml of glacial acetic acid with stirring, and then poured into 1 liter of diethyl ether with vigorous stirring. The precipitated solids were collected by filtration and were crystallized from either H_2O or ethanol/H_2O (4:5, vol/vol) with decolorizing carbon as necessary to afford 50% yields of the respective O^6-benzylated guanine. O^6-(*p*-Chlorobenzyl)-guanine: UV, λ_{max} (pH 7) 239,283; ^{1}H NMR, δ5.48 (s, 2, ArCH_2), 6.29 (br s, 2, NH_2, exchange with ^2H_2O), 7.50 (dd, 4, Ar), 7.83 (s, 1, 8-*H*), 12.43 (br s, 1, 9-*H*, exchanges with ^2H_2O); +ve FAB MS, *m/z* 276 ([M + H]$^+$), 152 ([guanine + H]$^+$), 125 ([C_7H_6Cl]$^+$). *Analysis.* Calculated for $C_{12}H_{10}N_5OCl\cdot\frac{1}{2}H_2O$: C, 50.62; H, 3.89; N, 24.60. Found: C, 51.02; H, 3.82; N, 25.15. O^6-(*p*-Methylbenzyl)guanine: UV, λ_{max} (pH 7) 241,283; ^{1}H NMR, δ2.31 (s, 3, ArCH_3), 5.43 (s, 2, ArCH_2), 6.28 (br s, 2, NH_2) 7.30 (dd, 4, Ar), 7.80 (s, 1, 8-*H*), 12.41 (br s, 1, 9-*H*, exchanges with ^2H_2O); +ve FAB MS, *m/z* 256 ([M + H]$^+$), 152 ([guanine + H]$^+$), 105 ([C_8H_9]$^+$). *Analysis.* Calculated for $C_{13}H_{13}N_5O$: C, 61.17; H, 5.13; N, 27.43. Found: C, 61.05; H, 5.13; N, 27.14.

Cell Culture. The human colon carcinoma line HT29 (originally obtained from Leonard C. Erickson, Department of Medicine, Loyola University of Chicago, Maywood, IL) was maintained in Dulbecco's modified Eagle's medium containing 36 mM $NaHCO_3$ supplemented with 10% fetal bovine serum, gentamycin (50 μg/ml), and 3% glutamine in a humidified atmosphere of 5% CO_2 at 37°C. Cells were seeded weekly at 2.5×10^5 cells per 75-cm^2 flask.

Three days prior to treatment, HT29 cells were plated at a density of 5×10^6 cells per 150-cm^2 flask. After growth for 2–3 days, the medium on cells was replaced with fresh medium or medium containing O^6-alkylguanine derivatives. The treatment conditions such as concentration and time of exposure are indicated in the figure legends. The cells were then harvested, collected by centrifugation at $1000 \times g$ in medium, and washed with ice-cold 0.01 M phosphate-buffered saline (PBS) and the cell pellets were stored at −80°C until assaying for alkyltransferase. Extracts were prepared as described previously (39).

Cell colony-forming efficiency was determined by plating HT29 cells at a density of 1×10^6 cells per 25-cm^2 flask 24 hr prior to replacing medium with fresh medium or medium containing 10 μM O^6-benzylguanine for 2 hr. Cells were then treated with various concentrations of chloroethylating agent for 2 hr at 37°C. The medium was then replaced with the same medium as that used for the pretreatment for an additional 18 hr, and the cells were replated at densities between 100 and 3200 per 25-cm^2 flask. The cells were allowed to grow for 10–12 days and colonies were then washed with 0.9% saline, stained with 0.5% crystal violet in ethanol, and counted.

Alkyltransferase Assay and Inactivation. Crude extracts from cells or tissues for alkyltransferase assay were prepared as described previously (39, 40). Extracts containing alkyltransferase were incubated with various concentrations of O^6-benzylguanine or O^6-methylguanine as indicated. The possible reversal of inactivation was investigated by dialysis against 50 mM Tris·HCl, pH 7.5/0.1 mM EDTA/5 mM dithiothreitol for 16 hr following incubation of 25 μM O^6-benzylguanine in HT29 extract for 1 hr at 37°C. Alkyltransferase activity was measured as removal of O^6-[^{3}H]methylguanine from a [^{3}H]methylated DNA substrate which was prepared by allowing [^{3}H]methylnitrosourea to react with calf thymus DNA. The extracts were incubated with [^{3}H]methylated DNA 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 hydrolyzed in 0.1 M HCl at 70°C for 30 min (39). The modified bases were separated by reverse-phase HPLC (27). Protein was determined by the method of Bradford (41), and the results are expressed as fmol of O^6-methylguanine released from the DNA per mg of protein.

RESULTS

We and others demonstrated previously that O^6-methylguanine is a weak substrate for the alkyltransferase, leading to its inactivation on prolonged incubation (25, 26). Since benzyl groups are usually more readily displaced in bimolecular displacement reactions than methyl groups (33–35), we decided to test whether O^6-benzylguanine might be an effective substrate and hence a more efficient inactivator of the alkyltransferase. When alkyltransferase from human HT29 cells was incubated with 20 nM of an oligodeoxynucleotide duplex [5′-d(GTGGGCGCTGa^{6}GAGGCG)-3′ with its complement (36), in which a^{6}G represents either O^6-methylgua-

nine or O^6-benzylguanine] for 20 min, all alkyltransferase activity was lost (results not shown). Since such oligodeoxynucleotides containing O^6-methylguanine are known to be good substrates for the alkyltransferase (42–44), the rapid loss of activity in response to the oligodeoxynucleotides containing O^6-benzylguanine provides support for the hypothesis that this adduct might be a good substrate for the mammalian alkyltransferase. This is further supported by the results of experiments in which HT29 cell extracts were incubated with 0.25 μM O^6-benzylguanine for 30 min. This led to a loss of 55% of the alkyltransferase activity. Exposure to 2.5 μM O^6-benzylguanine or higher concentrations completely inactivated the protein within 60 min (Fig. 1). The loss of alkyltransferase activity was irreversible. Dialysis for 16 hr at 4°C of extracts inactivated by reaction with 25 μM O^6-benzylguanine led to no restoration of activity. The related compounds O^6-(*p*-chlorobenzyl)guanine and O^6-(*p*-methylbenzyl)guanine were as active in reducing alkyltransferase as O^6-benzylguanine itself but 7-benzylguanine had no inhibitory effect, and, in agreement with previous reports (25, 28), O^6-methylguanine was only weakly active, with only a 56% reduction after exposure to 200 μM for 60 min. Inactivation of the alkyltransferase by O^6-benzylguanine and the *para*-substituted analogues was rapid, with 50% of the activity lost within 10 min of exposure to 0.5 μM and 90% of the activity lost within 10 min of incubation with 2.5 μM solutions (Fig. 1). It can also be seen in Fig. 1 that the presence of 2.5 μM O^6-benzylguanine in the assay medium decreased the measured alkyltransferase activity by 57%. This inhibition, which occurred in the absence of any preincubation of the alkyltransferase with the drug, indicates that O^6-benzylguanine is able to inactivate the alkyltransferase even in the presence of the methylated DNA substrate.

Exposure of HT29 cells to O^6-benzylguanine led to the efficient depletion of alkyltransferase activity (Fig. 2). Complete loss of activity was seen in response to addition of 5 μM or higher concentrations of this compound to the culture medium for 4 hr, while 100 μM concentrations of O^6-methylguanine were needed to achieve a maximal response, which was only an 80% reduction in activity (Fig. 2*A*). As illustrated in Fig. 2*B*, the depletion was dose dependent in the range of 0–2.5 μM for the three O^6-benzylguanine derivatives, while 7-benzylguanine exhibited no effect. This latter observation together with the data from cell extracts described above establishes that the inhibitory effect of the O^6-benzylguanines is the result of the attachment of the respective benzyl group to the O^6 position of the purine and is not a general result of exposure to any benzylated guanine. The data of Fig. 2*B* also indicate that at very low concentrations (i.e., 10–100 nM) the *p*-chlorobenzyl and *p*-methylbenzyl derivatives were somewhat more potent than O^6-benzylguanine, but at doses of 0.5 μM or higher all three compounds were equally active.

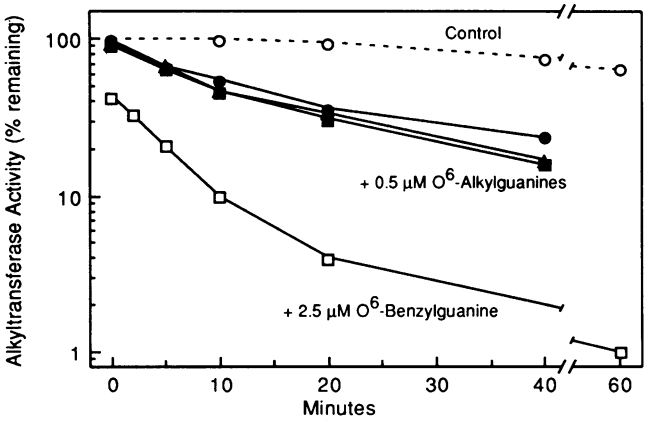

FIG. 1. Rate of inactivation of human alkyltransferase by O^6-benzylguanine and related compounds. The alkyltransferase from HT29 cells was incubated without addition (○) or with 2.5 μM O^6-benzylguanine (□), 0.5 μM O^6-benzylguanine (■), 0.5 μM O^6-(*p*-chlorobenzyl)guanine (▲), or 0.5 μM O^6-(*p*-methylbenzyl)guanine (●) in a total volume of 0.99 ml for the time shown and then the residual alkyltransferase activity was determined in a 30-min assay after the addition of 0.01 ml containing the [^{3}H]methylated DNA substrate. The alkyltransferase activity was then determined and expressed as the percentage remaining based on that present in untreated controls (approximately 300 fmol/mg of protein). Results are the mean values for two to four estimations, which agreed within ±10%.

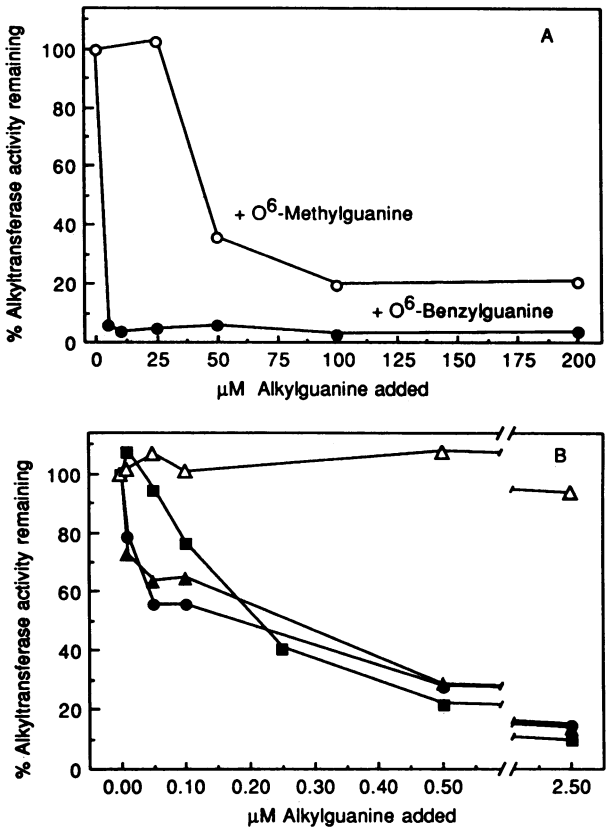

FIG. 2. Loss of alkyltransferase activity in HT29 cells exposed to O^6-benzylguanine. In the experiment shown in *A* the cells were exposed to O^6-benzylguanine or O^6-methylguanine at concentrations of 5–200 μM as shown for 4 hr. In the experiment shown in *B*, the cells were exposed to O^6-benzylguanine (■), O^6-(*p*-chlorobenzyl)-guanine (▲), O^6-(*p*-methylbenzyl)guanine (●), or 7-benzylguanine (△) at concentrations of 0.01–2.5 μM for 4 hr. The cells were then rinsed with PBS and extracts were prepared for alkyltransferase analysis. The results were expressed as a percentage of the alkyltransferase activity present at the time of addition of the drugs and indicate a representative experiment which was repeated several times with identical results.

The time course for alkyltransferase depletion in HT29 cells by both O^6-benzylguanine and O^6-methylguanine is illustrated in Fig. 3. The presence of O^6-benzylguanine at 100 μM in the culture medium caused a complete loss of alkyltransferase activity within 15 min and this was maintained over the entire 8-hr period studied. The identical exposure to O^6-methylguanine produced only an 80% reduction and the maximal effect required 4 hr of exposure (Fig. 3). The rate of recovery of alkyltransferase activity was analyzed in HT29 cells by changing the medium to remove the base after exposure to 10 μM O^6-benzylguanine or 400 μM O^6-methylguanine for 2 hr. The alkyltransferase activity remained below 4% of control values for up to 8 hr after the period of exposure to O^6-benzylguanine. In contrast, as

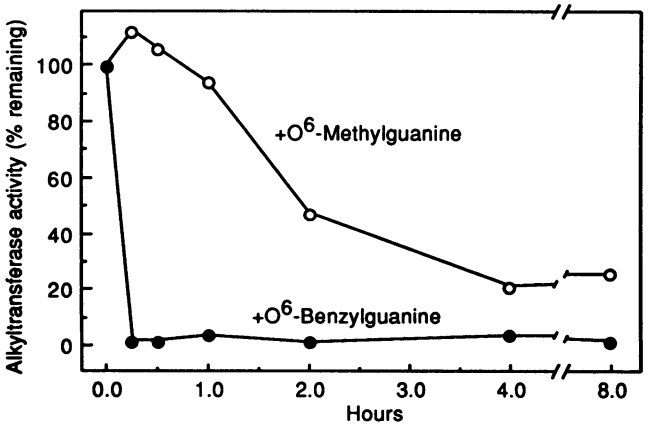

FIG. 3. Time course of the loss of alkyltransferase activity in HT29 cells. The cells were exposed to 100 μM O^6-benzylguanine (●) or O^6-methylguanine (○) for the time indicated. Other details were the same as in Fig. 2.

previously reported (25, 30), after the same time of exposure to O^6-methylguanine there was a steady rise in alkyltransferase activity which reached 34% at 8 hr. The rate of recovery of activity which occurs in this experiment after removal of the exogenous base reflects the synthesis rate of the alkyltransferase and the extent to which the residual O^6-guanine derivative remains in the washed cells. Although we cannot rule out the possibility that O^6-benzylguanine leads to a change in the rate of protein synthesis, the most likely explanation for the difference is that there is sufficient O^6-benzylguanine in the washed cells to continue to suppress the alkyltransferase activity.

The reduction of alkyltransferase in HT29 cells by exposure to 10 μM O^6-benzylguanine for 2 hr led to a very marked increase in the cytotoxicity of either Clomesone (Fig. 4*A*) or CCNU (Fig. 4*B*). The HT29 cells were very resistant to these drugs over the dose range used but after pretreatment with O^6-benzylguanine the cells were readily killed by them. Exposure to O^6-benzylguanine alone showed no toxic effects at doses below 100 μM for 24 hr.

To be a clinically or experimentally useful tool for manipulating alkyltransferase levels it is necessary that O^6-benzylguanine should be active *in vivo*. This was tested by administering the drug to CD-1 mice. Treatment by intraperitoneal injection at a dose of 10.2 mg/kg led to a more than 95% reduction in the alkyltransferase activity in the liver and kidney within 2 hr after injection. The effect was then slowly reversed over the next 15 hr. When O^6-benzylguanine was given at a dose of 126 mg/kg alkyltransferase activity was abolished for at least 24 hr (results not shown).

DISCUSSION

Our data demonstrate that O^6-benzyl-, O^6-(*p*-chlorobenzyl)-, or O^6-(*p*-methylbenzyl)guanine can be used successfully to deplete the alkyltransferase in mammalian cells and tissues. While the *para*-substituted derivatives appeared to be somewhat more effective than O^6-benzylguanine at low concentrations in HT29 cells, this may be due to an improved uptake of these compounds, since the rate at which they inactivated the alkyltransferase in HT29 cell extracts *in vitro* was virtually the same as that for the benzyl analogue. All of the O^6-benzylguanine derivatives were much more active than O^6-methylguanine (24–27) or other derivatives such as O^6-(*n*-butyl)guanine (25) in terms of the concentration needed to inactivate as well as the rate and completeness of alkyltransferase inactivation. It is well documented that the rate of repair of DNA adducts by the alkyltransferase decreases with

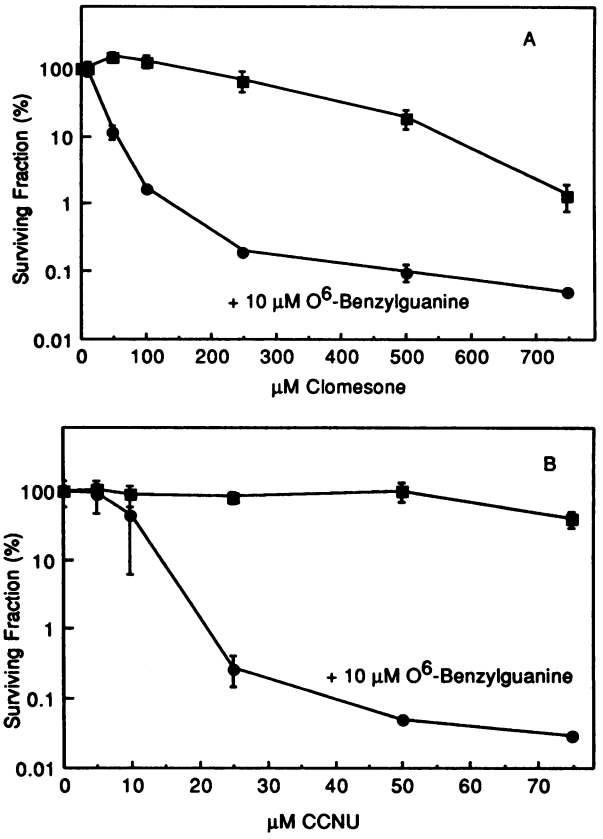

FIG. 4. Enhancement of cytotoxicity of chloroethylating agents by O^6-benzylguanine. Cultures of HT29 cells were exposed to fresh medium or medium containing 10 μM O^6-benzylguanine for 2 hr and then both sets were treated with the concentration shown of Clomesone (*A*) or CCNU (*B*) for 2 hr. Colony-forming efficiency is expressed as percent survival (relative to zero drug addition). Results are shown ±SD. Where no error bar is shown the variation was less than the size of the symbol.

the size of the alkyl group and that the bulkier branched chain products such as O^6-isobutylguanine react very slowly or not at all with the protein (6–8, 10, 11, 45, 46). This is consistent with the well-known effects of changes in alkyl group structure on the rates of bimolecular displacement reactions—i.e., the greater the bulk of the alkyl group the lower its rate of displacement, since the incoming nucleophile (e.g., sulfhydryl group) is sterically impeded from interacting favorably with the alkyl reaction center. The steric impediment to reaction rates posed by the benzene ring in a benzyl group is usually more than offset by the ability of the benzene ring to delocalize charge in the transition state for a displacement reaction. Consequently, the usual reactivity order for displacement reactions follows the sequence benzyl > methyl > ethyl > *n*-propyl > isobutyl (33–35). This is the same order we have observed for the alkyltransferase reactions. It is very likely that the mechanism by which O^6-benzylguanine inactivates the alkyltransferase involves its acting as a substrate for the reaction leading to the transfer of the benzyl group to the cysteine acceptor site on the alkyltransferase protein. In preliminary experiments, we have observed the production of labeled guanine when O^6-benzyl[^{3}H]guanine was incubated with the transferase. The ability of O^6-benzylguanine to act as a low molecular weight substrate for the alkyltransferase may prove useful for the design of convenient assay procedures for this activity.

Chemotherapeutic chloroethylating agents react with guanine residues in DNA to generate O^6-chloroethylguanine residues that can subsequently rearrange to O^6,N^1-etha-

noguanine and react with cytosines on the complementary strand, forming potentially lethal cross-links (47–51). The alkyltransferase reaction with either the O^6-chloroethylguanine (6, 47, 52) or O^6,N^1-ethanoguanine (5, 53) residues removes these vital cross-link precursors and thereby limits the therapeutic effectiveness of the drug. Pretreatment with O^6-methylguanine to deplete cells of alkyltransferase enhances the effectiveness of chloroethylating drugs towards cultured tumor cells (25–29). However, very large doses of this compound are needed to obtain alkyltransferase reductions *in vivo* (31, 32) and the limited extent of these reductions together with the poor solubility of O^6-methylguanine at the required doses offers little hope that this compound will be very useful for animal or clinical studies.

In contrast, our data suggest that O^6-benzylguanines might be more effective at depleting alkyltransferase levels than any agent studied so far. Even at low doses of the order of 10 mg/kg, O^6-benzylguanine completely depleted the alkyltransferase activity in mouse liver and kidneys. Although larger doses were required to achieve longer-lasting reductions, it should be noted that a transient decrease is all that is needed to enhance the effectiveness of chloroethylating agents. Furthermore, there were no obvious toxic effects of much larger doses which led to a long-lasting reduction in alkyltransferase. Treatment with O^6-benzylguanine may also be a useful way in which to evaluate the role of the alkyltransferase activity in protection against the mutagenic and carcinogenic actions of alkylating agents.

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