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# INCREASED KILLING OF PROSTATE, BREAST, COLON, AND LUNG TUMOR CELLS BY THE COMBINATION OF INACTIVATORS OF $O^6$-ALKYLGUANINE-DNA ALKYLTRANSFERASE AND *N,N'*-BIS(2-CHLOROETHYL)-*N*-NITROSOUREA

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**Abstract**—The ability of a number of compounds that act as inactivators of $O^6$-alkylguanine-DNA alkyltransferase (AGT) to sensitize human tumor cell lines to the effects of *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) were examined. The AGT inactivators tested included $O^6$-benzylguanine (BG) and its 8-aza-, 8-bromo-, 8-methyl-, 8-oxo, and 8-amino-derivatives and $O^6$-[*p*-(hydroxymethyl)benzyl]guanine. All of these compounds except the 8-amino-derivative were active in greatly increasing the killing of HT29 colon, Du145 prostate, MCF-7 breast and A549 lung tumor cells by BCNU. Their activities were comparable to those of BG. Two pyrimidines, 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine and 2,4-diamino-6-benzyloxy-5-nitropyrimidine, were found to be considerably more potent than BG in enhancing BCNU-induced cell killing. The addition of a steroid group to the 9-position of BG forming either $O^6$-benzyl-9-[3-oxo-4-androsten-17β-yloxycarbonyl)methyl]guanine or $O^6$-benzyl-9-[3-oxo-5α-androstan-17β-yloxycarbonyl)methyl]guanine also produced compounds effective in enhancing the cytotoxicity of BCNU when added at 10 μM. These results indicate that a range of potent compounds with potentially different pharmacokinetics is available to test the hypothesis that inactivation of AGT overcomes the resistance of many tumor cells to nitrosoureas.

*Key words:* $O^6$-alkylguanine-DNA alkyltransferase; $O^6$-benzylguanine; 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine; 2,4-diamino-6-benzyloxy-5-nitropyrimidine; *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; cancer chemotherapy

Chloroethylating agents including BCNU[‖] have been used for the treatment of malignant disease for a considerable period but have had only limited success [1–3]. Many tumors are resistant to killing by these agents. A significant factor in this resistance is the presence of the DNA repair protein AGT [4–7]. This protein repairs adducts in DNA at the $O^6$-position of guanine by transferring them to a cysteine residue located within the AGT protein sequence. The DNA is thus restored to its original state in a single step.

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‖ Abbreviations: BCNU, *N,N'*-bis(2-chloroethyl)*N*-nitrosourea; AGT, $O^6$-alkylguanine-DNA alkyltransferase (EC 2.1.1.63); BG, $O^6$-benzylguanine; 8-aza-BG, 8-aza-$O^6$-benzylguanine; 8-amino-BG, 8-amino-$O^6$-benzylguanine; 8-bromo-BG, $O^6$-benzyl-8-bromoguanine; 8-methyl-BG, $O^6$-benzyl-8-methylguanine; 8-oxo-BG, $O^6$-benzyl-7,8-dihydro-8-oxoguanine; AND-BG, $O^6$-benzyl-9-[3-oxo-4-androsten-17β-yloxycarbonyl)methyl]guanine; DHT-BG, $O^6$-benzyl-9-[3-oxo-5α-androstan-17β-yloxycarbonyl)methyl]guanine; HM-BG, $O^6$-[*p*-(hydroxymethyl)benzyl]guanine; 5-amino-BP, 2.4,5-triamino-6-benzyloxypyrimidine; 5-nitroso-BP, 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine; 5-nitro-BP, 2,4-diamino-6-benzyloxy-5-nitropyrimidine; and 4-desamino-5-nitro-BP, 2-amino-4-benzyloxy-5-nitropyrimidine.

The predominant pathway by which chloroethylating agents kill tumors cells is via the formation of 1-(3-cytosinyl)-2-(1-guanyl)ethane interstrand cross-links in cellular DNA [4, 8–10]. Such cross-links are very effective lesions for cell killing. However, the initial adduct formed in DNA leading to these cross-links is $O^6$-(2-chloroethyl)guanine. This adduct undergoes an intramolecular cyclization to form 1, $O^6$-ethanoguanine, which, in turn, reacts with the 3-position of cytosine in the complementary strand to form the cross-link. Cross-link production is prevented by the removal of the chloroethyl adduct from the $O^6$-position by the action of AGT prior to cross-link formation. Tumor cell lines having the $Mer^-$ phenotype fail to express AGT activity, and such lines are highly sensitive to the action of chloroethylnitrosoureas [4–9]. Unfortunately, the great majority of primary tumors are either $Mer^+$ and are resistant to these drugs or contain a significant fraction of $Mer^+$ cells that allows for the regrowth of a resistant tumor after treatment [9, 11, 12].

We discovered that BG can inactivate mammalian AGT and render $Mer^+$ cells sensitive to chloroethylating agents [13]. BG is a low molecular weight alternative substrate for the AGT. The action of AGT on BG results in the formation of guanine and *S*-benzylcysteine at the cysteine-acceptor site, which inactivates the protein [14]. Pretreatment with BG was found to greatly sensitize $Mer^+$ brain and colon tumor cell lines to killing by

BCNU and other chloroethylating agents *in vitro* and to improve the therapeutic index of BCNU when these tumors were grown as xenografts in nude mice [11–13, 15–19]. Clinical trials of this combination have just commenced. BG is clearly the most promising candidate drug for modulation of AGT activity presently available, but there are several reasons for attempting to find a more effective inactivator of AGT.

Possible problems with BG may result from: (a) its limited water solubility and rapid clearance by metabolism and excretion [19, 20]; (b) the facile production by point mutations of alterations in the human AGT protein sequence, which result in mutant proteins that are resistant to BG [21, 22]; and (c) the lack of selectivity towards inactivation of AGT in tumor cells as compared with normal cells. Furthermore, the value of the combination of BG with chloroethylating agents has only been tested in a limited number of tumor types (predominately brain or colon), even though this approach would be expected to have more general utility.

In attempts to provide improved AGT inactivators, we have synthesized more than 60 analogs of BG and measured their capacity to inactivate AGT from HT29 colon tumor cells [23–25]. In the present studies, some of the more active of these compounds have been examined for their abilities to sensitize a range of tumor cell lines to BCNU.

## MATERIALS AND METHODS

### *Materials*

The synthesis of all of the AGT inactivators used has been described previously [13, 23–25]. HT29 colon adenocarcinoma cells were provided by Dr. L. C. Erickson (Loyola University of Chicago, Maywood, IL). MCF-7 breast adenocarcinoma cells were obtained from Dr. A. Manni (Department of Medicine, Pennsylvania State University). The prostate adenocarcinoma cells (LNCaP, Du145 and PC-3) and A549 lung carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD.

### *Cell culture*

MCF-7 and Du145 cells were grown in MEM medium containing 36 mM $NaHCO_3$, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. HT29 cells were grown in Dulbecco's modified Eagle's medium containing 36 mM $NaHCO_3$ supplemented with 10% fetal bovine serum plus 3% glutamine and gentamycin (50 μg/mL). A549 cells were grown in Ham's F12 medium plus 3% glutamine, 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. LNCaP cells were grown in RPMI 1640 medium with 10% fetal bovine serum. Crude extracts containing AGT were prepared as previously described [26]. The AGT activity of the cells was then determined as described below and expressed as femtomoles of $O^6$-methylguanine removed per milligram of protein present in the cell extracts. Protein was determined by the method of Bradford [27].

### *Assay of loss of AGT activity*

The putative inactivators were added to the culture medium of HT29 cells at concentrations from 5 nM to 20 μM, and the cells were harvested 4 hr later. Extracts were prepared and AGT activity was determined as described by Dolan *et al.* [13] by incubation for 30 min at 37° of crude extract with a [$^3$H]methylated calf thymus DNA substrate. The substrate was made by reaction of DNA with *N*-[$^3$H]methyl-*N*-nitrosourea (Amersham Inc., Arlington Heights, IL). The extent of removal of $O^6$-methylguanine was calculated, and the results were expressed as the percentage of AGT activity remaining compared with that in untreated cells. The concentration of inhibitor required to produce 80% AGT inactivation was determined from plots of the extent of inactivation against the concentration of compound added.

To examine the persistence of AGT inactivation, either BG or DHT-BG was added at 2 μM to the medium of HT29 cell cultures. Sample dishes were harvested at 4 hr for measurement of the decrease in AGT activity. The drugs were removed from other dishes by removing the medium, washing the cells once with PBS at 37° and then replacing with fresh medium without drugs. Dishes were then harvested at various times for the next 23 hr and AGT levels were determined.

### *Killing of cells by BCNU*

Cell killing was determined using a colony-forming assay as previously described [28]. The cells were plated using $10^6$ cells/25 $cm^2$ flask and grown for 24 hr. After 2 hr of incubation with the AGT inactivator alone, 40 μM BCNU was added for 2 hr. The medium was replaced with fresh medium also containing the AGT inactivator, and the cells were left at 37° for an additional 18 hr. The cells were then replated at densities of 100–4000 cells in 5 mL medium (without inhibitors) in 25 $cm^2$ flasks and grown for 7–12 days until discrete colonies could be stained and counted. The colonies were washed with 0.9% saline, stained with crystal violet and counted.

## RESULTS

### *Loss of AGT activity in HT29 cells*

Several potent inactivators of AGT have been produced based on the lead compound BG [3, 23–25]. Compounds used in this report are illustrated in Fig. 1. These inhibitors can be divided into three classes: benzyloxypyrimidine derivatives related to 5-nitro-BP; BG derivatives with alterations at the 8-position; and other BG derivatives. Table 1 shows the concentrations of these compounds required to give 80% inactivation of the AGT in HT29 cells after a 4-hr exposure.

The most potent inactivators of the AGT were the 5-nitro- and the 5-nitroso-BP analogs for which only 50 nM was needed to produce an 80% reduction in the AGT activity in HT29 cells exposed for 4 hr. Removal of the amino group at the 4-position, forming 4-desamino-5-nitro-BP, reduced the activity slightly. This compound and the 8-aza- and 8-bromo-derivatives of BG were slightly more active than BG itself (150 nM needed for 80% inhibition compared with 200 nM), whereas the 8-methyl-, 8-oxo- and $O^6$-[*p*-(hydroxymethyl)benzyl]-guanine derivatives were slightly less active (300–400 nM). The other compounds tested, 5-amino-BP where the 5-nitro group is replaced by an amino group, BG derivatives with steroid groups attached to the 9-position and the 8-amino derivative of BG, were markedly less

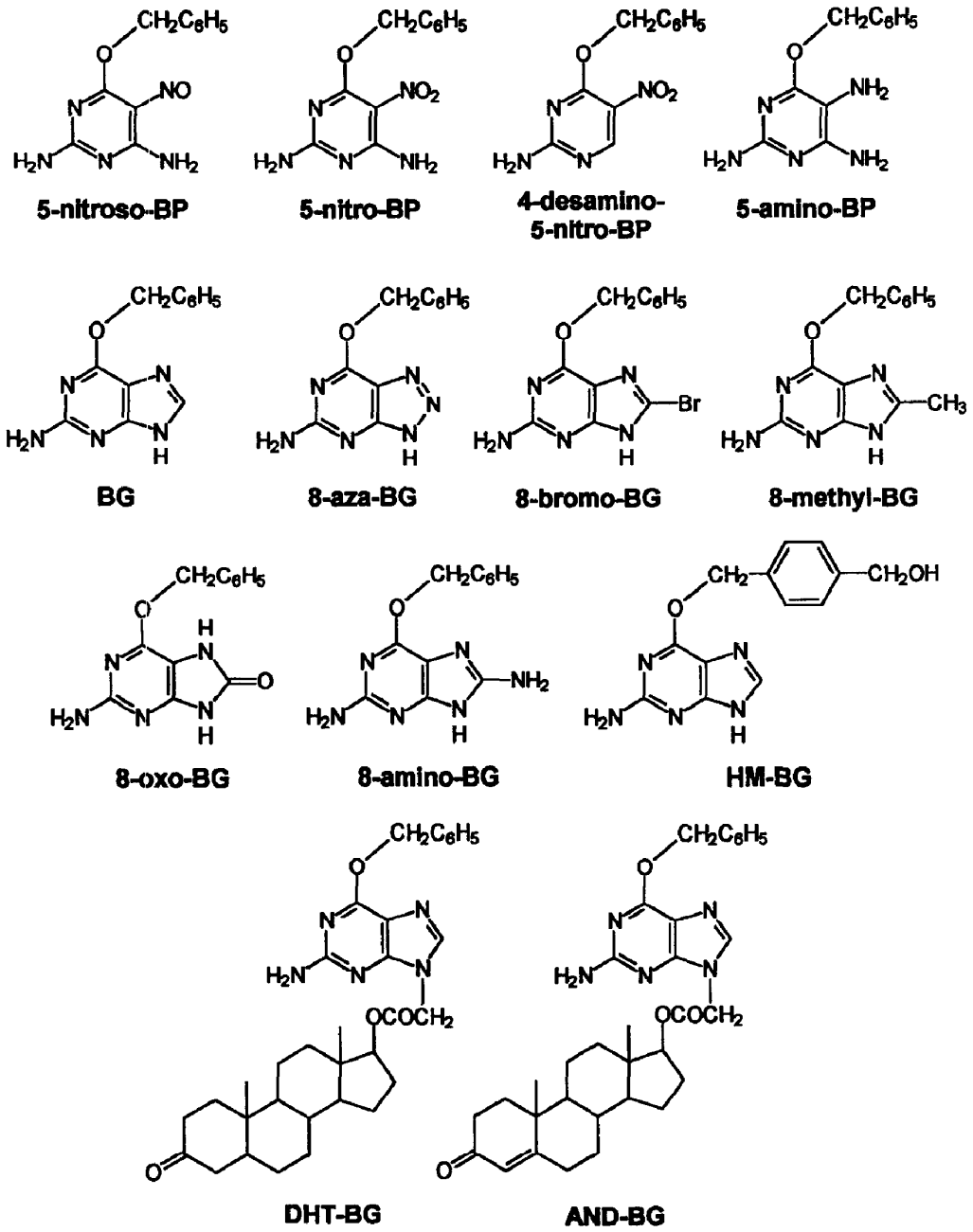


Fig. 1. Structures of AGT inactivators.

active than BG requiring 1–6 μM for 80% AGT inactivation (Table 1).

*Activity of AGT in tumor cell lines*

Measurements of the AGT activity in untreated HT29, Du145, MCF-7 and A549 cells confirmed that all of these cell lines were $Mer^+$. The AGT activities were 461 fmol/mg protein for HT29, 513 fmol/mg protein for Du145, 260 fmol/mg protein for MCF-7, and 318 fmol/mg protein for A549, respectively.

*Effect of 8-substituted derivatives of BG on killing by BCNU*

Both HT29 colon carcinoma cells and Du145 prostate carcinoma cells were, within the limits of experimental error, completely resistant to killing by 40 μM BCNU (Table 2). When pretreated with 10 μM BG, these cell lines were greatly sensitized to the effects of BCNU. All of the 8-substituted derivatives of BG also greatly sensitized the cells to BCNU when added at 10 μM except for the 8-amino derivative. There was no effect on cell survival of the BG derivatives alone. The effects of the active BG derivatives were only marginally apparent when the concentration was reduced to 1 μM, and there was no indication that any of the compounds were better than BG alone at this concentration.

MCF-7 breast tumor cells and A549 lung carcinoma cells were marginally sensitive to 40 μM BCNU with 15 and 30% loss in colony-forming efficiency, respectively (Table 3). When added at 10 μM, all of the 8-substituted

Table 1. Concentration of inhibitor needed to inactivate alkyltransferase by 80% in HT29 cells

| Compound* | Concentration producing 80% reduction in AGT (μM) |
|---|---|
| 2,4-Diamino-6-benzyloxy-5-nitrosopyrimidine (5-nitroso-BP) | 0.05 |
| 2,4-Diamino-6-benzyloxy-5-nitropyrimidine (5-nitro-BP) | 0.05 |
| 8-Aza-$O^6$-benzylguanine (8-aza-BG) | 0.15 |
| $O^6$-Benzyl-8-bromoguanine (8-bromo-BG) | 0.15 |
| 2-Amino-4-benzyloxy-5-nitropyrimidine (4-desamino-5-nitro-BP) | 0.15 |
| $O^6$-Benzylguanine (BG) | 0.20 |
| $O^6$-[*p*-(Hydroxymethyl)benzyl]guanine (HM-BG) | 0.30 |
| $O^6$-Benzyl-8-methylguanine (8-methyl-BG) | 0.30 |
| $O^6$-Benzyl-7,8-dihydro-8-oxoguanine (8-oxo-BG) | 0.40 |
| 2,4,5-Triamino-6-benzyloxyprimidine (5-amino-BP) | 1 |
| $O^6$-Benzyl-9-[(3-oxo-5α-androstan-17β-yloxycarbonyl)methyl]guanine (DHT-BG) | 2 |
| $O^6$-Benzyl-9-[(3-oxo-4-androsten-17β-yloxycarbonyl)methyl]guanine (AND-BG) | 2 |
| 8-Amino-$O^6$-benzylguanine (8-amino-BG) | 6 |

* The compounds shown were added to the culture medium of HT29 cells at various concentrations, and the cells were harvested 4 hr later.

derivatives of BG sensitized the cells to BCNU except for the 8-amino derivative, which had some effect on the MCF-7 cells but had no effect on A549. When added at 1 μM, all of the derivatives except 8-amino-BG also had a significant but not maximal effect, increasing killing up to 85–91% for MCF-7 and up to 87–95% for A549.

*Effects of other derivatives of BG on killing by BCNU*

HM-BG was active in increasing the effects of BCNU on MCF-7 or Du145 cells (Table 4), but 10 μM was needed to get a maximal effect and, at lower concentrations, it was somewhat less effective than BG itself (compare Tables 3 and 4).

Although the attachment of a steroidal function to the 9-position of BG decreased the AGT inactivation by a factor of about 10 when assayed in HT29 cells (Table 1 and Ref. 24), there was a possibility that these compounds might be retained or accumulated better in steroid-responsive cells. They were tested in the breast and prostate cell lines for their ability to enhance killing by BCNU. As shown in Table 4, both DHT-BG and AND-BG were effective in sensitizing MCF-7 breast and Du145 prostate cells to killing by BCNU when added at 10 μM, but were not active at 1 μM. They were, therefore, slightly less active than BG itself (compare Table 3 and Table 4).

The possibility of prolonged retention of DHT-BG was examined by exposing Du145 and HT29 cells to 2 μM or DHT-BG for 4 hr, and then removing the drug from the culture medium and following the return of AGT activity (Fig. 2). There was no difference in the rate of return of activity, which had reached 60–80% of starting values within 27 hr. Since DHT-BG is considerably less potent than BG (Table 1), the similar rate of return of AGT activity suggests that more DHT-BG than BG is available for AGT inactivation after removal of the drugs from the medium.

Table 2. Effects of 8-substituted benzylguanine derivatives on killing of colon and prostate tumor cells by BCNU

| | Colony formation/1000 cells plated | | | |
|---|---|---|---|---|
| | HT29 colon | | Du145 prostate | |
| Addition | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU |
| None | 435 ± 63 | 442 ± 34 | 453 ± 81 | 394 ± 76 |
| 10 μM BG | 435 ± 33 | 8 ± 4* | 462 ± 68 | 28 ± 5* |
| 10 μM 8-aza-BG | 537 ± 48 | 2 ± 1* | 452 ± 72 | 28 ± 5* |
| 10 μM 8-bromo-BG | 401 ± 22 | 1 ± 1* | 493 ± 90 | 16 ± 3* |
| 10 μM 8-oxo-BG | 413 ± 34 | <1* | 379 ± 34 | 34 ± 3* |
| 10 μM 8-methyl-BG | 513 ± 76 | <1* | 357 ± 43 | 50 ± 7* |
| 10 μM 8-amino-BG | 504 ± 30 | 430 ± 41 | 380 ± 36 | 295 ± 45† |
| 1 μM BG | ND‡ | 277 ± 25† | ND | 299 ± 18† |
| 1 μM 8-aza-BG | ND | 423 ± 42 | ND | 248 ± 21† |
| 1 μM 8-bromo-BG | ND | 299 ± 30† | ND | 267 ± 39† |
| 1 μM 8-oxo-BG | ND | 221 ± 15† | ND | 329 ± 43 |
| 1 μM 8-methyl-BG | ND | 230 ± 51† | ND | 306 ± 157 |
| 1 μM 8-amino-BG | ND | 475 ± 26 | ND | 435 ± 70 |

Results are the means ± SD for at least four estimations.
* Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.001$.
† Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.05$.
‡ ND, not determined.

Table 3. Effects of 8-substituted benzylguanine derivatives on killing of breast and lung tumor cells by BCNU

| | Colony formation/1000 cells plated | | | |
|---|---|---|---|---|
| | MDF-7 cells | | A549 cells | |
| Addition | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU |
| None | 426 ± 78 | 364 ± 60* | 630 ± 91 | 440 ± 98* |
| 10 μM BG | 455 ± 63 | 4 ± 2† | 654 ± 54 | 6 ± 1† |
| 10 μM 8-aza-BG | 483 ± 27 | 2 ± 1† | 688 ± 69 | 84 ± 8† |
| 10 μM 8-bromo-BG | 380 ± 109 | 3 ± 1† | 673 ± 43 | 2 ± 1† |
| 10 μM 8-oxo-BG | 522 ± 78 | 4 ± 2† | 650 ± 58 | 12 ± 2† |
| 10 μM 8-methyl-BG | 376 ± 76 | 2 ± 1† | 673 ± 31 | 4 ± 1† |
| 10 μM 8-amino-BG | 432 ± 36 | 95 ± 8† | 559 ± 74 | 539 ± 37 |
| 1 μM BG | ND‡ | 45 ± 14† | ND | 57 ± 15† |
| 1 μM 8-aza-BG | ND | 63 ± 6† | ND | 84 ± 8† |
| 1 μM 8-bromo-BG | ND | 38 ± 15† | ND | 29 ± 7† |
| 1 μM 8-oxo-BG | ND | 46 ± 7† | ND | 78 ± 16† |
| 1 μM 8-methyl-BG | ND | 54 ± 16† | ND | 44 ± 12† |
| 1 μM 8-amino-BG | ND | 373 ± 63 | ND | 495 ± 85 |

Results are the means ± SD for at least four estimations.
* Significantly different from no BCNU, $P < 0.05$.
† Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.001$.
‡ ND, not determined.

The ability of the BG derivatives containing steroids attached at the 9-position to inactivate AGT in different cell lines was examined by determining the amount of compound needed to inactivate their AGT by 80% within 4 hr of exposure in HT29, MCF-7, Du145, PC-3 and LNCaP cells. These results showed only small differences in the $EC_{80}$ values, which were in the range of 1.2 to 2.4 μM for AND-BG, 1.2 to 3.5 μM for DHT-BG and 0.2 to 0.4 μM for BG. The LNCaP cells, which express androgen receptors, had the highest value for DHT-BG.

*Effect of benzyloxypyrimidine derivatives on killing by BCNU*

When added at 10 μM, all of the benzyloxypyrimidine derivatives increased the killing by 40 μM BCNU in all of the cell lines tested. Even 5-amino-BP the least active of the compounds, led to an 87–99% reduction in the colony-forming efficiency when added at a concentration of 10 μM with 40 μM BCNU (Table 5). When the amount of compound was reduced to 1 μM, 5-amino-BP was inactive and 4-desamino-5-nitro-BP had only a partial effect (43–90% reduction), but 5-nitroso-BP and 5-nitro-BP were highly active (Table 5).

A more detailed comparison of the effects of 5-nitroso-BP and 5-nitro-BP with those of BG was carried out using the compounds over a range of 0.1 to 2.5 μM. The results shown in Fig. 3 indicate that both of these compounds were considerably more potent than BG in sensitizing all of the $Mer^+$ tumor cell lines tested to killing by 40 μM BCNU. The most active compound was 5-nitroso-BP, which produced a more than 90% reduction in colony-forming efficiency at 0.5 μM or less. The 5-nitro-BP was only slightly less efficient requiring about 1 μM or less, whereas BG required 2.5 μM for all the cells lines except Du145 where 5–10 μM was needed (Fig. 3 and Table 1).

Table 4. Effects of $O^6$-[*p*-(hydroxymethyl)benzyl]guanine and of steroid substitutions on $O^6$-benzylguanine on killing of breast and prostate tumor cells by BCNU

| | Colony formation/1000 cells plated | | | |
|---|---|---|---|---|
| | MCF-7 cells | | Du145 cells | |
| Addition | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU |
| None | 426 ± 78 | 364 ± 60 | 453 ± 81 | 394 ± 76 |
| 10 μM DHT-BG | 305 ± 53 | 21 ± 3* | 470 ± 114 | 79 ± 4* |
| 5 μM DHT-BG | ND† | 134 ± 45* | ND | 101 ± 48* |
| 1 μM DHT-BG | ND | 322 ± 63 | ND | 312 ± 90 |
| 10 μM AND-BG | 427 ± 129 | 6 ± 3* | 423 ± 43 | 95 ± 35* |
| 1 μM AND-BG | ND | 273 ± 68 | ND | 362 ± 39 |
| 10 μM HM-BG | 394 ± 40 | 3 ± 1* | 400 ± 64 | 35 ± 9* |
| 5 μM HM-BG | ND | 199 ± 58 | ND | 296 ± 73 |
| 1 μM HM-BG | ND | 392 ± 35 | ND | 384 ± 100 |

Results are the means ± SD for at least four estimations.
* Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.001$.
† ND, not determined.

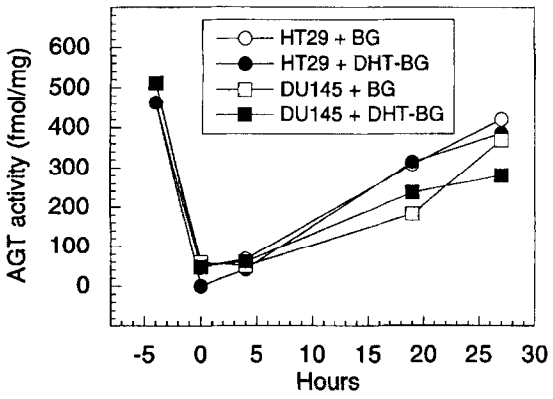


Fig. 2. Loss of AGT activity and subsequent recovery after exposure to BG and DHT-BG. Cultures of HT29 cells (○, ●) or Du145 cells (□, ■) were exposed to 2 μM BG (○, □) or 2 μM DHT-BG (●, ■) for 4 hr. At this time, indicated by 0 on the graph, the cells were then washed, the medium was replaced with fresh medium lacking the drug, and cultures were incubated at 37° for a further 27 hr. At the times shown, dishes were harvested, and the AGT activity was determined. Results are the means of triplicate observations.

## DISCUSSION

These results suggest that all of the potent AGT inactivators tested here have the potential to be used to increase the therapeutic index of chloroethylating agents. When present at 10 μM, all of the compounds tested except 8-amino-BG increased the cell killing by BCNU to more than 95%. Based on the pharmacokinetic data with BG in animals ([29, 30]; Roy SK, Gupta E, and Dolan ME, unpublished observations), plasma levels of 5–10 μM should be readily achievable. The fact that 8-oxo-BG was as active as BG itself in sensitizing tumor cells to killing by BCNU (Tables 2 and 3) is of particular interest since this compound is a major metabolite of BG [20].

The presence of the AGT is well known to be a major factor in resistance to chloroethylation [4–12]. In agreement with this, the HT29 and Du145 cell lines, which had the highest levels of AGT of the cells used in these experiments, were slightly more resistant to BCNU and required higher amounts of BG for full sensitization than the A549 and MCF-7 cells. This difference was less apparent when 5-nitro-BP and 5-nitroso-BP were used, but it is possible that these pyrimidines are metabolized differently between the cell types.

The two most active benzyloxypyrimidines (5-nitroso-BP and 5-nitro-BP) were significantly more potent than BG in increasing the cytotoxic effects of BCNU (Fig. 3), but, at present, little is known about their metabolism and disposition. However, even if these compounds are more rapidly degraded or excreted than BG, they may still have utility for purposes where tumor-selective delivery of the compound can be effected (e.g., intrathecal administration). An additional potential advantage of these compounds is that they are active at concentrations below 10 μM as inactivators of mutant forms of the human AGT that are resistant to BG (Crone TM, Moschel RC, and Pegg AE, unpublished observations).

The inactivation data shown in Table 1 is in good agreement with that previously published [23–25] where the data were presented in terms of the amount of compound needed to inactivate the AGT by 50%. There is a reasonably good correlation between these published $EC_{50}$ values, and the amount needed for 80% inactivation since the inactivation curves have similar shapes for all of the compounds tested. In general, AGT-inactivating potency could be used to predict the effectiveness of an agent in sensitizing cells to BCNU since the most potent AGT inactivators (5-nitroso-BP and 5-nitro-BP) were most active sensitizers and the least potent AGT inactivator (8-amino-BG) was only marginally active. However, smaller differences in AGT-inactivating potency, as exhibited by 8-aza-BG and 8-oxo-BG, were not reflected in differences in sensitization to BCNU.

The level of the AGT inactivators needed to achieve enhancement of the effects of BCNU was considerably greater than that needed for 80% inactivation of the AGT. These results indicate that virtually complete inactivation of the AGT is needed to bring about this sensitization. This finding is consistent with other studies using BG or its deoxyribonucleoside, which indicated that prolonged depletion of the AGT activity is needed to maximize the potentiation of the effects of BCNU [31, 32]. This suggests that it is necessary to inactivate vir-

Table 5. Effects of benzyloxpyrimidines on killing of tumor cells by BCNU

| | Colony formation/1000 cells plated | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HT29 cells | | Du145 cells | | A549 cells | | MCF-7 cells | |
| Addition | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU | No BCNU | +40 μM BCNU |
| None | 435 ± 63 | 442 ± 34 | 453 ± 81 | 394 ± 76 | 549 ± 74 | 440 ± 98 | 426 ± 78 | 364 ± 60 |
| 10 μM 5-amino-BP | 453 ± 59 | 3 ± 1* | 429 ± 101 | 57 ± 7* | 548 ± 37 | 33 ± 6* | 448 ± 12 | 12 ± 4* |
| 1 μM 5-amino-BP | ND† | 487 ± 3 | ND | 378 ± 60 | ND | 301 ± 36 | ND | 329 ± 27 |
| 1 μM 4-desamino-5-nitro-BP | 423 ± 43 | 250 ± 77‡ | 400 ± 64 | 192 ± 17‡ | 440 ± 98 | 57 ± 15* | 432 ± 36 | 124 ± 28* |
| 1 μM 5-nitroso-BP | 528 ± 64 | 19 ± 4* | 403 ± 35 | 25 ± 4* | 579 ± 63 | 4 ± 1* | 447 ± 87 | 4 ± 2* |
| 1 μM 5-nitro-BP | 428 ± 25 | 45 ± 4* | 407 ± 80 | 59 ± 6* | 485 ± 86 | <2* | 314 ± 69 | 11 ± 9* |

Results are the means ± SD for at least four estimations.
* Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.001$.
† ND, not determined.
‡ Significantly different from 40 μM BCNU with no AGT inactivator, $P < 0.05$.

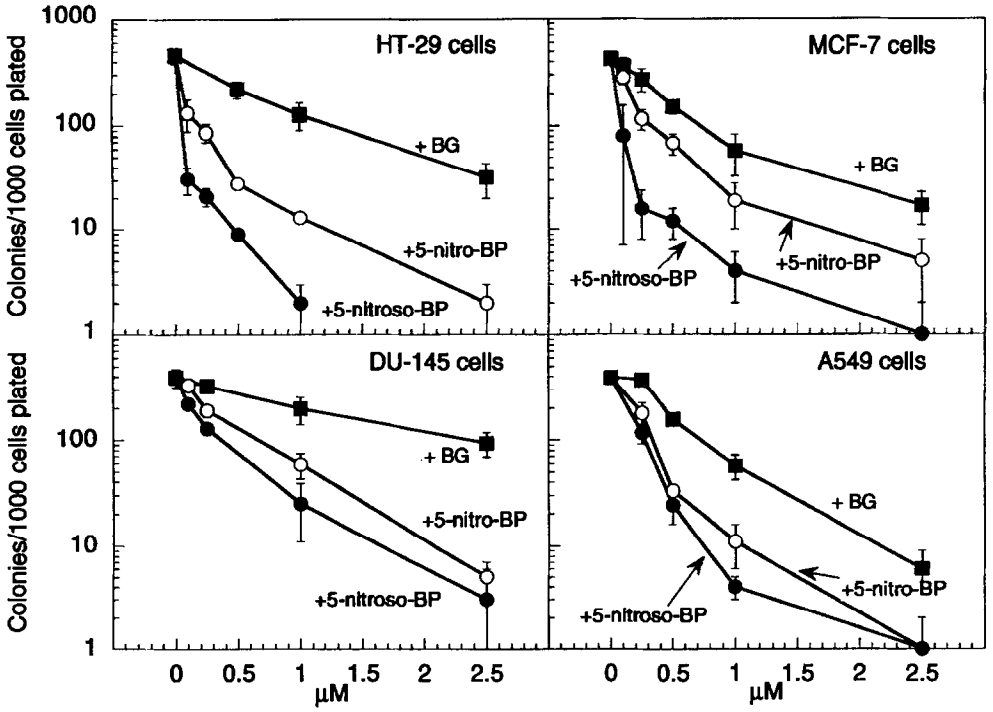


Fig. 3. Effects of different concentrations of BG, 5-nitro-BP and 5-nitroso-BP on killing of cells by BCNU. Cultures of HT29, MCF-7, Du145 and A549 cells were treated with BG (■), 5-nitro-BP (○) and 5-nitroso-BP (●) at the concentrations indicated for 2 hr, and the cells were then exposed to 40 μM BCNU for 2 hr. After a further period of 18 hr in the presence of the AGT inhibitors but not BCNU, the cells were replated at low densities, and colonies were determined. Results are the means ± SD for at least four estimations.

tually all of the cellular AGT activity prior to treatment with BCNU as well as to inactivate any AGT that is synthesized during the period in which the BCNU-derived AGT-sensitive adducts are present in the DNA. Since the $O^6$-(2-chloroethyl)guanine in DNA is likely to be a much better substrate for the AGT than BG or other inactivators, a higher concentration of the inactivator is needed to ensure that such newly synthesized AGT reacts with the inhibitor rather than repairs the DNA. The actual concentration of the inactivator required has to be determined by experiment, but it appears that about 10 to 20-fold higher concentrations than those producing 80% inactivation are needed for most of the compounds tested in our experiments. A possibly significant exception is the 9-steroidal derivatives where only a 5-fold higher concentration was required. This could be due to the better retention of these compounds in the cells after removal from the medium. As shown in Fig. 2, the recoveries of AGT activity after exposure to DHT-BG and BG were similar, even though DHT-BG is a less active inhibitor.

The better retention of DHT-BG is not likely to be due to the interaction with specific hormone receptors since it occurred in HT29 cells that are not androgen responsive and there was no clear difference related to the presence of androgen receptors in the AGT inactivation when a range of hormone-sensitive and -insensitive cells were treated with DHT-BG for 4 hr. The lack of interaction could be due to the site of attachment on the steroid, preventing recognition by receptors. A more likely explanation for the retention of the DHT-BG is that the highly hydrophobic nature of this derivative renders it very difficult to wash out completely from the cells by a simple medium change. The fact that HM-BG was less active in sensitizing cells to BCNU than expected from its ability to inactivate the AGT, requiring 50-fold higher levels, is consistent with this interpretation since it is the least hydrophobic of the compounds examined in this study.

Sensitization of BCNU by a number of AGT-inactivating agents occurred in HT29, Du145, A549 and MCF-7 human tumor cell lines tested and is likely to apply to all other $Mer^+$ tumor cells. Although our studies have been carried out only with BCNU, it is also very likely that the results shown here can be extended to all of the chloroethylating agents currently in use or under development as well as those methylating agents such as dacarbazine and temozolomide for which the mechanism of action involves modification of the $O^6$-position of DNA guanine residues [reviewed in Refs. 7, 11 and 12]. It remains to be seen whether the altered metabolic or pharmacokinetic properties of these AGT-inactivating compounds offer significant improvement over BG. The current Phase I trial of BG should allow a better understanding of the potential problems and advantages of BG as an AGT inactivator.

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## REFERENCES

1. Mitchell EP and Schein PS, Contribution of nitrosoureas to cancer treatment. *Cancer Treat Rep* **70:** 31–42, 1986.

2. Johnston TP and Montgomery JA, Relationships of structure to anticancer activity and toxicity of the nitrosoureas in animal systems. *Cancer Treat Rep* **70:** 13–30, 1986.
3. Colvin M and Chabner BA, Alkylating agents. In: *Cancer Chemotherapy: Principles and Practice* (Eds. Chabner BA and Collins JM), pp. 276–333. J. B. Lippincott, Philadelphia, 1990.
4. Brent TP, Isolation and purification of $O^6$-alkylguanine-DNA alkyltransferase from human leukemic cells. Prevention of chloroethylnitrosourea-induced cross-links by purified enzyme. *Pharmacol Ther* **31:** 121–140, 1985.
5. Pegg AE, Mammalian $O^6$-alkylguanine-DNA alkyltransferase: Regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* **50:** 6119–6129, 1990.
6. Mitra S and Kaina B, Regulation of repair of alkylation damage in mammalian genomes. *Prog Nucleic Acid Res Mol Biol* **44:** 109–142, 1993.
7. Pegg AE, Dolan ME and Moschel RC, Structure, function and inhibition of $O^6$-alkylguanine-DNA alkyltransferase. *Prog Nucleic Acid Res Mol Biol*, in press.
8. Ludlum DB, DNA alkylation by the haloethylnitrosoureas: Nature of modifications produced and their enzymatic repair or removal. *Mutat Res* **233:** 117–126, 1990.
9. Pegg AE and Byers TL, Repair of DNA containing $O^6$-alkylguanine. *FASEB J* **6:** 2302–2310, 1992.
10. Gonzaga PE, Potter PM, Niu T, Yu D, Ludlum DB, Rafferty JA, Margison GP and Brent TP, Identification of the cross-link between human $O^6$-methylguanine-DNA methyltransferase and chloroethylnitrosourea-treated DNA. *Cancer Res* **52:** 6052–6058, 1992.
11. Pegg AE, Dolan ME, Friedman HS and Moschel RC, Inhibition of $O^6$-alkylguanine-DNA alkyltransferase as a means to enhance the effectiveness of chemotherapeutic alkylating agents. *Proc Am Assoc Cancer Res* **34:** 565, 1993.
12. Gerson SL, Liu L, Phillips WP, Zaidi NH, Heist A, Markowitz S and Wilson JKV, Drug resistance mediated by DNA repair: The paradigm of $O^6$-alkylguanine DNA alkyltransferase. *Proc Am Assoc Cancer Res* **35:** 699–700, 1994.
13. Dolan ME, Moschel RC and Pegg AE, 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. *Proc Natl Acad Sci USA* **87:** 5368–5372, 1990.
14. Pegg AE, Boosalis M, Samson L, Moschel RC, Byers TL, Swenn K and Dolan ME, Mechanism of inactivation of human $O^6$-alkylguanine-DNA alkyltransferase by $O^6$-benzylguanine. *Biochemistry* **32:** 11998–12006, 1993.
15. Dolan ME, Mitchell RB, Mummert C, Moschel RC and Pegg AE, Effect of $O^6$-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res* **51:** 3367–3372, 1991.
16. Mitchell RB, Moschel RC and Dolan ME, Effect of $O^6$-benzylguanine on the sensitivity of human tumor xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea and on DNA interstrand cross-link formation. *Cancer Res* **52:** 1171–1175, 1992.
17. Friedman HS, Dolan ME, Moschel RC, Pegg AE, Felker GM, Rich J, Bigner DD and Schold SC Jr, Enhancement of nitrosourea activity in medulloblastoma and glioblastoma multiforme. *J Natl Cancer Inst* **84:** 1926–1931, 1992.
18. Gerson SL, Zborowska E, Norton K, Gordon NH and Willson JKV, Synergistic efficacy of $O^6$-benzylguanine and BCNU in a human colon cancer xenograft completely resistant to BCNU alone. *Biochem Pharmacol* **45:** 483–491, 1993.
19. Dolan ME, Pegg AE, Moschel RC, Vishnuvajjala BR, Flora KP, Grever MR and Friedman HS, Biodistribution of $O^6$-benzylguanine and its effectiveness against human brain tumor xenografts when given in polyethylene glycol or cremophor-EL. *Cancer Chemother Pharmacol* **35:** 121–126, 1994.
20. Dolan ME, Chae MY, Pegg AE, Mullen JH, Friedman HS and Moschel RC, Metabolism of $O^6$-benzylguanine, an inactivator of $O^6$-alkylguanine-DNA alkyltransferase. *Cancer Res* **54:** 5123–5130, 1994.
21. Crone TM and Pegg AE, A single amino acid change in human $O^6$-alkylguanine-DNA alkyltransferase decreasing sensitivity to inactivation by $O^6$-benzylguanine. *Cancer Res* **53:** 4750–4753, 1993.
22. Crone TM, Goodtzova K, Edara S and Pegg AE, Mutations in $O^6$-alkylguanine-DNA alkyltransferase imparting resistance to $O^6$-benzylguanine. *Cancer Res* **54:** 6221–6227, 1994.
23. Moschel RC, McDougall MG, Dolan ME, Stine L and Pegg AE, Structural features of substituted purine derivatives compatible with depletion of human $O^6$-alkylguanine-DNA alkyltransferase. *J Med Chem* **35:** 4486–4491, 1992.
24. Chae M-Y, McDougall MG, Dolan ME, Swenn K, Pegg AE and Moschel RC, Substituted $O^6$-benzylguanine derivatives and their inactivation of human $O^6$-alkylguanine-DNA alkyltransferase. *J Med Chem* **37:** 342–347, 1994.
25. Chae M-Y, Swenn K, Kanugula S, Dolan ME, Pegg AE and Moschel RC, 8-Substituted $O^6$-benzylguanine, substituted 6(4)-benzyloxypyrimidine and related derivatives as inactivators of human $O^6$-alkylguanine-DNA alkyltransferase. *J Med Chem* **38:** 359–365, 1995.
26. Domoradzki J, Pegg AE, Dolan ME, Maher VM and McCormick JJ, Correlation between $O^6$-methylguanine-DNA-methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Carcinogenesis* **5:** 1641–1647, 1984.
27. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
28. Dolan ME, Young GS and Pegg AE, Effect of $O^6$-alkylguanine pretreatment on the sensitivity of human colon tumor cells to the cytotoxic effects of chloroethylating agents. *Cancer Res* **46:** 4500–4504, 1986.
29. Berg SL, Godwin K and Balis FM, Pharmacokinetics and cerebrospinal fluid penetration of $O^6$-benzylguanine in the nonhuman primate. *Proc Am Assoc Cancer Res* **35:** 2543, 1994.
30. Rogers TS, Rodman LE, Tomaszewski JE, Osborn BE and Page JG, Preclinical toxicology and pharmacokinetic studies of $O^6$-benzylguanine (NSC-637037) in mice and dogs. *Proc Am Assoc Cancer Res* **35:** 1953, 1994.
31. Marathi UK, Kroes RA, Dolan ME and Erickson LC, Prolonged depletion of $O^6$-methylguanine DNA methyltransferase activity following exposure to $O^6$-benzylguanine with or without streptozotocin enhances 1,3-bis(2-chloroethyl)-1-nitrosourea sensitivity *in vitro*. *Cancer Res* **53:** 4281–4286, 1993.
32. Marathi UK, Dolan ME and Erickson LC, Extended depletion of $O^6$-methylguanine-DNA methyltransferase activity following $O^6$-benzyl-2'-deoxyguanosine or $O^6$-benzylguanine combined with streptozotocin treatment enhances 1,3-bis(2-chloroethyl)-1-nitrosourea cytotoxicity. *Cancer Res* **54:** 4371–4375, 1994.