Minireview

**O⁶-Benzylguanine and Its Role in Chemotherapy**¹

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Abstract

The presence of the DNA repair protein, O⁶-alkylguanine-DNA alkyltransferase (AGT) in tumor cells is a significant source of resistance to chemotherapeutic alkylnitrosoureas and alkyltriazenes. O⁶-Benzylguanine provides a means to effectively inactivate the AGT protein and increase the chemotherapeutic effectiveness of chloroethylnitrosoureas and methylnitrosoureas. O⁶-Benzylguanine has been proposed as a mechanism of resistance through: (a) conjugation to nitrosoureas, reducing DNA interstrand cross-links and cytotoxicity (28, 29); or (b) the glutathione-dependent denitrosation of nitrosoureas, a reaction preferentially catalyzed by one of the rat liver glutathione S-transferase μ enzymes (30). Although there have been some studies supporting the involvement of glutathione and related enzymes in nitrosourea resistance in certain cell lines (28–32), others have suggested that the role glutathione plays is minor at best (33, 34).

Because DNA is a critical target of alkylating agents, the repair of DNA has been considered an important mechanism of resistance to these agents. The DNA repair protein, AGT, plays a major role in the resistance to alkylating agents (27, 35–39). Although there is a vast amount of data demonstrating the role of AGT in protecting tumors from alkylating agent toxicity in cells and in animals, more recently studies evaluating AGT levels in patients receiving BCNU therapy have supported this concept. Following an evaluation of 226 high-grade astro-

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3 The abbreviations used are: BCNU, carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea; DTIC, 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide; AGT, O⁶-alkylguanine-DNA alkyltransferase; CSF, cerebrospinal fluid.
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with higher AGT activity tended to be less responsive to Tumors cytoma patients receiving BCNU therapy. Belanich et al. (40) reported that low AGT content in tumors correlates with better response to treatment and greater survival. Conversely, the presence of a subpopulation of cells in a tumor with elevated AGT is correlated with poor prognosis. In a separate study of 42 malignant gliomas, 7 samples (16.7%) had low AGT activity. Tumors with higher AGT activity tended to be less responsive to postoperative therapy, including chloroethylnitrosoureas (41).

Chloroethylnitrosoureas and methylating agents destroy tumor cells by reacting covalently with DNA. Methylating agents react with at least 12 nucleophilic sites on DNA, including the O6-position of guanine. Persistence of the O6-methylguanine lesion has been correlated with cytotoxicity (42). The chloroethylnitrosoureas form a number of adducts on DNA, including an interstrand cross-link between guanine and cytosine on the opposite strand of DNA (43-45). This lesion is formed by an initial attack at the O6-position of guanine by the chloroethylnitrosourea to produce 2-chloroethylnitrosourea, followed by an intramolecular rearrangement to form 1,06-ethanoguanine. Within about 10-12 h, this adduct reacts with the opposite cytosine to form the 1-(3-cytosinyl)-2-(1-guanyl)ethane cross-link. There is a strong correlation between the number of cross-links formed and cell kill (44).

The AGT protein protects cells from damage by removing methylating and chloroethylating damage from the O6-position of guanine. Cross-links are prevented from forming following exposure to chloroethylating agents by two reactions with the AGT protein: (a) removal of the chloroethyl lesion from the O6-position prior to rearrangement; and (b) reaction with the intermediate, 1,06-ethanoguanine, to form a cross-link between DNA and the repair protein (46, 47). There are no other proteins or cofactors involved in this reaction, and the protein is inactivated in the repair process (36). These unique properties make the AGT protein ideal for modulation. The high degree of correlation that exists between alkyltransferase activity and sensitivity to nitrosoureas in vitro and in animal models suggests that the elimination of this protein may reverse resistance (reviewed in Refs. 27, 35, 36, and 45-47).

Modulation of Alkyltransferase

Because the mechanism of repair involves inactivation of the protein, and because lower levels of AGT correlate with increased sensitivity to alkyltransferase, efforts to potent AGT substrates would have considerable potential in chemotherapy. The first modulation experiments used a combination of methylating and chloroethylating agents (48-51). The rationale for this combination is that pretreatment of cells with methylating agents decreases alkyltransferase levels by introducing O6-methylguanine residues in DNA, which are then repaired by the alkyltransferase (48-53). Exposure of tumor cells in culture to N-methyl-N'-nitro-N-nitrosoguanidine or streptozotocin resulted in the formation of sufficient O6-methylguanine DNA adducts to deplete alkyltransferase activity (48). This transient depletion resulted in an increase in BCNU-induced interstrand cross-linking and a 2-3-log enhancement of BCNU cytotoxicity in vitro (49, 51). Human tumor xenograft studies in nude mice demonstrated that although depletion of AGT activity could be demonstrated in tissues and xenograft tumors with methylating agents, this did not result in an increase in the therapeutic index of BCNU (54, 55).

On the basis of in vitro observations and the fact that methylating agents were Food and Drug Administration-approved agents, clinical trials of the combination were initiated. Administration of patients with either streptozotocin or DTIC resulted in a depletion in AGT levels of peripheral blood mononuclear cells, suggesting that the approach was feasible (53, 56). Following these studies, Phase I trials combining streptozotocin or DTIC with BCNU in patients were initiated (57-59). The dose-limiting toxicity was hematological, although there were also reports of significant hepatic and pulmonary toxicity from several Phase I and II studies (57-61). There was an absence of clinical activity of the combination of streptozotocin and BCNU in two Phase II studies directed at refractory melanoma (60) and metastatic colon cancer (61). However, in a study combining DTIC with a nitrosourea, fotemustine, considerable activity in malignant melanoma was reported (62). In this same study, 6 of 107 patients developed rapidly progressive and fatal respiratory failure (62). Some of the factors contributing to the lack of effectiveness of this combination include enhanced toxicity by the addition of the methylating agent as well as the possibility that although the lymphocyte AGT activity decreased to some extent, depletion in the tumor was not significant to overcome clinical resistance to BCNU. In support of this, a recent study by Willson et al. (61) demonstrated the lack of AGT inactivation in metastatic colorectal cancer following administration of streptozotocin. Equally important to the added toxicity is the mutagenic and carcinogenic properties of the methylating agents (63). This combination might be expected to result in a high incidence of secondary leukemias. The enthusiasm for this combination has been moderated by these limitations.

More recently, direct substrates for the alkyltransferase protein have been designed and tested for their ability to effectively inactivate the protein. Exposure of cells or cell extracts to millimolar amounts of the free base, O6-methylguanine, for 4 h results in a loss of AGT activity and subsequent increase in the sensitivity of tumor cells to alkylating agents (64-66). Although preliminary results in cells looked promising, there was no enhancement of the therapeutic index of BCNU when combined with O6-methylguanine to treat mice carrying human tumor xenografts (67). Most likely, this was due to poor solubility of the drug, only partial inhibition of AGT activity, and poor affinity of the AGT for the substrate. Effective modulation requires a prolonged period of inactivation to allow the full conversion of the O6-(2-chloroethyl)guanine adducts to interstrand cross-links (68, 69). O6-Methylguanine never entered clinical trials.

Design and Testing of O6-Benzylguanine

Effective modulation of the alkyltransferase protein, resulting in an increase in the therapeutic index of BCNU, required a more potent and selective substrate. O6-Benzylguanine was designed based on an understanding of the bimolecular displacement reaction between the AGT protein and the leaving group at the O6-position of guanine (64, 70). Benzyl groups are known to
enter more readily into bimolecular reactions compared to alkyl groups because the electron charge stabilizes the benzyl group in the transition state. Fig. 1 illustrates the reaction between the AGT protein and O\textsuperscript{6}-benzylguanine. Administration of human tumor cells with micromolar concentrations of O\textsuperscript{6}-benzylguanine for 2 min resulted in a complete depletion of the alkyltransferase protein, rendering cells more sensitive to agents that alkylate the O\textsuperscript{6}-position of guanine (70). Loss of AGT led to a marked enhancement of the sensitivity to alkylating agents in vitro. Detailed studies of the inactivation by O\textsuperscript{6}-benzylguanine using the purified human alkyltransferase revealed that upon reaction of this compound with the active site of the mammalian protein, S-benzylcysteine is formed (71). Transfer of the benzyl group to the AGT protein leads to a very marked decrease in the stability of the protein in HT29 cells (73). Although the cellular fate of the alkylated form of the protein is not well understood, it appears that a change in configuration occurs upon alkylation of the active site cysteine. This change in configuration reduces the affinity of the protein for DNA, increases its sensitivity to proteases (73–75), and also renders the AGT protein subject to ubiquitination (76). These changes facilitate the degradation of the protein. Even on prolonged incubation in vitro under a variety of conditions, no reactivation of the benzylated form of AGT formed by reaction with O\textsuperscript{6}-benzylguanine has been observed. The rapid degradation of the benzylated form in vivo renders it most unlikely that the cellular activity can be regenerated in the cell. Thus, once depletion occurs, restoration of AGT activity requires the synthesis of new protein.

Inactivation of the alkyltransferase protein in a variety of human tumor cells expressing the alkyltransferase protein by nontoxic doses of O\textsuperscript{6}-benzylguanine renders these cells more sensitive to the cytotoxic effects of alkylating agents including BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, chlorozotocin, clomesone, streptozotocin, and 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide (77–79). The proliferating activity of transplanted brain tumor cells was significantly inhibited after treatment with intracarotid 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloromethyl)-3-nitrosourea with both O\textsuperscript{6}-methylguanine and O\textsuperscript{6}-benzylguanine substrates, although an even greater effect was observed when O\textsuperscript{6}-benzylguanine was used (80). There is an even greater effect if the time schedule is optimized for sensitization of tumor cells, with continuous exposure to O\textsuperscript{6}-benzylguanine after BCNU being much superior to short-term exposure (68–70). Fig. 2 illustrates the greater enhancement of BCNU in HT29 cells following long-term exposure to O\textsuperscript{6}-benzylguanine (2 h prior to BCNU and an additional 16 h following BCNU) compared to short-term exposure to O\textsuperscript{6}-benzylguanine (2 h prior to BCNU).

Several studies have demonstrated a strong correlation between the degree of enhancement and the level of AGT activity, with little or no enhancement observed in cells with undetectable levels of AGT activity and the greatest enhancement in cells with high AGT activity (77–80). Depletion of AGT by O\textsuperscript{6}-benzylguanine has no sensitizing effect on alkylating agents that do not produce a toxic lesion at the O\textsuperscript{6}-position of guanine in DNA, such as cisplatin and melphalan (77, 81).

Sarker et al. (82) evaluated the effect of O\textsuperscript{6}-benzylguanine combined with hypoxia on the cytotoxicity of BCNU and found that the cytotoxicity of BCNU was four times higher in O\textsuperscript{6}-benzylguanine-treated hypoxic cells than in oxic cells treated with BCNU alone. Thus, the cytotoxicity produced by BCNU would be expected to be greater in hypoxic regions of a tumor after treatment with O\textsuperscript{6}-benzylguanine than in oxic regions of a tumor.

**Antitumor Effect of O\textsuperscript{6}-Benzyguanine in Animals**

Encouraging *in vitro* results led to an evaluation of O\textsuperscript{6}-benzylguanine plus alkylating agents in animal tumor models. The effect of O\textsuperscript{6}-benzylguanine on AGT levels in tissues of hamsters and mice and on the growth of human tumor xenografts in nude mice was investigated. Treatment of nude mice carrying SF767 human brain tumor xenografts with O\textsuperscript{6}-benzylguanine prior to 1-(2-chloroethyl)-3-methylcyclohexyl-1-nitrosourea or BCNU leads to a significant inhibition of tumor growth as compared to chloroethylating agent alone (83, 84).
Although the toxicity of BCNU was enhanced with \( O^6 \)-benzylguanine requiring the administration of a lower dose of BCNU, the combination was clearly better than the maximally tolerated dose of BCNU alone.

Upon administration of \( O^6 \)-benzylguanine prior to BCNU to mice carrying the D341Med or D456MG brain tumors, 8 of 10 and 10 of 10 tumor regressions were observed compared to 0 of 10 and 1 of 10 for animals treated with BCNU alone, respectively (85). Using the intracranial D341MED medulloblastoma model, Felker et al. (86) demonstrated a significant increase in median survival in animals treated with \( O^6 \)-benzylguanine prior to BCNU compared to BCNU alone. \( O^6 \)-Benzylguanine has been shown to reverse resistance in several colon tumor models (84, 87, 88). Growth inhibition was also observed in the Dunning rat prostate model after treatment with \( O^6 \)-benzylguanine and BCNU, which was not observed in animals treated with BCNU alone (89). Marathi et al. (68, 69) suggested that optimal reversal of BCNU resistance might require complete inactivation of the AGT protein for at least 24 h following BCNU administration. This can be achieved by higher doses of \( O^6 \)-benzylguanine or longer exposure or the addition of streptozotocin to the regimen. The three-drug combination, \( O^6 \)-benzylguanine-streptozotocin-BCNU, produced greater cell kill than any two-drug regimen in vitro (68, 69), warranting further testing. Unfortunately, nude mouse studies indicated that the addition of streptozotocin to the \( O^6 \)-benzylguanine and BCNU combination did not result in a greater antitumor activity than observed with \( O^6 \)-benzylguanine and BCNU (55). It is possible that a different schedule and dose would produce a therapeutic advantage.

The major dose-limiting toxicity of nitrosoureas is bone marrow suppression (1–8). Recently, Fairbairn et al. (90) reported an increase in the sensitivity of human primary bone marrow cells to the cytotoxic effects of the methylating agent, temozolomide, after treatment with \( O^6 \)-benzylguanine. Furthermore, it has been shown that the dose of BCNU must be lowered in rats and mice when administered in combination with \( O^6 \)-benzylguanine, indicating an increase in toxicity. The enhancement of the antitumor activity was greater than the enhancement of toxicity; thus, the therapeutic index was greater for the combination than for BCNU alone (84, 85).

**\( O^6 \)-Benzylguanine Formulation**

Many of the original animal experiments used a cremophor vehicle to dissolve \( O^6 \)-benzylguanine because of its low solubility in an aqueous medium (83–89). Because cremophor is not suitable for human clinical trials, the formulation was changed to a polyethylene glycol 400-based vehicle for toxicological testing and human clinical trials. This formulation was effective in sensitizing D456MG glioblastoma xenografts in nude mice to BCNU (91) at doses of \( O^6 \)-benzylguanine less than those used in the original experiments. Presumably, this is due to a greater bioavailability in the PEG 400 compared to cremophor. The drug is administered to humans in a solution of 40% PEG 400 in phosphate buffer (pH 8) and mannitol.

**Toxicity of \( O^6 \)-Benzylguanine and BCNU**

Toxicological evaluation of \( O^6 \)-benzylguanine and BCNU has been performed on mice, rats, and dogs. Mice received single i.v. doses of \( O^6 \)-benzylguanine alone, single i.v. doses of BCNU alone, or combinations of both drugs. BCNU was administered 1 h after \( O^6 \)-benzylguanine administration. Drug-related mortality, loss of body weight, and clinical signs of toxicity were noted primarily in mice receiving both drugs. Drug-related histopathological lesions occurred in the bone marrow, thymus, intestine, forestomach, Kupffer cells, testes, and ovaries (92). The maximally tolerated dose in mice was 30 mg/kg of \( O^6 \)-benzylguanine in combination with 16 mg/kg of BCNU. Similar histopathological results were obtained in Copenhagen rats receiving BCNU alone or the combination \( O^6 \)-benzylguanine and BCNU. There was a decrease in all types of bone marrow cells, loss of intestinal crypts, and a decreased number of lymphocytes in the spleen (89).

Male and female Beagle dogs received 1-h i.v. infusions of \( O^6 \)-benzylguanine alone, single i.v. doses of BCNU alone, or combinations of both drugs. BCNU was administered 1 h after the completion of the \( O^6 \)-benzylguanine infusion. \( O^6 \)-Benzylguanine inhibited alkylation transferase activity in peripheral blood mononuclear cells at 2 and 24 h after the start of infusion (93). A drug-related, reversible leukopenia occurred in dogs receiving combinations of \( O^6 \)-benzylguanine and BCNU. Reversible, drug-related histopathological lesions occurred in the testes and epididymes of dogs treated with \( O^6 \)-benzylguanine alone or in combination with BCNU (93). The maximally tolerated dose of the combination in dogs was 5 mg/kg of \( O^6 \)-benzylguanine and 0.25 mg/kg of BCNU.

**Metabolism of \( O^6 \)-Benzylguanine in Animals**

To prepare \( O^6 \)-benzylguanine for clinical trials, its metabolic fate was evaluated in mice, rats, and monkeys. A major plasma and urinary metabolite of \( O^6 \)-benzylguanine found in
mice (94), rats (94), and monkeys (95) is the oxidized derivative, 8-benzyl-8-oxoguanine (Fig. 3). The debenzylated metabolites, guanine and 8-oxoguanine, were found in rats, but the mechanism by which the benzyl group is lost is unknown. Acetylation at the N-2 position is a species-specific reaction because N2-acetyl-8-benzylguanine, N2-acetylated guanine, and N2-acetyl-8-benzyl-8-oxoguanine were found in rats but not mice or monkeys. Acetylation resulted in metabolites that were much less potent than 8-benzylguanine, in contrast to oxidation at the 8-position, which resulted in a slightly less potent derivative.

To determine whether P-450 enzymes were responsible for the conversion to 8-benzyl-8-oxoguanine, rats were administered phenobarbital prior to 8-benzylguanine. This resulted in a 17–19-fold increase in the amount of oxidized product in the urine, indicating a role for CYP450s in the oxidation of 8-benzylguanine. The cytosolic enzyme aldehyde oxidase also contributed to the oxidation of 8-benzylguanine, as determined by the use of inhibitors such as menadione and allopurinol (96).

### Pharmacokinetics of 8-Benzylguanine

The disposition and pharmacokinetic parameters of i.v. 8-benzylguanine have been evaluated in rats, dogs, and non-human primates (monkeys). Table 1 illustrates results of half-life, clearance, Vd, and the ratio of the AUC of 8-benzyl-8-oxoguanine to the AUC of 8-benzylguanine following i.v. administration of 8-benzylguanine. The plasma profile of 8-benzylguanine was best described by a one-compartment model for each species (95–97). The plasma half-life of 8-benzylguanine was 1.6 h, 3.4–6.3 h, and 1.6 h for rats, dogs, and monkeys, respectively. 8-Benzylguanine was rapidly cleared from the plasma, which could be partially accounted for by oxidation to 8-benzyl-8-oxoguanine. Approximately, 37% of parent drug was converted to 8-benzyl-8-oxoguanine in rats (96). Furthermore, the urinary excretion of 8-benzylguanine was only 8 and 1% of the administered 8-benzylguanine to rats and monkeys, respectively. Only 2% of the total 8-benzylguanine dose was excreted as 8-benzyl-8-oxoguanine in the first 8 h in monkeys, suggesting that 8-benzyl-8-oxoguanine undergoes extensive further metabolism, although biliary excretion cannot be ruled out. The fate of 8-benzyl-8-oxoguanine has not been established in any non-human primates.

Berg et al. (95) reported that the oxidized metabolite, 8-benzyl-8-oxoguanine, penetrated in the CSF to a much greater extent than did 8-benzylguanine. The overall CSF exposure to 8-benzyl-8-oxoguanine was 90-fold higher than that of 8-benzylguanine. The high degree of CSF penetration of the active metabolite suggests that 8-benzylguanine would be potentially useful in enhancing the antitumor effects of nitrosoureas in brain tumors or neoplastic meningitis.

### Human Metabolism of 8-Benzylguanine

Prior to clinical trials, the oxidation of 8-benzylguanine was examined using human liver cytosol, microsomes, and several P-450 isozymes (98). Incubation of 8-benzylguanine with human liver cytosol resulted in the formation of 8-benzyl-8-oxoguanine, which was inhibited by menadione, a potent inhibitor of aldehyde oxidase. Inhibition by allopurinol (a xanthine oxidase inhibitor) was less dramatic. Oxidation of 8-benzylguanine also occurred with pooled human liver microsomes and was inhibited by both furafylline and troleandomycin, selective inhibitors of CYP1A2 and CYP3A4, respectively. Human P-450s CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4 expressed in HepG2 hepatoma cells using vaccinia virus vectors were incubated with 8-benzylguanine. 8-Benzylguanine was oxidized primarily by CYP1A2 and to a lesser extent by CYP3A4. CYP1A2 exhibits a more than 200-fold higher relative catalytic activity (Vmax/Km) compared to CYP3A4. Therefore, at therapeutically relevant concentrations of 8-benzylguanine, CYP1A2 could be primarily involved in its oxidation because it shows a much lower Km (1.3 μM) compared to that of CYP3A4 (52.2 μM) and cytosol (81.5 μM). However, one would expect interindividual variation in the extent of oxidation of 8-benzylguanine, depending on the levels of these enzymes.

### Phase I Clinical Trial of 8-Benzylguanine

Phase I clinical trials of the combination of 8-benzylguanine and BCNU are ongoing at the University of Chicago (99) and Case Western Reserve University (100). 8-Benzylguanine is administered for over 1 h as an i.v. infusion, followed by BCNU 1 h after completion of the 8-benzylguanine infusion. Preliminary data from both studies indicate that 8-benzyl-8-oxoguanine is present in human plasma at a much higher concentration than 8-benzylguanine, indicating rapid oxidation in humans. Furthermore, the half-life of 8-benzyl-8-oxoguanine is approximately 8-fold slower than that of 8-benzylguanine.
(99). The oxidized metabolite in humans may be the primary determinant of the extent and duration of AGT depletion (99). Results from the Phase I trial at Case Western indicate that 18 h after infusion of 17.7 mg/m² O′-benzylguanine, mean inactivation of AGT in tumor tissue was 95% (100). This study demonstrates that biochemical modulation of tumor tissue AGT activity is feasible, and O′-benzylguanine holds promise as a mechanism to overcome clinical resistance to nitrosoureas.

**Resistance to O′-Benzylguanine**

AGT proteins have been isolated from many different species (36). Although these proteins show significant homology with 22 absolutely conserved residues in the core domain, which is about 80 amino acids in length (located from amino acids 94 to 172 in the human AGT, which is 207 amino acids in total), there were striking species variations in sensitivity to O′-benzylguanine. The yeast and Escherichia coli Ada-C alkyltransferases were totally resistant to inactivation, and the E. coli Ogt alkyltransferase was much less sensitive than the human protein (72). These results raised the possibility that quite minor changes in the human AGT structure might render the protein resistant to O′-benzylguanine. This proved to be the case because several point mutations produced by site-directed mutagenesis (101, 102) increased the ED₅₀ for inactivation by O′-benzylguanine, which is 0.2 µM for the wild-type AGT. Altering the prolines present at positions 138 or 140 to alanines increased the ED₅₀ to 2.5 and 5 µM, respectively. Changing the glycine at position 156 to alanine or tryptophan increased the ED₅₀ to 60 or 80 µM (102). Combinations of these mutations produced even larger increases, and the P140A/G156A double mutant was totally resistant to O′-benzylguanine. The recent identification of the crystal structure for the Ada-C alkyltransferase (103) provides a mechanistic explanation for these results. The space at the cysteine acceptor site in Ada-C is very limited and is not sufficient to accommodate the bulky O′-benzylguanine group. Mutations in the Ada-C sequence that increase this space by inserting a proline at position 316 and removing a large tryptophan at position 336 render it sensitive to O′-benzylguanine (104). A homology model of the human AGT structure suggests that the space at the active site is larger than in Ada-C but can be reduced by the mutations that produce resistance (105).

Thus, the reasons for lack of reaction with O′-benzylguanine appear to be steric restrictions on the size of the active site. Although at present no such mutations have been identified in AGT isolated from tumors, it is possible that they may occur during therapy when there would be a strong selection pressure in favor of them. This possibility is strengthened by very recent findings that a mutant G160R which has the change of glycine 160 to arginine in human AGT sequence, significantly reduced the inactivation by O′-benzylguanine with at least a 20-fold increase in the ED₅₀ (106). It has been reported that a polymorphism in the human AGT gene exists, with about 15% of the population studied having arginine at codon 160 instead of glycine: therefore, this “mutant” is likely to occur in a fraction of the patient population (107). These results indicate that it is likely that a subpopulation of patients resistant to O′-benzylguanine may exist.

Higher doses of this drug or additional alkyltransferase inhibitors capable of inactivating these resistant forms of the alkyltransferase may, therefore, be needed. A definitive crystal structure of the human AGT rather than the current homology model would be of great benefit in such rational drug design. Expression of the G156A and P140A mutant AGT proteins in CHO cells did render these cells resistant to BCNU and, as expected, O′-benzylguanine was much less effective in restoring sensitivity to BCNU (108). The G156A mutation provided the greater amount of resistance to O′-benzylguanine, and the CHO cells expressing this mutant AGT were not effectively killed by the O′-benzylguanine plus BCNU combination (108). Such cells can be used to assess the effectiveness of drugs designed to overcome these limitations.

**Protection of Hematopoiesis against the Cytotoxic and Mutagenic Effects of O′-Benzylguanine and BCNU**

Although the results obtained using mutant AGTs raise the possibility that resistance to therapy with O′-benzylguanine and chloroethylating agents may arise by the selection of tumor cells expressing a mutated AGT, they also provide a potential means of improving the therapeutic effectiveness of agents forming O′-alkylguanine adducts. The main limitation in the clinical use of alkylating agents is their dose-related acute toxicity to the hematopoietic system (1–8). In animal studies (92, 93, 97) of the combination of O′-benzylguanine and BCNU, enhanced bone marrow toxicity has been noted. This may also be true in humans, because AGT activity in hematopoietic progenitor cells has been shown to be very low (109, 110). Furthermore, O′-benzylguanine pretreatment markedly sensitized hematopoietic progenitor cells to BCNU (110). Alkylating agents carry the added risk of inducing acute myeloid leukemia in patients surviving for long periods after successful therapy of the primary tumor (111). Because myelosuppression increases the rate of DNA synthesis in a progenitor population prone to development of secondary leukemias, depletion of the AGT activity by O′-benzylguanine may enhance the risk of mutational events and perhaps the risk of drug-related leukemias in these patients.

One way to overcome enhanced hematopoietic toxicity and mutagenicity would be to use gene therapy to express an alkyltransferase gene in the relevant marrow stem cells. The feasibility of this approach has been demonstrated in animal models using retroviral transduction techniques (112–116). Expression of the G156A mutant AGT in hematopoietic progenitor cells by gene therapy techniques could be used to increase their AGT activity and provide a form that was resistant to O′-benzylguanine (117). Such resistance would also provide a way to select in vitro for cells expressing the inserted gene and thus improve the delivery of the protective gene. Expression of an O′-benzylguanine-resistant AGT in normal bone marrow would provide an increase in the therapeutic index for treatment of tumors that would have an AGT activity sensitive to O′-benzylguanine. Although the bacterial Ada-C could also be used for this pur-
pose, a modified mammalian protein is more likely to provide maximal protection of the nuclear DNA.

**Analogues of O^6^-Benzylguanine**

Following up on the discovery of the potent inactivation of AGT by O^6^-benzylguanine, a considerable number of additional compounds have been tested for AGT inactivation potential and in some cases for the ability to sensitize cells to the effects of chloroethylating agents (118–125). A wide range of activities have been reported, with some compounds being more potent than O^6^-benzylguanine and others being much less active. Quite minor changes such as the addition of methyl groups to the N-2 or N-7 position of O^6^-benzylguanine (119, 120) leads to a huge loss of AGT inactivating activity, whereas other changes, such as the addition of an 8-bromo or 8-aza substitution increase potency (120). The published information on more than 60 putative AGT inhibitors provides a useful data base for modeling studies to evaluate the interaction of such low molecular weight pseudo substrates with the AGT protein.

Many compounds have now been made that inactivate AGT at levels that suggest they would be useful for chemotherapeutic inactivation (118–120, 122, 125). Although none of these have yet been tested against isolated tumor cells and tumor xenografts as extensively as O^6^-benzylguanine, differences between them and O^6^-benzylguanine may offer advantages. Some examples of compounds that may prove to be improvements are: (a) O^6^-benzyl-2'-deoxyguanosine (69, 118), which is much more soluble in aqueous solutions than O^6^-benzylguanine. Despite the 10-fold lower potency of O^6^-benzyl-2'-deoxyguanosine compared to O^6^-benzylguanine in *vitro*, these compounds were equally effective *in vivo* (126); (b) O^6^-benzyl-8-oxoguanine (94, 95, 120), which has been described as the primary plasma metabolite in humans, monkeys, rats, and mice. Direct administration of this compound would be expected to reduce the variability of AGT inactivation observed upon administration of O^6^-benzylguanine; (c) other 8-substituted O^6^-benzylguanine derivatives, such as 8-aza-O^6^-benzylguanine or O^6^-benzyl-8-bromoguanine (110), which are more active than O^6^-benzylguanine, and will differ in metabolism; (d) 2,4-diamino-6-benzoxypyrimidines substituted at the 5-position with a strong electron withdrawing group such as nitro or nitroso, which are the most potent AGT inhibitors yet described (120, 124); and (e) O^6^-allylguanine (118), which is able to inactivate some of the AGTs described above that are resistant to O^6^-benzylguanine.

**Summary**

There is overwhelming evidence that the presence of AGT renders tumor cells resistant to killing by agents that attack the O^6^-position of guanine in DNA. Other mechanisms of resistance may also exist, particularly for methylating agents, where a lack of mismatch repair leads to tolerance of the presence of O^6^-methylguanine in DNA. But there is no doubt that the presence of AGT activity is a significant source of resistance to killing by chloroethylyating and methylating agents. These compounds will, therefore, exhibit their maximum effectiveness only if AGT activity is eliminated.

Treatment with O^6^-benzylguanine provides a means to moderate AGT activity, but it remains to be determined to what extent such AGT depletion enhances the chemotherapeutic effectiveness of these agents. The current clinical trials should provide useful information on this and have already demonstrated that effective modulation of AGT can be produced by O^6^-benzylguanine. On the basis of studies with human tumor xenografts carried in nude mice, the use of O^6^-benzylguanine is expected to produce an increase in the therapeutic index of these alkylating agents. However, it remains possible that additional measures to enhance the tumor versus normal cell selectivity of these combinations will be needed to achieve a significant clinical effect.

Such selectivity may be obtained in several ways: (a) regional therapy with O^6^-benzylguanine or a related compound may provide a selective depletion in the tumor; (b) derivatives of O^6^-benzylguanine with more specificity toward tumor cell AGT may be developed; (c) the use of gene therapy to express an O^6^-benzylguanine-resistant AGT in hematopoietic cells, reducing the dose-limiting toxicity; and (d) the use of newer chloroethylyating agents that themselves have greater tumor specificity. Many such chloroethylyating agents have been made, but their development has stalled or been abandoned during preliminary testing due to a lack of efficacy. The wide distribution of AGT in tumors is likely to have been a major factor in limiting their action. Their combination with O^6^-benzylguanine or second-generation AGT inhibitors should be tested in detail before discarding any of these potentially useful agents. Effective inhibition of tumor AGT along with protection of bone marrow progenitors by expression of O^6^-benzylguanine-resistant proteins is expected to bring on a new era for the clinical use of nitrosoureas.

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