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Protection of CHO Cells by Mutant Forms of O^6-Alkylguanine-DNA Alkyltransferase from Killing by 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU) Plus O^6-Benzylguanine or O^6-Benzyl-8-oxoguanine

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ABSTRACT. O^6-Benzylguanine (BG) is an inactivator of human O^6-alkylguanine-DNA alkyltransferase (AGT) currently undergoing clinical trials to enhance cancer chemotherapy by alkylating agents. Mutant forms of AGT resistant to BG *in vitro* were expressed in CHO cells to determine if they could impart resistance to killing by the combination of BG and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). All the BG-resistant mutant proteins tested (P140A, P140K, P138M/V139L/P140K, G156A, P140A/G160R, and G160R) showed a reduced rate of reaction with methylated DNA substrates *in vitro*. However, when expressed in equal amounts in CHO cells, mutants P140A, P140K, P138M/V139L/P140K, and G160R gave levels of protection from the chloroethylating agent BCNU equivalent to that of wild-type AGT. This indicates that a 10-fold reduction in rate constant did not prevent their ability to repair chloroethylated DNA in the cell. AGT activity was readily lost when CHO cells expressing wild-type AGT were exposed to BG or its 8-oxo metabolite (O^6-benzyl-8-oxoguanine), but cells expressing mutants P140A or G160R required 30-fold higher concentrations and cells expressing mutants P140K or P138M/V139L/P140K were totally resistant. When cells were treated with 80 μM BCNU plus BG or 8-oxo-BG, those expressing wild-type AGT were killed when inhibitor concentrations of up to 500 μM were used, whereas cells expressing P140K or P138M/V139L/P140K showed no effect, and cells expressing P140A or G160R showed an intermediate resistance. These results suggest that: (i) appearance of BG-resistant mutant AGTs may be a problem during therapy, and (ii) the P140K mutant AGT is an excellent candidate for gene therapy approaches where expression of a BG-resistant AGT in hematopoietic cells is used to reduce toxicity. BIOCHEM PHARMACOL **58**;2:237–244, 1999. © 1999 Elsevier Science Inc.

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Inactivation of the DNA repair protein AGT† by BG has been shown in animal models to enhance the cancer chemotherapeutic effects of chloroethylating agents such as BCNU and methylating agents such as temozolomide [1–4]. Phase I clinical trials of this approach have indicated that adequate suppression of AGT occurs to permit the use of BG to test the hypothesis that AGT is a significant factor in resistance to these drugs [3, 5–7]. However, the myelosuppression produced by these alkylating agents was enhanced by BG treatment, and it remains possible that the reduction in dose necessitated by this toxicity may limit the value of such chemotherapy. It is also possible that effective use of BG to treat primary tumors may lead to an increase in secondary tumor formation, as this is a well-known consequence of successful therapy by alkylating agents [8–10].

A second problem with the development of BG as a therapeutic agent may be the appearance of BG-resistant forms of AGT due to either selection of pre-existing resistant forms or the generation of such forms because of the mutagenic activity of the therapeutic alkylating agents. As described below, several such resistant forms have been isolated by site-directed mutagenesis or by screening for BG resistance in mutated AGT cDNA libraries expressed in *Escherichia coli*. The purpose of the experiments described in the present paper was to determine if these BG-resistant AGT mutants were fully active in repairing DNA in a mammalian cell background.

Although these BG-resistant AGT mutants may necessitate the development of additional inhibitors if they are fully active, a highly active and potent BG-insensitive mutant protein would be very useful for gene therapy

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† *Abbreviations*: AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; 8-oxo-BG, O^6-benzyl-8-oxoguanine; and BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea.

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approaches to preventing the hematopoietic toxicity of alkylating therapy. Expression of such a mutant form of AGT in bone marrow stem cells would allow the use of higher doses of BG plus alkylating therapy. A considerable number of BG-resistant mutant AGTs have been identified [11–16]. Selection of the most appropriate mutant for use in such gene therapy approaches is of obvious importance.

It should be noted that the Phase I clinical trials of BG have shown that this drug is modified extensively to the 8-oxo derivative, which is much longer lived in the body than BG itself [6, 7]. This metabolite, 8-oxo-BG, is practically as active as BG in inactivating wild-type AGT *in vitro* [17], and it appears likely that the metabolite contributes substantially to the inactivation of AGT in patients treated with BG. Therefore, when selecting useful mutants for gene therapy, in addition to the obvious considerations of stability, activity, and resistance to BG it is essential that the resistance also is sustained when 8-oxo-BG is used as an inactivator.

Several mutants of human AGT that were resistant to inactivation by BG were found by screening mutations introduced by site-directed mutagenesis. These include P140A, G156A, and G160R [12, 13]. The resistance of the P140A and G156A mutants may be explained by steric effects limiting the size of the binding pocket for BG [18, 19]. The observation that the combination of these two mutations to form P140A/G156A led to an even more resistant form [12] is consistent with this hypothesis, but the very poor stability and activity of the P140A/G156A mutant render it unlikely to be optimal for approaches involving gene therapy. Although encouraging results with this mutant were reported by one group [20, 21], this protection was not observed in studies by others, although the P140A mutant was effective [22]. In fact, even the G156A mutant AGT, which has shown promising results in preliminary experiments aimed at expression in the bone marrow [23–25], appeared to be less active than wild-type AGT when expressed in mammalian cells [26]. When transfected CHO cells expressing equal AGT activities were compared, a much larger amount of AGT protein was present in cells expressing the G156A mutant compared with the wild-type protein [26]. This suggests that the G156A protein is not fully active and/or is incorrectly folded.

The resistance of the G160R mutant is more likely to arise from a different mechanism than steric limitation of the active site, since the even more bulky alteration in the G160W mutant did not lead to BG resistance [13, 27]. It has been suggested that the presence of the charged arginine residue disrupts a hydrophobic binding site for the BG [13]. One objective of the experiments described in this paper was to examine whether AGTs rendered resistant to BG by this mechanism were more suitable for use to protect cells deficient in AGT.

More recent studies have identified BG-resistant mutants, using a screening procedure in which libraries of plasmids containing random alterations to the AGT sequence are expressed in bacteria [14–16]. Such proteins can provide resistance to an *E. coli* strain deficient in endogenous AGT and, by carrying out the selection in BG-permeable strains and in the presence of BG, resistant mutants were identified. Many of these mutants contain multiple mutations, and it is possible that several alterations contribute to the resistance. However, even the single alteration in mutant P140K rendered AGT totally resistant to BG [16]. It is possible that this mutation combines both the steric limitation caused by mutating this proline and the disruption of the hydrophobic binding pocket by the charged side chain of the lysine. The present experiments, therefore, were designed to test whether these mutant AGT molecules retained full activity and resistance to BG and 8-oxo-BG when expressed in mammalian cells and whether multiple mutations provide any advantage over such single alterations.

MATERIALS AND METHODS

Materials

BG and 8-oxo-BG were synthesized as previously described [28, 29] and were provided by Dr. R. C. Moschel (National Cancer Institute-Frederick Cancer Research Development Center). BCNU was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. *N*-[*methyl*-^{3}H]*N*-Nitrosourea was purchased from Amersham, Inc.

Oligodeoxynucleotides were purchased from GIBCO-BRL Life Technologies, Inc. Other reagents for molecular biology, cell culture, and AGT assays were obtained from GIBCO-BRL Life Technologies, the Sigma Chemical Co., Atlanta Biologicals, New England Biolabs, Perkin Elmer/Cetus, and Promega.

Plasmid Constructions

The recombinant AGT mutant proteins (except for C145A and R128A) were expressed in *E. coli* using the pQE-30 vector, which adds a 12-amino-acid sequence [MRGS[H]$_6$GS-] to the amino terminus of the AGT protein allowing purification by immobilized metal affinity chromatography [13, 16]. The C145A and R128A mutant proteins were expressed without the (His)$_6$-tag from the pIN vector and purified as previously described [11, 30]. The plasmid pCMV-Neo-Bam was used to express human AGT and its mutants under the control of a CMV promoter in CHO cells [26]. The original plasmid contained a single *Bam*HI site for the insertion of the expressed cDNA. The plasmid pCMV-AGT containing the wild-type AGT sequence was mutagenized to convert the *Bam*HI site at the 3′ end of the inserted cDNA sequence to an *Nhe*I site to facilitate the further constructions.

The pCMV-G160R plasmid was constructed by polymerase chain reaction (PCR) using *Pfu* polymerase and pQE-30-G160R as template DNA. The 5′ primer for the PCR [which eliminates the (His)$_6$ tag sequence and regenerates

the normal AGT sequence at the amino terminus] was 5′-CTCACTATAGGATCCAAA**ATG**GACAAG-GAT-3′ with a *Bam*HI site (underlined type) preceding the start codon (bold type). The 3′ primer was 5′-GGATCTATCAACAGGAGTGCTAGCTCAGC-3′, which was designed to create the underlined *Nhe*I restriction site. (The use of this primer introduces 9 additional restriction sites from pQE-30 into the pCMV vector.) The fragment obtained by cutting this PCR product with *Bam*HI and *Nhe*I then was inserted into pCMV-AGT cut with the same enzymes to form pCMV-G160R.

The pCMV-P140K and the pCMV-P138M/V139L/P140K plasmids were made by replacing the 422-bp section of the AGT coding region in pCMV-G160R located between the *Eco*NI (located at position 99 in the coding region) and *Age*I (located at 521) sites. The entire AGT protein-coding region for all plasmids was sequenced to ensure that no additional mutations were present.

Cell Culture

CHO cells were grown in α-MEM containing 36 mM $NaHCO_3$, 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained by seeding at 2.5×10^5 cells/75-cm^2 flask at weekly intervals. The CHO clones expressing AGT or its mutants were grown in the presence of 0.5 mg/mL of geneticin (GIBCO-BRL) to prevent the loss of the plasmids.

Transfection and Selection Procedure

The plasmids described above were used for the transfection of CHO cells to obtain clones expressing the mutant forms of AGT essentially as previously described for selection of CHO cells expressing the AGT mutant G156A [26]. Briefly, CHO cells were transfected using Lipofectin (GIBCO-BRL) according to the manufacturer's protocol for stable transfection of adherent cells using 5 μg DNA and 10 μL Lipofectin. Cells were incubated for 5 hr, and then the medium was replaced with the growth medium. After 24 hr, cells that had taken up the plasmid were selected with geneticin at a final concentration of 1 mg/mL. Clones from individual cell colonies were isolated, examined for the level of AGT protein expression, and used for further experiments. A control clone was also established using the pCMV vector plasmid without an insert coding for AGT.

Cytotoxicity Assay

The colony-forming ability of cells was determined using a colony-forming assay [26]. The CHO cells were plated using 10^6 cells/25-cm^2 flask and grown for 24 hr. Cells were incubated for 2 hr with different concentrations of freshly prepared solutions of BCNU. (The BCNU was first dissolved in absolute ethanol at a concentration of 8 mM, then diluted with the same volume of PBS, and applied immediately to the cells.) The medium was replaced with fresh medium, and the cells were left to grow for an additional 16–18 hr. Then the cells were replated at densities of 100-1000 cells/25-cm^2 flask and grown for 7–8 days until discrete colonies could be stained and counted. The colonies were washed with 0.9% saline solution, stained with 0.5% crystal violet in ethanol, and counted. The plating efficiency for CHO cells not treated with drugs was about 50%. In experiments to assess the effect of AGT inactivation on sensitivity to killing by BCNU, cells were treated with BG or 8-oxo-BG 2 hr before exposure to 80 μM BCNU. Then the medium was replaced with fresh medium containing the AGT inhibitor. The AGT inhibitor was added to the medium with which the cells were incubated for 16–18 hr after treatment with BCNU to ensure that inhibitor was present during the entire period in which DNA adducts formed by BCNU at the O^6-position of guanine existed in the cell [4, 31].

AGT Purification and Activity Assays

The mutant AGT proteins were purified to homogeneity using immobilized metal affinity chromatography as previously described [13].

The rate of repair of methylated DNA was determined by incubating the protein for various time periods at 37° with a [^{3}H]methylated DNA substrate prepared by reaction of calf thymus DNA with *N*-[*methyl*-^{3}H]*N*-nitrosourea and calculating the second-order rate constant [32]. This was determined by measuring the appearance of the [^{3}H]methylated AGT at various time points using concentrations of AGT protein determined in preliminary experiments to give readily measurable rates under the assay conditions. The reaction mixture (1 mL) for each time point contained AGT (4.5×10^{-10} M wild type, 2.7×10^{-9} M P140A, 6.5×10^{-9} M P140K, 3.8×10^{-9} M P138M/V139L/P140K, 4.5×10^{-9} M G160R, 2.5×10^{-9} M P140A/G160R, 5.1×10^{-9} M G156A, or 9.1×10^{-7} M R128A), 3.6×10^{-10} M O^6-[^{3}H]methylguanine in [^{3}H]methylated DNA substrate, and 50 μg of unlabeled calf thymus DNA in a buffer containing 50 mM Tris–HCl (pH 7.6), 5 mM dithiothreitol, and 0.5 mM EDTA.

The rate constant was determined by the following equation

$$K_t = 1/C_a^0 - C_b^0 \cdot \ln[C_b^0(C_a^0 - C_c)/C_a^0(C_b^0 - C_c)]$$

where C_a^0 is the initial concentration of the AGT protein, C_b^0 is the initial concentration of the methylated DNA substrate, and C_c is the concentration of methylated AGT formed at a given time *t*.

The loss of AGT activity in response to BG or 8-oxo-BG was determined by incubation of purified AGT with the inhibitor for 30 min at 37° in the presence of calf thymus DNA. The residual AGT activity then was determined, and loss of activity was plotted against the drug concentration. The EC_{50} value representing the amount of drug

TABLE 1. Properties of purified AGT mutant proteins

Mutant AGT	EC_{50} for BG* (μM)	Rate constant (% of wild type)
Wild-type	0.1	100†
P140A‡	4	42
P140K§	> 1200	10
P138M/V139L/P140K§	> 1200	10
G160R‖	4.5	17
P140A/G160R	65	13
G156A¶	60	4
C145A‡	—††	0
R128A**	—††	< 0.1

*The EC_{50} shown represents the concentration of BG required to produce 50% inactivation of the AGT content after incubation for 30 min *in vitro*.

†The 100% value for wild type was $38 \times 10^6\ M^{-1} \cdot min^{-1}$.

‡–**These mutants have been described previously: ‡ [11]; § [16]; ‖ [13]; ¶ [12]; ** [30].

††The EC_{50} cannot be calculated due to insufficient activity of the mutants.

needed to inactivate 50% of the AGT activity during the 30-min incubation was determined from these graphs. There was no significant loss of AGT activity on incubation under these conditions in the absence of drug.

Inactivation of Cellular AGTs by BG or 8-oxo-BG

Inactivation of AGT by BG or 8-oxo-BG was determined in cells that reached about 80% confluence after 4 hr of exposure to the AGT inhibitor. Cells were harvested and washed with PBS, and the cell pellets were stored at −80° until assayed. Cell extracts were prepared as previously described [26], and residual AGT activity was measured by incubation for 30 min at 37° with the [^{3}H]methylated DNA substrate and either measuring the loss of O^6-methylguanine from the substrate DNA after DNA hydrolysis and HPLC separation of the bases [28] or by measuring the [^{3}H]methylated protein formed, which was collected on nitrocellulose filters [16]. The results were expressed as the percentage of the AGT activity present in the control cells to which no drugs were added.

Western Blot Analysis

The expression of R128A, C145A, P140K, and P138M/V139L/P140K proteins in transfected CHO cells was determined using immunoblots. After SDS–PAGE in 12.5% acrylamide gels, the protein immobilized on the nitrocellulose membrane was determined with a Vistra ECF Western Blotting Kit (Amersham) using antibody MAP-1 [33]. The intensity of chemiluminescence was measured using a FluorImager (Molecular Dynamics).

RESULTS

Several novel mutations in the human AGT sequence have been described recently that lead to a reduced sensitivity to inactivation by the drug BG. Some of these mutations are summarized in Table 1. The mutations P140A and G160R produced a moderate increase in resistance to BG (40- to 45-fold), whereas the mutations G156A and the double mutation P140A/G160R (not described previously) gave a greater increase (600- to 650-fold). However, the greatest effect was obtained with mutant P140K and the triple mutant P138M/V139L/P140K, which include the proline to lysine change in P140K, giving a > 12,000-fold increase in resistance. It is noteworthy that the combination of the P140A and G160R mutations increased the resistance to BG substantially over either mutation alone. However, the P140A/G160R double mutant was still much less resistant to BG than the P140K single mutant.

Although these BG-resistant mutant AGT proteins are clearly active in bringing about the removal of methyl groups from O^6-methylguanine in DNA, the mutations did reduce the rate of repair of methylated DNA substrates. When the rate constant for the reaction was measured, it was found that this was decreased by a factor of 2.5 (for P140A) up to 25 (for G156A) (Table 1).

To test the ability of the BG-resistant P138M/V139L/P140K, P140K, and G160R AGT mutants to protect CHO cells from alkylating agents, the cDNAs were placed in an expression vector under the control of the CMV promoter, and the plasmids were transfected into CHO cells. Clones were isolated, and the AGT protein expression was analyzed by western blot analysis (results not shown). Clones representing all of these mutants, the wild-type AGT, and two inactive AGT mutants (C145A and R128A) that had similar levels of AGT protein were selected and used for further experiments, in which they were compared with CHO cells containing the pCMV plasmid without an insert. Except for the C145A and R128A mutant AGTs that lack activity *in vitro* (Table 1), all of the clones selected on the basis of a similar level of AGT protein expression had a similar level of AGT activity when this was tested using crude cell extracts with methylated DNA substrates in reactions that were run to completion (30 min).

As previously reported, CHO cells, which lack detectable levels of endogenous AGT expression, are very sensitive to killing by BCNU (<0.1% survival at 40 μM BCNU). The expression of the wild-type AGT and the mutant AGT proteins P140A or G156A protected these cells from BCNU [26]. As shown in Fig. 1, the expression of the P140K, P138M/V139L/P140K, or G160R mutant AGT proteins also protected the cells to about the same extent as wild-type AGT. These results indicate that the reduction in the rate of repair of DNA by these AGT mutants (shown in Table 1) does not prevent them from ameliorating the toxic effects of BCNU when expressed in CHO cells. However, the protection clearly is due to the ability of the AGT protein to repair alkylated DNA, since no significant protection was provided by the mutants C145A and R128A (Fig. 1). The C145A mutant is totally inactive, since the cysteine acceptor site for the alkyl group is removed [11], and the R128A mutant has a reduction of more than 1000-fold in the rate constant for DNA repair due to a reduced ability to bind to DNA [30].

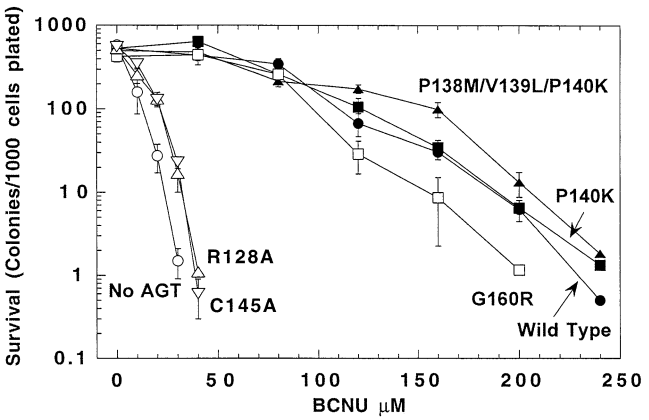

FIG. 1. Ability of BG-resistant AGT mutants to protect CHO cells from killing by BCNU. CHO clones expressing similar levels of wild-type, R128A, P140K, C145A, G160R, or P138M/V139L/P140K mutant AGTs were treated with the concentration of BCNU shown for 2 hr, and the survival was measured. Results are shown as the means ± SD for at least 5 estimations.

The AGT activity present in the CHO cells transfected with wild-type AGT was highly sensitive to inactivation by either BG or its 8-oxo-derivative, and more than 90% of the activity was lost with 10 μM levels of the inhibitors (Fig. 2 and Table 2). In contrast, there was no loss of activity at all in the CHO cells transfected with the P138M/V139L/P140K and P140K mutant AGTs even when 200 μM was used. The AGT activity in the cells expressing the G160R and P140A mutants was reduced by BG and 8-oxo-BG, but only when considerably higher concentrations were used than were needed to inhibit the wild-type AGT (Fig. 2). The EC_{50} values for the loss of AGT activity in these cells were increased by 25- to 30-fold over those for cells expressing wild-type AGT (Table 2).

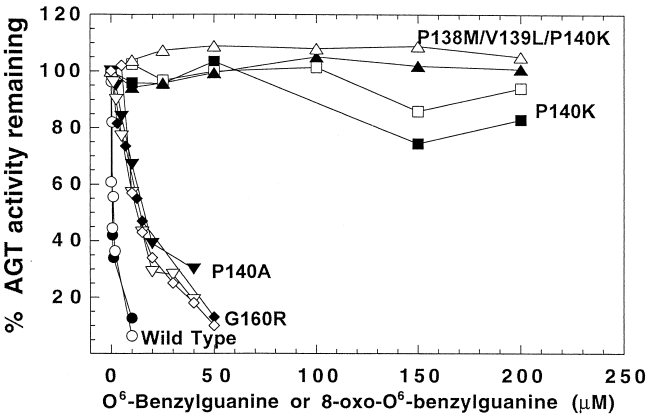

FIG. 2. Loss of AGT activity in CHO cells expressing wild-type and mutant AGTs upon treatment with BG or 8-oxo-BG. CHO clones expressing similar levels (11 pmol/mg protein) of wild-type, P140A, P140K, G160R, or P138M/V139L/P140K mutant AGTs were treated with the concentration of BG (open symbols) or 8-oxo-BG (closed symbols) shown for 4 hr, and the residual AGT activity was measured. Results are shown as the means ± SD for at least 4 estimations.

TABLE 2. Sensitivity of AGT activity in CHO cells expressing mutant AGTs to inactivation by BG or 8-oxo-BG

	EC_{50}* (μM) in CHO cells treated with:	
Mutant AGT	BG	8-oxo-BG
Wild-type	0.6†	0.4
P140A	15†	13
P140K	≫200	≫200
P138M/V139L/P140K	≫200	≫200
G160R	16	13
G156A	30†	ND

*The EC_{50} represents the concentration of compound required to produce 50% inactivation of the AGT content after exposure for 4 hr. This value was determined from graphs in Fig. 2 of cellular AGT activity remaining as a function of concentration of BG or 8-oxo-BG.

†Previously published [26].

This increase was slightly less than the 50-fold increase seen with mutant G156A that was reported previously [26].

To test the effects of BG on the protection of CHO cells from BCNU by mutant AGTs, the cells were treated with 80 μM BCNU and differing concentrations of BG (Fig. 3A). More than 99% of the cells expressing wild-type AGT

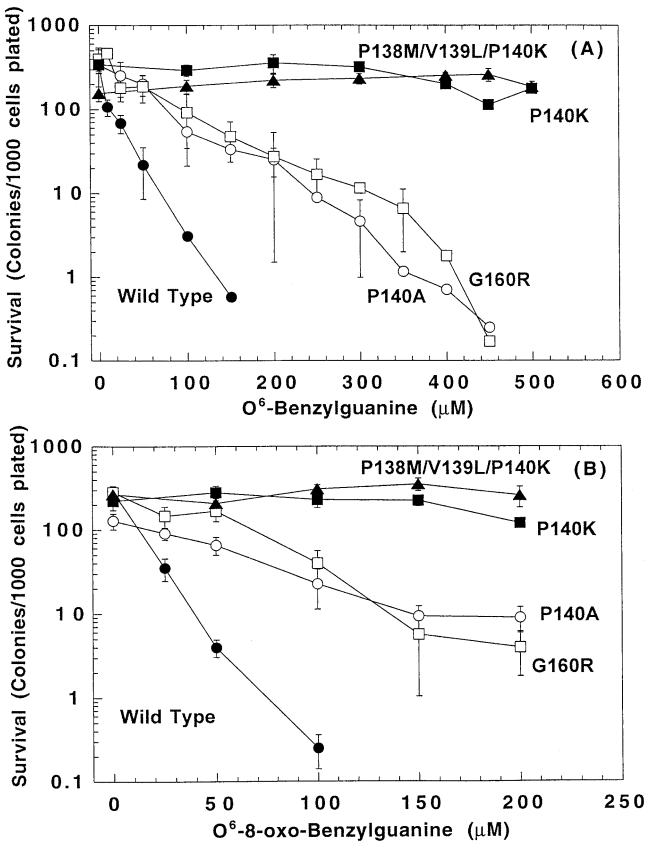

FIG. 3. Effect of treatment with BG or 8-oxo-BG on survival of CHO cells expressing wild-type and mutant AGTs after treatment with BCNU. CHO clones expressing similar levels of wild-type, P140A, P140K, G160R, or P138M/V139L/P140K mutant AGTs were treated with 80 μM BCNU and the concentration of BG (panel A) or 8-oxo-BG (panel B) shown, and the survival was measured. Results are shown as the means ± SD for at least 6 estimations.

were killed by 80 μM BCNU when 100 μM or higher concentrations of BG were used. All of the BG-resistant mutants tested allowed survival in the presence of much higher concentrations of BG. In fact, there was no significant reduction in cell viability with BG at 500 μM when cells expressing P140K or P138M/V139L/P140K mutant AGTs were used. These mutants were more effective than either P140A or G160R (Fig. 3A).

Essentially similar results were seen when the cells were treated with 8-oxo-BG instead of BG (Fig. 3B). The cells expressing P140K or P138M/V139L/P140K were unaffected by 200 μM 8-oxo-BG (the highest concentration that could be tested due to the limited solubility of 8-oxo-BG). Mutants P140A and G160R were less effective but still provided substantial protection, whereas the ability of wild-type AGT to protect from 80 μM BCNU was lost completely with 100 μM 8-oxo-BG (Fig. 3B).

DISCUSSION

Our studies have examined in more detail the properties of human AGT mutants reported to be resistant to BG [11–13, 16]. Although the initial studies showed no reduction in the activity of these mutants towards methylated DNA, the AGT reaction is normally very fast and is stoichiometric in the sense that one molecule of protein can repair only one molecule of a DNA lesion. The standard assays of AGT activity, therefore, do not normally measure the rate of methyl transfer and, unless the activity is so greatly impaired that complete repair does not occur during the assay period, alterations would not be detected. The more detailed studies involving the measurement of the rate constants carried out here and shown in Table 1 provide evidence that all of the BG-resistant human AGT mutants show a reduced rate of repair of methylated DNA. This reduction presumably also applies to the repair of O^6-chloroethylguanine, which represents the primary lesion acted upon by AGT to prevent the toxicity of chloroethylating agents such as BCNU [34, 35], although the chemical instability of this adduct prevents direct investigation of this process. However, the results presented here in Fig. 1 and previous results for mutants P140A and G156A [26] show that the reduction in rate constant for DNA repair does not prevent the BG-resistant mutant AGTs from providing protection equal to that of wild-type AGT in resistance of CHO cells to BCNU.

These results suggest that the reduced rate of repair is not a critical factor in limiting the effectiveness of AGT to prevent the formation of lethal interstrand cross-links in DNA [31, 36]. There is good evidence that the key lesion in cell killing by chloroethylnitrosoureas is an interstrand cross-link between guanine and cytosine on the opposite strand of DNA. This adduct is formed by an intramolecular rearrangement from O^6-chloroethylguanine to form 1,O^6-ethanoguanine which, in turn, reacts with the opposite cytosine to form the 1-(3-cytosinyl)-2-(1-guanyl)ethane cross-link [34–36]. This process takes several hours to complete, and both the initial O^6-chloroethylguanine and the 1,O^6-ethanoguanine are substrates for AGT. The wild-type AGT repair action is extremely fast compared with the rate of chemical reaction of the O^6-chloroethylguanine described above and with the rate of cell division in CHO cells, so that it appears that the reduction in rate seen with the mutants does not interfere significantly with AGT-mediated protection.

A potential problem with BG-resistant mutants that were selected on the basis of studies of recombinant proteins expressed in *E. coli* is that they may not localize correctly to the nucleus when expressed in mammalian cells. Very little is known about the factors needed to direct AGT to the nucleus, although preliminary evidence of a possible nuclear recognition sequence located at positions 124–128 has been described [37]. Our results provide good evidence that none of the mutations imparting BG resistance prevents the action of AGT on nuclear DNA adducts.

Our results also indicate that it is likely that the BG-resistant mutants studied are correctly folded and reasonably stable in mammalian cells. All of the experiments were carried out with clones that gave levels of protein and AGT activity similar to those of wild-type AGT. Although we did not directly measure the half-life of the AGT protein in the cell, it is unlikely that this is greatly different between the mutants and the wild type, since it was quite easy to select clones that had similar levels of expression. If any of the mutants was degraded much more rapidly than the wild-type AGT, a much greater synthesis rate would be needed to obtain a similar steady-state level of protein.

It may be noted that in past studies carried out with mutant G156A expressed in CHO cells, when clones with similar levels of AGT activity were selected, the amount of AGT protein detected by western blotting was much greater in this G156A clone than in the wild type [26]. These results suggest that the mutant G156A is less active than wild-type AGT in repairing cellular DNA. This could be due both to the reduced rate constant [which was decreased by 25-fold (Table 1), a larger reduction than that of any of the other BG-resistant mutants tested] and also to the instability or incorrect folding of part of the G156A protein in the cell.

Despite these problems with the G156A mutant, experiments by Gerson and colleagues have shown that its expression from a retroviral vector is quite effective in protecting mouse and human hematopoietic cells from the toxicity of BCNU plus BG [23–25]. The P140A mutant AGT has also been found to be active in such experiments [22]. However, neither of these mutants nor mutant G160R gave a maximal level of protection from BG and 8-oxo-BG in CHO cells (Fig. 3 and Ref. 26). It is possible that the level of protection afforded by mutants P140A and G160R is adequate to allow survival from the physiologically attainable doses of BG and BCNU. These mutants do also have the possible advantage that the rate constant for

repair is reduced by only 2.5- to 5-fold. However, our results show that the P140K AGT mutant is a logical choice for continued study of such gene therapy to enhance the tolerance to treatment with alkylating agents plus BCNU. This mutant gave equal protection from BCNU as wild-type and P140A AGT in CHO cells expressing the same level of protein, and this protection was unaffected by BG and 8-oxo-BG at all concentrations tested.

The alternative approach of using AGT mutants with multiple mutations to achieve greater BG resistance raises the increased possibility that protein stability and folding will be a limiting factor and may also render the expressed AGT more likely to elicit an immune response. Although Margison and colleagues [20, 21, 38] have reported that the double mutant P140A/G156A was active in protecting K562 cells and human bone marrow cells transduced with a retroviral vector, this mutant was not effective in other studies by another group [22]. In our experiments, which first identified the BG resistance of this double mutant [12], its limited stability and activity suggested that it would not prove suitable for expression studies.

The P140K mutant has a $\gg$ 1000-fold increase in resistance to BG and 8-oxo-BG when expressed in either CHO cells (Table 2) or *in vitro* (Table 1 and results [for 8-oxo-BG] not shown). Despite a 10-fold reduction in reaction rate in repairing methylated DNA, this mutant was as active as wild-type AGT in conferring protection from BCNU on CHO cells, and this protection was unaffected by BG or 8-oxo-BG at all concentrations tested. These results suggest that the P140K mutant AGT should be tested for use in gene therapy approaches to enhance the therapeutic effectiveness of BG combined with BCNU or with temozolomide.

Although no BG-resistant AGT mutants have yet been reported to arise in tumors treated with BCNU plus BG, our results emphasize that such resistance is potentially a significant problem in the use of BG for cancer chemotherapy. Efforts to provide inactivators of the resistant mutants would be greatly aided by determination of the crystal structure of wild-type and BG-resistant AGTs. Such studies are in progress. One approach that shows some promise is to use short oligodeoxynucleotides as inactivators. Preliminary studies have shown that the binding of these molecules to mutant AGTs presents the benzyl group in a favorable configuration for reaction even with the moderately BG-resistant mutants such as G156A and P140A [39]. However, it remains to be seen if this approach will be able to provide good inhibitors of the mutants, such as P140K, which are shown here to be totally unaffected by BG.

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References

1. 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.
2. Pegg AE, Dolan ME and Moschel RC, Structure, function and inhibition of O^6-alkylguanine-DNA alkyltransferase. *Prog Nucleic Acid Res Mol Biol* **51:** 167–223, 1995.
3. Dolan ME, Inhibition of DNA repair as a means of increasing the antitumor activity of DNA reactive agents. *Adv Drug Delivery Rev* **26:** 105–118, 1997.
4. Dolan ME and Pegg AE, O^6-Benzylguanine and its role in chemotherapy. *Clin Cancer Res* **3:** 837–847, 1997.
5. Friedman HS, Kokkinakis DM, Pluda J, Friedman AH, Cokgor I, Haglund MM, Ashley DM, Rich J, Dolan ME, Pegg AE, Moschel RC, McLendon RE, Kerby T, Herndon JE, Bigner DD and Schold SC Jr, Phase 1 trial of O^6-benzylguanine for patients undergoing surgery for malignant glioma. *J Clin Oncol* **16:** 3570–3575, 1998.
6. Dolan ME, Roy SK, Fasanmade A, Paras PR, Schilsky RL and Ratain MJ, O^6-Benzylguanine in humans: Metabolic, pharmacokinetic and pharmacodynamic findings. *J Clin Oncol* **16:** 1803–1810, 1998.
7. Stefan TL, Ingalls ST, Minkler PE, Willson JKV, Gerson SL, Spiro TP and Hoppel CL, Simultaneous determination of O^6-benzylguanine and 8-oxo-O^6-benzylguanine in human plasma by reversed-phase high-performance liquid chromatography. *J Chromatogr* **704:** 289–298, 1997.
8. Sorsa M, Hemminki K and Vainio H, Occupational exposure to anticancer drugs. Potential and real hazards. *Mutat Res* **154:** 135–149, 1985.
9. Pedersen-Bjergaard J, Long-term complications of cancer chemotherapy. *J Clin Oncol* **13:** 1534–1536, 1995.
10. Smith MA, McCaffrey RP and Karp JE, The secondary leukemias: Challenges and research directions. *J Natl Cancer Inst* **88:** 407–418, 1996.
11. 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.
12. 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.
13. Edara S, Kanugula S, Goodtzova K and Pegg AE, Resistance of the human O^6-alkylguanine-DNA alkyltransferase containing arginine at codon 160 to inactivation by O^6-benzylguanine. *Cancer Res* **56:** 5571–5575, 1996.
14. Christians FC, Dawson BJ, Coates MM and Loeb LA, Creation of human alkyltransferases resistant to O^6-benzylguanine. *Cancer Res* **57:** 2007–2012, 1997.
15. Encell LP, Coates MM and Loeb LA, Engineering human DNA alkyltransferases for gene therapy using random sequence mutagenesis. *Cancer Res* **58:** 1013–1020, 1998.
16. Xu-Welliver M, Kanugula S and Pegg AE, Isolation of human O^6-alkylguanine-DNA alkyltransferase mutants highly resistant to inactivation by O^6-benzylguanine. *Cancer Res* **59:** 1936–1945, 1998.
17. 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.
18. Goodtzova K, Kanugula S, Edara S, Pauly GT, Moschel RC and Pegg AE, Repair of O^6-benzylguanine by the *Escherichia coli* Ada and Ogt and the human O^6-alkylguanine-DNA alkyltransferase. *J Biol Chem* **272:** 8332–8339, 1997.
19. Crone TM, Kanugula S and Pegg AE, Mutations in the Ada O^6-alkylguanine-DNA alkyltransferase conferring sensitivity to inactivation by O^6-benzylguanine and 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine. *Carcinogenesis* **16:** 1687–1692, 1995.
20. Chinnasamy N, Rafferty JA, Hickson I, Lashford LS, Long-

hurst SJ, Thatcher N, Margison GP, Dexter TM and Fairbairn LJ, Chemopreventive gene transfer II: Multilineage *in vivo* protection of haemopoiesis against the effects of an antitumour agent by expression of a mutant human O^6-alkylguanine-DNA alkyltransferase. *Gene Ther* **5:** 842–847, 1998.
21. Hickson I, Fairbairn LJ, Chinnasamy N, Lashford LS, Thatcher N, Margison GP, Dexter TM and Rafferty JA, Chemopreventive gene transfer I: Transduction of human haemopoietic progenitors with O^6-benzylguanine-resistant O^6-alkylguanine-DNA alkyltransferase attenuates the toxic effects of O^6-alkylating agents *in vitro*. *Gene Ther* **5:** 835–841, 1998.
22. Maze R, Kurpad C, Pegg AE, Erickson LC and Williams DA, The P140A, but not P140A/G156A, mutant form of O^6-methylguanine-DNA methyltransferase protects hematopoietic cells against O^6-benzylguanine and chloroethylnitrosourea treatment. *J Invest Med* **44:** 318A, 1998.
23. Reese JS, Koc ON, Lee KM, Liu L, Allay JA, Phillips WP Jr and Gerson SL, Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O^6-benzylguanine plus 1,3-bis(chloroethyl)-1-nitrosourea. *Proc Natl Acad Sci USA* **93:** 14088–14093, 1996.
24. Davis BM, Reese JS, Koc ON, Lee K, Schupp JE and Gerson SL, Selection for G156A O^6-methylguanine DNA methyltransferase gene-transduced hematopoietic progenitors and protection from lethality in mice treated with O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Res* **57:** 5093–5099, 1997.
25. Koc ON, Reese JS, Davis BM, Liu L, Majczenko KJ and Gerson SL, ΔMGMT transduced bone marrow infusion increases tolerance to O^6-benzylguanine and BCNU and allows intensive therapy of BCNU resistant human colon cancer xenografts. *Human Gene Therapy* **10:** 1021–1030, 1999.
26. Loktionova NA and Pegg AE, Point mutations in O^6-alkylguanine-DNA alkyltransferase prevent the sensitization by O^6-benzylguanine to killing by *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea. *Cancer Res* **56:** 1578–1583, 1996.
27. Rafferty JA, Wibley JEA, Speers P, Hickson I, Margison GP, Moody PCE and Douglas KT, The potential role of glycine-160 of human O^6-alkylguanine-DNA alkyltransferase in reaction with O^6-benzylguanine as determined by site-directed mutagenesis and molecular modelling comparisons. *Biochim Biophys Acta* **1342:** 90–102, 1997.
28. 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.
29. 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 O^6-alkylguanine-DNA alkyltransferase. *J Med Chem* **38:** 359–365, 1995.
30. Kanugula S, Goodtzova K, Edara S and Pegg AE, Alteration of arginine-128 to alanine abolishes the ability of human O^6-alkylguanine-DNA alkyltransferase to repair methylated DNA but has no effect on its reaction with O^6-benzylguanine. *Biochemistry* **34:** 7113–7119, 1995.
31. Erickson LC, Laurent G, Sharkey NA and Kohn KW, DNA cross-linking and monoadduct repair in nitrosourea-treated human tumor cells. *Nature* **288:** 727–729, 1980.
32. Goodtzova K, Kanugula S, Edara S and Pegg AE, Investigation of the role of tyrosine-114 in the activity of human O^6-alkylguanine-DNA alkyltransferase. *Biochemistry* **37:** 12489–12495, 1998.
33. Pegg AE, Wiest L, Mummert C and Dolan ME, Production of antibodies to peptide sequences present in human O^6-alkylguanine-DNA alkyltransferase and their use to detect this protein in cell extracts. *Carcinogenesis* **12:** 1671–1677, 1991.
34. Ludlum DB, DNA alkylation by the haloethylnitrosoureas: Nature of modifications produced and their enzymatic repair or removal. *Mutat Res* **233:** 117–126, 1990.
35. 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.
36. Erickson LC, Bradley MO, Ducore JM, Ewig RA and Kohn KW, DNA cross-linking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. *Proc Natl Acad Sci USA* **77:** 467–471, 1980.
37. Ayi TC, Loh KC, Ali RB and Li BFL, Intracellular localization of human DNA repair enzyme methylguanine-DNA methyltransferase by antibodies and its importance. *Cancer Res* **52:** 6423–6430, 1992.
38. Hickson I, Fairbairn LJ, Chinnasamy N, Dexter TM, Margison GP and Rafferty JA, Protection of mammalian cells against chloroethylating agent toxicity by an O^6-benzylguanine-resistant mutant of human O^6-alkylguanine-DNA alkyltransferase. *Gene Ther* **3:** 868–877, 1996.
39. Pegg AE, Kanugula S, Edara S, Pauly GT, Moschel RC and Goodtzova K, Reaction of O^6-benzylguanine-resistant mutants of human O^6-alkylguanine-DNA alkyltransferase with O^6-benzylguanine in oligodeoxyribonucleotides. *J Biol Chem* **273:** 10863–10867, 1998.