Resistance of the Human $O^6$-Alkylguanine-DNA Alkyltransferase Containing Arginine at Codon 160 to Inactivation by $O^6$-Benzylguanine

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Abstract

Inactivation of $O^6$-alkylguanine-DNA alkyltransferase by $O^6$-benzylguanine renders tumor cells more sensitive to killing by methylating and chloroethylylating agents, and $O^6$-benzylguanine is currently undergoing clinical trials for development as an agent to enhance chemotherapy. It has been reported recently that a polymorphism in the human $O^6$-alkylguanine-DNA alkyltransferase gene exists, with about 15% of the population studied having arginine at codon 160 instead of glycine (Y. Imai et al., Carcinogenesis (Lond.), 16: 2441—2445, 1995). We have studied the effects of mutations of this glycine to arginine, tryptophan, or alanine on the interaction of human alkyltransferase with $O^6$-benzylguanine using direct determination of the amount of activity remaining after incubation with various concentrations of the inhibitor and measurement of the rate of production of $[8-3^H]guanine from$ $O^6$-benzyl$[^3H]guanine as assays. These mutations had little effect on the alkyltransferase activity in repairing $O^6$-methylguanine in methylated DNA. Alteration of glycine 160 to tryptophan or alanine slightly increased the sensitivity to $O^6$-benzylguanine (by up to 4-fold). However, alteration of glycine 160 to arginine drastically reduced the inactivation by $O^6$-benzylguanine with at least a 20-fold increase in the $ED_{50}$ value and a similar reduction in the production of guanine whether inactivation was carried out in the absence or presence of DNA. These results raise the possibility that a subpopulation of patients may be resistant to $O^6$-benzylguanine and that higher doses or additional alkyltransferase inhibitors capable of inactivating this form of the alkyltransferase will be necessary.

Introduction

It is now well established that the presence of AGT (EC 2.1.1.63) is an important factor in imparting resistance to the killing of tumor cells by methylating agents such as temozolomide and dacarbazine and by chloroethylylating agents such as BCNU and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (1, 2). One possible approach to overcoming this resistance would be to inactivate the alkyltransferase protein, and $O^6$-benzylguanine has been developed as an inhibitor for this purpose (3). Treatment of nude mice carrying human tumor xenografts with $O^6$-benzylguanine has been shown to improve the therapeutic index of BCNU at equitoxic doses (4—6) but it remains to be seen whether these results can be duplicated in human patients. Phase I clinical trials are currently underway to determine an effective dose of $O^6$-benzylguanine for alkyltransferase inactivation and the maximum tolerated dose of $O^6$-benzylguanine and BCNU.

Although human AGT has been reported to be very sensitive to $O^6$-benzylguanine with an $ED_{50}$ value of 0.2 $\mu$M (3), the equivalent proteins from a number of other species are less sensitive despite substantial sequence similarity (7). There is a particularly striking difference with the Escherichia coli Ada and the yeast alkyltransferases which are not inhibited by 1 mM. This difference is at least partially explained by steric factors which limit the size of the active site pocket in these proteins, thus excluding $O^6$-benzylguanine. Mutations to increase the size of this pocket by introducing a proline residue present at position 140 in mammalian AGTs and by removing a bulky tryptophan residue allow the Ada-C protein to react with $O^6$-benzylguanine (8). However, the mutated Ada-C protein is still substantially less sensitive to $O^6$-benzylguanine than the human AGT and other residues must also contribute to the ability to react well with this free base.

Point mutations in the human AGT sequence at proline 138, proline 140, and glycine 156 (9, 10) increase the $ED_{50}$ of $O^6$-benzylguanine by factors of 10, 20, and 240, respectively. Also, the mouse AGT, which is quite similar to the human AGT in amino acid sequence and contains all of these residues, has been reported to be 4—5-fold (11) or 12-fold (12) less sensitive to inactivation. These results suggest that variants of the human AGT having a lowered sensitivity to $O^6$-benzylguanine may exist and be selected for during exposure to this drug plus an alkylating agent. Such variants might be produced by the mutagenic action of alkylating agents or exist naturally due to polymorphism in the alkyltransferase gene.

It is unlikely that all of the positions at which the AGT can be altered to modify sensitivity to $O^6$-benzylguanine have yet been identified, and many of the residues forming the pocket containing the cysteine acceptor site are likely candidates for such sites. It was therefore of considerable interest that a polymorphism of codon 160 was reported in which about 15% (6/40) of the subjects tested had arginine substituted for glycine at this position (13). Although it was reported that this alteration had little or no effect on the activity of the alkyltransferase (when this was expressed as a bacterial fusion protein with glutathione S-transferase), the assays were conducted only for the ability to repair methylated DNA (13). In the experiments described below, we have examined the effect of alterations at codon 160 on the ability to react with $O^6$-benzylguanine. Replacement of glycine 160 with arginine renders the protein much less reactive toward this drug, whereas replacement with tryptophan or alanine increases reactivity.

Materials and Methods

Materials. Oligodeoxynucleotides were made in the Macromolecular Core Facility, Hershey Medical Center, by using a Milligen 7500 DNA synthesizer. GWR109 cells (14) were kindly provided by Dr. L. Samson (Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA). Restriction enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD) and New England Biolabs (Beverly, MA). The pQE-30 plasmid was obtained from Qiagen (Chatsworth, CA) and the Talon Metal Affinity Resin was purchased from Clontech (Palo Alto, CA). Ampicillin,
kanamycin, isopropyl β-D-thiogalactopyranoside, hemocyanin, and most other biochemicals were purchased from Sigma (St. Louis, MO). N²-[3H]-methyl-N²-nitrosourea (5.9 mCi/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). O²-Benzylguanine, synthesized as described (3), was generously provided by Dr. R. C. Moschel (ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). O²-Benzyl-[8-3H]guanine (0.34 mCi/mmol) was prepared by catalytic tritium exchange of O²-benzylguanine with tritiated water by Amersham Corporation and was purified as described previously (7).

Expression and Purification of AGT and Mutants G160A, G160W, and G161A. Homogeneous preparations of recombinant human AGT were obtained by expressing the protein from the pINAGT plasmid in GWR109 cells as described previously (7, 9, 10). This plasmid contains the human AGT sequence in the E. coli expression vector pIII-33 [bp³⁻]. The means of construction changes the amino terminal sequence by the addition of five amino acids, giving a sequence MKGKLL in place of M⁻⁻. The G160A, G160W, and G161A AGT mutant proteins were prepared in the same way. The mutations were introduced into pINAGT by PCR as described previously (9, 10) using the following primers (with the altered codon sequence underlined): for G160A, 5'-GGCAACTACTCCGAGCTGGCC-3'; for G160W, 5'-GGCAACTACTCCGGAGCTGGCC-3'; and for G161A, 5'-GGCAACTACTCCGAGCTGGCC-3'.

Construction of pQE30-hAGT Plasmid for Expression and Purification of Wild-Type AGT and Mutant G160R Protein. The G160R mutant AGT and a control AGT preparation were expressed and purified to homogeneity using the pQE30 vector to place a (His)₉ tag at the amino terminus of the protein, allowing purification by immobilized metal affinity chromatography. The plasmid construction adds the 12-amino acid sequence MRGS(H₉)GS⁻⁻ to the amino terminus of the protein.

The coding region of human AGT protein flanked by BamHI and KpnI sites was amplified by PCR using PGMAGT (10) as template DNA and the oligonucleotides 5'-GCGGATCCGCGGGTACCGAGGAT-TGT-3' with BamHI site (underlined) preceding the start codon (italics) at the N-terminal end and 5'-GACTCTAGACTTCACTTACGTTCTCC-3' with a KpnI site (underlined) following the stop codon (italics) at the C-terminal end. The PCR product was used to transform JM109 cells.

Asel:Fig. 1. Plasmid used for the production of (His)₉-tagged AGT and G160R mutant.

JM109 cells transformed with pQE30-hAGT or pQE30-G160R were grown in a 1-liter culture inoculated with an overnight grown culture at a 1:50 dilution. At a cell density equivalent to A₅₅₀ of 0.5, 0.3 mM isopropyl O-D-thiogalactopyranoside, was added and the cells were harvested 4 h later. The pellet was suspended in 30 ml of 20 mM Tris-HCl (pH 8.0) and 250 mM NaCl and disrupted using a French Press. After centrifugation at 17,000 × g for 45 min at 4°C to remove cell debris, the supernatant was applied to a 2-ml column of Talon IMAC resin (Clontech) equilibrated with 20 mM Tris-HCl (pH 8.0) and 250 mM NaCl, and the column was washed with this buffer containing 10 mM imidazole. The AGT protein was then eluted using 200 mM imidazole, and the fractions found to contain the AGT using SDS-PAGE were pooled and dialyzed immediately against 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM DTT, and 0.1 mM EDTA. The yield of protein was about 8 mg/liter of culture, and the purity was >90% as judged by SDS-PAGE.

Assay of Inactivation by O²-Benzylguanine. The purified AGT protein and mutants were incubated with O²-benzylguanine in 0.5 ml of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5.0 mM DTT in the presence or absence of calf thymus DNA as indicated for 30 min at 37°C. When DNA was omitted, 50 μg of hemocyanin were added instead. (Under these conditions with either DNA or hemocyanin added, the AGT activity was completely stable with <5% loss of activity in the 30-min incubation). The residual alkyltransferase activity was then determined by a 30-min incubation with a 32P-labeled methylated DNA substrate which had been methylated by reaction with N²-[3H]-methyl-N²-nitrosourea essentially as described (3, 15). The results were expressed as the percentage of the alkyltransferase activity remaining. The graphs of activity remaining against inhibitor concentration were used to calculate an ED₅₀ value representing the amount of inhibitor needed to produce a 50% loss of activity.

Reaction of AGT with O²-Benzyl-[8-3H]guanine. Measurements of [8-3H]guanine formation from O²-benzyl-[8-3H]guanine were carried out using an assay mixture consisting of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5.0 mM DTT in a volume of 0.25 ml in the presence or absence of DNA as indicated. The reaction was incubated for up to 2 h at 37°C, and at various times, aliquots were removed and the reaction stopped by the addition of 0.6—0.8 ml of the same buffer containing 0.2 mM of guanine and 0.2 mM of O²-benzylguanine. These aliquots were then separated by reversed-phase high-pressure liquid chromatography to determine the amount of guanine formed (16). The results were expressed as cpm of [8-3H]guanine formed per μg of protein per min. The amounts of protein and times of incubation used were such that [8-3H]guanine production was proportional to both the time of incubation and to the amount of protein added.

Results

Human AGT and mutants at glycine 160 and glycine 161 were expressed in E. coli from both the pIN and the pQE30 vectors as
indicated in Table 1 and purified to homogeneity. There was no significant difference in the repair of methylated DNA or in the inactivation by \( \text{O}^6 \)-benzylguanine between the control preparations isolated using the pIN and PQE30 systems, indicating that the addition of the (His)\(_6\) tag to the amino terminus of the protein by the PQE30 vector to facilitate purification did not affect the properties of the protein.

The mutations at glycine 160 and glycine 161 did not affect the level of expression and the specific activity of the purified preparations were all similar, indicating that these mutations do not destabilize the protein. They also had little or no effect on the ability of the protein to repair methylated DNA. The rate of repair of double-stranded methylated DNA by the AGT at 37°C is very fast and it is very difficult to measure accurately but, as shown in Table 1, comparison of the repair of methylated DNA by very small amounts of protein indicated that mutants G161A and G160R reduced the rate by at most 2-fold and mutations G160A and G160W had no effect at all on repair of methylated DNA. Our results with the G160R mutant confirm the previous report that this alteration had little or no effect on the activity of the AGT (13).

Comparison of the mutants of AGT in which glycine 160 was altered to a tryptophan or an alanine residue showed that these mutations increased the sensitivity of the protein to inactivation by \( \text{O}^6 \)-benzylguanine (Fig. 2A). The ED\(_{50}\) value was reduced by about a factor of 4 by these mutations from 0.4 to 0.1 \( \mu \text{M} \) (Table 1). In contrast, the conversion of glycine 160 to an arginine residue rendered the protein much more resistant than the control AGT (Fig. 2B), increasing the ED\(_{50}\) value by >20-fold to 9 \( \mu \text{M} \) (Table 1). The alteration of glycine 161 to alanine also increased the ED\(_{50}\) value for inactivation by \( \text{O}^6 \)-benzylguanine but only slightly, by 2.5-fold to 1 \( \mu \text{M} \).

Previous studies have shown that the reaction of AGT with \( \text{O}^6 \)-benzylguanine is increased in the presence of DNA (16) and it is possible that in vivo, the inactivation of AGT is enhanced in this way. However, as shown in Fig. 2C, the addition of DNA did not alter the large difference between the control and the G160R mutant AGT, although the ED\(_{50}\) values were lowered to 0.2 and 4 \( \mu \text{M} \), respectively, under these conditions.

The inactivation of AGT with \( \text{O}^6 \)-benzylguanine is caused by the protein recognizing the \( \text{O}^6 \)-benzylguanine as a substrate and converting it to guanine at the same time forming S-benzylcysteine at the active site (7, 17). The ability of AGTs to react with \( \text{O}^6 \)-benzylguanine can therefore be studied directly by measuring the initial rate of formation of guanine from \( \text{O}^6 \)-benzylguanine under conditions where the inhibitor is in excess. When these studies were carried out with the control and the mutant AGTs, the results confirmed that mutants G160A and G160W reacted slightly more rapidly with \( \text{O}^6 \)-benzylguanine than the control AGT and that mutants G161A and G160R reacted considerably more slowly (Fig. 3A). The difference was particularly striking for mutant AGT and where guanine production was reduced by >90% irrespective of whether the assay was carried out in the presence (Fig. 3B) or absence of DNA (Fig. 3A). The presence of DNA increased guanine formation by all of the AGTs tested but the increase was less for mutants G160A and G160W and the DNA addition abolished the difference between these mutants and the control AGT (Fig. 3).

**Discussion**

Our results show clearly that the presence of the basic arginine residue at position 160 in place of the neutral glycine found in the predominant form of human AGT substantially reduces the ability of the protein to react rapidly with \( \text{O}^6 \)-benzylguanine. This reduction cannot be attributed to a general decrease in the efficiency with which the protein carries out alky group transfer as repair of \( \text{O}^6 \)-methylguanine in DNA was reduced by at most a factor of 2 (Table 1). It is therefore likely to result from a decreased affinity of the protein for the \( \text{O}^6 \)-benzylguanine pseudosubstrate.
Ada-C protein described above, there is a more likely explanation for the striking effect of replacing glycine with arginine.

This is that the charged nature of the side chain is itself responsible for decreasing the affinity of binding of O°-benzylguanine. There is good evidence from comparisons of potency of inhibition by various O°-benzylguanine analogues that this binding is facilitated by hydrophobic interactions between the protein and the inhibitor (2, 21—23). The insertion of a charged arginine residue into this environment could therefore greatly weaken the binding of O°-benzylguanine. This explanation would also be consistent with the finding that insertion of an alanine or tryptophan at position 160 did not reduce the interaction with O°-benzylguanine but, in fact, slightly increased it. The fact that the very bulky tryptophan side chain can be tolerated suggests that adequate space exists at this position to allow binding. The increased sensitivity of the G160A and G160W mutants could be due to increased hydrophobic interactions.

The concept that the presence of an additional charged hydrophilic residue may reduce the ability to react with O°-benzylguanine as a free base could also provide an explanation for the decreased sensitivity of the mouse AGT compared to the human and rat AGT (11, 24). The mouse AGT has a charged histidine residue at the position equivalent to residue 157 in the human AGT. This position is a neutral asparagine in the more sensitive human and rat AGTs.

Irrespective of the means by which the effect is brought about, the finding that a naturally occurring variant of the human AGT with a decreased sensitivity to O°-benzylguanine exists must be taken into account in the design and interpretation of clinical trials with this drug. It is possible that a significant fraction of the population will show a decreased response to O°-benzylguanine. Several individuals who were homozygous for the arginine 160 form of AGT were identified by Imai et al. (13). They also found some subjects who were heterozygous for the G160R polymorphism. In such heterozygous patients, tumor cells expressing the resistant variant of the AGT protein may be selected by the treatment with O°-benzylguanine plus an alkylating agent. Our results therefore place additional emphasis on the synthesis and design of additional inactivators of AGT active against both the control and O°-benzylguanine-resistant forms of the protein.

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References


Fig. 3. Rate of formation of guanine from O°-benzylguanine by control and mutant AGTs. The rate of [8-3H]guanine formation from O°-benzyl[8-3H]guanine was measured as cpm of [8-3H]guanine formed per μg protein per min as described in "Materials and Methods." A, results for assay in the absence of DNA and B, results for assays in the presence of 50 μg of calf thymus DNA. Results are shown as the means for assays involving four or more estimations or as the mean for assays carried out in duplicate or triplicate. Bars, SE.


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