Mutations in Human O^6-Alkylguanine-DNA Alkyltransferase Imparting Resistance to O^6-Benzylguanine

Tina M. Crone, Karina Goodtova, Suvarchala Edara, and Anthony E. Pegg

Departments of Cellular and Molecular Physiology and Pharmacology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

ABSTRACT

O^6-Benzylguanine is an inactivator of O^6-alkylguanine-DNA alkyltransferases (AGT) which is currently entering clinical trials as an agent improving the cancer chemotherapeutic activity of chloroethylnitrosoureas and other alkylating agents. O^6-Benzylguanine acts by virtue of its ability to serve as a substrate for the AGT forming S-benzylcysteine at the cysteine acceptor site. The effects of a number of mutations in the human AGT sequence on the reaction with O^6-benzylguanine were investigated by two methods: (a) by measuring the loss of the ability of the AGT to repair a methylated DNA substrate after preincubation with O^6-benzylguanine; and (b) by measuring the production of guanine from O^6-benzylguanine by the AGT proteins. Both assays gave similar results and showed that mutations of the proline residues at positions 138 and 140 and of the glycine residue at position 156 significantly reduced the ability to react with O^6-benzylguanine. The combination of these mutations gave even greater resistance. Thus, the 50% effective dose for O^6-benzylguanine was increased from 0.25 μM in the control AGT to 29 μM by mutations P138K/PJ4OA, to 60 μM by mutation G156A and to >380 μM by mutations P140A/G156A. Truncation of the AGT at the carboxyl end, removing either 31 or 23 amino acids did not affect the activity or the ability to react with O^6-benzylguanine, but removal of the 36 carboxyl terminal amino acids, which includes a highly conserved glutamic acid residue, led to the loss of all activity. The rate of the reaction between the AGT and O^6-benzylguanine was increased when DNA was present. This increase amounted to about 6-fold with the control AGT and the carboxyl-truncated mutants but was reduced to only 2-fold with G156A mutant and increased to 11–18-fold with the mutations of proline residues at 138 and 140. These results indicate that several residues in the AGT sequence affect the access of the active site to O^6-benzylguanine and that these residues are located in at least two regions on either side of the active site cysteine, which is located at residue 145. Mutations in these regions may occur during therapy with alkylating agents and O^6-benzylguanine. The development of other AGT inactivators which are still able to inactivate the resistant mutants may be necessary to maximize the potential of AGT inhibition for cancer chemotherapy.

INTRODUCTION

The repair of adducts at the O^6 position of guanine in alkylated DNA is brought about by the action of AGT(1–6). This protein acts by transferring the alkyl group to a cysteine acceptor molecule within its own sequence. The content of active AGT is therefore a critical factor in the sensitivity of cells to the toxic and mutagenic effects of exposure to agents forming O^6-alkylguanine. Many studies have shown that cells lacking AGT are more sensitive to mutagenesis by methylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine and are more sensitive to killing by therapeutic methylating agents such as procarbazine, dacarbazine, and temozolomide and by chloroethylnitrosoureas such as BCNU, 1-(4-amino-2-methyl-5-pyrimidiyl)methyl-3-(2-chloroethyl)-3-nitrosourea, trans-1-(2-chloroethyl)-3-(4-methyl-cyclohexyl)-1-nitrosourea, and clomesome (3, 7, 8).

Although a significant fraction of the human tumor cell lines tested exhibit the Mer^- phenotype and fail to express AGT, the great majority of primary tumors contain Mer^+ cells (1, 3, 6). The presence of active AGT in these cells, therefore, imparts significant resistance to cancer chemotherapy using chloroethylnitrosoureas or methylating agents.

In order to overcome this resistance, a series of compounds able to inactivate the AGT have been synthesized (9–13). The most studied of these is O^6-benzylguanine. This compound was found to be a potent inhibitor of the human AGT, and theoretical considerations and time-dependent characteristics of the inactivation suggested that the O^6-benzylguanine acts as a low molecular weight substrate for the protein (9). More recent studies have fully confirmed this model and shown that inactivation is accompanied by the stoichiometric formation of S-benzylcysteine at the active site of the AGT protein and the release of guanine from the O^6-benzylguanine (14, 15). The AGTs from other mammalian species including mice, rats, and Syrian hampsters have also been shown to be rapidly inactivated by <5 μM concentrations of O^6-benzylguanine (14, 16–18). In contrast, the AGTs from Escherichia coli (14, 18, 19), Saccharomyces cerevisiae (14) and Bacillus subtilis are very resistant to inactivation by O^6-benzylguanine with no inhibition occurring on incubation with concentrations of >200 μM (14, 18, 19). This finding is quite surprising since all of these AGTs have the same sequence, (I/V)PCHR(WI), surrounding the cysteine acceptor site and significant other similarities (5, 6, 20, 21).

These results raise the possibility that mutations in the sequence coding for the human AGT could lead to the protein becoming resistant to O^6-benzylguanine. This possibility was supported by our preliminary finding that mutation of proline-140 to alanine in the human AGT sequence did indeed produce a protein with a significantly lower ability to react with O^6-benzylguanine (22). Thus, additional AGT inhibitors may be needed in order to ensure that such resistance can be overcome.

Furthermore, although treatment with O^6-benzylguanine did sensitize a variety of Mer^- human tumor cells in cell culture to killing by methylating and by chloroethylnitrosoureas and improved the chemotherapeutic index when these agents were used to treat human tumors carried as xenografts in nude mice (9, 13, 16, 23–32), this compound is not an ideal therapeutic agent. It is relatively insoluble in water, necessitating formulation in a PEG-300 vehicle, it is rapidly excreted, and the doses needed for effective inactivation of the AGT in vivo are quite high (13, 32–34). Therefore, more information on the mechanism of action of the AGT is needed in order to design improved AGT inhibitors.

In order to obtain more data on the structural features that are important for the AGT to react with low molecular weight pseudosub-

Received 6/29/94; accepted 9/30/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Cancer Institute Grants CA-18137 and CA-57725.

2 To whom requests for reprints should be addressed, at Penn State University, Milton Hershey Medical Center, Cellular and Molecular Physiology, 500 University Drive, Hershey, PA 17033.

3 The abbreviations used are: AGT, O^6-alkylguanine-DNA alkyltransferase; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; PCR, polymerase chain reaction; cDNA, complementary DNA; dNTP, deoxynucleotide triphosphate; ED_50, the concentration of O^6-benzylguanine needed to reduce the activity by 50%; RF-HPLC, reverse phase high pressure liquid chromatography.

4 T. M. Crone and A. E. Pegg, unpublished observations.
strates such as Oβ-benzylguanine and with alkylated DNA, we have produced a series of mutations in the human AGT sequence, expressed the mutated proteins in *E. coli*, and studied the activity of the recombinant proteins. The results show that alterations of amino acid residues in regions on either side of the active site sequence can lead to a striking change in the ability to react with Oβ-benzylguanine, and on the stimulation of this reaction by DNA, which results from a change in conformation in the AGT protein on binding DNA (35).

**MATERIALS AND METHODS**

**Preparation of AGT Mutants.** Plasmid pINAGT, which expresses the human AGT in *E. coli*, was produced by inserting the human cDNA-coding sequence into the *E. coli* expression vector pNIII-A3 (pSP5-3) (36) using the EcoRI and BamHI sites in the vector and PCR to generate the appropriate sites in the cDNA as described previously (14). The pINAGT was digested with EcoRI and BamHI, and the resulting fragment containing the human AGT amino acid coding sequence was inserted into pGEM-3Zf (+). This plasmid, termed pGEMAGT, was used to generate the mutations as described below.

Mutants W100A, P140A, and G156A were produced as described previously (22). Alkyltransferase mutants ΔI-19, ΔD2-97, P138K, H146A, R147A, and G156A were made in a similar way by site-directed mutagenesis using purified, single stranded DNA from pGEM-AGT and Oligonucleotide-directed Mutagenesis System (Ver. 2.1; Amersham, Amersham, UK), according to manufacturer’s instructions. The following oligodeoxynucleotides (with mismatches underlined if present) were used to generate the mutants indicated: ΔI-19, 5'-GGGAGAAGCGAATTCGCTCGGTTGAGC-3'; ΔD2-97, 5'-CACCATTCCCTCCGGAGGTGCATATGTTGACTG-3'; P138K, 5'-GCAAGTGGAGACTAAGGCTACCCATCTATCCGG-3'; H146A, 5'-CCTCAATTTGCTGGCGAGTTCGTCG-3'; R147A, 5'-CATCCCGGCTCCAGGTGTTGCTGAGC-3'; G156A, 5'-AGCGAGGACGTGCGCC-3'; E172Q, 5'-CGACTACCTTGG-3'; E172Stop, 3'-GAA-GACCGGOTAAAGCGGAGcCGTGGçCAACTACrCCGGA-3'; G156W, 5'-GCCGTGTGGA-3'; P138A, 5'-GTGAAATGAAACGCAGAATfCTGGACAGC-3'; P138K/G156A, G156W, E172Q, E172Stop, Gl77Stop, Sl85Stop, and PJ4OA/Sl85Stop. The sense primer specific to the 11 promoter, which lies 5' to the AGT cDNA sequence, was used as the template-specific primer for production of all mutations except ΔI-19. The antisense primer specific to the SP6 promoter, which lies 3' to the AGT cDNA sequence, was used as the template-specific primer for production of ΔI-19.

Mutations P138A, P138A/PJ4OA, P138K/PJ4OA, P140A/G156A, G156W, and E172Q were constructed by asymmetric primer extension using two rounds of PCR (37) with pGEMAGT as the template for P138A, G156W and E172Q, and pGEMAGT containing the P140A mutation for the others. The reaction mixture for the first round contained 5 ng template, 50 pmol mutagenic primer, 75 pmol SP6 template-specific primer, 250 μM dNTPs, 10 μM of 10× reaction buffer, and 1.0 unit Vent DNA polymerase in a total volume of 100 μl. The PCR product of the first round corresponding to a band of <300 base pairs was isolated on a 2.0% agarose gel, purified, and suspended in 150 μl water yielding approximately 0.4 pmol/μl. The reaction mixture for the second round included 5 ng template, 10 pmol purified PCR product, 70 pmol T7 template-specific primer, 250 μM dNTPs, 10 μM of 10× reaction buffer and 1.0 unit Vent DNA polymerase in a total volume of 100 μl. A fragment between 0.6 and 0.7 kilobase was isolated using Gene Clean (Bio 100, La Jolla, CA) according to the manufacturer’s instructions.

Five μg of purified fragments from the PCR reactions were digested with EcoRI and BamHI, precipitated with ethanol and dissolved in water, and ligated into 100 ng EcoRI/BamHI-digested pINIII-A3 (pSP5-3) vector. One-third of the ligation reactions were electroporated into electrocompetent DH5α cells and plated on Luria-Bertani agar plates supplemented with 100 μg/ml ampicillin and plasmids were isolated. Mutant plasmids were verified through DNA sequencing. The insert fragments were removed by digestion with EcoRI and BamHI, purified and ligated into pNIII-A3 (pSP5-3) to generate the mutated pINAGT plasmids.

The human AGT mutants ΔI-19, P138A, P138A/PJ4OA, P138K/PJ4OA, P140A/G156A, G156W, E172Q, E172Stop, and P140A/ Sl85Stop were made by PCR. The following oligodeoxynucleotides (with mismatches underlined) were used to generate the mutants indicated: ΔI-19, 5'-GGGAGAAGCGAATTCGCTCGGTTGAGC-3'; ΔD2-97, 5'-CACCATTCCCTCCGGAGGTGCATATGTTGACTG-3'; P138K, 5'-GCAAGTGGAGACTAAGGCTACCCATCTATCCGG-3'; H146A, 5'-CCTCAATTTGCTGGCGAGTTCGTCG-3'; R147A, 5'-CATCCCGGCTCCAGGTGTTGCTGAGC-3'; G156A, 5'-AGCGAGGACGTGCGCC-3'; E172Q, 5'-CGACTACCTTGG-3'; E172Stop, 3'-GAA-GACCGGOTAAAGCGGAGcCGTGGçCAACTACrCCGGA-3'; G156W, 5'-GCCGTGTGGA-3'; P138A, 5'-GTGAAATGAAACGCAGAATfCTGGACAGC-3'; P138K/G156A, G156W, E172Q, E172Stop, Gl77Stop, Sl85Stop, and PJ4OA/Sl85Stop. The sense primer specific to the 11 promoter, which lies 5' to the AGT cDNA sequence, was used as the template-specific primer for production of all mutations except ΔI-19. The antisense primer specific to the SP6 promoter, which lies 3' to the AGT cDNA sequence, was used as the template-specific primer for production of ΔI-19.

**Expression and Assay of Recombinant AGT.** The recombinant human AGT and its mutants were expressed in *E. coli* GWR109 as described previously (14, 22). Bacteria containing the pINAGT plasmids were grown to an *A*<sub>oo</sub> of 1.0 in the presence of 0.3 μM isopropyl β-D-thiogalactopyranoside. The cells were then collected, and extracts prepared for the assay of AGT activity by resuspension in 2 ml of 50 mm Tris-HCl (pH 7.5)-0.1 mm EDTA-5 mm dithiothreitol and sonication for 8 min at 0°C using an Ultrasonic Cell Disruptor Model W-225-R on pulse setting 50% duty cycle. Cell debris was pelleted by centrifugation at 4°C for 30 min at 15,000 x g and the supernatant was used for assays. The AGT activity was determined by measuring the removal of Oβ-methylguanine from a calf thymus DNA which had been methylated by reaction with N<sup>6</sup>-(3H)methyl-N-nitrosourea as described (9).

**Purification of AGT.** Wild-type and mutant AGTs were purified using precipitation with polyvinyl P, ammonium sulfate fractionation, and chromatography on Mono S (Pharmacia) as described previously (14). The final specific activity of the preparations was ≥75% of that expected if the protein was homogeneous and all molecules were active (46 pmol/μg). The purified proteins were used for studies of the conversion of Oβ-benzylguanine to guanine as described below.

**Determination of the Sensitivity of the AGTs to Inactivation by Oβ- Benzylguanine.** The sensitivity of the AGTs by Oβ-benzylguanine was determined essentially as described previously for crude extracts from HT29 cells (10). Extracts containing the AGT were incubated with Oβ-benzylguanine for 30 min at 37°C in 50 mm Tris-HCl (pH 7.5)-1 mm dithiothreitol-0.1 mm EDTA in a volume of 0.5 ml. A further 0.5 ml of the same buffer containing the methylated DNA substrate was then added, and the remaining AGT activity was measured over a further 30-min incubation period. Results were expressed as the ED<sub>50</sub>. In some experiments, the ability of Oβ-benzylguanine to act as a competitive inhibitor of the AGT was determined by adding the Oβ-benzylguanine directly to the assay medium containing the Oβ-methylated DNA substrate, starting the reaction by the addition of the AGT, and incubating for 30 min at 37°C.

**Formation of Guanine from Oβ-Benzylguanine.** Measurements of guanine formation from Oβ-benzylguanine were carried out using various amounts of the purified AGT protein in an assay buffer consisting of 0.7 μM Oβ-benzyl-[<sup>3</sup>H]guanine and 50 mm Tris-HCl (pH 7.5), 0.1 mm EDTA, and 5 mm dithiothreitol in an assay volume of 0.25 ml (14, 35). Various amounts of calf thymus DNA were added to these assays as indicated. The formation of labeled product was stopped by the addition of 0.6–0.8 ml of the same buffer containing 0.2 μM of guanine and 0.2 μM of Oβ-benzylguanine. Aliquots were then separated by RP-HPLC on a Beckman Ultrasphere ODS column (25 cm x 4.6 mm) using isocratic elution at a temperature of 36°C and a buffer of equal parts methanol and 0.05 M ammonium formate (pH 4.5). Guanine was eluted...
at 3 min and 0\(^{-6}\)-benzylguanine at 12 min. The eluate from the HPLC was monitored by radioactivity by mixing with 3.5 volumes of Flow Scint CIII and passing through a Radiomatic Flo-One/Beta A-140 A radioactivity monitor (Packard Instruments). The efficiency of counting was 38%. Assays were carried out under conditions where the amount of guanine was proportional to the amount of AGT protein added. Different amounts of protein from the AGT preparations had to be used in order to ensure that assays were carried out under conditions where the amount of guanine formed was proportional to the amount of protein added. Results were expressed as cpm in guanine formed/µg of AGT protein added.

In some experiments, the ability of the AGTs to form 2'-deoxyguanosine from 0\(^{-6}\)-benzyldeoxyguanosine was determined. This was carried out in a similar way replacing the 0\(^{-6}\)-benzyl-[8-\(^{3}\)H]guanine with 0.23 µM 0\(^{-6}\)-benzyl-[8-\(^{3}\)H]deoxyguanosine. The reaction products were separated by RP-HPLC on a Beckman UltraspHERE ODS column (25 cm x 4.6 mm) using isoionic elution at a temperature of 36\(^{\circ}\)C with a buffer of methanol:0.05 M ammonium formate, pH 4.5 (35:65). In this system, 2'-deoxyguanosine eluted at 4 min and 0\(^{-6}\)-benzyldeoxyguanosine at 33 min.

Materials. All oligodeoxynucleotides were made in the Macromolecular Core Facility, Hershey Medical Center, by using a Milligen 7500 DNA synthesizer. GWR109 cells (38) were generously provided by Dr. L. Samson (Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA). DH5α MCR cells were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Restriction enzymes were purchased from GIBCO-BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA). Ampicillin, kanamycin, isopropyl \(\beta\)-D-thiogalactopyranoside, and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

A series of mutations were made in the human AGT cDNA and the proteins were expressed in E. coli GWR-109 cells. Extracts from the cells were used for the determination of AGT activity using the standard assay in which labeled 0\(^{-6}\)-methylguanine is removed. A representative inhibition curve for control AGT and human SPPGRW-207 is shown in Fig. 2. Mutations of human AGT retaining activity towards methylated DNA substrates. The mutations made are indicated above the human AGT sequence. The sequence of the carboxyl-terminal domain of the E. coli Ada AGT is shown for comparison.

These active mutant alkyltransferase proteins were tested for inhibition by 0\(^{-6}\)-benzylguanine using an assay in which the extracts from E. coli expressing the respective AGT were incubated at 37\(^{\circ}\)C for 30 min with increasing amounts of the inhibitor in a total volume of 0.5 ml. An additional 0.5 ml of a solution containing a \(^{3}\)H-methylated DNA substrate was then added and the remaining AGT activity was determined. The results were plotted and a value for the ED\(_{50}\) was determined. A representative inhibition curve for control AGT and mutants G156A, P138K/P140A, and P140A/G156A is shown in Fig. 3A, and ED\(_{50}\) values for all of the mutants are shown in Table 1.

Sensitivity to 0\(^{-6}\)-benzylguanine was not affected by mutation W100A or by truncating the carboxyl end of the protein at position 177 or 185. Mutation of the proline residue at amino acid 138 to either alanine or lysine increased the resistance to 0\(^{-6}\)-benzylguanine by 8-10-fold. A somewhat greater increase (20-fold) was produced by changing the proline at position 140 to alanine or lysine. The double mutation G156A, P138A, or 185 increased the resistance to 0\(^{-6}\)-benzylguanine by about 100-fold. The single mutation that made the greatest difference to the response to 0\(^{-6}\)-benzylguanine was changing the glycine at position 156 to either an alanine or a tryptophan. This increased the ED\(_{50}\) value 240-fold from 0.25 µM to 60 or 80 µM. When the G156A mutation was combined with a mutation at proline 140, the ED\(_{50}\) value was increased to more than 300 µM and could not be determined accurately owing to the limited solubility of 0\(^{-6}\)-benzylguanine (Table 1, Fig. 3A).

These changes in the ability to react with 0\(^{-6}\)-benzylguanine detected in this assay are not due to a general reduction in the rate at which the mutant AGT proteins react with all substrates. There was no difference in the rate of repair of methylated DNA by these mutants, which was very rapid, such that the reaction went to completion within less than 5 min (results not shown). Furthermore, when a competitive assay was carried out and the 0\(^{-6}\)-benzylguanine was added at the same time as the DNA substrate, there was still a very large increase in resistance produced by the G156A and P138K/P140A mutants (Fig. 3B).
Table 1 Effect of mutations in AGT on sensitivity to O6-benzylguanine

<table>
<thead>
<tr>
<th>Alkyltransferase</th>
<th>ED50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.25</td>
</tr>
<tr>
<td>W100A</td>
<td>0.25</td>
</tr>
<tr>
<td>P138K</td>
<td>2.00</td>
</tr>
<tr>
<td>P140A</td>
<td>2.50</td>
</tr>
<tr>
<td>G156A</td>
<td>5.00 a</td>
</tr>
<tr>
<td>G156W</td>
<td>60.00</td>
</tr>
<tr>
<td>P138K/P140A</td>
<td>80.00</td>
</tr>
<tr>
<td>P138K/P140A</td>
<td>22.00</td>
</tr>
<tr>
<td>P140A/G156A</td>
<td>29.00</td>
</tr>
<tr>
<td>P140A/S185Stop</td>
<td>&gt;300.00</td>
</tr>
<tr>
<td>G177Stop</td>
<td>5.00</td>
</tr>
<tr>
<td>S185Stop</td>
<td>0.30</td>
</tr>
</tbody>
</table>

a The ED50 value was calculated as described under "Materials and Methods" using assay results obtained as described in Fig. 3A.

b These mutants were reported previously (22).

Fig. 3. Effect of O6-benzylguanine on activity of AGT and its G156A, P140A/G156A, and P138K/P140A mutants. (A) Results in the standard assay in which the AGTs were incubated with the concentration of O6-benzylguanine shown for 30 min in a volume of 0.5 ml prior to the addition of another 0.5 ml containing the 3H-methylated DNA substrate. Residual AGT activity was then determined after a further 30-min incubation in the presence of the substrate and expressed as the percentage of the activity found when no O6-benzylguanine was added. (B) Results when O6-benzylguanine was added directly to the AGT assay medium at the concentration shown.

Fig. 4. Formation of guanine from O6-benzylguanine by AGT mutants. Results are shown for the amount of guanine formation in assays containing 0.7 µM O6-benzyl-[8-3H]guanine, 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol; plus 6 µg of AGT from control, W100A, G177Stop, and S185Stop; 40 µg of P138K, P140A, and P140A/S185Stop; 100 µg of G156A; and 200 µg of P140A/G156A in an assay volume of 0.25 ml. Dark shaded bar, results for assays carried out in the absence of calf thymus DNA. Total bar, results for assays carried out in the presence of 5 µg of DNA for AGT from control, W100A, G177Stop, and S185Stop; and in the presence of 10 µg of DNA for assays with AGT from 40 µg of P138K, P140A, P140A/G156A, P140A/S185Stop, and G156A. The actual values as cpm (× 103)/µg protein were: control, 2.49; W100A, 2.67; P138K, 0.77; P140A, 0.29; G156A, 0.05; P138A/P140A, 0.06; P140A/G156A, 0.004; P140A/S185Stop, 0.35; G177Stop, 2.63; S185Stop, 2.23. purified mutant AGTs to react with O6-benzylguanine was carried out. The AGTs were purified, and the purified proteins were incubated with O6-benzyl-[8-3H]guanine. We have shown previously that inactivation of the AGT protein results from the formation of S-benzylcysteine at the active site of the AGT with the stoichiometric formation of guanine (14, 15). When the active AGT is incubated with O6-benzyl-[8-3H]guanine, [3H]guanine is produced (14, 35). As shown in Fig. 4, the formation of guanine by the purified AGTs was not affected by the mutations W100A, G177Stop, and S185Stop, was reduced by 70% by mutation P138K, by 89% by mutations P140A and P140A/S185Stop, by 98% by mutations G156A and P138A/P140A, and by >99.8% by mutation P140A/G156A. These results are in good agreement with the ability of the AGTs to act on O6-benzylguanine as determined by the ED50 values in Table 1.

The ability of the control AGT and of most of the mutant AGTs to form [3H]guanine from O6-benzyl-[8-3H]guanine was stimulated by DNA (Figs. 4 and 5). However, even in the absence of DNA, the mutations at positions 138, 140, and 156 greatly reduced the ability to bring about this conversion (Fig. 4). The extent of stimulation of [3H]guanine production by DNA was investigated in more detail (Fig. 5). The control AGT and the AGT truncated at position 185 were stimulated by about 6-fold. In mutants W100A and G177Stop, there was a small reduction in this stimulation to about 4-fold but mutant
Table 2 Conversion of O\(^6\)-benzyldeoxyguanosine to deoxyguanosine by AGT mutants

<table>
<thead>
<tr>
<th>Alkyltransferase</th>
<th>Deoxyguanosine formed (cpm/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assayed − DNA</td>
</tr>
<tr>
<td>Control</td>
<td>452</td>
</tr>
<tr>
<td>P138K</td>
<td>134</td>
</tr>
<tr>
<td>P140A</td>
<td>25</td>
</tr>
<tr>
<td>G156A</td>
<td>28</td>
</tr>
<tr>
<td>S185Stop</td>
<td>409</td>
</tr>
</tbody>
</table>

G156A was much less responsive to DNA with only a 2.5-fold stimulation (Fig. 5A). In contrast, the P138K, P140A, and P140A/S185Stop mutants were stimulated to a much greater extent than the control AGT, by 11-, 18-, and 16-fold, respectively (Fig. 5B). The P138A/P140A double mutant was stimulated very slightly by DNA but the formation of guanine by this mutant was very small and the accuracy of the measurements with this mutant is questionable.

We have shown previously that O\(^6\)-benzyldeoxyguanosine is also able to inactivate the mammalian AGT (10) and that the inactivation is accompanied by the formation of deoxyguanosine (35). The ability of some of the mutants to form [\(^8\)-\(^3\)H]deoxyguanosine from O\(^6\)-benzyl-[\(^8\)-\(^3\)H]deoxyguanosine was determined (Table 2). It was found that mutant S185Stop did not differ from the wild-type AGT in this reaction but mutants P138K, P140A, and G156A were much less active on O\(^6\)-benzyldeoxyguanosine (30, 6, and 6%, respectively, when assayed in the presence of calf thymus DNA). This indicates that these mutations also impart resistance to inactivation by O\(^6\)-benzyldeoxyguanosine. In contrast to the reaction with the free base, the rate of reaction of the AGTs with the deoxynucleoside was inhibited by DNA except for the G156A mutant, which was not significantly affected by DNA. These results are consistent with the hypothesis that the binding of O\(^6\)-benzyldeoxyguanosine to the DNA-activated form of the AGT is reduced (10).

DISCUSSION

AGT cDNAs have been isolated and the protein sequence derived from a number of sources including, E. coli, Salmonella typhimurium, B. subtilis, S. cerevisiae, human, rat, mouse, Chinese hamster (reviewed in Refs. 5, 6, 20) and rabbit (21). There is a significant degree of homology between these proteins. Included among the 22 invariant residues is the sequence PCHR containing the active site cysteine. The mechanism for alkyl transfer is thought to involve acid-catalyzed S\(_2\)2 chemistry with a cysteine thiolate acting as the nucleophile (4, 39).

The basic histidine and arginine residues may aid in the generation or chemistry with a cysteine thiolate acting as the nucleophile (4, 39). Recently, the crystal structure of the carboxyl-terminal domain of the E. coli ada gene product (AdaC) has been determined (20). The crystal structure shows that the active site cysteine is buried within the structure and that a significant conformational change must occur on binding DNA in order to permit access of the substrate. It has been proposed that a movement about residues 325−335 would swivel the COOH-terminal helix formed by residues 340−350 to expose the cysteine. This movement would also break the bond between glutamic acid-348 and histidine-322 allowing the histidine to rotate and facilitate the formation of the needed thiole anion (20). These residues are conserved in all known AGT sequences and the equivalent residues in the human AGT are histidine-146 and glutamic acid-172. The lack of activity of the mutants H146A, R147A, E172Q, and E172Stop (Fig. 1) (40, 41) supports the concept that these residues, which are all conserved in all AGTs, are involved in the mechanism of alkyl transfer.

The hypothesis that the binding of DNA causes a conformational change in the AGT that activates the cysteine at the active site is strongly supported by experiments showing that the rate of reaction with O\(^6\)-benzylguanine is enhanced by the addition of DNA (35) (Figs. 4 and 5). Unfortunately, more direct evidence for the reaction mechanism is not currently available because there is no information on the location of the binding site for the O\(^6\)-alkylguanine or of the residues involved in this binding since the only crystal structure available is that of the protein. The use of low molecular weight substrates such as O\(^6\)-benzylguanine to study the reaction may be of value in such analysis but, as described below, the Ada AGT does not act on this substrate.

It should be noted that the apparent loss of AGT activity against methylated DNA substrates with the mutations summarized in Fig. 1 and described as inactive may be due to either the formation of an inactive AGT or to a lack of accumulation of the mutant AGT protein in the E. coli strain containing the expression plasmid. The C145A mutation clearly leads to a loss of activity since this protein is present in similar levels to the wild-type AGT (22) and there was no detectable activity. However, all of the other mutations shown in Fig. 1 lead to a substantial reduction in the amount of AGT protein as determined by Western blotting analysis. This is probably due to the instability of the AGT protein containing these mutations. Ling-Ling et al. (40) also noted that most replacements of histidine-146 led to a loss of AGT activity and that mutation of this site led to a loss of protein stability. Our results with the mutations E172Q and E172Stop are in agreement with a recent report of Rafferty et al. (41), who also found that changing the glutamic acid at this position to glutamine led to the loss of activity and a reduction in the amount of AGT protein present. Because of the very low level of AGT protein accumulated, the apparent lack of activity of this mutant should not be regarded as complete proof that it is nonfunctional.

In contrast, the mutant AGTs described as active on the basis of the activity against methylated DNA substrates and tested for sensitivity to O\(^6\)-benzylguanine showed stabilities and activities comparable to the wild-type protein in E. coli cells and extracts. This includes the mutants that were found to be highly resistant to O\(^6\)-benzylguanine and suggests that such mutations would produce an AGT in mammalian cells that would be active in repairing chloroethyl adducts from DNA but could not be inactivated by O\(^6\)-benzylguanine. It is possible that these mutants would have reduced stability or activity within mammalian cells. Experimental proof that these mutant AGTs do produce the expected phenotype requires the expression of the mutant proteins in a Mer− tumor cell line, and these experiments are in progress.

Our results (Table 1, Fig. 3) show that quite minor changes in the human AGT structure involving at least three different amino acids (prolines at 138 and 140 and the glycine at 156) can lead to a substantial increase in resistance of the human AGT to inactivation with O\(^6\)-benzylguanine. Furthermore, these effects are independent in the sense that combination of the mutations leads to an additional increase in resistance. As shown in Fig. 2, these residues in the human sequence are not present in the E. coli Ada AGT, which is totally refractory to O\(^6\)-benzylguanine (14, 19). In fact, glycine-156 is not present in any of the microbial AGTs which react poorly or not at all with O\(^6\)-benzylguanine (14, 18, 19). Proline-140, but not proline-138, is present in the E. coli Ogt protein, which is slightly sensitive to the inhibitor (18, 19). It is clear that it is the absence of proline rather than the presence of lysine at position 138 which imparts resistance since changes P138A and P138K gave equivalent results.

The most probable explanation for the striking difference in sensitivity to O\(^6\)-benzylguanine between the AGTs is a steric restriction on the active site.
the access to the active site cysteine. If this is the case, the loss of either or both of the prolines at positions 138 and 140 may reduce the access to the active site since prolines can alter the direction of the polypeptide chain (42, 43). The greater effect of adding DNA on the rate of reaction of these mutants with O6-benzylguanine is consistent with a change in the protein structure at the active site. The reason for the large effect of mutations of glycine-156 on sensitivity could also be related to an opening of the access to the active site. This residue is located in the region equivalent to the region in the Ada protein postulated to act as a hinge, causing the displacement of the terminal helix region on binding of DNA (20). It is conceivable that the alteration of this residue changes the configuration to favor a greater access to the active site. The results of the studies in which the effect of DNA on the conversion of O6-benzylguanine to guanine was measured are consistent with this interpretation. The G156A mutant was stimulated very slightly by DNA (Fig. 5), suggesting that the protein does not show the same activation on DNA binding.

Our results do not support the suggestion by Morgan et al. (44) that the carboxyl-terminal tail region of the human AGT, which is not present in the Ada AGT (see Fig. 2) is important for the reaction with O6-benzylguanine. Although they reported a 5-fold change in sensitivity to inhibition by O6-benzylguanine when a mutant AGT truncated to remove the terminal 28 residues was used, we found no difference in the inactivation of the AGT or in the rate of conversion of O6-benzylguanine to guanine with mutants lacking the 31 or 23 residues from the carboxyl terminus. A major difference between our experimental protocols that may account for this discrepancy is that they used a GST fusion protein as the source of AGT (44). Such a fusion protein may have an altered structure and/or be unable to show the normal conformational change of the AGT in response to binding DNA. Our results show clearly that there is no effect of the extreme carboxyl-terminal domain on the activity of the unmodified human AGT to react with O6-benzylguanine or on the extent of stimulation of this process by DNA.

The full activity of the carboxyl-truncated AGT mutants, in which 23 or 31 residues have been removed from the COOH terminus, is in agreement with other studies in which 22 or 28 amino acids were removed from the mammalian AGT sequence leaving an active protein (44, 45). This carboxyl domain is poorly conserved in the mammalian AGT sequences and is completely absent in all of the microbial AGTs and also from the rabbit AGT (21), further supporting the idea that it does not play a significant role in the action or in the stability of the AGT.

The fact that human AGTs with appreciable resistance to O6-benzylguanine can be generated by point mutations is clearly of concern for the clinical use of this agent. The possibility of the selection of such a preexisting mutant or the formation of such a mutant during therapy with alkylating agents followed by selection is raised by these results. The limited solubility and rapid clearance of O6-benzylguanine renders it unlikely that AGTs inhibited by this compound with ED50 values of more than 2.5 μM will be completely inactivated in vivo. One way to deal with this problem is the synthesis of additional AGT inactivators that are sufficiently potent against the mutant AGTs so that complete inactivation can be maintained. Although there was less difference between the inactivation of the control and P140A AGTs by the smaller O6-alkylguanine than by O6-benzylguanine, the former compound is not likely to be useful due to its lack of potency. O6-Alkylguanine was a considerably less effective inactivator with an ED50 value of 20 μM for the control AGT and about 80 μM for the P140A AGT (22). Similarly, the more water-soluble derivatives, O6-(p-hydroxybenzyl)guanine (11) and O6-benzyl-2'-deoxyguanosine (10), may not be sufficiently active to block the mutant AGTs activity although a larger dose can be given. Therefore, new compounds are likely to be needed.

Another possible problem in the use of O6-benzylguanine combined with BCNU for chemotherapy is the possibility of increasing the known toxicity of BCNU toward the bone marrow and other sites due to the depletion of AGT in these tissues. The expression, from a suitable vector, of a resistant AGT in such normal cells would be one way to deal with such toxicity. Although the microbial AGTs have the desired insensitivity to O6-benzylguanine, it remains to be seen whether they have sufficient stability and access to the nuclear environment that is needed to give good activity in mammalian cells. The production of a mutant human AGT such as the G156A(W) or P140A/G156A may be a preferable way to produce resistance in normal cells.

ACKNOWLEDGMENTS

We thank Dr. R. C. Moschel for the synthesis of O6-benzylguanine and O6-benzyldeoxyguanosine and L. Wiest for the purification of many of the mutant alkyltransferases.

REFERENCES


Mutations in Human $O^6$-Alkylguanine-DNA Alkyltransferase Imparting Resistance to $O^6$-Benzylationanine

Tina M. Crone, Karina Goodtzova, Suvarchala Edara, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/23/6221

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.