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Interaction of mammalian O^6-alkylguanine-DNA alkyltransferases with O^6-benzylguanine

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Abstract

Human O^6-alkylguanine-DNA alkyltransferase (hAGT) activity is a major factor in providing resistance to cancer chemotherapeutic alkylating agents. Inactivation of hAGT by O^6-benzylguanine (BG) is a promising strategy for overcoming this resistance. Previous studies, which have focused on the region encompassed by residues Pro138 to Gly173, have identified more than 100 individual mutations located at 23 discrete sites at which alterations can render AGT less sensitive to BG. We have now extended the examination of possible sites in hAGT at which alterations might lead to BG resistance to include the residues from Val130 to Asn137, which also make up part of the binding pocket into which BG is postulated to fit. A further 21 mutations located at positions Gly132, Met134, Arg135, and Gly136 were found to lower sensitivity to BG. Mutants R135L, R135Y, and G136P were the most strikingly resistant, with a 50-fold increase in the amount of BG needed to obtain 50% inactivation. These results therefore increase the number of sites at which BG resistance can occur in response to a single amino acid change to 27. Although mammalian AGTs are very similar in amino acid sequence, mouse AGT (mAGT) is significantly less sensitive to BG than rat AGT (rAGT) or hAGT. Construction of chimeric proteins in which portions came from the rAGT and the mAGT indicated that the difference in inactivation resided solely in the amino acids located in the sequence from residues 150 to 188. Individual mutations of the three residues where rAGT and mAGT differ in this region showed that the principal reason for the reduced ability of the mAGT to react with BG was the presence of a histidine residue at position 161, which is occupied by asparagine in rAGT and hAGT. These experiments indicate that many minor changes in amino acids forming all parts of the nucleoside binding pocket of AGT can alter its ability to react with BG and that the possibility that polymorphisms or variants may occur reducing the effectiveness of combination therapy with BG and alkylating agents must be considered.

Keywords: Alkyltransferase; O^6-Benzylguanine; Alkylating agents; DNA repair; Drug resistance

1. Introduction

Alkylating agents that form adducts at the O^6-position of guanine in DNA are an important class of mutagens and carcinogens, and some, such as temozolomide, dacarbazine, and BCNU, are also valuable anti-tumor agents. The major cellular defense against these agents is the presence of a unique DNA repair protein, AGT [1–5]. It acts to restore the original DNA structure in a single step by transfer of the alkyl group from the DNA to a Cys residue in the AGT protein itself. The *S*-alkylcysteine formed at the active site of AGT is not converted back to Cys so the protein can act only once. The alkylated form of the AGT protein undergoes rapid degradation [6,7].

The activity of mammalian AGT is readily inactivated by compounds such as BG [8]. The mechanism of inactivation is well understood: BG acts as a substrate for the protein, and the transfer of a benzyl group to the active site Cys leads to the irreversible loss of activity [9,10]. Several derivatives of BG including 8- and 9-substituted compounds [11–13] and O^6-(4-bromothenyl)guanine [14] also have been shown to be potent inactivators. These compounds presumably act by the same mechanism, although the mechanism has not been studied in the same detail as that of BG. Removal of AGT activity by exposure to BG or these related compounds has been demonstrated to render

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Abbreviations: BCNU, *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; AGT, O^6-alkylguanine-DNA alkyltransferase; hAGT, human AGT; mAGT, mouse AGT; rAGT, rat AGT; BG, O^6-benzylguanine; Ada-C, the *E. coli* alkyltransferase formed by the C-terminal domain of the Ada gene product; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PCR, polymerase chain reaction.

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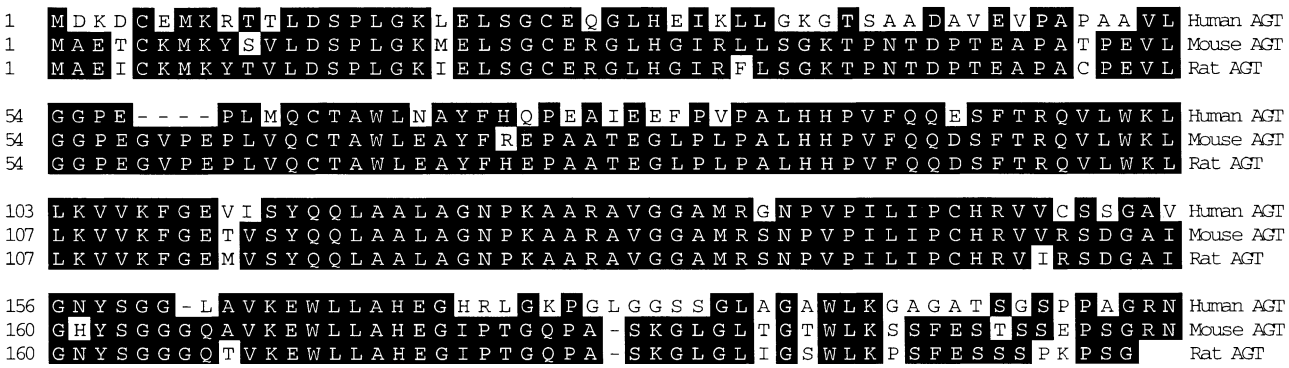

Fig. 1. Comparison of sequences of hAGT, mAGT, and rAGT. Residues identical in 2 or 3 of the sequences are shaded in black.

cultured tumor cells much more sensitive to killing by BCNU, temozolomide, and similar drugs (reviewed in [4,15–18]). These studies have led to tests in experimental tumor xenograft model systems, which have shown that a considerable enhancement of the therapeutic index of alkylating agent therapy is achieved when AGT is inactivated [4,16,19]. That conclusion led to clinical trials of the combination of AGT inhibitors with either BCNU or temozolomide [20–23].

The appearance of resistance to cancer chemotherapeutic agents is an extremely common result of treatment with anti-tumor drugs. Although such resistance can arise in various ways, one mechanism is through alteration in the target site to render it less susceptible to the drug. The possibility that this may occur with BG has been raised in several papers in which it has been shown that mutant forms of hAGT with an impaired ability to react with BG can be obtained readily [24–29]. This work has identified 23 sites at which point mutations can render hAGT resistant to inactivation by BG [18]. These studies were carried out either by targeting residues that are not conserved between hAGT and the *Escherichia coli* alkyltransferase Ada-C (which is highly resistant to BG [9,30,31]) or by inserting random sequences into the region between Pro138 and Gly173 in hAGT and selecting mutants from this library by screening for resistance to MNNG in the presence of BG.

The availability of the crystal structures of both hAGT [32,33] and Ada-C [34] has provided a mechanistic basis for these changes, which all occur in amino acid residues whose side chains project into the active site pocket within which BG must bind in order to act as a substrate for the alkyl transfer reaction. However, the crystal structure of hAGT also shows that part of this binding pocket appears to be generated by some of the residues present in the helix H6, which precedes the Asn-hinge formed by residues from Asn137 to Pro144 [32,33]. Therefore, we have extended the examination of possible sites in hAGT at which mutations or polymorphisms might lead to BG resistance to residues from Val130 to Asn137 that make up this part of the H6 helix. Four additional sites (Gly132, Met134, Arg135, and Gly136) at which BG resistance can occur were found in this region, increasing the number of sites in hAGT at which BG resistance can arise from a single amino acid change to 27.

Although mammalian AGTs are very similar in amino acid sequence (Fig. 1), it has been reported that there is a significant difference in the ability of the mAGT, compared with the rAGT or hAGT, to react with BG [31,35]. The extent, if any, to which this reduction contributes to the success of BG treatment in improving the therapeutic index of alkylating agent therapy towards human xenograft tumors carried in mice is unknown. The molecular basis underlying the difference in ability to interact with BG among these closely related mammalian AGTs is also unknown. By constructing chimeras between sections of the mAGT and the rAGT to reduce the size of the region that needed to be examined and then constructing mutants of all the residues in this region in which the mAGT and rAGT differ, we determined that the difference predominantly responsible for the resistance of the mAGT was the presence of a His residue at position 161, which is equivalent to Asn161 and Asn157 in rAGT and hAGT, respectively. These results provide a more complete picture of the active site pocket of AGT and the requirements for binding of BG and subsequent inactivation.

2. Materials and methods

2.1. Materials

All oligodeoxyribonucleotides were purchased from GIBCO-BRL Life Technologies, Inc. Ampicillin, kanamycin, MNNG, and most of the other reagents for molecular biology and AGT assays were obtained from the Sigma Chemical Co., Perkin Elmer/Cetus, and Promega. *N*-[*methyl*-^{3}H]-*N*-Nitrosourea (5.9 mCi/mmol) was obtained from Amersham, Inc. O^{6}-Benzyl[^{3}H]guanine (0.34 mCi/mmol) was prepared by catalytic tritium exchange of BG by Amersham, Inc. and purified as described [9]. Restriction enzymes were obtained from New England Biolabs and GIBCO-BRL Life Technologies, Inc. *Pfu* polymerase was from Stratagene. T4-ligase and the "ligation express kit" were purchased from Clontech.

JM109, XL1-Blue bacterial strains, and competent cells were purchased from Stratagene. *E. coli* strain TRG8 [26] was derived from strain GWR109 [36] that was provided by Dr. L. Samson, Department of Molecular and Cellular Toxicology, Harvard School of Public Health. The DNA isolation kits were from Qiagen. BG [8], O^6-allylguanine, and O^6-benzylhypoxanthine [37] were provided by Dr. R.C. Moschel (ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center). The pQE-30 plasmid was obtained from Qiagen, and the Talon Metal Affinity Resin was from Clontech. Plasmids pUC-AGTm and pUC-inAGT have been described previously [26,28].

2.2. Construction of random libraries containing individual mutations in codons from Val130 to Asn137

The random library was designed to contain a single amino acid substitution at only one codon at a time [29]. The DNA inserts were created by PCR using *Pfu* polymerase. pUC-AGTm, which had been made previously by replacing a 337 bp fragment of the human AGT-coding region in pUC-hAGT between the *Eco*NI and *Dra*III sites with the equivalent modified AGT sequence containing silent mutations providing four additional restriction sites [26], was used as a template. Eight individual PCR products were synthesized using the sense primer, 5′-CCAGCAAGAGTCGTTCACGCGTCAGGTG-3′, containing a *Mlu*I site (underlined) at position 283 and mutagenic antisense primers each containing a *Dra*III site at position 436 and one codon replaced with random nucleotide sequence (Table 1). The PCR products were individually gel purified using the Gel Extraction Kit (Qiagen), quantified, combined in equal amounts, digested with *Mlu*I and *Dra*III enzymes, and ligated into pUC-inAGT plasmid digested with the same enzymes. The coding region of hAGT in the pUC-inAGT had been disrupted by inserting a 1200-bp irrelevant sequence between the *Dra*III and the *Afl*II (at position 307) sites [28]. This disruption of the AGT sequence was made to eliminate contamination of the libraries by partially digested wild-type AGT sequences.

After electroporation of ligated products into XL-1 Blue cells, an aliquot of the transformation mixture was grown on LB plates supplemented with 50 μg/mL of ampicillin to determine the number of bacteria containing plasmids. The remainder of the culture was amplified by being grown overnight. The plasmid DNA was isolated from the overnight cultures and electroporated into TRG8 cells. An aliquot of this transformant mixture was plated to determine the total number of available transformants, and the rest of the mixture was amplified overnight in LB medium containing ampicillin and kanamycin (50 μg/mL each). Aliquots of the library were used for screening or stored at −70°.

Individual Gly131X, Met134X, and Arg135X libraries were constructed the same way using corresponding antisense primers (Table 1). Antisense primer, 5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCT**GAA**GG–CGCCTCC-3′, was used to create the M134F mutant (*Eco*NI site underlined, altered codon sequence in bold).

2.3. Contraction and purification of rAGT, mAGT, and their mutants

All recombinant AGT proteins (rAGT, mAGT, and their mutants) were expressed in *E. coli* JM109 cells using the pQE-30 vector, which adds 12 amino acids by replacing M- with MRGHS$(H)_6$GS- to the amino terminus of the AGT protein, and purified by immobilized metal affinity chromatography as described previously [38]. All PCR reactions were carried out using *Pfu* polymerase. The entire AGT protein-coding region for all constructs was sequenced to ensure that no additional mutations were present.

The rAGT cDNA was obtained by nested PCR from a rat liver cDNA library in the λZAPII vector using a sense primer, 5′-GTTTCTGCACATATGGCAAACTGG-3′, 20 bp upstream from the start codon of rAGT and antisense primer (Primer 1), 5′-GTCATTCAAAAGCTTACTCAATT**TCA**-GCC-3′, complementary to the flanking cDNA sequence and creating a *Hin*dIII restriction site (underlined) after the stop codon (bold). The PCR gave two products of similar size, which were isolated and sequenced. The second round of PCR was performed using the PCR product containing the rat AGT cDNA sequence as a template, antisense Primer 1, and sense primer (Primer 2), 5′-GGAGGAAGGATCC**ATG**GCTGAG-3′, creating a *Bam*HI site

Table 1
Primers used for construction of the mutant hAGTs

Mutant	Sequence
Val130X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCTCATGGCGCCTCC**SNN**TGCTCGCGC-3′
Gly131X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCTCATGGCGCC**SNN**CACTGCTCG-3′
Gly132X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCTCATGGC**SNN**TCCCACTGC-3′
Ala133X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCTCAT**SNN**GCCTCCCAC-3′
Met134X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCFTCT**SNN**GGCGCCTCC-3′
Met134Phe	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCCTCT**GAA**GGCGCCTCC-3′
Arg135X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATTGCC**SNN**CATGGCGCC-3′
Gly136X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGGATT**SNN**TCTCATGGCGCC-3′
Asn137X	5′-GCTGCAGACCACTCTGTGGCACGGGATGAGGATGGGGACAGG**SNN**GCCTCTCATGGCGCC-3′

*Dra*III restriction sites are underlined; mismatches are in bold.

(underlined) directly upstream from the start codon (bold). The PCR product was cloned into pQE-30 between the *Bam*HI and *Hin*dIII restriction sites (pQE-rAGT).

Plasmid pSS600 containing the mAGT cDNA from mouse liver was a gift from Dr. S. Mitra (Sealy Center for Molecular Genetics, University of Texas) [39]. Mouse AGT cDNA was subcloned first into the multiple cloning site of the pGEM-3Zf(+) vector between the *Eco*RI and *Pst*I sites. PCR was performed to create the *Bam*HI and *Hin*dIII restriction sites necessary to insert mAGT cDNA into pQE-30 (pQE-mAGT) using Primer 2 and antisense primer (Primer 3), 5′-GCATTAAAGCTTACGCATCC-**TCA**ATTTCG-3′ (*Hin*dIII site underlined, stop codon in bold type).

Both pQE-rAGT and pQE-mAGT have a unique restriction site for *Eco*NI, which cuts after Cys149, the alkyl acceptor site of the protein. *Eco*NI recognizes the sequence: $CCT\underline{(N)_2}/(N)_3AGG$ (position 149 is underlined). The nucleotide sequences at this region in pQE-mAGT and pQE-rAGT are identical, making it possible to ligate mouse and rat cDNAs forming chimeric constructs. Thus, pQE-rAGT and pQE-mAGT were digested with *Bam*HI and *Eco*NI, and fragments were separated on agarose gels, purified, and ligated to create pQE-(rat$^{1–149}$/mouse$^{150–211}$) and pQE-(mouse$^{1–149}$/rat$^{150–209}$) chimeras.

The cDNAs encoding rat, mouse, mouse$^{1–149}$/rat$^{150–209}$, and rat$^{1–149}$/mouse$^{150–211}$ AGT proteins were used to create four additional pQE-30 plasmids containing cDNAs for chimeric rat$^{1–188}$/mouse$^{189–211}$, mouse$^{1–188}$/rat$^{189–209}$, rat$^{1–149}$/mouse$^{150–188}$/rat$^{189–209}$, and mouse$^{1–149}$/rat$^{150–188}$/mouse$^{189–211}$ proteins. The constructs were made by replacing the 196-bp section of the 3′-coding region located between the *Bst*XI (a unique restriction site located at position 564 of the AGT coding sequence) and *Nhe*I located in the pQE-30 multiple restriction site. *Bst*XI recognizes the sequence $\underline{CCA}(N)_3/(N)_1\underline{TGG}$. The region between the underlined nucleotides differs at only 1 bp in rat and mouse cDNAs, making it possible to form chimeric constructs.

The pQE-(H161N)-mAGT mutant was made in the pQE-30 vector by site-directed mutagenesis using antisense Primer 3 and sense primer (Primer 4), 5′-CTCATCC-CCTGCCACAGGGTGGTTCGCAGTGACGGTGCCAT-CGGC**AAT**TACTCCG-3′, which had the altered codon sequence (bold) and the *Eco*NI restriction site (underlined). The PCR product and pQE-30-mAGT were digested with *Eco*NI and *Hin*dIII, ligated, and transformed into JM109 cells.

Three single mutants (I153V, N161H, and T168A) and three double mutants (I153V/N161H, I153V/T168A, and N161H/T168A) were made in pQE-rAGT by site-directed mutagenesis, using PCR. In all cases, the antisense primer was Primer 1, and the sense primers contained an *Eco*NI restriction site. The sense primers and templates used for construction of these mutants are shown in Table 2.

2.4. Selection of BG-resistant hAGT mutants

To select active and BG-resistant hAGT mutants, TRG8 cells were treated with MNNG in the presence or absence of BG. An aliquot of the library in TRG8 cells was grown at 37° until it reached $A_{600} = 0.5$; then 50 μM BG was added to half of the culture for 1.5 hr at 25° [26] followed by 20 or 40 μM MNNG for 30 min at 25°. After that aliquots were grown on LB plates containing antibiotics and 50 μM BG. Plasmid DNA from individual colonies surviving after treatment with 40 μM MNNG and 50 μM BG (20 μM MNNG and 50 μM BG in the case of the G131X library) was isolated and sequenced.

2.5. Assays of AGT activity and inactivation by BG

Crude bacterial cell extracts and purified rAGT, mAGT, and hAGT proteins and mutants were incubated with a [^{3}H]methylated DNA substrate prepared by reaction of calf thymus DNA with *N*-[*methyl*-^{3}H]-*N*-nitrosourea, and the alkyltransferase activity was determined by measuring the formation of [^{3}H]methylated protein, which was collected on nitrocellulose filters [26]. The reaction was carried out with the amount of protein needed to remove 80% of the O^6-methylguanine (500 fmol) from the [^{3}H]methylated calf thymus DNA. Inactivation of the AGT activity by BG was determined by incubating aliquots of crude bacterial cell extracts with different concentrations of BG at

Table 2
Primers and templates used for the construction of rat mutants by PCR

Primer	Template	Product
Primer 4	pQE-rAGT	pQE-(I153V)-rAGT
Primer 4	pQE-(I153V/N161H/T168A)-rAGT	pQE-(I153V/T168A)-rAGT
Primer 5	pQE-(I153V/T168A)-rAGT	pQE-(T168A)-rAGT
Primer 5	pQE-(I153V/N161H/T168A)-rAGT	pQE-(N161H/T168A)-rAGT
Primer 6	pQE-rAGT	pQE-(I153V/N161H)-rAGT
Primer 5	pQE-(I153V/N161H)-rAGT	pQE-(N161H)-rAGT

In all cases the antisense primer was Primer 3. Primer 4: 5′-CTCATCCCCTGCCACAGGGTGGTTCGCAGTGACGGTGCCATCGGCAATTACTCCG-3′. Primer 5: 5′-CCTCATCCCCTGCCACAGGGTGATTCGCAG-3′. Primer 6: 5′-CCTCATCCCCTGCCACAGGGTGGTTCGCAGTGACGGTGC-CATTGGCCATTACTCTGG-3′. The *Eco*NI restriction site in Primers 4–6 is underlined.

37° for 30 min in 0.5 mL of 50 mM Tris–HCl (pH 7.6), 5 mM dithiothreitol, 0.1 mM EDTA, 50 μg hemocyanin, and 10 μg of calf thymus DNA. When using purified proteins, calf thymus DNA was replaced by 200 mM NaCl and 25% glycerol to stabilize AGT proteins. The residual AGT activity was measured, and the results were expressed as the percentage of the AGT activity remaining. The graphs of the remaining AGT activity against concentration of BG were used to calculate the EC_{50} (concentration needed to reduce the alkyltransferase activity by 50% in a 30-min incubation at 37°).

The measurement of guanine formation from BG was carried out by incubating various amounts of purified mammalian proteins with 0.7 μM O^6-benzyl[^{3}H]guanine in 50 mM Tris–HCl, pH 7.6, 5 mM dithiothreitol, and 0.1 mM EDTA in the presence of 50 μg hemocyanin and 25% glycerol (total volume 0.25 mL). After incubation at 37° for 30 min, the reaction was stopped by the addition of 0.5 mL of the same buffer containing 0.2 mM guanine and 0.2 mM BG. The formation of [8-^{3}H]guanine was determined by HPLC as described previously [40].

3. Results

3.1. Effect of mutations in the region from codons 130–137 on activity of hAGT and resistance to BG

A preliminary selection of the library of mutants in hAGT in the region Val130 to Asn137 was carried out using exposure to MNNG and BG to obtain active mutants able to provide protection to the *E. coli* strain TRG8 under these conditions. Fifty-six different colonies were sequenced and gave 5 wild-type sequences, 6 mutants encoding wild-type AGT with an altered codon, and 45 mutant AGTs representing 23 different mutants. There were: no mutants at Gly131 or Asn137; only one mutation, the highly conservative change to Ile at Val130; two conservative changes to Thr or Ile at Ala133; and two conservative changes to Leu and Ala at Met134. These results are consistent with comparative studies of known AGT sequences from different species. In 48 AGT sequences in the database, Val130 is changed in only 5 (to Ala, Ile, or Phe) and Gly131 is changed in only 8 (to Ala in every case). Asn137 is a totally conserved residue, which plays a critical role in maintaining AGT structure and stability [41,42].

In the present study, the greatest numbers of mutants were found at position Gly136 (17; 9 different replacements) and Gly132 (14; 5 different replacements). To increase the numbers of mutants at other positions, the individual libraries for positions Gly131, Met134, and Arg135 were re-examined using individual libraries at these sites. This analysis confirmed that Gly131 is critical for activity: no colonies of mutants at this site survived treatment with 40 μM MNNG and 50 μM BG and seven out of eight mutants that survived 20 μM MNNG plus BG had altered codons still coding for Gly with the only alteration being a Cys replacement. Two additional changes at positions Met134 and three additional changes at Arg135 were also obtained.

A complete analysis of the sensitivity of the mutants to BG is shown in Table 3. Using an EC_{50} value for BG equal to a 3-fold increase over that of wild-type hAGT as a cut-off for the determination of resistance, there were 21 mutants that were clearly resistant to inactivation by BG. These were at positions Val130 (1 resistant/1 total), Gly132 (1/5),

Table 3
Sensitivity of hAGT mutants in the region between codons 130 and 138 to BG

Codon	Number of times found[a]	Replacement	μg Protein used for assay[b]	EC_{50} (μM)
WT[c]			2.5	0.2
Val130	3	Ile	5	1.0*
Gly131	1[d]	Cys	5	0.2
Gly132	10	Ser	5	0.6*
Gly132	1	Asn	1	0.2
Gly132	1	Ile	1	0.2
Gly132	1	Phe	50	0.2
Gly132	1	Val	2.5	0.4
Ala133	1	Ile	1	0.2
Ala133	1	Thr	1	0.2
Met134	1[e]	Phe	25	5.0**
Met134	2	Thr	5	3.0**
Met134	5	Leu	5	1.0*
Met134	3	Ala	1	0.6*
Met134	1	Val	2.5	0.6*
Arg135	1	Leu	40	125.0***
Arg135	2	Tyr	10	17.0***
Arg135	2	Val	5	1.6*
Arg135	1	Met	7.5	1.5*
Arg135	1	Phe	7.5	1.5*
Arg135	2	Thr	5	1.0*
Arg135	1	Cys	7.5	0.2
Arg135	1	Ser	5	0.2
Gly136	2	Pro	50	17.0***
Gly136	2	Phe	2.5	1.7*
Gly136	1	Val	10	1.6*
Gly136	3	Ala	2.5	1.0*
Gly136	1	Thr	1.0	0.8*
Gly136	5	Arg	2.0	0.6*
Gly136	1	His	1.0	0.6*
Gly136	1	Ser	2.5	0.6*

Resistant to BG: * (3- to 10-fold); ** (11- to 50-fold); and *** (>50-fold).

[a] Colonies were found after selection with 40 μM MNNG and 50 μM BG.

[b] Amount of crude protein extract needed to get 80% removal (500 fmol) of O^6-methylguanine from [^{3}H]methylated DNA in the standard assay.

[c] Extracts from bacteria expressing mutant V130V was used for this analysis.

[d] G131C mutant was found in the library after selection with 20 μM MNNG and 50 μM BG.

[e] M134F was not found after screening of the library and was constructed separately.

Met134 (5/5), Arg135 (6/8), and Gly136 (8/8). Mutants R135L, R135Y, and G136P had more than a 50-fold increase in the EC_{50} value, and mutants M134F and M134T showed a >10-fold rise. Therefore, mutations in residues making up the distal portion of the H6 helix, whose side chain substituents form part of the active site pocket, can lead to BG resistance.

3.2. Differential sensitivity of mammalian AGTs to BG

It has been reported that mAGT is less sensitive to BG than either hAGT or rAGT [31,35]. Our studies carried out with His-tagged purified preparations of these mammalian AGTs confirm this finding (Table 4 and Fig. 2). In our experiments, the purified rAGT (and some of the chimeras described below) were unstable unless incubated in the presence of either DNA or glycerol. The presence of DNA is known to increase the rate of reaction of BG with AGTs and to decrease the differences between the mammalian AGTs [26]. Therefore, we incubated proteins with BG in the presence of 25% glycerol and 200 mM NaCl. Under these conditions, the AGT activities of rat, mouse, and the chimeric proteins were the same as those of the hAGT (12.5 nmol/mg protein), and the loss of AGT activity during the 30-min preincubation without BG did not exceed 5%. There was a 9-fold difference in EC_{50} values between the hAGT and the mAGT. Although hAGT and rAGT have been reported to show identical sensitivity to BG [31], our studies did find a small difference (Table 4). However, our results also confirmed that rAGT, which is extremely similar to mAGT with only 16 differences in 209 amino acids, clearly is considerably more sensitive to this inhibitor than mAGT.

To understand the reason for this difference, chimeras of rAGT and mAGT were constructed using a unique *Eco*NI restriction site, which cuts the rat and mouse cDNA sequences after the codon for the Cys149 alkyl acceptor site (Fig. 1). The EC_{50} value for the mouse$^{1–149}$/rat$^{150–209}$ AGT was identical to that for rAGT, whereas the EC_{50} value for BG of the rat$^{1–149}$/mouse$^{150–211}$ was markedly higher and similar to that for mAGT (Fig. 2). These results show that the residues responsible for the difference in sensitivity to BG are present in the C-terminal region of the protein.

To narrow further the region imparting altered sensitivity to BG, a unique *Bst*XI restriction site in rat and mouse AGT cDNAs was used to construct four additional chimeras in the pQE-30 vector: pQE-(rat$^{1–188}$/mouse$^{189–211}$), pQE-(mouse$^{1–188}$/rat$^{189–209}$), pQE-(rat$^{1–149}$/mouse$^{150–188}$/rat$^{189–209}$), and pQE-(mouse$^{1–149}$/rat$^{150–188}$/mouse$^{189–211}$). Chimeric proteins mouse$^{1–188}$/rat$^{189–209}$ and rat$^{1–149}$/mouse$^{150–188}$/rat$^{189–209}$ were as resistant to BG as mAGT, whereas rat$^{1–188}$/mouse$^{189–211}$ and mouse$^{1–149}$/rat$^{150–188}$/mouse$^{189–211}$ were more sensitive to BG (Table 4). These results allowed us to conclude that the amino acid sequence encoded by the sequence between the *Eco*NI and *Bst*XI restriction sites corresponding to amino acids 150–188 is responsible for the protein being resistant or sensitive to BG.

There are only three differences in the amino acid sequences between rAGT and rat$^{1–149}$/mouse$^{150–188}$/rat$^{189–209}$AGT (Fig. 1). These are Ile153 (which is Val in mAGT), Asn161 (His in mAGT), and Thr168 (Ala in mAGT). To determine which amino acid residue is responsible for BG resistance, we constructed and purified six single and double mutants of rAGT (I153V, N161H, T168A, I153V/N161H, I153V/T168A, and N161H/T168A). The EC_{50} values for BG are shown in Table 4. These results show clearly that the critical alteration is that at Asn161. All of the rAGT mutants in which the N161H change was present, including the single N161H change, were more resistant to BG. Further confirmation of this was provided by the construction and assaying of the reverse mutant H161N in mAGT, which increased the sensitivity of the mAGT to BG (Table 4 and Fig. 2).

Since AGT inactivation by BG is brought about by its reaction as a substrate to form *S*-benzylcysteine at the active site and release guanine, the reaction can be followed by measuring the production of [8-^{3}H]guanine from [8-^{3}H]BG [40]. As shown in Table 5, this assay confirmed the results shown above. The formation of guanine was

Table 4
Sensitivity of purified AGT proteins to inactivation by BG

AGT used	EC_{50} BG (μM)
hAGT	0.5
rAGT	1.5
mAGT	4.5
Mouse$^{1–149}$/Rat$^{150–209}$AGT	1.5
Rat$^{1–149}$/Mouse$^{150–211}$AGT	5.0
Mouse$^{1–188}$/Rat$^{189–209}$AGT	6.0
Rat$^{1–149}$/Mouse$^{150–188}$/Rat$^{189–209}$AGT	5.0
Rat$^{1–188}$/Mouse$^{189–211}$AGT	2.5
Mouse$^{1–149}$/Rat$^{150–188}$/Mouse$^{189–211}$AGT	2.0
(I153V)-rAGT	3.5
(I153V/T168A)-rAGT	2.0
(T168A)-rAGT	2.0
(N161H)-rAGT	5.0
(I153V/N161H)-rAGT	5.0
(N161H/T168A)-rAGT	4.0
(H161N)-mAGT	2.0

Table 5
Formation of guanine from BG by rAGT, mAGT, and mutants

AGT used	Formation of [8-^{3}H]guanine from [8-^{3}H]BG (cpm/μg/min)
rAGT	15.8 ± 4.0
mAGT	8.0 ± 2.2
Mouse$^{1–149}$/Rat$^{150–209}$AGT	19.4 ± 3.6
Rat$^{1–149}$/Mouse$^{150–211}$AGT	6.2 ± 2.0
(H161N)-mAGT	19.1 ± 4.1

Results are means ± SD for 7–11 estimations for each mutant.

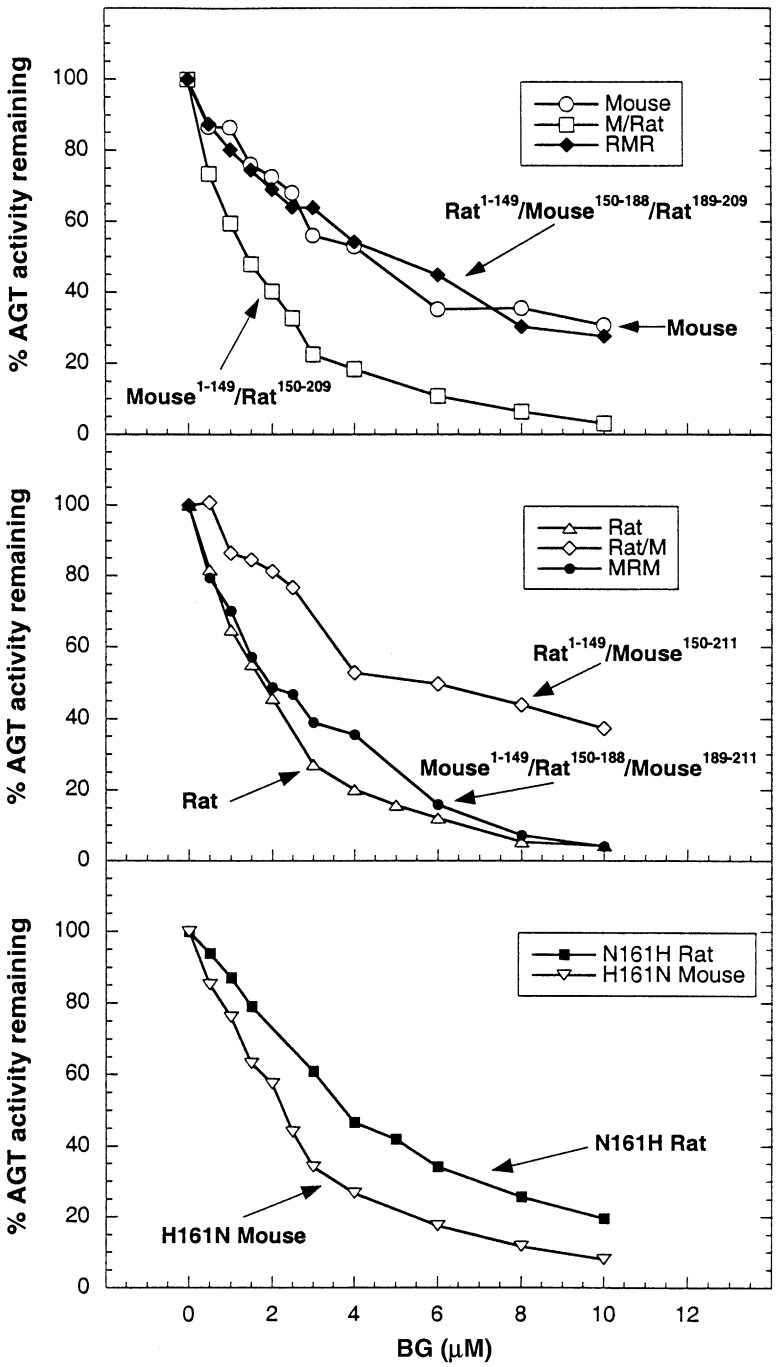

Fig. 2. Inactivation of purified AGT proteins by BG. The inactivation reaction was carried out as described in Section 2 by incubation of the His-tagged purified mammalian proteins indicated with the BG concentrations shown for 30 min in the presence of 25% glycerol and 200 mM NaCl. The remaining AGT activity was then determined by the addition of a [^{3}H]methylated DNA substrate and additional incubation for 30 min. The experiment was performed four times with essentially identical results.

greater by rAGT and mouse$^{1–149}$/rat$^{150–209}$AGT than for mAGT or rat$^{1–149}$/mouse$^{150–211}$AGT. The single H161N mutant in mAGT increased the ability to form [8-^{3}H]gua-^{3}H]guanine from [8-^{3}H]BG to that found with rAGT and mouse$^{1–149}$/rat$^{150–209}$AGT.

A similar difference in sensitivity between rAGT and mAGT was also seen when the less potent inhibitors, O^6-allylguanine or O^6-benzylhypoxanthine, were used (Table 6 and Fig. 3). The same region in the protein from amino acids 150–188 was responsible for this difference. The introduction of the H161N mutation into the mAGT rendered the mAGT more sensitive to these inhibitors, and the converse introduction of the N161H mutation into the rAGT led to an increase in resistance. Therefore, the presence of the His161 residue in mAGT also imparts a lowered sensitivity to these other inhibitors.

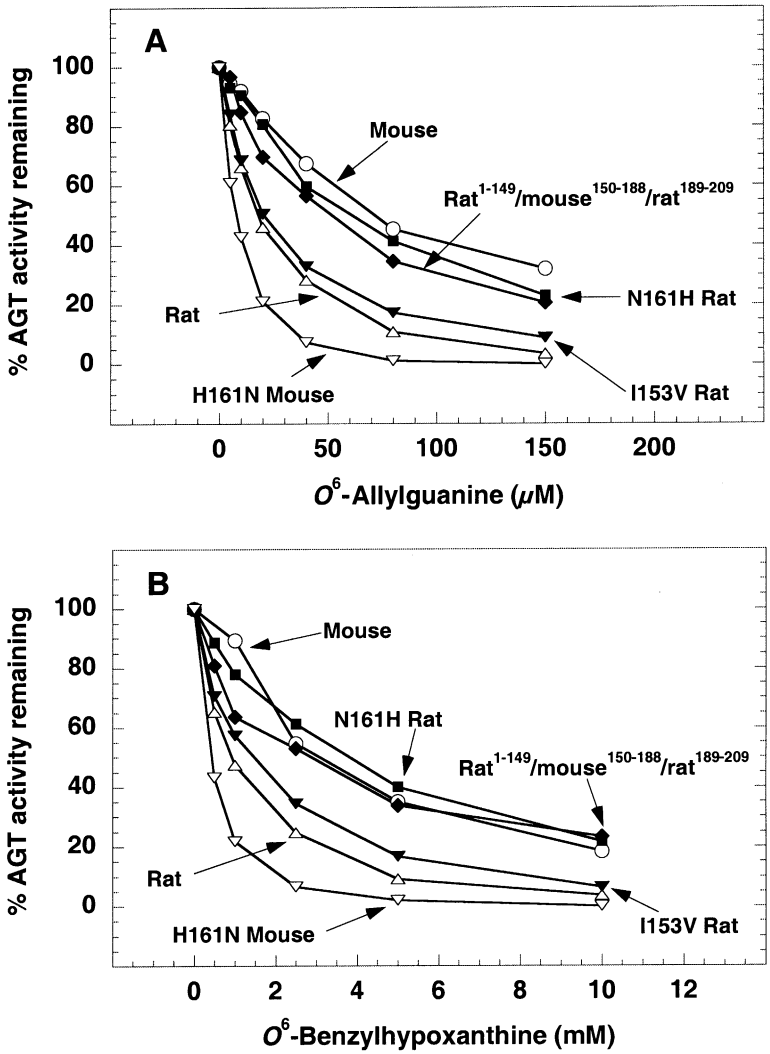

Fig. 3. Inactivation of purified AGT proteins by O^6-allylguanine or O^6-benzylhypoxanthine. The inactivation reaction was carried out as described in Section 2 and in the legend of Fig. 2. The experiment was performed twice with essentially identical results.

Table 6
Sensitivity of purified AGT proteins to inactivation by O^6-allylguanine or O^6-benzylhypoxanthine

AGT used	EC_{50}	
	O^6-Allylguanine (μM)	O^6-Benzylhypoxanthine (mM)
rAGTs	20	1.0
mAGT	70	3.0
$Rat^{1–149}$/$Mouse^{150–188}$/ $Rat^{189–209}$AGT	50	3.0
(I153V)-rAGT	20	1.5
(N161H)-rAGT	60	3.5
(H161N)-mAGT	10	0.5

4. Discussion

The molecular mechanism of DNA repair by AGT is not understood fully, but highly plausible models have been proposed on the basis of the crystal structures of Ada-C [34] and hAGT [32,33]. In these models, the DNA is bound via a winged helix-turn-helix DNA binding domain, and the target O^6-methyldeoxyguanosine is flipped out of the DNA helix and placed in a binding pocket that contains the Cys acceptor site. This active site (Cys145) in hAGT is part of an extensive hydrogen-bonding network involving a well-ordered water molecule and residues Tyr158, His146, and Glu172. Proton abstraction to facilitate nucleophilic attack by Cys145 is likely to occur through this hydrogen-bond network. The activated cysteine residue then attacks the O^6-methyl group forming *S*-methylcysteine and restoring the DNA structure.

Models of the interaction of BG with hAGT, based on the crystal structure of both the native protein and the protein in which Cys145 is converted to *S*-benzylcysteine after reaction with BG [33], show that BG is able to serve as a substrate for hAGT even though there is no interaction with the DNA binding domain because it fits well into the binding pocket for the ''flipped'' base and is held there in the correct orientation for reaction. A major factor in the binding of BG is an interaction between its hydrophobic

phenyl ring and the side chain of residue Pro140. Additional interaction with the aromatic ring of Tyr158 may also be important in the productive binding of BG. In agreement with these models, mutations of Pro140 (to many different amino acids) [24,26,27] or of Tyr158 (to His, the only change except for Phe which is compatible with activity) [28] lead to major reductions in the ability of the hAGT to be inactivated by BG. For example, the EC_{50} values for mutants P140K and Y158H are >1200 and 620 μM, respectively.

Previous studies in which BG-resistant hAGT mutants were selected from libraries in which random sequences were inserted into the region between Pro138 and Gly173 have identified multiple other sites at which resistance can occur [25,26,28,29]. Most of these produce smaller effects than the mutants at Pro140 and Tyr158 described above, but many changes at Gly156 or Lys165 give increases in the EC_{50} of more than 100-fold; in all, an additional 21 sites were identified and a total of 111 BG-resistant point mutants at the 23 sites were obtained. The BG resistance of most of these mutants can be attributed to steric effects whereby the mutations either distort the binding pocket or have side-chains intruding into the space that needs to be occupied by BG. Since the O^6-substituent of BG requires considerably more space than a simple methyl group or even a linear 2-chloroethyl group, many such changes can be accommodated without greatly affecting the ability of the AGT to repair methylated or chloroethylated DNA, which are the substrates provided by therapeutic O^6-alkylating agents.

The models based on the crystal structure predict that part of the binding pocket for BG is made up by some of the residues present in the C-terminal part of helix H6. The results presented in Table 3 confirm that BG resistance is also provided by alterations of residues Gly132, Met134, Arg135, and Gly136, which are present in this region. These results therefore increase the number of known BG-resistant point mutations in hAGT to 134, and the individual sites where BG resistance can occur in response to a single amino acid change to 27. They also provide excellent supporting evidence of the models derived from the crystal structure of the residues making up the binding pocket into which BG is postulated to fit. Although the studies reported here were carried out with extracts from *E. coli* cells expressing the mutant hAGT proteins rather than with the purified proteins, previous studies have shown that such assays do give an accurate indication of the ability of the protein to interact with BG [24–29].

These results raise a serious question as to whether the potential benefits of clinical therapy with BG and alkylating agents might be counteracted by the appearance of tumor cells expressing mutant forms of AGT with reduced sensitivity to the inhibitor. Such mutations could arise directly as a result of therapy with alkylating agents. Another possibility is that existing genetic variants in hAGT could impart resistance. Several groups have reported on the occurrence of variant forms of AGT protein, which include W65C, L84F, I143V, G160R, and K178R [43–47], and others may be discovered. One of these known variants, G160R, does indeed render hAGT less susceptible to inactivation by BG [38]. When expressed in CHO cells, this hAGT variant renders the cells resistant to killing by combinations of BCNU with BG that kill CHO cells expressing wild-type hAGT [48].

Once alkylated, hAGT is degraded rapidly [6,7]. The alteration in structure, brought about by the addition of an alkyl group to Cys145, leads to the protein becoming destabilized [33,49]. It is probable that some of the alterations produced by mutations in the active site pocket causing BG resistance have a destabilizing effect. As shown in Table 3, considerably larger amounts of cell extract were needed for the assays of some of the mutant forms of hAGT, even though expression was provided by the same vector. Previous studies have shown that this is likely to be due to a reduced stability of the hAGT protein [29,50]. This does not affect the EC_{50} measurement since all assays were carried out under *in vitro* conditions in which hAGT activity was constant over the 30-min incubation unless BG was added. Furthermore, studies in which BG-resistant AGT mutants of reduced stability, such as G156A or K165A, have been expressed in mammalian cells show that this instability does not impair their ability to protect cells from alkylating agents even in the presence of BG [50–53]. Therefore, the mutants identified here such as R135L and G136P are very likely to be able to provide similar protection.

Mammalian AGTs are very similar in amino acid composition (Fig. 1), and there may be only very minor differences in the three-dimensional structures since the crystal structures of Ada-C [34] and the AGT from *Pyrococcus kodakaraensis* [54] closely resemble that of hAGT, particularly in the DNA binding domain and active site pocket regions [32,33]. However, the finding that many point mutations in hAGT render it less able to react with BG emphasizes that quite minor differences are sufficient to impart this phenotype. It is therefore not surprising that mAGT, in this respect, shows a significant difference from hAGT and rAGT.

The results in Tables 4 and 5 and Fig. 2 show that the major factor in this difference is the presence of a charged His residue in place of Asn at position 161. This could discourage BG binding either by steric effects or by an insertion of a positive change into the binding site pocket. It was suggested previously that the presence of Leu at position 180 in mAGT rather than Arg, which is the corresponding residue in hAGT, may account for the difference between them in response to BG [35]. However, our studies disprove this hypothesis. Due to a strain difference in the AGT sequence, this Leu is not present in our mAGT clone, which, like the rAGT, has Pro at position 180. Since the rAGT and the mAGT differ in reaction with BG, this position cannot be critical. Furthermore,

residue-180 does not form part of the substrate-binding pocket and is in a part of the protein that is not needed for activity [4].

Although present in the substrate binding pocket of hAGT, Asn157 is unlikely to interact directly with either the N^2 amino group or the alkyl group of BG. It is probable that the replacement of Asn157 with His discourages BG binding by reducing the already weak interactions with the purine ring. The findings that mAGT is also resistant to O^6-allylguanine or O^6-benzylhypoxanthine and that this is due to the presence of a histidine residue in this position (Table 6 and Fig. 3) are consistent with this hypothesis, since these inhibitors differ from BG in the alkyl group and the absence of an N^2 amino group, respectively.

It is quite possible that the difference between mAGT and hAGT contributes to the increased therapeutic index when BG is used in conjunction with alkylating agents to treat human xenograft tumors carried in nude mice (reviewed in [4,16,19]). The doses of BG used in these studies are high enough to inactivate both the tumor hAGT and the mAGT present in normal tissues, but the recovery of activity may occur more rapidly in the mouse tissues as the BG is eliminated. This recovery could reduce the toxicity of the treatment. It appears from the preliminary evidence available from clinical trials with BG that the enhanced toxicity particularly towards the bone marrow may prevent the use of sufficient doses of the alkylating agents to achieve a therapeutic effect [20–23]. Regional therapy with BG may therefore be needed.

An alternative approach would be to protect the bone marrow using gene therapy to increase hAGT activity by expressing a hAGT mutant that is resistant to BG. Several groups have published preliminary data showing the potential value of this protocol using mutants P140K [55,56] or G156A [57] and some AGTs containing multiple mutations [58,59]. Although the mutants R135L, R135Y, and G156P identified here are as effective in producing resistance as G156A and would therefore be equally good candidates for such studies, they offer no obvious advantage over P140K, which seems to be the single mutant of choice based on resistance and stability.

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