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Alteration of the Conserved Residue Tyrosine-158 to Histidine Renders Human O^6-Alkylguanine-DNA Alkyltransferase Insensitive to the Inhibitor O^6-Benzylguanine[1]

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ABSTRACT

The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) protects cells from alkylation damage. O^6-Benzylguanine (BG) is a potent inactivator of human AGT (ED_{50} of 0.1 μM) that is currently undergoing clinical trials to enhance chemotherapy by alkylating agents. In a screen of AGT mutants randomly mutated at position glycine-160, we found that the double mutant Y158H/G160A protected *Escherichea coli* from killing by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) even in the presence of BG and that the AGT activity of this mutant was strongly resistant to BG (ED_{50} of 180 μM). Because the single mutant G160A was not resistant to BG, this suggested that the presence of the charged histidine residue at position 158 was responsible. This hypothesis was confirmed by the construction of the single mutation Y158H. The Y158H-mutant AGT was slightly less active than wild-type AGT for the repair of methylated DNA *in vitro*, but it protected *E. coli* from killing by MNNG even in the presence of BG and had an ED_{50} for the inactivation by BG of 620 μM. In contrast, mutant Y158F had an ED_{50} of 0.2 μM. Previous studies (M. Xu-Welliver *et al*., Cancer Res., *58:* 1936–1945, 1998) have shown that mutant P140K is highly resistant to BG (ED_{50} of >1200 μM). Models of human AGT suggest that the side chain of the lysine inserted into this mutant is close to tyrosine-158 and that the positively charged lysine side-chain may interfere with BG binding. The double mutants P140K/Y158H and P140K/Y158F resembled P140K and Y158H in being highly resistant to BG, but the use of a sensitive assay for reaction of BG with AGT indicated that their abilities to react were in the order P140K/Y158H < P140K < P140K/Y158F. These results confirm that the presence of a positively charged residue close to the active site of human AGT renders it highly resistant to BG without substantially affecting activity toward methylated DNA substrates. Such mutants may limit the value of BG therapy if they arise in malignant cells during chemotherapy, but the mutant sequences may be useful for gene therapy approaches in which BG-resistant human AGTs are used to prevent hematopoietic toxicity. At least 28 AGT sequences (from 25 species) have now been described. In 25 of these, the position equivalent to 158 in the human AGT is also a tyrosine, and in the other 3, it is a phenylalanine. The importance of an aromatic ring side chain at this position is emphasized by previous studies (S. Edara *et al*., Carcinogenesis, *16:* 1637–1642, 1995), which show that the replacement by alanine renders human AGT inactive. Our results show that histidine can also substitute for tyrosine at this position.

INTRODUCTION

An important site of damage to DNA produced by reaction with alkylating agents is the O^6 position of guanine (1–4). This adduct is removed by the action of the DNA repair protein AGT.[4] AGT acts to transfer the alkyl group to a cysteine acceptor site (located at residue 145 in human AGT) and to restore the DNA in a single step. The presence of AGT is known to protect cells from alkylating agent-mediated toxicity including mutagenesis, cell killing, and carcinogenesis. However, the presence of AGT in tumors provides resistance to therapeutic alkylating agents (5–7). Human AGT is strongly inhibited by BG (8). BG acts as a substrate for AGT. It is bound at the active site, and AGT-mediated transfer of the benzyl group to the cysteine acceptor site causes its inactivation (9). The combination of BG and BCNU is currently undergoing clinical trials for cancer chemotherapy (6, 7, 10).

AGT appears to be a very widely distributed protein, and sequences are now known from at least 25 different species. Some of the microbial AGTs have been found to be very resistant to inactivation by BG (9). Several human AGT mutants with a reduced ability to react with BG have been obtained either by site-directed mutagenesis (11–14) or by screening cDNA libraries containing random insertions in the AGT sequences for the ability to protect *E. coli* from killing by MNNG in the presence of BG (14–16).

Such resistant mutants are of interest for several reasons: (*a*) they suggest that resistance to BG therapy may arise relatively readily, and it will be necessary to obtain additional AGT inhibitors able to inactivate these resistant mutants; (*b*) the comparison of BG-sensitive and BG-resistant AGT mutants provides more information about the interaction of the protein and the drug that should prove valuable in designing improved inhibitors with greater potency and specificity; and (c) the dose-limiting toxicity of therapy with BG plus alkylating agents is myelosuppression, which is due to the very low level of AGT in bone marrow progenitor cells (17, 18). The use of a BG-resistant AGT-mutant protein for gene therapy approaches in which an AGT is expressed from a suitable vector in hematopoietic cells may overcome this toxicity and improve the chemotherapeutic index of the treatment because the tumor would remain sensitive whereas the bone marrow would be resistant (19–22).

We (12–14) and others (15, 16) have, therefore, been attempting to produce a panel of BG-resistant mutants and to identify the major factors involved in permitting AGT to continue to work on DNA adducts but to be inactive on BG. A screening method to obtain such mutants was set up by using the ability of expressed human AGT to protect a BG-permeable *E. coli* strain lacking endogenous alkyltransferase (TRG8) from killing by MNNG (14). This protection was abolished by BG. We have shown that this screen can be used to identify BG-resistant mutants in human AGT containing a random sequence inserted in place of the codons corresponding to amino acids 138–140 (14). This screen indicated that the conversion of proline-140 to lysine (mutant P140K) produced by far the greatest resistance of any point mutation, increasing the ED_{50} value for inactivation by more than 12,000-fold.

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[4] The abbreviations used are: AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; ED_{50}, concentration of BG needed to reduce the alkyltransferase activity by 50% in a 30 min incubation at 37°C; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; LB, Luria-Bertani medium.

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Another position at which human AGT can be altered to provide resistance to BG is at glycine-160. This was discovered by the comparison of the properties of a naturally occurring variant G160R that was found in a minority of Japanese subjects (23) with wild-type AGT (13). The G160R AGT was slightly more resistant to BG (20-fold increase in ED_{50}), whereas the mutants G160A and G160W, which were made as controls, were found to be more sensitive than wild type (13). The finding that G160W was sensitive to BG was later confirmed by Rafferty *et al.* (24).

In the course of studies of the possible-BG resistance of an AGT-mutant population in which the codon at position 160 was replaced by NNS to produce a complete library covering replacements with all amino acids, we have now found a highly BG-resistant mutant. However, sequencing of this mutant revealed that it contained the double change Y158H/G160A. The G160A mutation was unlikely to explain the properties of this mutant because previous work had shown that G160A mutant AGT is not resistant but is slightly more sensitive than wild-type AGT (13). Also, position 158 in AGT sequences is highly conserved with 25 of 28 known AGT sequences having tyrosine and the other three having the closely related phenylalanine.

We have, therefore, constructed the Y158H-mutant AGT by site-directed mutagenesis and carried out a detailed study of this and related AGT mutants. These studies show that the Y158H AGT is active in repairing DNA and is highly resistant to BG. Molecular modeling studies suggest that the charged residues on the side chains of either the P140K or the Y158H mutants may be present in the same vicinity at the AGT active site. The presence of a charged hydrophilic residue in this position may prevent the binding of the hydrophobic BG.

MATERIALS AND METHODS

Materials. All oligodeoxynucleotides were purchased from Life Technologies, Inc. (Gaithersburg, MD). *E. coli* strain TRG8 was derived from strain GWR109 (25) that was kindly provided by Dr. L Samson (Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA). pQE30 plasmid and the DNA isolation kits were obtained from Qiagen (Chatsworth, CA). Restriction enzymes and *Pfu* polymerase were purchased from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA). T4-ligase and the Talon Metal Affinity Resin were purchased from Clonetech (Palo Alto, CA). Ampicillin, kanamycin, isopropyl β-D-thiogalactopyranoside, hemocyanin, and most of the other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). BG was synthesized (8) and generously provided by Dr. R. C. Moschel (ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). [8-^{3}H]BG (0.34 mCi/mmol) was prepared by catalytic tritium exchange of BG with tritiated water by Amersham Corporation (26).

Plasmids for Expression of AGT. AGT was expressed in the pUC-18 vector for selection and studies in *E. coli* extracts. Expression from the pQE or pIN vectors was used for purification of the recombinant protein. Plasmids pUC-AGT (14), pQE-AGT (13), pQE-P140K (14), pIN-Y158F, and pIN-Y158A (27) have been described previously.

The pUC-Y158H mutant was made by PCR by using primer 1 (5′–CCGTTTTCCAGCAAGAGTCG-3′) matching nt 310 to nt 432 in the pQE-AGT plasmid and primer 2 (5′–CCTTCACG*GCTAG*CCCTCCGGAGT*G*GT-TGCCCACGGCTCC-3′) for the mutation of the codon for tyrosine-158 to histidine (mismatch is underlined and *Nhe*I site is shown in italics). The PCR reaction was done using *Pfu* polymerase (Stratagene) with pQE-AGT plasmid as template under the following conditions: (*a*) initial denaturation for 2 min at 92°C; (*b*) 25 cycles of denaturation (30 s at 92°C); (*c*) annealing (30 s at 52°C); and (*d*) extension (30 s at 72°C) followed by a final extension at 72°C for 5 min. The PCR product (236 bp) was purified, digested with *Mlu*I and *Nhe*I and ligated into the pUC-inAGT2 plasmid (described below in the next section of "Materials and Methods") digested with the same enzymes to form pUC-Y158H. Plasmid pQE-Y158H was made by ligation of the 243-bp DNA containing the sequence for the Y158H mutation (obtained by digestion of pUC-Y158H with *Mlu*I and *Age*I) with pQE-AGT digested with the same enzymes.

The P140K/Y158H mutant AGT was generated by using pQE-P140K and pQE-Y158H. The 1.2-kb fragment between the *Dra*III (at 436) and the *Xba*I (at 1600) restriction sites from pQE-Y158H was ligated with the 2.8-kb fragment from pQE-P140K digested with the same enzymes to form pQE-P140K/Y158H.

The P140K/Y158F mutant was generated by using the pQE-P140K and pIN-Y158F DNA constructs. The 390-bp fragment from pIN-Y158F generated by *Dra*III digestion (which cuts the pIN-AGT plasmid at two sites at 436 and 826 because of a site in the vector sequence) was isolated. This fragment was further digested with the *Age*I restriction enzyme (at 521), dephosphorylated, and ligated with the large fragment from pQE-P140K digested with *Dra*III (at 436) and *Age*I (at 521) to form pQE-P140K/Y158F.

The entire AGT coding region of all of the plasmids used for AGT expression and activity measurements were sequenced to ensure no secondary mutations were formed.

Construction and Screening of AGT Library with a Random Sequence Corresponding to AGT Codon 160. A 1040-bp irrelevant inactivating sequence was inserted into the AGT cDNA in pGEMAGT2 (14) between the *Dra*III (at 436) and the *Nhe*I (at 483) sites to prevent contamination of the library with wild-type AGT. The DNA insert was created by PCR using *Pfu* polymerase and pCM9 (28) as template with sense primer 5′-GGAAGCTG-*CACAGAGTG*TCGAAGGGACCGAGAAGC-3′ (mismatches underlined) to create the *Dra*III site (shown in italics) and the antisense primer 5′-CGACTCTA*GCTAGC*ATCCACCACC-3′ (mismatches underlined) to create the *Nhe*I site (shown in italics). The PCR product was purified by the PCR Purification kit (Qiagen), digested with *Dra*III and *Nhe*I, and ligated into pGEM-AGT2 plasmid digested with the same enzymes to form pGEM-inAGT2. From this plasmid, the 1078-bp piece fragment between *Dra*III and *Age*I sites was isolated and inserted into the large fragment (3 kb) from pUC-AGT plasmid digested with the same enzymes to form pUC-inAGT2. The resulting plasmid, pUC-inAGT2, was then used for insertion of the random sequences. The DNA insert containing the random sequences was created by PCR covering the AGT coding region from 420 to 540, including the *Dra*III site (at 436) and the *Age*I site (at 521), using as the sense primer, 5′-CCGTGCC*ACAGAGTG*GTCTGCAGCAGCGGAGCCGTGGGCAACTA-CTCC*NNS*GGGCTAGCCGTGAAGG-3′, and as the antisense primer, 5′-GGCTTCCCC*AACCGG*TGGCC-3′, (mismatches underlined, and restriction sites shown in italics). The PCR reaction was carried out using *Pfu* polymerase and pUC-AGT as the template under the same conditions as described above. The PCR product was gel-purified, digested with *Dra*III and *Age*I enzymes, and ligated into pUC-inAGT2 digested with the same enzymes. The ligated products were used to transform XL1-Blue cells. An aliquot of the transformation mixture was plated on LB plates supplemented with 50 μg/ml ampicillin to determine the total number of plasmid-containing bacteria, which was found to be about 2.5×10^5. The remainder of the transformation mixture was amplified by growing overnight. The plasmid DNA was isolated, subjected to sequencing analysis to confirm that the randomized sequence was present, and introduced into the TRG8 strain by electroporation. An aliquot of the sample was plated to determine the efficiency of transformation (2×10^8 transformants/μg DNA), and the rest was amplified overnight in LB medium containing ampicillin and kanamycin (50 μg/ml each). Aliquots of this library were either subjected to the screening process or stored at −70°C for further use.

The screening for active and BG-resistant AGT mutants was carried out as described previously (14) using treatment with 50 μM BG for 1.5 h followed by 40 μg/ml MNNG for 0.5 h at 25°C. Crude extracts for the determination of AGT activity were prepared from the independent mutants grown overnight in 10 ml of LB medium by sonicating the bacterial pellet resuspended in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM DTT for 2 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 15 min at 15,000 × *g*, and the supernatant was used to determine the AGT activity and its sensitivity to BG.

MNNG Survival Assay in the Presence of BG. TRG8 bacteria containing plasmids expressing wild-type Y158H or Y158H/G160A AGT were grown in 5 ml of LB media containing 50 μg/ml ampicillin and 50 μg/ml kanamycin with agitation at 250 rpm in a water bath at 37°C until the A_{600} was 0.5. The cultures were then pelleted, washed, resuspended in 1 ml of LB media and

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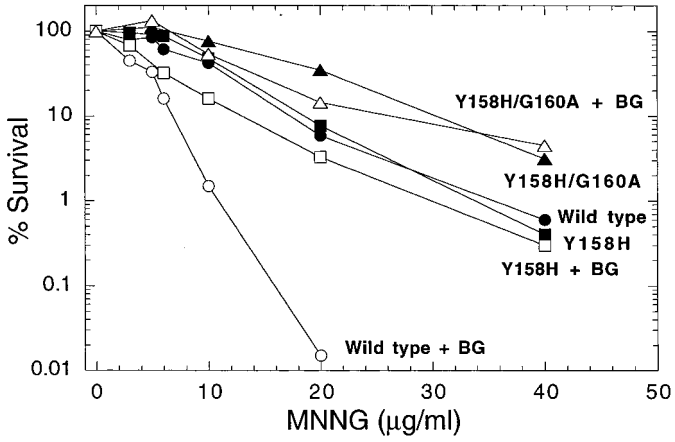

Fig. 1. The effect of mutations Y158H and Y158H/G160A on the survival of MNNG-treated TRG8 cells in the presence and absence of BG. The survival of TRG8 cells expressing the AGT shown after treatment with the MNNG concentration indicated in the presence of 250 μM BG (*open symbols*) or in the absence of BG (*closed symbols*) was measured. Results are shown for cells expressing wild-type AGT (○, ●), mutant Y158H (□, ■), and Y158H/G160A (△, ▲).

divided into two 0.5-ml aliquots, one of which was supplemented with 250 μM BG. The tubes were agitated at 250 rpm for 1.5 h at 25°C before an additional 2 ml of media with or without BG was added to restore the original volume. After BG treatment, the cultures were exposed to MNNG (0–40 μg/ml) for 30 min. The reactions were stopped by diluting small aliquots of the bacteria cultures in M9 media on ice. The bacteria were further diluted and spread on LB plates containing 50 μg/ml ampicillin and 50 μg/ml kanamycin with or without 100 μM BG. The plates were incubated at 37°C for 16 h, and the colony numbers were determined. The percentage of survival was determined by using the colony number/ml of culture exposed to MNNG divided by the colony number/ml of culture when MNNG was absent.

Purification of AGT. The wild type and all of the mutants except Y158F were expressed using the pQE vector, which adds a 12-amino acid sequence [MRGS $(H)_6$GS-) to the NH_2 terminus of the AGT protein, and were purified by immobilized metal affinity chromatography as described previously (13, 14). The recombinant Y158F mutant protein was produced using the pIN-Y158F plasmid and was purified as described (27).

Determination of Effects of BG on AGT Activity. Inactivation of the AGT activity by BG in crude bacterial extracts or purified protein preparations was measured by incubating aliquots of the protein with different concentrations of BG at 37°C for 30 min in 0.5 ml of 50 mM Tris-HCl (pH 7.6), 5 mM DTT, and 0.1 mM EDTA containing 50 μg hemocyanin and 10 μg calf thymus DNA (14). The residual AGT activity was determined, and the results were expressed as the percentage of the AGT activity remaining. Graphs of AGT activity remaining against inhibitor concentration were used to calculate an ED_{50} value representing the amount of inhibitor needed to produce a 50% loss of activity.

Measurements of the formation of [8-^{3}H]guanine from [8-^{3}H]BG were carried out as described previously (26) using various amounts of the purified AGT proteins in an assay buffer consisting of 0.7 μM O^6-benzyl-[^{3}H]guanine, 25 μg of calf thymus DNA, 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, and 5 mM DTT in an assay volume of 0.25 ml.

Kinetics of Repair by AGT. The rate constant for the AGT reaction was determined by measuring the appearance of the [^{3}H]methylated AGT at various times using concentrations of AGT protein determined in preliminary experiments to readily measurable rates under the assay conditions (29). The reaction mixture (1 ml) for each time point contained AGT (4.5×10^{-10} M for wild type, 2.75×10^{-9} M for Y158H, 6.5×10^{-9} M for P140K, 1.1×10^{-8} M for P140K/Y158H or 3.4×10^{-9} M for P140K/F158F), 3.6×10^{-10} M O^6-[^{3}H]methylguanine in [^{3}H]methylated DNA substrate and 50 μg of cold calf thymus DNA in a buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM DTT, and 0.5 mM EDTA.

RESULTS

A library of AGT mutants containing an NNS codon replacing codon 160 in the human AGT was placed in the pUC18 vector and expressed in TRG8 cells that lacked endogenous AGT activity and were permeable to BG (14). The library was then selected by exposure to MNNG and BG, colonies were isolated, and the AGT sequence present was determined. One of the colonies contained a sequence coding for the double mutation Y158H/G160A. The origin of the second mutation producing the Y158H change is obscure but the mutation was an A:T to G:C transition that could have been derived from the mutagenic effects of the MNNG used for selection. The ability of the cells carrying this mutant AGT to survive the selection procedure suggested that the Y158H/G160A was likely to be active in repairing methylated DNA and to be resistant to BG. This was confirmed by direct measurement of the killing of the cells by MNNG in the presence and absence of BG (Fig. 1) and by assays using crude extracts from the TRG8 cells (Fig. 2).

Cells containing the Y158H/G160A mutant or wild-type AGT were resistant to killing by MNNG. When 250 μM BG was also added, the protection by wild-type AGT was abolished, but that by Y158H/G160A was unaffected (Fig. 1). Direct proof that the Y158H/G160A mutant was resistant to inactivation by BG was obtained using crude extracts from the cells and measuring AGT activity after exposure to the inhibitor (Fig. 2). The Y158H/G160A mutant was more than 1000 times more resistant to BG than wild type, with an ED_{50} value of ~180 μM as compared with ~0.1 μM.

Previous studies with the purified protein have shown that the single mutation G160A does not render AGT resistant to BG and, in fact, slightly increases sensitivity to this drug (13). It, therefore, seemed likely that this mutation was not contributing to the resistance of the Y158H/G160A double mutant, although it might aid in maintaining the activity of the protein when the highly conserved tyrosine-158 was replaced by histidine. To test this, the single Y158H mutant AGT sequence was made and expressed in TRG8 cells. The cells expressing this protein showed similar protection from MNNG to cells expressing wild-type AGT, and this protection was not abolished by BG (Fig. 1). Also, the Y158H-mutant AGT in crude extracts from the TRG8 cells was actually significantly more resistant to BG inactivation than the double mutant (Fig. 2).

This resistance was confirmed using AGT purified to homogeneity

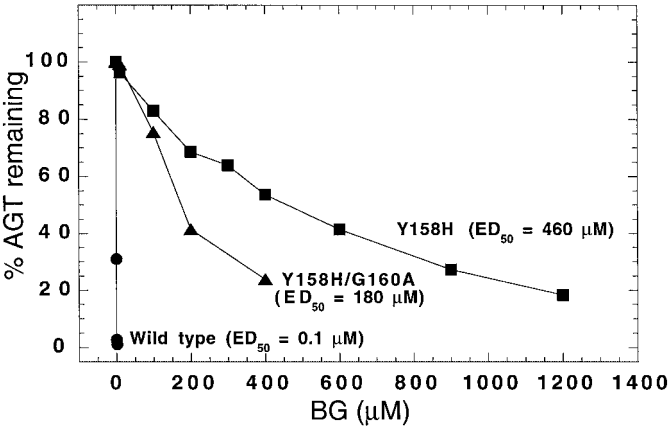

Fig. 2. The sensitivity of AGT in crude cell extracts to BG. AGT inactivation studies were carried out as described under Methods and Materials by incubation of the crude cell extracts from TRG8 cells expressing the AGT shown with the BG concentrations indicated for 30 min in the presence of 10 μg of calf thymus DNA. The residual AGT activity was then determined by addition of a [^{3}H]methylated DNA substrate and a further incubation for 30 min. There was no significant loss of AGT activity on incubation in the absence of BG.

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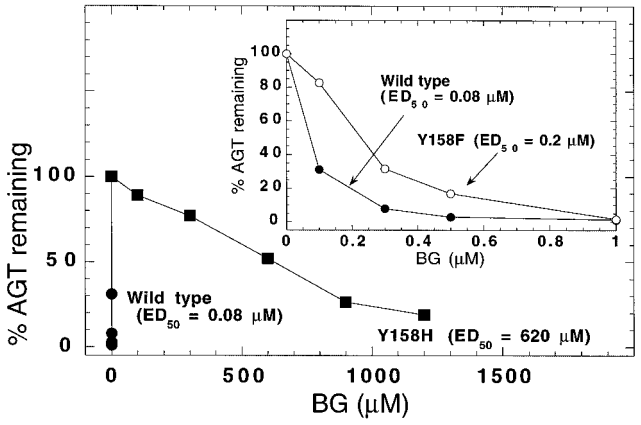

Fig. 3. The effect of BG on purified AGT proteins. Inactivation studies were carried out as described in the legend to Fig. 2 using purified AGT proteins. Results for Y158H and wild type AGT are shown in the main figure. The insert shows results with wild-type and Y158F-mutant AGT proteins.

(Fig. 3 and Table 1). The Y158H mutant AGT was expressed using the pQE vector, which places a $(His)_6$-tagged sequence at the NH_2 terminus. The protein was then purified using immobilized metal affinity chromatography. As shown in Fig. 3, the ED_{50} for inactivation by BG of the Y158H AGT was greatly increased, whereas the Y158F mutation had little significant effect on the reaction with BG. The ED_{50} value for Y158H was increased by more than 6000-fold over wild-type AGT from 0.1 μM to 620 μM (Table 1).

Only one other point mutation in AGT is associated with a greater increase in resistance to BG. This is the mutation P140K, which renders the ED_{50} value more than 1200 μM (14). Models based on the known crystal structure of the related *E. coli* Ada-C protein suggested that the side chain of the lysine in the P140K mutant may be in close proximity to the hydroxyl group of tyrosine-158 (Fig. 4). The presence of a positive charge in this location may, therefore, strongly discourage the binding of the hydrophobic BG.

This concept was examined by the construction of the double mutants P140K/Y158F and P140K/Y158H. These mutants and P140K were tested for inactivation by BG, and all of them were so highly resistant that ED_{50} values could not be measured, although Y158F/P140K showed a slight reduction of activity with 1.2 mM BG (Table 1). Measurement of the rate of guanine production when AGT acts on BG provides a more sensitive method for determining the interaction of AGT with BG (26). Such studies were, therefore, carried out using [8-^{3}H]BG as a substrate in a 20 min reaction. The production of [8-^{3}H]guanine by the mutants P140K, Y158H and Y158F was consistent with the ED_{50} values (Table 1). As reported previously (14), the formation of [8-^{3}H]guanine was greatly reduced by the P140K mutation. The Y158H mutation was slightly less effective but it also greatly reduced [8-^{3}H]guanine formation, whereas the Y158F mutation had only a small effect (Table 1). The P140K/Y158F and P140K/Y158H double mutants had a highly impaired ability to convert [8-^{3}H]BG to [8-^{3}H]guanine but P140K/Y158F was actually more reactive than P140K, whereas P140K/Y158H gave no detectable guanine formation even when 0.6 mg of AGT protein was used (Table 1).

Although the Y158H and P140K mutations clearly did not prevent the ability of AGT to repair methylated DNA, they did produce a significant effect on the rate of the reaction. As shown in Table 1, there was an approximately 10-fold reduction in the rate constant for DNA repair. These reductions may reflect the distortion at the active site. The combination of the P140K mutation with the Y158H mutation that may produce an even greater distortion reduced the rate constant by a greater amount (40-fold) but the combination P140K/Y158F gave a smaller reduction of only 4-fold. This may indicate that the loss of the hydroxyl group from the side chain of residue 158 may allow the lysine inserted at position 140 to be accommodated with less of an alteration in the structure of the active site.

DISCUSSION

It is possible that the G160A alteration in the Y158H/G160A double mutant that was selected in our screening procedure contributes to an increased activity of this mutant compensating for the 10-fold reduction in rate constant produced by the Y158H change. However, this was not investigated in our studies because the Y158H/G160A was not obtained in a purified form suitable for such experiments. Although there was apparently a slight difference in resistance to killing by MNNG between the cells expressing wild-type or Y158H AGT and those expressing the Y158H/G160A double mutant with the latter showing a greater level of protection (Fig. 1), these results should not be taken to indicate that the double mutant is more active. The observed difference may be an experimental artifact because the cells expressing the double mutant were those surviving the treatment with BG plus MNNG. These cells may have acquired additional mutations in the bacterial genome during the selection procedure, and they grew more slowly than the original untreated TRG8 cells in which the wild type and the Y158H mutants were expressed. This difference in growth rate or other effects of unknown mutations could contribute to the difference observed.

Our results show that, although the position in all of the alkyltransferases equivalent to 158 in the human AGT is highly conserved in all of the known AGTs and is restricted to tyrosine or occasionally phenylalanine, the substitution with histidine also gives rise to an active protein. It is possible that these amino acids are the only ones tolerated at this position because mutant Y158A was inactive (27), and no AGTs with other alterations at position 158 have been found in screens of mutated AGT libraries. The ability of histidine to replace tyrosine-158 and to provide an active AGT is also suggested by recent results of the screening using BG and MNNG for the selection of plasmid libraries in which random mutagenesis was used for substitutions of positions 150–172 of AGT (16). All of the BG-resistant mutants found had multiple substitutions with 3–5 amino acids differing from wild type, but 5 of 8 of these mutants had the Y158H

Table 1 *Properties of AGT mutant proteins*

Mutant	Inactivation by BG (ED_{50}, μM)	Conversion of [8-^{3}H]BG to [8-^{3}H]guanine (cpm/μg protein)	Rate constant for reaction with methylated DNA ($M^{-1}\cdot min^{-1}$)
Wild type	0.1	13,879	38×10^6
Y158F	0.2	7,653	Not measured
Y158H	620	30.1	3.4×10^6
Y158A	Inactive	Inactive	Inactive
P140K	>1200 (99% remaining at 1.2 mM)	2.0	3.6×10^6
P140K/Y158H	>1200 (99% remaining at 1.2 mM)	<0.1	1.0×10^6
P140K/Y158F	>1200 (88% remaining at 1.2 mM)	7.0	9.4×10^6

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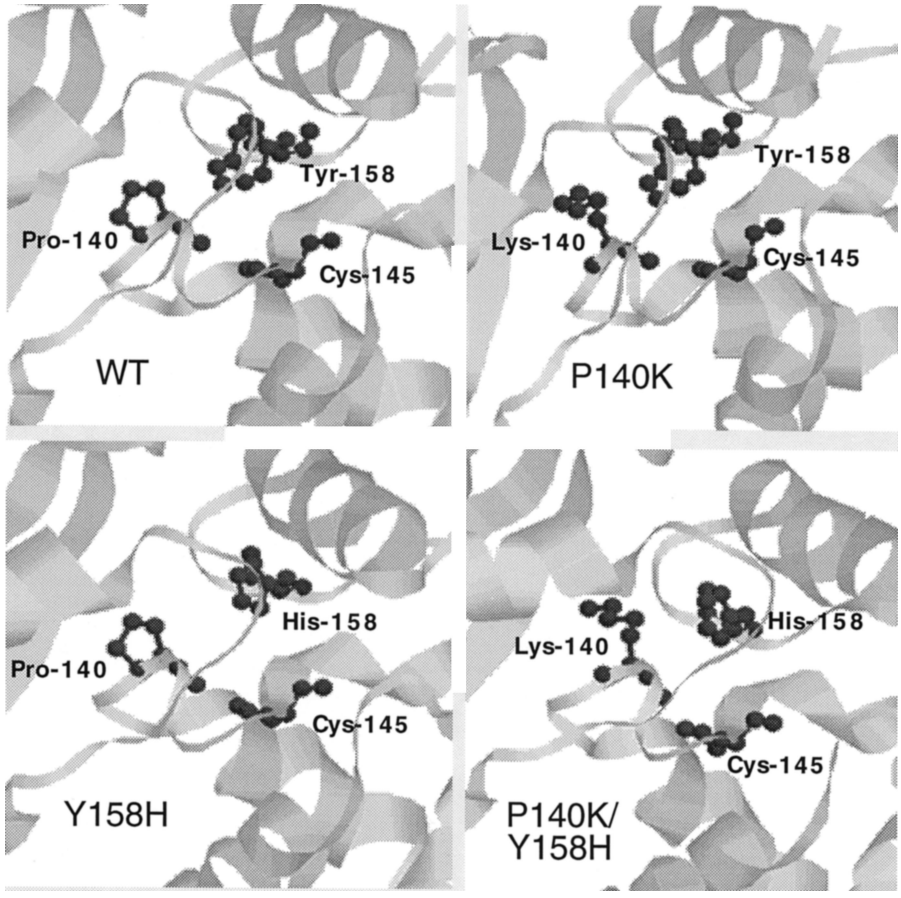

Fig. 4. Model of partial structure of P140K and Y158H mutant AGTs. The figures were generated using the program Ras-Mol using the coordinates for the model of human AGT (36) and inserting the point mutations with the Swiss protein data base viewer program. The spatial arrangement of the protein chain at the active site is shown with the side chains of residues 140, 145, and 158 shown as *ball and stick structures*.

change. Our results suggest that this alteration was the major reason for the resistance of these mutants to BG.

Several microbial AGTs including the Ada-C protein from *E. coli* are virtually unaffected by BG (9). Studies by site-directed mutagenesis and comparison with the known crystal structure for this protein (30, 31) have indicated that the resistance of the *E. coli* Ada-C AGT is due to a steric restriction of the size at the active site that prevents the binding of the bulky BG (32, 33). This restriction is caused by the combination of the absence of a proline and the presence of a tryptophan residue in the active site pocket. The mutation of both of these residues to form proline and alanine, respectively, renders Ada-C able to react with BG (32, 33).

The inability of the first reported BG-resistant mutants of the human AGT (P140A and G156A) to react well with this drug also seems to be due to steric factors inasmuch as these alterations would be expected to reduce the size of the active site pocket in the human AGT (11, 12). However, the discovery that the G160R mutant was also resistant, whereas G160A and G160W were not (and were actually more sensitive), suggested that another class of resistant mutants might be formed by the presence of a hydrophilic group in the active site pocket (13).

The finding that the Y158H-mutant AGT is highly resistant to inactivation by BG (ED_{50} of 620 μM) provides strong support for this hypothesis. Its resistance can be explained by the presence of a charged residue in the active-site pocket that discourages the binding of the hydrophobic BG. The other two single amino acid changes that have the largest effect in rendering AGT resistant to BG are P140K (ED_{50} of $>$ 1200 μM) and P140R (ED_{50} of 190 μM; Ref. 14). These changes are also likely to lead to placing a charged residue in the active site and may also influence the reaction by the steric effect caused by replacing the proline described above.

The combination of the P140K and Y158H mutations forming mutant P140K/Y158H rendered human AGT totally unable to react with BG. The limit of detection in the assays shown in Table 1 is such that the reduction in rate of reaction of this double mutant when compared with wild type is by a factor of more than 10^5. This P140K/Y158H mutant was considerably less active with BG than the single P140K mutant. Although a small part of this reduction may be due to the difference in rate constant between these two mutants, this was altered by only a factor of 3.6, whereas the reduction in the rate of guanine formation from BG was more than 20-fold (Table 1). In contrast, the increased ability of the P140K/Y158F to react with BG when compared with P140K alone may be due solely to the alteration in the rate constant.

Although both the P140K- and the Y158H-mutated AGT proteins have a significant reduction in the rate constant for the repair of methylated DNA, this does not prevent them from being highly effective in protecting *E. coli* from killing by MNNG (Fig. 1 and reference (14)) or, in the case of P140K, from protecting mammalian cells from the therapeutic agent BCNU (34). This is probably due to the very rapid rate of repair by the wild-type AGT. A moderate reduction in this rate, therefore, has little physiological effect. In studies with other mutants of AGT in the DNA binding domain that are more severely compromised in their activity, a greater than 35-fold reduction was required before any effect was observed. Even mutant Y114A, which had almost a 1000-fold reduction in rate constant, provided some protection (29).

The protection from killing by alkylating agents afforded by mutants Y158H and P140K was not reduced by BG even at concentrations far in excess of those likely to be achievable in patients treated with this drug. Therefore, these mutants are excellent choices for gene therapy approaches in which the expression of a BG-resistant mutant

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AGT in hematopoietic progenitor cells is used to protect the bone marrow and improve the therapeutic index of such therapy.

Conversely, the relatively facile production of BG-resistant mutants that is revealed in these and other experiments suggests that the formation or selection for such forms of AGT in tumor cells during therapy may be a significant problem although these forms have lower-than-wild-type-AGT activity. This focuses attention on the development of novel inhibitors that could be used to inactivate such forms. The use of short single-stranded oligodeoxynucleotides containing an O^6-benzyldeoxyguanosine may be useful in this regard because the binding of these to the AGT-active site involves the DNA binding domain and is much stronger than the binding of the free base BG (35).

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Alteration of the Conserved Residue Tyrosine-158 to Histidine Renders Human O^6-Alkylguanine-DNA Alkyltransferase Insensitive to the Inhibitor O^6-Benzylguanine

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