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Role of Codon 160 in the Sensitivity of Human O^6-Alkylguanine-DNA Alkyltransferase to O^6-Benzylguanine

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ABSTRACT. O^6-Alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein that provides protection from alkylating agents such as dacarbazine, temozolomide, and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), which are used for cancer chemotherapy. O^6-Benzylguanine (BG) is an inhibitor of AGT that sensitizes tumors to these agents. BG is currently in clinical trials. It is possible that the presence of resistant forms of AGT may limit the effectiveness of this strategy. Previous studies have shown that the AGT mutant G160R, which may occur naturally as a result of a polymorphism in the *AGT* gene, is resistant to BG, whereas the mutants G160W and G160A are actually more sensitive to the inhibitor. To examine other mutations at this site, a random sequence was placed at codon 160 in the AGT cDNA, and a plasmid library was constructed to express these sequences in *Escherichia coli*. After selection with BG and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, BG-resistant mutants were obtained and analyzed. Eleven different amino acid substitutions were found to impart BG resistance by this assay. The most resistant mutants contained histidine or arginine, which had EC_{50} values of 12 and 4.7 μM, respectively, compared with the wild-type EC_{50} of 0.08 μM, but nine other alterations led to at least a 10-fold rise in the EC_{50} value. Three additional mutations at codon 160 were constructed by site-directed mutagenesis, and these led to 6- to 11-fold increases in resistance to BG. Comparisons of the properties of mutants G160R and G160E showed that the presence of DNA enhanced the reaction with BG much more strongly when an acidic residue was present at this position. This may account for the lack of selection of the G160E mutation even though it did impart resistance to BG. These results indicate that many alterations of AGT at position 160 can lead to significant resistance to BG. BIOCHEM PHARMACOL **58**;8: 1279–1285, 1999. © 1999 Elsevier Science Inc.

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The DNA repair protein AGT‡ repairs DNA containing O^6-guanine adducts by a stoichiometric transfer mechanism in which the alkyl group is transferred to a cysteine acceptor site in the AGT protein. BG inactivates AGT by acting as a substrate for the protein. BG is bound to the active site, and the benzyl group is transferred to the cysteine, causing irreversible loss of AGT activity [1]. BG is a very potent inhibitor of human AGT, and elimination of this activity in tumors causes increased killing by methylating and chloroethylating agents [2–5]. BG currently is undergoing clinical trials to examine the feasibility of this approach to enhancing the chemotherapeutic effectiveness of agents such as BCNU and temozolomide [6–8].

However, BG is much less active against the alkyltransferases present in some other organisms such as *Escherichia coli* and yeast, despite a high level of similarity between these proteins and the human AGT [1, 9]. This suggests that minor alterations in the AGT structure may lead to resistance to BG, and several resistant mutants have been reported. These include alterations at positions Pro-138, Pro-140, Gly-156, and Gly-160 [10–15]. The alteration of Gly-160 was of particular interest for two reasons. First, it has been reported that the mutant G160R actually exists in some individuals as a result of a polymorphism in the *AGT* gene [16], and mutant G160R has been found to be strongly resistant to BG [12]. Selection of a resistant form during therapy, even from heterozygotes, is much more likely than mutational generation of such mutants. Second, two other mutations at codon 160, mutants G160W and G160A, are

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‡ *Abbreviations*: AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; PCR, polymerase chain reaction; and EC_{50}, concentration of O^6-benzylguanine needed to reduce the alkyltransferase activity by 50% in a 30-min incubation at 37°.

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not resistant to BG and, in fact, were found to be more sensitive to inactivation [12, 17]. These are the only AGT mutants reported thus far to have increased reactivity with BG.

In the present studies, we have carried out a more extensive examination of the results of replacing the Gly-160 position in human AGT with other amino acids. The results show that at least 14 alterations at this site rendered the protein resistant to BG.

MATERIALS AND METHODS

Materials

BG was synthesized as previously described [2] and was provided by Dr. R. C. Moschel (National Cancer Institute-Frederick Cancer Research Development Center). BCNU was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. *N*-[*methyl*-^{3}H]*N*-Nitrosourea was purchased from Amersham, Inc. O^6-Benzyl[8-^{3}H]guanine was produced by catalytic [^{3}H]exchange as previously described [18]. Reagents for molecular biology, cell culture, and AGT assays were obtained from Life Technologies, the Sigma Chemical Co., Stratagene, New England Biolabs, Perkin Elmer/Cetus, Qiagen, Clontech, and Promega.

Preparation of Purified AGT Mutants

The methods for protein production and purification and the plasmids for the preparation of purified, recombinant wild-type AGT and mutants G160A, G160R, and G160W have been described previously [12]. The pQE30-G160E plasmid for production of G160E was prepared by three-round PCR exactly as described for pQE-G160R [12] using the primer 5′-GGCAACTACTCC**GA**AGGGCTAGC-CGTG-3′, where the mismatches set in boldface introduce the G160E mutation. The pQE30-P140A/G160R plasmid for production of P140A/G160R was constructed by replacing the 337-bp section of DNA located between the sites for restriction enzymes *Eco*NI and *Dra*III in plasmid pQE-G160R [12] with the same fragment isolated from the pGEM-P140A plasmid [10]. The entire DNA coding sequences of all plasmids for mutant preparation were verified to ensure that no additional mutations were present. Recombinant G160E and P140A/G160R proteins were purified by immobilized metal affinity chromatography as described for G160R [12].

Construction of a Plasmid Library Containing Human AGT cDNA Sequence with Random Insertions at Codon 160

The library was constructed in the pUC-AGTm plasmid, which contains the sequence coding for AGT in an *E. coli* expression vector [14].

To eliminate interference in the library construction from contamination by partially digested wild-type AGT sequences, the coding region for AGT in the pGEM-AGT2 vector [14] was disrupted by inserting a 1040-bp irrelevant sequence between the *Dra*III (at 436) and the *Nhe*I (at 483) sites. The DNA insert was created by PCR using *Pfu* polymerase and pCM9 [19] as template with the sense primer 5′-GGAAGCTGCACA***G*****A*****G***T***G***TCGAAGGGA-CCGAGAAGC-3′ (mismatches in boldface) to create the *Dra*III site (shown in italics) and the antisense primer 5′-CGACTCTAG***CT***AGCATCCACCACC-3′ (mismatches in boldface) to create the *Nhe*I site (shown in italics). The PCR product was purified with 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 fragment between the *Dra*III and *Age*I site was cut out and inserted into the large fragment (3 kb) from the pUC-AGTm plasmid digested with the same enzymes to form pUC-inAGT2. The ligated product was transformed into the XL1-Blue strain and spread onto LB plates containing 50 μg/mL of ampicillin, and DNA from the transformants was purified and analyzed by restriction digestion and sequencing analysis. The resulting plasmid, pUC-inAGT2, then was used for insertion of the random sequences.

The random sequence replacing Gly-160 was created by PCR using as the mutagenic sense primer 5′-CCGTGCCA-CAGAG*T*GGTCTGCAGCAGCGGAGCCGTGGGCA-ACTACTCC**NNS**GGGCTAGCCGTGAAGG-3′, matching the pUC-AGTm plasmid from nucleotides 420 to 487 (mismatches are in boldface, and the *Dra*III site is shown in italics), and the antisense primer 5′-GGCTTCCCCAAC-CGGTGGCC-3′ matching pUC-AGTm plasmid from nucleotides 540 to 520 (*Age*I site shown in italics). The PCR reaction was carried out using *Pfu* polymerase and pUC-AGTm as template under the following conditions: initial denaturation for 2 min at 92°; 30 cycles of denaturation (1 min at 92°); annealing (1 min at 50°); extension (1 min at 72°); followed by a final extension at 72° for 5 min. The PCR product was agarose gel-purified using the Gel Extraction kit (Qiagen), digested with *Dra*III and *Age*I enzymes, and ligated into pUC-inAGT2 digested with the same enzymes. The ligated products were transformed into XL1-Blue cells. An aliquot of the transformation mixture was cultured on LB plates supplemented with 50 μg/mL of ampicillin to determine the number of plasmid-containing bacteria (which was found to be 15,000), and the remainder 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 TRG-8 strain by electroporation. An aliquot of the mixture 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° for further use.

Preparation of Mutants G160S and G160T by Site-Directed Mutagenesis

The pUC-G160S and pUC-G160T mutants were created in pUC-AGTm by PCR using *Pfu* polymerase and pQE-AGT as template with the sense primer 5′-CCCGTTTTCCAGCAAGAGTCGTTCA-3′ matching nucleotides from 259 to 284 and the mutagenic antisense primers (a) 5′-CCTTCACGGC*T*AGCCC**ACT**GGAGTAGTTGCC3′ for G160S or (b) 5′-CCTTCACGGC*T*AGCCC**TGT**GGAGTAGTTGCC-3′ for G160T, matching nucleotides from 496 to 465 (mismatches in boldface and the *Nhe*I site shown in italics). The PCR reactions were carried out under the same conditions as described above. The PCR products were gel purified using the Gel Extraction kit (Qiagen), digested with *Mlu*I (at 283) and *Nhe*I (at 483) enzymes, and ligated into the pUC-inAGT2 large fragment digested with the same enzymes. The ligated products were transformed into XL1-Blue cells and cultured on LB plates supplemented with 50 μg/mL of ampicillin. The plasmid DNA was subsequently purified and subjected to sequencing analysis to ensure that no secondary mutations were introduced.

Selection and Identification of BG-Resistant Mutants

TRG-8 cells, which lack endogenous alkyltransferases and are permeable to BG [14], were transformed with plasmids encoding the randomized-codon-160 AGT library. These cells then were treated during exponential growth with 50 μM BG for 1.5 hr followed by 40 μg/mL of MNNG for 0.5 hr at 25°. The culture was then diluted in M9 medium and grown on LB plates containing 50 μg/mL of ampicillin, 50 μg/mL of kanamycin, and 50 μM BG. The plates were incubated overnight at 37°. Plasmid DNA was isolated and sequenced from the surviving colonies. After sequencing, colonies corresponding to different mutants were grown overnight in 10 mL of LB medium containing 50 μg/mL of ampicillin and 50 μg/mL of kanamycin, and cells were pelleted by centrifugation. Then extracts were prepared by sonicating the bacterial pellet (resuspended in 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and 5 mM dithiothreitol) for 2 min at 0° using an ultrasonic cell disruptor model W-225-R on pulse setting 50% duty cycle. Cell debris was pelleted by centrifugation at 4° for 15 min at 15,000 *g*, and the supernatant was used to determine the AGT activity level and the sensitivity to BG.

The entire AGT coding region of all plasmids containing mutant AGT sequences was sequenced to ensure that only the mutations described in the text were present.

Determination of AGT Activity and Its Inactivation by BG

The EC_{50} values for the inactivation of the AGT activity by BG were determined by incubations of bacterial extracts or purified protein preparations with different concentrations of BG at 37° for 30 min in 0.5 mL of 50 mM Tris–HCl, pH 7.6, 5 mM dithiothreitol, 0.1 mM EDTA containing 50 μg of hemocyanin and 10 μg of calf thymus DNA. The residual AGT activity then was measured by the addition of 0.5 mL of 50 mM Tris–HCl, pH 7.6, 5 mM dithiothreitol, 0.1 mM EDTA containing a [^{3}H]methylated-DNA substrate. After incubation for 30 min at 37°, the amount of [^{3}H]methylated protein formed was determined [14]. The results were expressed as the percentage of the AGT activity remaining, and the graphs of AGT activity remaining against inhibitor concentration were used to calculate an EC_{50} value representing the amount of inhibitor needed to produce a 50% loss of activity exactly as described previously [10–12]. The assays were set up with an amount of protein that transferred approximately 80% of the radioactivity present in O^6-[^{3}H]methylguanine to the protein in the absence of BG. Incubation without BG produced no loss of AGT activity under the conditions used.

Rate constants for the repair of methylated DNA by purified wild-type G160R and G160E AGTs were measured as previously described [20].

Formation of Guanine from BG

Measurements of guanine formation from BG were carried out using various amounts of the purified wild-type or mutant AGT proteins in an assay buffer consisting of 0.7 μM O^6-benzyl[^{3}H]guanine, 50 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, and 5 mM dithiothreitol in an assay volume of 0.25 mL in the presence of either 25 μg of calf thymus DNA or 50 μg of hemocyanin. After incubation at 37° for 20 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, and the extent of formation of [8-^{3}H]guanine was determined by HPLC as previously described [18].

RESULTS

To examine the importance of the Gly-160 position in AGT in detail, an AGT cDNA expression library in the pUC plasmid was constructed in which the codon for the 160 position was mutated randomly to NNG/C to allow for any of the 20 amino acids to be present. The use of the NNG/C sequence reduces the chances of inserting a stop codon and also provides for a more even representation of the different amino acids. However, three of the amino acids (L, R, and S) are still each represented by 3 codons, five amino acids (A, G, P, T, and V) are represented by 2 codons, and the remaining twelve possibilities have only 1 codon each. There is also possibly some bias in the frequency due to the preference of the DNA polymerase used for PCR. However, the library contained 15,000 members and should, therefore, provide an adequate source for all of the 20 possible substitutions. This library then was expressed in an *E. coli* strain (TRG-8) deficient in endogenous alkyltransferase activity and permeable to BG [14]. Selection of BG-resistant mutants was carried out using BG and MNNG under conditions previously determined to kill

TABLE 1. Inactivation by BG of AGT mutant proteins altered at position Gly-160

Amino acid	% Found after screen*	Protein†	EC_{50} + DNA‡ (μM)	Protein extract used for assay§ (μg)
Histidine	8	Extract	12.0	7
Agginine	29	Extract	4.7	20
Arginine		Pure	4.0‖	
Proline	12	Extract	4.2	15
Methionine	4	Extract	3.1	9
Lysine	27	Extract	2.9	18
Leucine	6	Extract	2.9	5
Glutamine	6	Extract	2.5	6.5
Asparagine	2	Extract	2.2	15
Aspartic acid	2	Extract	1.6	20
Threonine	0	Extract	1.3	3.7
Serine	0	Extract	1.0	2.6
Tyrosine	2	Extract	0.9	5
Cysteine	2	Extract	0.8	5
Glutamic acid	0	Pure	0.5	
Glycine (wild type)	2	Extract	0.08	1.2
Glycine (wild type)		Pure	0.13	
Alanine	0	Pure	0.08¶	
Tryptophan	0	Pure	0.05¶	
Isoleucine	0		Not tested	
Valine	0		Not tested	
Phenylalanine	0		Not tested	

*After screening with BG plus MNNG, 52 plasmids were obtained and sequenced.

†Assays were carried out either with pure AGT protein or with crude extracts from the bacteria expressing recombinant AGT as indicated.

‡Assays were carried out by incubation for 30 min in the presence of 10 μg calf thymus DNA using a range of concentrations of BG.

§The amount of bacterial cell extract used to get transfer of approximately 80% of the radioactivity present in O^6-[^{3}H]methylguanine in the DNA substrate to the protein in the absence of BG.

‖Published previously [12].

¶The sensitivity of these mutants to BG has been published previously [12] but the published assays described values for assays in the absence of DNA. These EC_{50} values were 0.4 μM for wild type and 0.1 μM for either G160A or G160W.

cells lacking AGT activity [14]. A total of 52 colonies that survived this selection were taken, and the plasmids containing AGT were sequenced. As shown in Table 1, 11 different mutants were found, as well as one plasmid that coded for a wild-type AGT amino acid sequence (although the codon 160 for glycine was different from that in wild-type cDNA). The ability of BG to inactivate the AGT activity expressed by these mutants was determined using extracts from the bacterial cells as a source of AGT protein. The amount of BG needed to produce 50% inactivation in a 30-min incubation in the presence of 10 μg of calf thymus DNA was determined (Table 1). All of the 11 mutants found were resistant to BG, with EC_{50} values ranging from 0.8 to 12 μM compared with wild-type, which is about 0.1 μM.

The most common mutant selected was G160R, which occurred 15 times and which we have shown previously to be resistant to BG [12]. The EC_{50} value found in crude extracts of 4.7 μM was in good agreement with the value of 4.0 μM previously reported for the pure protein. The mutants selected most frequently in the screen were alterations leading to the highest levels of resistance (>30-fold) with changes to histidine (EC_{50} of 12 μM), arginine (4.7 μM), proline (4.2 μM), and lysine (2.9 μM). These accounted for 39 of the 52 colonies. Other mutations occurring more than once were methionine (occurring twice with an EC_{50} value of 3.1 μM) and leucine (2.9 μM) and glutamine (2.5 μM), which both occurred three times. Four other mutants (asparagine, aspartic acid, tyrosine, and cysteine) occurred only once and had slightly lower levels of resistance, with the EC_{50} values being increased 8- to 20-fold over wild type. Although the prevalence of arginine, leucine, and proline substitutions in the mutants found may be related partially to their over-representation in the library discussed in the paragraph above, the absence of serine, valine, alanine, and threonine (which also are represented by multiple codons) and the presence of lysine, histidine, and methionine (which each are represented by only 1 codon) suggests that the screening method does identify BG-resistant mutants.

These results show clearly that a majority of the possible alterations at position 160 make AGT resistant to BG. The absence of alanine and tryptophan substitutions is consistent with our previous findings that these mutants are not BG-resistant [12], and it is possible that the absence of phenylalanine, valine, and isoleucine also can be explained in this way. However, the absence of these residues and of glutamic acid, serine, and threonine may be due simply to the limited number of mutants screened. The alterations G160S, G160T, and G160E, therefore, were constructed

TABLE 2. Effect of DNA on reaction of BG with AGT mutant proteins altered at position Gly-160

AGT protein	EC_{50} (μM) for BG assayed*		Ratio	[^{3}H]Guanine formation from [^{3}H]BG (% wild type assayed − DNA)†		Stimulation by DNA
	+ DNA	− DNA		+ DNA	− DNA	
Wild type	0.13	0.4	3.1	391	100	3.9
G160R	4.0	9.0	2.3	24	9	2.7
G160E	0.5	4.5	9.0	172	24	7.2
P140A/G160R	45	110	2.4	2.3	0.7	3.3

*Assays were carried out using pure AGT or mutant protein in the presence or absence of DNA as indicated.

†The formation of guanine was measured over a 20-min period, and results are expressed as cpm formed per min per μg of protein. The value for wild type protein assayed in the absence of DNA (2440 ± 600 cpm/μg protein) was then used as the 100% value, and all other values were expressed as a percentage of this. Assays were carried out with amounts of each protein that gave results proportional to the amount of protein added and produced more than 1000 cpm above the background in the guanine peak.

directly by site-directed mutagenesis. As shown in Table 1, these mutants also imparted resistance to BG that varied from 5-fold for glutamic acid to 13-fold for serine. Therefore, at least 14 of the 19 possible substitutions at position 160 rendered AGT resistant to BG.

One hypothesis to account for the resistance of the G160R mutant AGT to react with BG is that the presence of the positively charged side chain in arginine interferes with the binding of BG to a hydrophobic region at the active site pocket. The resistance of the G160H and G160K mutants also may be explained in this way. It might be expected that alterations to acidic residues would have a similar effect, but mutants G160D and G160E were considerably less resistant than G160R (Table 1). In particular, when crude cell extracts were used as a source of AGT, the EC_{50} value for inactivation of G160E by BG was increased by only 5-fold, whereas the EC_{50} value for inactivation of G160R was increased by 40-fold. However, it should be noted that the assays of EC_{50} carried out in the experiments shown in Table 1 were performed in the presence of DNA. DNA was added to these assays for two reasons. First, some AGT mutants at positions other than codon 160 are unstable in the absence of DNA, and activity in crude extracts is lost on incubation even in the absence of BG [14]. Second, the addition of DNA is known to increase the reaction of BG with AGT [18], and the crude cell extracts contain some DNA. The further addition of 50 μg of calf thymus DNA ensured that all assays were carried out under conditions where there was a saturating amount of DNA present.

Studies in the absence of DNA could be carried out with some purified AGT preparations. Wild-type AGT and mutants G160R, G160E, G160A, and P140A/G160R were purified to homogeneity and were all stable for 30 min when incubated in the absence of DNA but in the presence of hemocyanin as a carrier protein [21]. These mutants were tested with respect to the effect of DNA on BG resistance (Table 2, first three columns). The inactivation of wild-type AGT by BG, as measured by EC_{50}, was enhanced about 3-fold by the presence of DNA, and inactivation of G160R and P140A/G160R was enhanced slightly less (about 2.3-fold). In contrast, the inactivation of G160E was stimulated to a much greater extent (about 9-fold). When assays were carried out in the absence of DNA, the G160E mutant was 11 times more resistant to inactivation by BG than wild type, and the G160R mutant was 23 times more resistant. In the presence of DNA, the assays with purified protein showed that, when compared with wild type, there were 3.8- and 31-fold increases in resistance for G160E and G160R, respectively.

Therefore, the G160E mutation had a much more profound effect on the ability of AGT to react with BG when assays were conducted in the absence of DNA. It appears that the conformational change, which is known to occur in AGT when it binds DNA [22–24], partially overcomes the inhibitory effects of the introduction of glutamic acid into the active site on the reaction with BG. Direct measurement of the reaction of BG with the mutant AGT proteins (Table 2, last three columns) gave results that were entirely consistent with this conclusion. Both the G160E and the G160R mutations reduced the formation of guanine from BG by AGT, but the effect of the G160E mutation was much less pronounced when assays were carried out in the presence of DNA. This system, in which the rate of conversion of O^6-benzyl[^{3}H]guanine to [8-^{3}H]guanine was measured, was also used to examine the effect of DNA concentration on the rate of reaction with BG. The stimulation of the ability of wild type, G160E, and G160R to convert BG to guanine showed a similar dependence on DNA with respect to DNA concentration (results not shown), and only the magnitude of the stimulation was different, with G160E being stimulated 7.2-fold and G160R increasing only 2.9-fold at saturating DNA concentrations (Table 2). The absence of the G160E mutant in the list of mutants found in the library screening, therefore, may reflect the fact that in the bacterial cell, the AGT proteins are largely complexed with DNA.

Another possible reason for the absence of the G160E mutant from the mutants found in the screen would be that it repairs methylated DNA more slowly than wild-type or G160R AGT, but this is not the case. Measurements of the second order rate constant for the repair of methylated DNA by the purified G160E and G160R proteins by standard methods [20] showed virtually identical results of

4.0×10^6 and 6.4×10^6 $M^{-1} \cdot min^{-1}$, respectively. These values are about 11–16% of that of the wild-type protein, but modest reductions in the rate of DNA repair by AGT (which normally acts very rapidly) do not affect its ability to protect either mammalian or bacterial cells from alkylating agents [20, 25].

DISCUSSION

The screening method used for the identification of the BG-resistant mutants of AGT, which involved the exposure of the bacteria expressing the AGT mutants to 50 μM BG plus MNNG, was successful in the sense that 51 out of 52 clones isolated contained plasmids with mutant AGT sequences that provided resistance to BG. The great majority (forty-seven) of these clones represented seven mutants with EC_{50} values for inactivation by BG of 2.5 to 12 μM. The remaining four clones encoded four different AGTs with lesser resistance, having EC_{50} values of 0.8 to 2.2 μM. Since these mutants occurred only once, it is possible that other mutants with a similar level of resistance survived the selection to the same extent and were not found simply due to the number of clones analyzed. This does appear to be the case, since a further three BG-resistant mutants with EC_{50} values of 0.5 to 1.3 μM were found when constructed by direct site-directed mutagenesis. Therefore, bias in the library and the choice of 50 μM as the BG level for screening did not appear to prevent identification of the majority of the BG-resistant mutants. However, the prevalence of the more highly resistant mutants (which may be related to the use of 50 μM BG for the screening) greatly increases the total number of clones that must be sequenced in order to determine whether the absence of a particular amino acid is due to the fact that the AGT containing it is inactive or not BG-resistant.

Even if the three AGT mutants not examined in our studies were inactive, or, more likely, did not show BG resistance, our results demonstrated that most, if not all, of the possible substitutions for Gly-160 did give rise to active AGT and that the majority (fourteen) of these produced resistance to BG. Although we have studied only two of the BG-resistant mutants (G160R and G160E) with the purified proteins, all of the other mutants produced were sufficiently stable and active that they effectively protected *E. coli* from MNNG. A crude estimation of the reduction in activity/stability imparted by these mutants can be obtained from the amount of crude extract protein needed to give 80% removal of the DNA-bound O^6-[^{3}H]methylguanine present in the substrate during the standard assay (Table 1, last column). This was increased from 3- to 15-fold over the amount needed from cells expressing wild-type AGT. However, the extracts from G160R were actually the least active in this assay and, as shown above, the rate constant for repair by G160R was reduced only 8-fold compared with wild type. This suggests that none of the other mutations is likely to have a more profound effect on the rate of repair of methylated DNA. It should be noted that AGT reacts very rapidly with O^6-methylguanine lesions in DNA and that reductions in its rate constant of at least 30-fold had no significant effect on the ability of mutant forms of the protein to protect either *E. coli* from MNNG [20] or mammalian cells from BCNU [25].

The failure to detect mutant G160E in the library screening may reflect the fact that assays in the presence of DNA more accurately reflect the *in vivo* situation in *E. coli*. This contrasts with the situation in mammalian cells. Studies with BG derivatives that have bulky adducts at the N-9 position indicate that these are much less potent AGT inhibitors in the presence of DNA. However, they are effective inhibitors of AGT in HT29 cells, suggesting that AGT is not always DNA bound in the mammalian cell [21].

The substitution of Gly-160 with basic amino acids provided the greatest reduction in the ability to react with BG, which is consistent with the hypothesis that the introduction of a charged group disrupts the hydrophobic binding pocket for BG. However, other substitutions such as leucine and methionine also provided resistance and cannot be explained in this way. A steric interference with the binding of BG is likely to explain the results with these mutants. The effect of replacement with proline, which was among the most resistant mutants, is also likely to be due to alteration of the size of the binding site available for BG. However, since the very bulky tryptophan can be accommodated and actually increases reactivity with BG [12], it appears very likely that interactions between the component amino acids occur in the active site region when glycine is replaced, and these cannot readily be predicted theoretically. As pointed out by Rafferty *et al.* [17], Gly-160 may form a hinge in the structure of AGT. Replacement of glycine by any amino acid may disrupt the structure, altering the size of the pocket into which BG must bind and the rate of subsequent alkyl transfer. It should be emphasized that the structure of AGT is not known precisely but is only inferred from the similarity of AGT to the *E. coli* Ada-C, for which a crystal structure is available [26]. The lack of reliability of this model is well illustrated by the fact that Ada-C is totally resistant to BG, and a major component of this resistance is the presence of tryptophan at the position apparently equivalent to Gly-160 [9]. This is completely opposite to the situation with AGT, where G160W is more sensitive to BG [12, 17].

The other major factor imparting resistance of Ada-C to BG is the absence of a proline residue at the position equivalent to Pro-140 in AGT [9]. Previous studies [10, 11, 14] have shown that alteration of this proline in human AGT imparts BG resistance, and mutant P140A has an EC_{50} value of about 4 μM when assayed in the presence of DNA, which is similar to the 40-fold increase in resistance for G160R. As shown in Table 2, when this mutation was combined with the G160R mutation to make mutant P140A/G160R, the increase in resistance was more than 400-fold. This is consistent with the concept that the amino acids at positions 140 and 160 both play a major role in determining the conformation of the active site pocket.

In summary, our results demonstrated that the majority of replacements at codon 160 lead to active AGT proteins that are resistant to BG. Combination of alteration at this position with mutation at codon 140 led to an AGT that was very highly resistant to BG. These results and those showing that alterations of several other codons in the human AGT sequence also yielded BG-resistant forms of AGT suggest that the emergence of forms of the protein resistant to the drug may be a serious problem in the use of BG to enhance chemotherapy by alkylating agents. Although, at present, these BG-resistant AGT proteins have been observed only in the laboratory (with the exception of the G160R polymorphism [16]), the design of potent novel inactivators of AGT able to abolish activity of the resistant mutants is highly desirable in case they appear in a clinical setting.

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