Brain tumor cell lines resistant to O^6-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have O^6-alkylguanine-DNA alkyltransferase mutations

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Abstract

The chemotherapeutic activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU or carmustine) may be improved by the addition of O^6-benzylguanine (O^6-BG). The reaction of O^6-BG with O^6-alkylguanine-DNA alkyltransferase (AGT) prevents the repair of O^6-chloroethyl lesions caused by BCNU. In clinics, the combination of O^6-BG and BCNU is now being tested for the treatment of brain tumors. However, the effectiveness of this drug regimen may be limited by drug resistance acquired during treatment. To understand the possible mechanisms of resistance of brain tumor cells to the O^6-BG/BCNU combination, we generated medulloblastoma cell lines (D283 MED, D341 MED, and Daoy) resistant to the combination of O^6-BG and BCNU [O^6-BG/BCNU resistant (OBR)]. DNA sequencing showed that all of the parent cell lines express wild-type AGTs, whereas every OBR cell line exhibited mutations that potentially affected the binding of O^6-BG to the protein as evidenced previously by *in vitro* mutagenesis and structural studies of AGT. The D283 MED (OBR), Daoy (OBR), and D341 MED (OBR) cell lines expressed G156C, Y114F, and K165T AGT mutations, respectively. We reported previously that rhabdomyosarcoma TE-671 (OBR) also expresses a G156C mutation. These data suggest that the clonal selection of AGT mutants during treatment with O^6-BG plus an alkylator may produce resistance to this intervention in clinical settings. [Mol Cancer Ther 2004; 3(9):1127–35]

Introduction

Carmustine or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is one of the most widely used drugs in treating brain tumors because it can efficiently cross the blood-brain barrier (1). In aqueous environment, BCNU decomposes into chloroethyl isocyanate and chloroethyl diazohydroxide (2). The latter gives rise to chloroethyl carbonium intermediate, which reacts with the O^6 position of guanine (3). This initial adduction eventually leads to the formation of the cytotoxic lesion, which is the ethanyl cross-link between the N^1 of guanine and the N^3 of cytosine in the opposite strand (4).

The foremost problem in cancer chemotherapy is the development of drug resistance during treatment despite a promising initial response. A major resistance factor to O^6-guanine alkylating drugs such as BCNU is O^6-alkylguanine-DNA alkyltransferase (AGT; ref. 5), which acts as a suicide enzyme by removing the methyl or chloroethyl damage at the O^6 position of guanine. The repair of the O^6-(2-chloroethyl)guanine formed by BCNU prevents the formation of the interstrand cross-links described above. The activity of BCNU can also be hindered by glutathiones (6) and base excision repair (7, 8). The possibility of an interstrand cross-link repair mechanism has also been described (9).

O^6-benzylguanine (O^6-BG) is designed to inactivate AGT by acting as an alternate substrate (10–12). The combination of O^6-BG with either BCNU or temozolomide is currently being tested in clinical trials for the treatment of several types of cancer including adult and childhood brain tumors (13, 14). Through *in vitro* mutagenesis, it has been shown that the ability of O^6-BG to inhibit AGT may be altered by certain mutations (15). Therefore, a legitimate concern for oncologists is the possibility that these AGT mutations are present before or may arise during treatment. We established sublines of the medulloblastoma cell lines D283 MED and Daoy that are resistant to the O^6-BG/BCNU drug combination [O^6-BG/BCNU resistant (OBR)]. The parent and resistant cell lines were characterized for their AGT activity and genotype. In an earlier study, we reported that an OBR rhabdomyosarcoma (TE-671) harbors a G156C substitution in AGT (16). In that publication, we also described the generation of D341 MED (OBR), another medulloblastoma subline, with its *AGT* genotype included in this report.

Received 3/10/04; revised 6/3/04; accepted 7/6/04.

Grant support: NIH grants NS-30245, CA-71976, and NS-20023.

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Methodology

Drugs

BCNU was purchased from Sigma Chemical Co. (St. Louis, MO). O^6-BG was synthesized and purified according to published reports (10).

Cell Lines

D283 MED, D341 MED, Daoy, and TE-671 were grown in Improved MEM Zinc Option (Richter's modification, Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Invitrogen; ref. 17).

Generation of Drug-Resistant Cells

The generation of the D341 MED and TE-671 OBR sublines has been described previously (16). The drug-resistant cell lines derived from D283 MED and Daoy were generated in a similar fashion. The parental cell lines were exposed to 100 μmol/L O^6-BG for 10 minutes prior to treatment with 10 μmol/L BCNU for 1 hour. The cells were washed free of drugs and maintained in 10 μmol/L O^6-BG. The cells were treated with 10 μmol/L BCNU in combination with O^6-BG after every three passages until they showed increased survival to drug treatment. The BCNU dosage was subsequently increased by 5 to 10 μmol/L after every three passages to generate OBR cell lines.

Limiting Dilution Assay

The cytotoxicity of BCNU against D283 MED, D283 MED (OBR), Daoy, and Daoy (OBR) with and without O^6-BG was determined using limiting dilution assay as described (17).

AGT Assay

The AGT activity of the cells was measured as described (10). Briefly, the ^{3}H-methylated DNA substrate (prepared by reaction of calf thymus DNA with [^{3}H]methylnitrosourea) was incubated with cell-free extract for 30 minutes at 37°C. The substrate was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 mol/L and depurinated by incubating the precipitate with 500 μL of 0.1 mol/L HCl at 70°C for 30 minutes. The modified bases were separated by reverse-phase high-performance liquid chromatography and quantified by scintillation counting. The enzyme activity was defined as the femtomoles of O^6-[^{3}H]methylguanine removed from ^{3}H-methylated DNA per milligram of extracted protein. Protein concentration was determined using Bio-Rad protein assay with bovine serum albumin as standard (Bio-Rad, Hercules, CA).

AGT Sequencing

Total RNAs and genomic DNAs of the parent and OBR cells were prepared using TRIzol reagent (Invitrogen) and DNeasy tissue kit (Qiagen, Valencia, CA), respectively. The AGT cDNA was generated from total RNA by reverse transcription (using SuperScript II RNase H^- reverse transcriptase and random hexamer primers from Invitrogen) followed by PCR (using PCR Master Mix from Promega, Madison, WI). The PCR primers used were AGT-L (5′-GCGCGGATCCGGTACTTGGAAAAATGGACAAG-GATTG-3′) and AGT-R (5′-GCGCGAATTCCAAACATC-CATCCTACTGCACATAC-3′). For genomic DNA, 0.2 μg was used as template to amplify *AGT* exons 4 and 5 by PCR. For exon 4 amplification, the primer pair was AGTexon4-L (5′-GCGTTTCCTGTTTTGGGACT-3′) and AGTexon4-R (5′-CGCCATGAGAACTCACAGGA-3′). For exon 5, the primers AGTexon5-L (5′-CTTGACCCCAAAG-ACCTCGT-3′) and AGTexon5-R (5′-TGTCGCTCAAAC-ATCCATCC-3′) were used. For every PCR reaction, the conditions were as follows: (*a*) initial denaturation at 95°C for 2 minutes; (*b*) 40 cycles of 95°C for 30 seconds, 62°C for 1 minute, and 72°C for 1 minute; and (*c*) final extension at 72°C for 7 minutes. The PCR products were ethanol precipitated, dissolved in 15 μL of 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA, and electrophoresed on a 1.0% agarose gel (with 0.5 μg/mL ethidium bromide). The AGT DNA fragments (680 bp for cDNA, 246 bp for exon 4, and 306 bp for exon 5) were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). Sequencing of the DNA fragments (using BigDye Terminator Cycle Sequencing) was carried out at the DNA Sequencing Facility at Duke University Medical Center (Durham, NC). The AGT cDNA was sequenced using both AGT forward (5′-CTGGCTGAATGCCTATTTCC-3′) and AGT reverse (5′-ATTGCTCCTCCCACTGCTC-3′) sequencing primers. The PCR primers AGTexon4-L and AGTexon5-L were used to sequence exons 4 and 5, respectively.

Production of Recombinant Wild-type and Mutant AGT Proteins

Recombinant wild-type AGT was isolated and purified by immobilized affinity chromatography as described previously using the pQE vector, which adds a 12–amino acid sequence (MRGS [H]$_6$GS-) to the NH_2 terminus of the AGT protein (18). The generation and purification of the recombinant mutant Y114F protein has been described (19). The mutants G156C and K165T were isolated during the screening of a library of plasmids encoding AGT molecules in which a 24–amino acid region was subjected to random mutagenesis (15). These mutant proteins were also purified using the pQE vector. Mutant K165T, which was found previously to be very unstable in *Escherichia coli* (20), could be purified in this way albeit in very poor yield.

Determination of Effects of O^6-BG on Activities of Wild-type and Mutant Recombinant AGTs

Inactivation of the AGT activity by O^6-BG was measured by incubating aliquots of the protein with different concentrations of O^6-BG at 37°C for 30 minutes in 0.5 mL of 50 mmol/L Tris-HCl (pH 7.6), 5 mmol/L DTT, and 0.1 mmol/L EDTA containing 50 μg hemocyanin and 10 μg calf thymus DNA. The residual AGT activity was determined by incubating the samples with ^{3}H-methylated DNA substrate for 30 minutes at 37°C and measuring ^{3}H-methylated protein formed after collection on nitrocellulose filters (21). 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 ED_{50} value representing the amount of inhibitor needed to produce a 50% loss of activity.

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 yield readily measurable rates under the assay conditions. The reaction mixture (1 mL) for each time point contained wild-type or mutated AGT (1.5×10^{-9}–15×10^{-9} mol/L depending on the activity), 3.6×10^{-10} mol/L O^6-[^{3}H]methylguanine in ^{3}H-methylated calf thymus DNA substrate, and 50 μg cold calf thymus DNA in a buffer containing 50 mmol/L Tris-HCl (pH 7.6), 5 mmol/L DTT, and 0.5 mmol/L EDTA. The rate constant was determined as described (19).

Western Analysis

Cellular extracts were prepared by incubating cells with lysis buffer [50 mmol/L Tris-HCl (pH 7.8), 0.15 mol/L NaCl, 1% NP40, complete mini EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN)] at 37°C for 10 minutes and isolating the supernatant after centrifugation. Sixty to 100 μg of protein extracts were mixed with an equal volume of 2× SDS loading buffer [20% glycerol, 4% SDS, 0.2 mol/L DTT, 0.01% bromophenol blue, 0.125 mol/L Tris-HCl (pH 6.8)], electrophoresed in 12% Tris-glycine SDS-PAGE gel, and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). The anti-AGT monoclonal antibody clone 3.1 was used as described (22). Monoclonal antibody to α-tubulin (Calbiochem, San Diego, CA) was used as a loading control. The primary antibodies were diluted to 10 μg/mL (AGT) and 2 μg/mL (α-tubulin). Antibody binding was detected with the Enhanced Chemiluminescence Plus system (Amersham, Piscataway, NJ). Images were captured with a Kodak ImageStation 440CF and quantitated using 1D Image Analysis software (Kodak, Rochester, NY). Results for AGT were normalized to the values obtained for α-tubulin and were determined in at least three experiments for each cell line.

Results

BCNU Cytotoxicity

The surviving fraction of D283 MED (OBR) was almost 100 times higher than D283 MED at every BCNU dosage (Fig. 1A). The addition of 10 μmol/L O^6-BG did not increase the toxicity of BCNU for either cell line. BCNU toxicity for Daoy and Daoy (OBR) were similar (Fig. 1B). When AGT was inactivated by the addition of O^6-BG, enhanced killing of Daoy, but not Daoy (OBR), was observed. The survival curves for D341 MED/D341 MED (OBR) and TE-671/TE-671 (OBR) resembled those for Daoy/Daoy (OBR; ref. 16).

AGT Expression

The parental cell line D283 MED exhibited minimal AGT activity (20 fmol/mg protein; Fig. 2A), whereas D283 MED (OBR) was >5 times higher. The AGT activity for D283 MED (OBR) was measured in cells that were treated with 10 μmol/L O^6-BG. When the O^6-BG concentration was increased further to 20 μmol/L, the AGT activity measured in D283 MED (OBR) cell extracts decreased 35% to 74 fmol/mg. The expression of AGT was detectable in D283 MED (OBR) but not in the parental cell line when analyzed by Western blotting (Fig. 3). The results for D283 MED correlate very well with the limiting dilution data (Fig. 1A). The ineffectiveness of O^6-BG in increasing toxicity of BCNU in D283 MED is consistent with the very low level of AGT expression. The parental Daoy cells registered AGT activity of 687 fmol/mg (Fig. 2B). When these cells were exposed to O^6-BG, very little AGT activity was observed. As observed in the limiting dilution experiments, the ablation of active AGT by O^6-BG greatly enhanced the sensitivity of the Daoy cells to BCNU. In contrast, no AGT activity was measured in Daoy (OBR). However, there was AGT protein detected in proteins extracted from these cells on a Western blot (Fig. 3). A similar result was observed for D341 MED, wherein no AGT activity was measured (16) but AGT protein was observed in cell extracts (Fig. 3).

AGT Mutations in OBR Lines

The AGT cDNA of D283 MED, D283 MED (OBR), Daoy, Daoy (OBR), D341 MED, and D341 MED (OBR) was

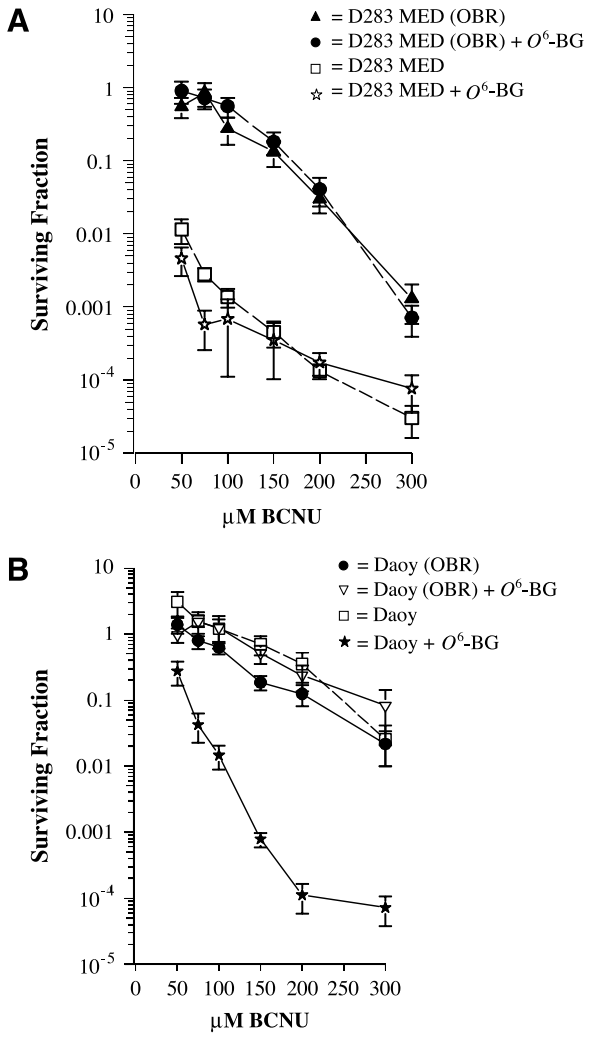


Figure 1. Survival of (**A**) D283 MED and D283 MED (OBR) and (**B**) Daoy and Daoy (OBR) after exposure to BCNU with and without O^6-BG. *Points,* average of at least three independent experiments; *bars,* SD.

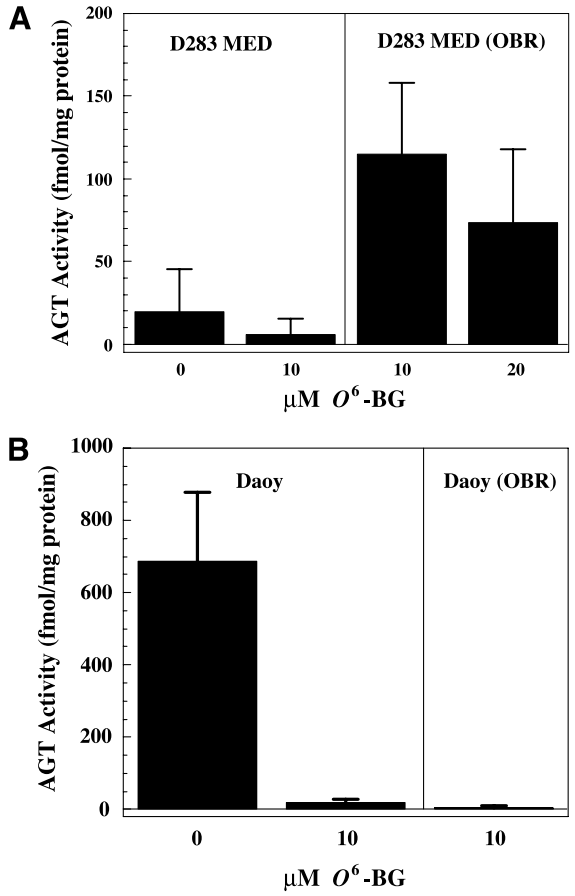


Figure 2. AGT activities of (**A**) D283 MED and D283 MED (OBR) and (**B**) Daoy and Daoy (OBR). AGT activities of D283 MED and Daoy with and without exposure to O^6-BG. D283 MED (OBR) and Daoy (OBR) were maintained in a medium with 10 μmol/L O^6-BG. Residual AGT activity in D283 MED (OBR) is still evident even when the O^6-BG concentration was increased to 20 μmol/L. *Bars,* SD.

amplified and sequenced. Results indicated a GGC (glycine) to TGC (cysteine) mutation at codon 156 for D283 MED (OBR), the same mutation found in TE-671 (OBR; ref. 16); TAC (tyrosine) to TTC (phenylalanine) at codon 114 for Daoy (OBR); and AAG (lysine) to ACG (threonine) at codon 165 for D341 MED (OBR; Fig. 4). Only the single base substitutions and not the wild-type sequences were observed in the AGT cDNA sequence. However, the corresponding genomic DNA sequences revealed that, unlike Daoy (OBR) and TE-671 (OBR), the *AGT* alleles of D283 MED (OBR) and D341 MED (OBR) were heterozygous. These results suggest that the AGT expression in D341 MED (OBR) and D283 MED (OBR) is limited to the mutated allele, whereas its wild-type counterpart is not expressed. *AGT* is among the several DNA repair genes with expression repressed by CpG island hypermethylation within their promoters (see ref. 23 for review). On the other hand, the lack of heterozygosity in genomic *AGT* mutations in Daoy (OBR) and TE-671 (OBR) suggests that both these cells possess a single *AGT* allele. Genomic *AGT* sequencing also revealed that D283 MED and D283 MED (OBR) have polymorphisms at codon 143 (ATC to GTC; Ile to Val) and codon 178 (AAG to AGG; Lys to Arg; Fig. 5). Apparently, this tandem polymorphism (24) occurs in the unexpressed *AGT* allele because these sequence variations are absent in both D283 (MED) and D283 MED (OBR) cDNA sequences.

Kinetic and Inhibition Studies of Recombinant Wild-type and Mutant AGTs

Compared with wild-type protein (ED_{50} for O^6-BG = 0.2 μmol/L), the mutants were not as easily inhibited by O^6-BG (Table 1). The recombinant G156C, Y114F, and K165T AGTs had ED_{50} values of 50, 73, and 36 μmol/L O^6-BG, respectively. The mutations also decreased the ability of AGT to repair methylated DNA. The rate constants of repair were 38×10^6, 2.9×10^6, 2.6×10^6, and 5.6×10^6 mol/L^{-1} min^{-1} for wild-type, G156C, Y114F, and K165T AGTs, respectively.

Discussion

According to the Goldie and Coldman (25, 26) model, the fraction of drug-resistant cancer cells will increase over time and is a function of the mutation rate and initial number of the cells. In this particular study, the selection process for the BCNU-resistant AGT mutant cell lines was driven by two key processes: (*a*) the mutational events that occurred at the *AGT* locus of the BCNU-treated parental cell line and (*b*) the selection and propagation of a clone expressing O^6-BG-resistant AGT. What determines the rate of mutation in the drug-exposed cells is the state of different DNA damage checkpoint (e.g., p53) and repair processes [e.g., mismatch repair (MMR) system] and, more significantly, the mutagenicity of BCNU. The loss of a functioning p53 has been correlated to increased mutations in cells exposed to DNA-damaging chemical and physical agents (27). DNA damage can lead to up-regulation of p53, which in turn activates expression of proteins involved in cell cycle arrest (28), DNA repair (29, 30), and apoptosis (31). Another important factor in maintaining genetic stability of cells is the MMR system. It has been shown that cancer cells with defective MMR have higher incidence of spontaneous (32–34) and DNA damaging agent–induced (35–37) mutations. When Liu et al. (38) treated

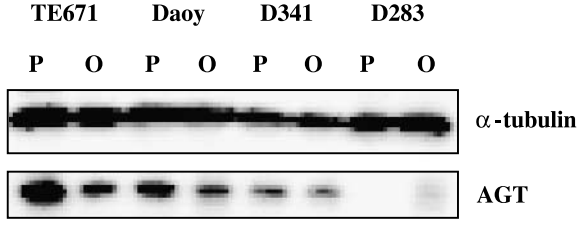
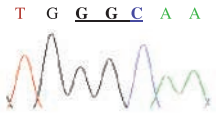
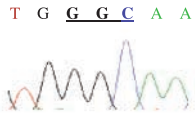
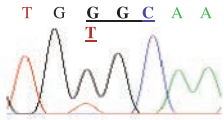
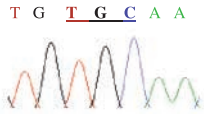
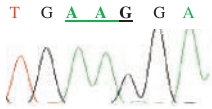
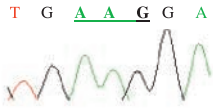
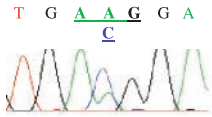
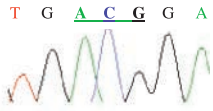
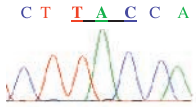
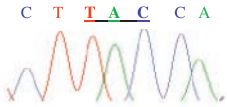
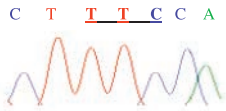
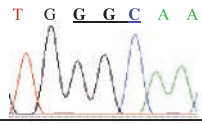
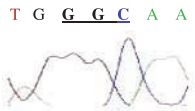
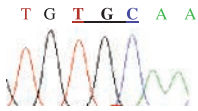
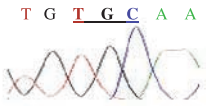


Figure 3. AGT expression of D283 MED and D283 MED (OBR), Daoy and Daoy (OBR), D341 MED and D341 MED (OBR), and TE-671 and TE-671 (OBR) by Western analysis. *P* and *O,* protein extracts from the parent and OBR cell lines, respectively. The monoclonal antibody for α-tubulin was used as a control.

Figure 4. *AGT* mutations in OBR cells. The relevant portions of the *AGT* genomic and cDNA sequences of D283 MED and D283 MED (OBR), D341 MED and D341 MED (OBR), Daoy and Daoy (OBR), and TE-671 and TE-671 (OBR).

Cell Line	*AGT* genomic sequence	*AGT* cDNA sequence	Relevant amino acid
D283 MED			Gly 156 (wt)
D283 MED (OBR)			Cys 156
D341 MED			Lys 165 (wt)
D341 MED (OBR)			Thr 165
Daoy			Tyr 114 (wt)
Daoy (OBR)			Phe 114
TE-671			Gly 156* (wt)
TE-671 (OBR)			Cys 156*

* cDNA sequence was reported previously (16)

colon cancer lines with three cycles of the combination of BCNU plus O^6-BG, the two MMR deficient cell lines became resistant and that resistance was also caused by mutations in the AGT protein. The MMR proficient cell line maintained sensitivity to the drug combination and expressed wild-type AGT protein. In contrast, we observed mutations in AGT in the MMR proficient (Daoy and TE-671) as well as MMR deficient (D341 MED and D283 MED) cell lines (summarized in Table 2). This difference probably reflects the fact that, in our experiments, the AGT was characterized after selection for resistance to the drug combination. Because D341 MED and D283 MED have MMR deficiencies, their corresponding OBR sublines were selected more rapidly than the MMR proficient cells. Generation and selection of the mutant AGTs in the OBR sublines may require that wild-type AGT is not present to repair the DNA damage produced by BCNU. Thus, of the four sublines, two have a single, mutated *AGT* allele and the other two, although heterozygous for *AGT*, express mRNA from only the mutated *AGT*. Silencing of the wild-type *AGT* could be a result of hypermethylation of the CpG islands present in the promoter of *AGT* (23) or of mutation of the regulatory regions of the wild-type *AGT* allele.

Obviously, the most significant source of AGT mutations would be the DNA damages caused by BCNU. This would be enhanced by the addition of O^6-BG, which eliminates the enzyme that repairs the mutagenic lesion at the O^6 position of guanine. We have made sublines resistant to

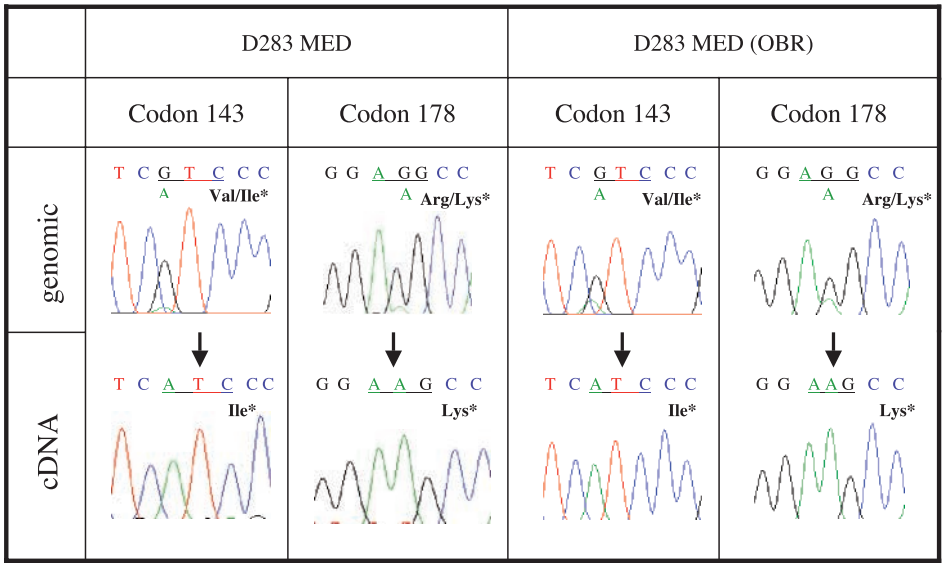


Figure 5. Tandem polymorphisms (heterozygous) at codon 143 (ATC to GTC; Ile to Val) and codon 178 (AAG to AGG; Lys to Arg) detected in the *AGT* gene of D283 MED and D283 MED (OBR).

* Non-polymorphic amino acid

BCNU in the absence of O^6-BG. These sublines are characterized by high levels of AGT expression (16). When the AGT cDNAs from these sublines were sequenced, each retained the wild-type sequence.[10] In Chinese hamster ovary cells that lack AGT, treatment with BCNU resulted in a mutation frequency in the adenine phosphoribosyl transferase (*aprt*) gene 6-fold above background (39). All possible transversions and transitions were seen, although the predominant mutations were G:C to T:A (51%) and G:C to A:T (20%). Overexpression of AGT in these cells reduced the frequency of both mutations, suggesting that they originate from initial O^6-chloroethyl lesions and T:A to G:C transversions became the predominant mutation (40). Two hotspots for mutation in the Chinese hamster ovary cells lacking AGT were identified: 5′-ACTGgAGGT-3′ and 5′-ACTAgAAAT-3′, which have similar helical properties on application of Calladine's rules (41). Two of the four OBR cell lines we isolated have a G:C to T:A transversion at 5′-TGgGCAA-3′, which, like the first hotspot, has the 5′-Gg-3′ sequence. The other two mutations resulted in A:T to C:G and A:T to T:A transversions, both of which have been identified as minor mutations in BCNU-treated cells.

The crystal structure of wild-type human AGT has been elucidated by two independent laboratories—the most significant difference being the absence (42) or the presence (43) of zinc in the NH_2-terminal region of the protein. Both studies provide a model on how O^6-BG binds prior to a S_N2 reaction with Cys^{145} and can be used as a basis for reasonable speculations on the reasons that the mutants described here have altered activity and resistance to O^6-BG. It is highly likely that alteration of Gly^{156} modifies the shape of the binding pocket to exclude O^6-BG due to the more restricted torsion angles allowed when other residues are placed at this site. Many different substitutions at Gly^{156} impart resistance (15). Similarly, interactions of Lys^{165}, which is a highly conserved residue in AGT sequences, play an important role in stabilizing the active site loop and all 19 changes at this site impart resistance to O^6-BG presumably by distorting the protein backbone (20). In the proposed reaction mechanism by Daniels et al. (43), the final step in removal of the benzyl group from guanine is the protonation of N^3 (of guanine) by the hydroxyl group of Tyr^{114}. This suggests that the loss of a hydroxyl group, which occurs in the Y114F mutant, would reduce the rate of reaction with O^6-BG. Our *in vitro* studies showed that these three mutations adversely affected both the inhibitory effect of O^6-BG (as evidenced in dramatic increase in ED_{50} values) and the ability of the protein to repair methylated

Table 1. Comparison of the ED_{50} (for O^6-BG) and rate constant for repair of methylated DNA in recombinant wild-type and mutant AGTs

Recombinant AGT	ED_{50} for O^6-BG, μmol/L	Rate Constant for Repair of Methylated DNA, $\times 10^6$ mol/L^{-1} min^{-1}
Wild-type	0.2	38
G156C	50*	2.9
Y114F	73	2.6†
K165T	36	5.6

*Previously reported as 49 μmol/L based on assays with crude extracts of *E. coli* expressing the G156C mutant (47).
†Previously reported (19).

[10] Unpublished results.

Table 2. Summary of characterization of parental and OBR cell lines

Cell Line	Parent Line AGT Activity, fmol/mg protein	Parent Line AGT Activity (10 μmol/L O^6-BG), fmol/mg protein	OBR Subline AGT Activity (10 mol/L O^6-BG), fmol/mg protein	Relative AGT Expression by Western (Parent)*	Relative AGT Expression by Western (OBR)*	AGT Mutation	MMR Phenotype†
D283 MED	20 ± 25	6 ± 10	115 ± 43	0	0.09 ± 0.06	G156C	–
D341 MED‡	2,232 ± 329	49 ± 31	0	0.83 ± 0.16	0.71 ± 0.10	K165T	–
Daoy	686 ± 191	20 ± 8	3.0 ± 6.0	1	0.61 ± 0.23	Y114F	+
TE-671‡	3,967 ± 341	18 ± 1	223 ± 36	1.68 ± 0.43	0.98 ± 0.52	G156C	+

*Relative to expression of D341 MED.
†Determined by *in vitro* MMR reaction using a G:T mismatch (48).
‡AGT activity data and the TE-671 AGT mutation are from Bacolod et al. (16).

guanine (the rate constants for repair all decreased significantly). However, these mutant proteins retained sufficient AGT activity to provide O^6-BG-treated *E. coli* protection from the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine during the selection of drug-resistant colonies (15).

The AGT activity of D283 MED (OBR; which is G156C AGT) increased noticeably compared with that of the parental cells, although the former was maintained in O^6-BG. The increase in AGT activity was consistent with the increased AGT expression (as confirmed by Western blotting). In contrast, Daoy (OBR; which is Y114F AGT) and D341 MED (OBR; which is K165T AGT) did not exhibit measurable AGT activity (16), although Western analysis indicated the presence of the protein. One possible explanation is the instability of K165T and Y114F AGTs and that those detected by Western analysis were actually a mixture of native and denatured forms. K165T AGT was observed to be very unstable *in vitro*, although it conferred resistance against O^6-BG in *E. coli* (20). Liu et al. (38) identified two OBR colon cancer lines with K165N and K165E mutations. Both Western and enzyme activity assays indicated that the mutant cell lines had lower levels of AGT expression compared with parental lines. However, the cells had equal amounts of AGT mRNAs, thus prompting the speculation that the mutant lines had unstable AGTs. When the mutant AGTs were transfected to non-AGT-expressing, BCNU-sensitive Chinese hamster ovary cells, the O^6-BG-treated cells became resistant to both BCNU and temozolomide. There is a possibility that the instability may lead to a new steady state at a lower amount of protein if degradation is enhanced and synthesis is not; however, if synthesis is also increased, the protein level would stay the same. These interpretations regarding the role of AGT in OBR are strongly supported by the limiting dilution results for Daoy (OBR; Fig. 1B) and D341 MED (OBR; ref. 16). The addition of O^6-BG increased BCNU cytotoxicity in D341 MED and Daoy but not their corresponding OBR sublines, indicating that these had sufficient levels of active, mutant AGTs to repair the O^6-chloroethyl lesions produced by BCNU. In TE-671 (OBR) and D283 MED (OBR), the role of the mutant AGT (both G156C) is more apparent. TE-671 (OBR) had measurable AGT activity even when O^6-BG dosage was increased to 60 μmol/L (16). D283 MED (OBR) had a higher AGT activity than its parent and this activity persisted in the presence of 20 μmol/L O^6-BG.

It is entirely possible that other mechanisms contribute to the drug-resistant phenotype of these cells. Initially, the selection of mutant AGTs may have been the most crucial factor for the survival of the O^6-BG/BCNU–treated cells. However, as the cells were passed and treated with increasing amounts of BCNU, other resistance factors may have started to play equal or more important roles than AGT. For example, further investigations are under way, exploring the increased expression of metallothioneins that were observed in D341 MED (OBR; ref. 16).

Considering the frequency (4 of 4) of AGT mutations in OBR lines, we believe that a potential hindrance to O^6-BG/BCNU chemotherapy in brain tumors may be the selection of clones with mutated O^6-BG-resistant AGT (regardless of the MMR ability of the tumor). This conclusion agrees with previous detailed studies using site-directed mutagenesis and the selection from libraries of plasmids expressing human AGT in *E. coli* in which critical regions of the coding sequence were replaced by random inserts to identify mutants resistant to O^6-BG. These studies have indicated that there are at least 28 individual amino acid residues in human AGT that can be altered to produce such resistance (44, 45). The current studies and those of Liu et al. (38) have identified mutations imparting resistance to O^6-BG enhancement of killing by BCNU at only three of these sites. Although this may be related to hotspots for the induction of mutations by this alkylating agent, it is more likely a much wider range of resistant sites will emerge with more extensive use of this drug combination. In addition, at least one of the alterations identified in the screens of AGT described above, namely, G160R, is a naturally occurring variant. Although this variant is rare (46), the presence of this allele would generate resistance to therapy with O^6-BG without need for mutagenesis. Our results therefore provide strong support for the development of second-generation inactivators of AGT that are able to inactivate those forms that are poorly affected by O^6-BG.

Acknowledgments

We thank Drs. Meng Xu-Welliver and Sreenivas Kanugula for originally generating the mutants and constructs used for recombinant protein expression and Dr. B.K. Ahmed Rasheed for technical advice.

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