Published in final edited form as:

Mol Carcinog. 2014 March ; 53(3): 201–210. doi:10.1002/mc.21964.

Tumor-Associated Mutations in O⁶-Methylguanine DNA-Methyltransferase (MGMT) Reduce DNA Repair Functionality

Kristy L. Lamb^{1,2}, Yanfeng Liu¹, Kimiko Ishiguro³, Youngho Kwon⁴, Nicolas Paquet⁴, Alan C. Sartorelli³, Patrick Sung^{1,4}, Sara Rockwell^{1,3}, and Joann B. Sweasy^{1,2,*}

¹Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut

²Department of Genetics, Yale University School of Medicine, New Haven, Connecticut

³Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

⁴Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut

Abstract

MGMT is the primary vehicle for cellular removal of alkyl lesions from the O-6 position of guanine and the O-4 position of thymine. While key to the maintenance of genomic integrity, MGMT also removes damage induced by alkylating chemotherapies, inhibiting the efficacy of cancer treatment. Germline variants of human MGMT are well-characterized, but somatic variants found in tumors were, prior to this work, uncharacterized. We found that MGMT G132R, from a human esophageal tumor, and MGMT G156C, from a human colorectal cancer cell line, are unable to rescue methyltransferase-deficient *Escherichia coli* as well as wild type (WT) human MGMT after treatment with a methylating agent. Using pre-steady state kinetics, we biochemically characterized these variants as having a reduced rate constant. G132R binds DNA containing an O⁶-methylguanine lesion half as tightly as WT MGMT, while G156C has a 40-fold decrease in binding affinity for the same damaged DNA versus WT. Mammalian cells expressing either G132R or G156C are more sensitive to methylating agents than mammalian cells expressing WT MGMT. G132R is slightly resistant to O⁶-benzylguanine, an inhibitor of MGMT in clinical trials, while G156C is almost completely resistant to this inhibitor. The impaired functionality of expressed variants G132R and G156C suggests that the presence of somatic variants of MGMT in a tumor could impact chemotherapeutic outcomes.

Keywords

O(6)-benzylguanine; O(6)-alkylguanine DNA alkyltransferase; MNNG; DNA repair; O-(6)-methylguanine

INTRODUCTION

Alkylation damage is ubiquitous, arising from endogenous metabolic processes and from environmental sources. The consumption of charred or processed meat and the inhalation of industrial and tobacco smoke are common sources of alkylation damage in the modern

world [1,2]. *O*⁶-methylguanine DNA methyltransferase (MGMT) is the primary mechanism for the removal of alkylation damage from the O-6 position of guanine. The O-6 position of guanine is one of several positions in DNA bases to which alkyl groups are attached in S_N1 alkylation reactions, and this repair has been well-characterized in mammalian cells and via MGMT homologs in bacteria and *Archaea* (reviewed in Refs. [3–6]). MGMT removes alkylation damage from the O-6 position of guanine, and to a lesser extent, the O-4 position of thymine, by a one-step irreversible transfer of the alkyl group to a conserved cysteine in the interior of the protein. Upon completion of this transfer step, the DNA strand is restored to the native state and MGMT is targeted for ubiquitination and ultimately degradation in the proteasome [7].

Alkylation damage from environmental sources such as processed meat, charred foods, and industrial smoke has been linked to an increased risk of a variety of human cancers that arise in tissues that come in contact with alkylating agents when they are ingested or inhaled, such as esophageal, lung, gastric, and colorectal cancer [8–11]. The removal of alkylation damage from DNA by MGMT safeguards the genome from the development of cancer-causing mutations. However, after cancer develops, the removal of DNA damage induced by treatment with alkylating agents such as carmustine (BCNU) or temozolomide (TMZ) may render these treatments ineffective by removing potentially toxic damage before DNA crosslinks form or mismatch repair signals apoptosis. *MGMT* is silenced via promoter methylation in as many as 45% of human glioblastomas [12]; this silencing sensitizes tumors to alkylating agents. *O*⁶-benzylguanine (*O*⁶BG) sensitizes cells to alkylating agents by providing a benzyl group for transfer to the cysteine acceptor at CYS145 of human MGMT [13]. As the transfer of an alkyl group to CYS145 is irreversible, MGMT molecules that react with *O*⁶BG are removed from the pool of MGMT available for repair. Replacing the CYS145 acceptor residue in MGMT with another amino acid renders MGMT inert [14].

Single amino acid substitutions in MGMT arising from single nucleotide polymorphisms have been identified in the germline and in cancer. MGMT germline variants are well characterized (reviewed in Ref. [4]). The L84F variant is found in up to 20% of the worldwide population. Residue 85, immediately adjacent, is one of four residues in the protein that bind to a zinc ion, which is not required for function but is believed to be important for overall structural integrity; zinc binding increases the rate of damage removal from *O*⁶-methylguanine (*O*⁶MeG). The I143V/K178R variant, found in almost exact linkage disequilibrium in the population, is also quite common. Despite proximity to the CYS145 acceptor residue, which excited great interest in the field, the I143V/K178R variant does not appear to differ from the WT protein in its ability to remove lesions. MGMT L84F and I143V/K178R both rescue MGMT-deficient cells in survival assays with alkylating drugs [15,16]. I143V/K178R can be inactivated by *O*⁶BG and cannot efficiently repair bulky adducts such as *O*⁶-(4-bromophenyl)guanine. L84F is not inactivated by *O*⁶BG as strongly as WT or I143V/K178R and is slightly better at repairing bulky adducts than I143V/K178R [17,18]. G160R, a rare variant found in Japanese populations, is resistant to *O*⁶BG and cannot fully rescue MGMT-deficient cells treated with an alkylating agent [19,20].

Most studies of MGMT in tumors have focused on characterizing the promoter methylation status of *MGMT* and searching for single nucleotide polymorphisms that are associated with methylated promoters. Only two studies have reported somatic sequence mutations of *MGMT*; both reported mutations that arose without chemotherapeutic drug treatment in patient tumors or tumor-derived cell lines [21,22].

A British group sequenced 88 colorectal tumors with matched normal tissues and 32 colorectal cell lines and reported a total of six variants that were not seen in matching normal tissue or other germline studies [21]. They identified the G156C somatic mutant

examined in this work in the SW48 cell line, a colon tumor cell line isolated from an 82-year-old Caucasian woman [23]. Only mutant RNA was expressed [21]. *MGMT* has five exons spanning 300 kb of the genome, with the area surrounding the active site in the fourth exon. A Chinese group sequenced this fourth exon in 70 esophageal tumors with matched normal tissues from patients in three cities in Northern China. They reported four distinct point mutations that result in amino acid substitutions in a total of seven tumors, including the G132R mutation characterized here [22]. G132R and G156C are both located near the active site of *MGMT* (Figure 1) [24]. Both arginine and cysteine are larger residues than the native glycine and may distort these key interactions.

These cancer-associated mutations in *MGMT* have not yet been characterized. Given the critical role of *MGMT* in chemotherapeutic outcomes, the attributes of these *MGMT* variants may provide important prognostic clues about tumor sensitivity to alkylating agents. Here, we characterize two cancer-associated *MGMT* mutants, G132R and G156C, and report that both are unable to repair damage as well as WT protein in both *Escherichia coli* and mammalian cell systems. These variants have a decreased ability to bind DNA and remove methyl damage from O⁶G. G132R and G156C are resistant to O⁶BG, an inhibitor of *MGMT* currently in clinical trials. Our studies have important implications for drug sensitivity in the treatment of a tumor bearing those mutations or other mutations that behave in a similar manner, and argue the need for characterization of *MGMT* in tumors beyond promoter methylation or expression studies.

METHODS

Constructs

Human *MGMT* cDNA was amplified from MGC-5186 (American Type Culture Collection, ATCC, Manassas, VA) and cloned using the TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA). *MGMT* was then subcloned into the pBAD His(A) (Invitrogen Corp.), pET28a(+) (Novagen, EMD Biosciences, Gibbstown, NJ), and pRVY-tet [25] expression vectors. Variants were generated using the Quikchange site-directed mutagenesis kit (Stratagene, now Agilent Technologies, Santa Clara, CA) and the following primers (Invitrogen Corp.), altered bases in lowercase: G132RF—5'-GCGAGCAGTGGGAaGAGCAATGAGAGGC-3', G132RR—5'-GCCTCTCATTGCTCtTCCCACTGCTCGC-3', G156-CF—5'-GTGGTCTGCAGCAGCtGcGCCGTGGGC-3', G156CR—5'-GCCACGGCgCaGCTGCTGCAGACCAC-3'.

Bacterial Survival

Human *MGMT* in pBAD His(A) was transformed into *E. coli* GWR111 [26], which was a gift from Leona Samson, MIT. This strain has the genotype: K-12, *argE3 hisG4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-1 thi-1 rpsL31 supE44 tsx-33 Dada-25 ogt-1::Kan^r*. Freezer stocks were generated and used to inoculate overnight cultures that were grown 15–17 h in Luria broth (LB)-Kan50-Amp100 at 37°C with shaking. Overnight cultures were diluted 1:50 in LB broth and grown to logarithmic phase at 37°C with shaking. L-arabinose was added to a final concentration of 0.04% to induce *MGMT* expression. After 30 min, cultures were treated with varying doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; National Cancer Institute (NCI) Chemical Carcinogen Reference Standards Repository, MRI Research, Kansas City, Missouri) for 30 min in the same growth conditions. Cultures were centrifuged to remove MNNG, resuspended in LB, serially diluted, and plated on LB-arabinose plates. Plates were incubated at 37°C overnight and colonies were counted approximately 16 h after plating.

Western Blots

Expression of MGMT in bacterial strains was confirmed by Western blot using a mouse monoclonal antibody against human MGMT (23.1) (AbCam, Cambridge, MA) at a 1:1,000 dilution. Mouse monoclonal antibody to RNA Pol Sigma70 (AbCam) at a 1:5,000 dilution was used to ensure equal loading of protein extract onto the gel. An anti-mouse monoclonal HRP-conjugated secondary (GE Healthcare BioSciences, Piscataway, NJ) was used at a 1:10,000 dilution. Following incubation of the blot with ECL Plus (Amersham Pharmacia Biosciences), blots were developed on film, scanned at 300 dpi, and grayscale files were quantitated using ImageJ Software [27].

MGMT Purification

C-terminally His-tagged [28] human MGMT was expressed from the pET28a (+) vector in BLR (pRARE) cells (Novagen). Fifty milliliters of seed cultures were inoculated in 2× LB (20 mg/ml tryptone, 10 mg/ml yeast extract, 10 mg/ml NaCl) containing 100 µg/ml ampicillin, and 17 µg/ml chloramphenicol and grown overnight. The seed culture was diluted 1:100 in 2.5 L 2× LB and grown for 6 h at 37°C to OD₆₀₀ = 0.8. At this time, 0.5 mM IPTG was added to induce protein expression and the culture was then incubated at 16°C overnight. Cultures were pelleted and frozen. Frozen pellets were resuspended in 30 ml cell breakage buffer (CBB) [50 mM Tris-HCl (pH 7.5), 10% sucrose, 10 mM EDTA, 600 mM KCl, 1 mM dithiothreitol, 0.01% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO)] in the presence of protein inhibitors (chymostatin, leupeptin, aprotinin, and pepstatin, 2 µg/ml at each, 1 mM phenylmethanesulfonyl fluoride) and sonicated at 50% power for five rounds of 10 s sonication followed by 10 s rest. The cell lysate was ultracentrifuged in a Ti70 rotor at 55k rpm for 1 h and the clarified supernatant was incubated with 10 mM imidazole and 1.5 ml Ni-NTA agarose (Qiagen) for 1 h. The mixture was poured into a column, washed with buffer T [25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Igepal CA-630] supplemented with 1 M KCl, 10 mM imidazole, 1 mM ATP, and 8 mM MgCl₂, then washed with buffer T supplemented with 150 mM KCl and 10 mM imidazole. Nickel-bound protein was eluted with buffer T containing 200 mM imidazole. Eluate was pooled and run on a 6 ml SP sepharose fast flow (GE Healthcare) column on the FPLC with a 100 ml gradient of 50–500 mM KCl in buffer T. The MGMT fractions were identified by SDS-PAGE, then pooled, concentrated, and aliquoted for storage at –80°C.

MGMT Activity Assay

Oligonucleotides for biochemistry as in [29] were synthesized by the Keck Oligonucleotide Synthesis Facility (Yale University, New Haven, CT): PvuIITopD—5'-GCCCGGCCAGCTXCAGTT-3', X = O⁶MeG (Glen Research, Sterling, VA); PvuIITopU—5'-GCCCGGCCAGCTGCAGTT-3'; PvuIIBottom—5'-AACTGCAGCTGGCCGGGC-3'. Oligonucleotides were gel-purified. PvuIITopD and PvuIITopU oligos were 5'-end labeled with [γ -³²P ATP] and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). After unreacted ATP was removed using Micro BioSpin p6 spin columns (BioRad, Hercules, CA) the top strands were separately annealed to unlabeled PvuIIBottom in a 1:1.5 ratio with 5 min at 95°C, a slow cool to 50°C over 30 min, and a 20 min incubation at 50°C. Annealing was checked on a non-denaturing PAGE gel. 0.53 nM kinased, annealed substrate was reacted with varying concentrations of MGMT in MGMT reaction buffer (final concentrations 50 mM Tris pH 7.8, 1 mM EDTA, 1 mM DTT, 1 mg/ml BSA) [29]. Reactions were conducted for various times and were then quenched with 82.5 µl 0.2% SDS (0.0015% final concentration) using the RQF-4 Rapid-Quench Apparatus (Kintek Corp., Austin, TX) [30]. Quenched reactions were microcentrifuged through Micro BioSpin p6 Spin Columns (BioRad) and digested with *PvuII* (New England Biolabs) for 1 h at 37°C.

Digested products were separated using 20% polyacrylamide sequencing gels run at 1,900 V for 1.5 h, exposed to a phosphor screen, scanned on a Storm 860 phosphorimaging system, and quantitated using ImageQuant Software (Molecular Dynamics, now GE Healthcare Life Sciences). Fraction reacted was normalized to a *Pvu*II-digested undamaged DNA control. Time courses were fit to a single exponential equation [$Y = Y_{\max}(1 - e^{-kX})$], where X = time in seconds, Y = fraction of MGMT reacted, and $K = k_{\text{obs}}$] to generate k_{obs} values, which were then plotted against concentration, and a single hyperbolic equation [$Y = (B_{\max} X)/(K_d + X)$], where X = concentration of MGMT, $Y = k_{\text{obs}}$, $B_{\max} = k_{\text{obs}}$ maximum, and K_d = equilibrium dissociation constant, or the MGMT concentration at which $k_{\text{obs}} = 0.5B_{\max}$] was fit to the data (GraphPad Prism, GraphPad, San Diego, CA) [30].

Mammalian Cell Lines

EMT6 cells [31] were infected with pRVY-tet empty vector or pRVY-tet containing *MGMT* (WT or variant) and polybrene for 3 h with shaking. After 24 h, pools were selected using media supplemented with 0.03 mg/ml hygromycin B (Invitrogen). Expression of MGMT in EMT6 cells was confirmed by Western blot using a rabbit antiserum raised against beta-actin (Cell Signaling Technology, Danvers, MA) at 1:10,000 and a mouse antiserum raised against MGMT (23.1) (Novus Biologicals, Littleton, CO) at 1:1,000 as primary, and anti-mouse at 1:5,000 for secondary (GE Healthcare BioSciences), developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), and visualized on a Geldoc XR+ (BioRad). Blots were quantitated using ImageJ. Pools were used prior to passage five.

Mammalian Survival With MNNG

EMT6 pools (see above) were seeded at 1×10^5 cells per 6 cm dish. Experiments were performed on pools within a range of three passages. Four days later, cells were treated with varying doses of MNNG (TCI America, Portland, OR) for 1 h. The cells were then plated at various dilutions and left undisturbed for 10 d. Plates were then stained with crystal violet and colonies were scored. A cluster of at least 50 cells was required to be scored as a surviving colony. Percent survival was calculated relative to untreated controls and graphed using GraphPad Prism.

Reaction With [Benzene-³H]O⁶-Benzylguanine (³H-BG) [32]

Briefly, 2×10^6 cells from EMT6 pools were incubated with tritiated ³H-BG in the presence or absence of excess unlabeled ³H-BG for 2 h, methanol precipitated, and washed to remove unbound O⁶BG, then samples were counted in a scintillation counter. To obtain the amount of benzyl residue transferred to MGMT, radioactivity in the presence of unlabeled O⁶BG (non-specific binding) was subtracted from that in the presence of labeled O⁶BG. Data are averages from three separate experiments and were analyzed using GraphPad Prism. Results were compared by one-way ANOVA and significance was calculated by Dunnett's multiple comparison test.

Circular Dichroism Spectroscopy Analysis

Circular dichroism spectra of 1.5 μ M purified human WT or variant MGMT in a 10 mM K₂HPO₄ solution were collected from 280 to 190 nm at 23°C using a Chirascan circular dichroism spectrometer (Applied Photophysics, Leatherhead, Surrey, UK).

RESULTS

The Tumor-Associated Variants Partially Complement MGMT-Deficient *E. coli*

It was previously shown that the MNNG sensitivity of the methyltransferase-deficient *E. coli* *GW111* strain could be complemented by the expression of human wild type (WT) MGMT [26]. To determine if tumor-associated variants G132R and G156C complement the MNNG sensitivity of the MGMT-deficient bacteria and permit cellular survival, we expressed the human WT and variant MGMTs in the bacterial strain and quantified survival using a colony forming assay [26]. We found that methyltransferase-deficient *E. coli* expressing MGMT G132R and MGMT G156C were more sensitive to MNNG than cells expressing WT MGMT (Figure 2B). Note that the Western blot shows approximately equal expression for the two variant MGMTs, which are clearly overexpressed relative to wild type; however, even with this abundance of MGMT they do not survive as well as WT (Figure 2A). These results suggest that the G132R and G156C tumor-associated variants are less active than WT MGMT in the removal of O⁶MeG. G132R and G156C are both in regions of the crystal structure surrounding the active site of MGMT (Figure 1) [24].

G132R and G156C are Dysfunctional MGMT Proteins

The reaction mechanism for removal of methylation damage from the O⁶ of guanine by MGMT has several discrete steps—DNA binding, base flipping, damage transfer, and release [30]. From the bacterial survival curves, it is not possible to discern at which step repair is impaired—the variants could bind to DNA poorly, the lesion may not fit into the pocket surrounding the active site of MGMT, or the variants may remove damage in a defective way. To further characterize the differences between MGMT WT, G132R, and G156C, we purified the proteins and performed in vitro biochemical assays to determine the overall rate of removal by MGMT and the efficiency of MGMT binding to DNA.

To determine if the variants exhibited gross structural alterations, we performed circular dichroism analysis of purified proteins and found no change in the overall shape of the spectra (Figure 3). We therefore concluded that the G132R and G156C somatic mutants do not create gross, global changes in the overall structure of MGMT. There may be slight alterations to parts of the protein that are not alpha helices or beta sheets, which would be undetectable via this method, but these slight alterations would not change the overall structure of MGMT.

To characterize the ability of MGMT variants to bind and repair the O⁶MeG lesion, we performed a pre-steady-state kinetic analysis [30]. We reacted varying concentrations of purified human WT and tumor-associated variant MGMTs with a constant concentration of DNA substrate on the Rapid-Quench apparatus. The 5'-radiolabeled 18 bp DNA substrate (see Methods Section) contained O⁶MeG within a *PvuII* restriction site. *PvuII* cannot cut unrepaired, methylated substrate but can cut repaired, unmethylated substrate (Figure 4A,B). By plotting the product formed over a time course at various concentrations of MGMT and fitting the data to a single exponential equation, we obtained an observed rate of reaction for each concentration (Figure 4C). We then plotted these observed reaction rates against the concentration of MGMT at which the rates were observed to find both an apparent rate of reaction, k_r , and binding affinity, K_d (Figure 4D). We found that G132R and G156C removed the methyl damage more slowly than WT MGMT, as shown in Figure 4 and Table 1. G132R removed lesions at an overall rate that was 16% slower than WT. G156C removed damage at less than one-third the rate of WT. We also found that G132R and G156C both bound DNA less tightly than WT MGMT. G132R had a K_d of 14×3 nM, meaning that it bound DNA half as tightly as WT ($K_d = 6.2 \times 0.7$ nM). This is interesting, given that G132R is located in the well-conserved canonical DNA binding domain for MGMT. G156C had a

K_d of 240×60 nM; it bound DNA 40 times less tightly than WT. This drastically reduced ability to bind DNA is likely responsible for the reduction in the overall rate of repair of O⁶MeG in DNA, as binding is a necessary first step for ultimately removing the lesion.

The Tumor-Associated Variants do not Confer Resistance to MNNG in EMT6 Mammalian Cells

Having observed phenotypic differences in bacterial survival and the biochemical mechanism of G132R and G156C, we next examined the response of mammalian cells expressing these variants to alkylating agents. Murine mammary epithelial EMT6 cells do not express MGMT [33], likely due to promoter methylation, which is prevalent in cell lines [34]. We infected EMT6 cells with the pRVY vector to generate pools of cells expressing WT or variant MGMT. Western blot analysis indicated that the EMT6 pools expressed similar amounts of WT, G156C or G132R MGMT proteins, as shown in Figure 5A.

Next, we assessed survival in the presence of MNNG using a colony formation assay. MGMT G132R and G156C did not completely complement the MGMT-deficient EMT6 cells to the level seen with WT MGMT, but cells expressing the variants were more resistant than EMT6 cells transfected with the empty expression vector, as shown in Figure 5B. At low doses of MNNG, the presence of any MGMT protein conferred a survival advantage to the cells. As the dose of MNNG, and hence amount of O⁶MeG, increased, G132R and G156C had roughly similar abilities to survive treatment with MNNG. Both G132R and G156C were unable to rescue the deficient cells at high doses to the same extent as WT MGMT ($P = 0.03$ and $P = 0.04$, respectively, Student's paired two-tailed t -test). These data suggest that tumors with either of these variants would be more sensitive to treatment with an alkylating agent than tumors with WT MGMT, but more resistant than tumors with methylated *MGMT* promoters.

Tumor-Associated Variants are Resistant to O⁶-Benzylguanine

Several single amino acid variants of MGMT have been reported to be resistant to O⁶-benzylguanine (O⁶BG), an inhibitor of MGMT that has been used in clinical trials [14,18–20,35–38]. MGMT binds the benzyl group and transfers it from O⁶BG to its CYS145 residue, consuming active MGMT. The rationale behind using this drug in trials is to decrease the amount of active MGMT present in cells before administration of an alkylating agent. Because both G132R and, to a greater extent, G156C have lower binding affinities for O⁶MeG, we asked whether they also had lower binding affinities for O⁶BG. To do this, we measured the transfer of tritiated O⁶BG to MGMT molecules in a constant number of cells from EMT6 pools expressing WT or variant MGMT, as previously described [32,39]. We found that O⁶BG did not transfer benzylguanine to cells expressing G156C any better than it transferred benzylguanine to MGMT-deficient cells; G156C appears to be entirely resistant to O⁶BG (Figure 6). This is consistent with the dramatically decreased binding affinity of G156C shown in our biochemical assays. G132R accepts the benzylguanine from O⁶BG, but not to the same extent as WT MGMT. From this test alone, G132R appears to be partially resistant to O⁶BG. Within an organism, however, the G132R molecules that do not react with O⁶BG will be available to repair DNA damage, a challenge not present in this direct transfer test.

These data suggest that a tumor with unmethylated MGMT promoters and positive MGMT expression, which would currently be assumed to have a unfavorable prognosis [40], may actually be sensitive to alkylating agents such as temozolomide due to single amino acid substitutions in MGMT arising in the tumor. While O⁶BG is in clinical trials as an inhibitor of MGMT, there many be patients who would be unresponsive to this inhibitor due to single amino acid substitutions.

DISCUSSION

MGMT plays an important role in safeguarding genomic integrity from damage to the O-6 position of guanine and the O-4 position of thymine and is fairly well conserved, so we hypothesized that alterations in *MGMT* found in cancer cells might attenuate or ablate repair. We found that two cancer-associated mutants of MGMT, G132R and G156C, repair methylation damage more slowly than WT MGMT and have a decreased ability to bind DNA. In our assays, we saw that bacterial and mammalian cells expressing G132R and G156C are more sensitive to methylating agents than cells expressing WT MGMT. We also found that these variants are resistant to O⁶BG, an inhibitor of MGMT that has been used in clinical trials.

G132R Exhibits Slight Reduction in Binding to O⁶MeG

The G132R variant, identified in esophageal tumors [22], has a slight reduction in its affinity for O⁶MeG and a slight decrease in reaction rate compared to WT. This is consistent with the location of G132 in the conserved DNA recognition helix (Ala127–Gly136) of a helix-turn-helix motif responsible for MGMT binding to the minor groove (Figure 1) [24]. A screen for O⁶BG-resistant mutants in *E. coli* isolated G132S 10 times while four other G132 mutants were isolated once, but only G132S was determined to be truly resistant to O⁶BG [41]. G132R was not pulled out of this screen; however, serine, like arginine, is nucleophilic, and arginine is bulkier than serine, so we hypothesized that G132R was O⁶BG-resistant as well. As we predicted based upon G132S resistance to O⁶BG [41], we found that G132R is also resistant to O⁶BG.

G156C Exhibits a Very Low Binding Affinity for O⁶MeG

We found that G156C, identified in a colorectal cancer cell line [21], has a drastic decrease in affinity for O⁶MeG and a reduced reaction rate compared to WT. Our finding of a reduced rate of damage removal by G156C is consistent with published data characterizing G156A as having a specific activity around half that of WT MGMT [37]. After MGMT binds to the minor groove of DNA, bases are flipped into a pocket of MGMT. G156 is in the loop surrounding this pocket, at the bottom of which the C145 cysteine acceptor residue resides (Figure 1) [24]. Interfering with the solvent-exposed areas of this pocket may create difficulties for fitting damaged DNA residues, as hypothesized by the group that found both cancer-associated variant G156C and germline variant G160R in *E. coli* screens for O⁶BG resistant mutants [20,37,38]. G156A was first identified in one of these screens and binds O⁶BG poorly, likely due to changes in the conformation of the binding pocket. G156A was further characterized in the same study as unstable [37]. Alternatively, as G156C is a mutation to a cysteine, the mutant residue may form disulfide bonds with other cysteine groups in the protein, notably the CYS145 acceptor residue or another binding pocket residue, CYS150. A molecular modeling study published before the report of G156C examined then-known tumor and germline variants of MGMT and found that the structurally proximal G160R mutation likely results in an abnormal local high energy region, giving rise to structural changes in the base-flipping region without alterations to the global-scale protein structure [42]. We tested this hypothesis by examining the CD spectra for purified G156C and found that it does not differ from the spectra for G132R or WT MGMT, suggesting that there are in fact no global-scale changes to protein structure.

G156A has been shown to be resistant to O⁶BG [37]. Since the slight change from one small amino acid, glycine, to another, alanine, altered O⁶BG sensitivity, we hypothesized that the even larger and nucleophilic addition of a sulfur molecule via the cysteine R-group may also provide resistance to O⁶BG. The results of our tritiated O⁶BG assay suggest that G156C is in fact resistant to O⁶BG, as we see the same level of successful transfer in G156C-

containing cells that we see in MGMT-deficient cells. G156C may cause steric hinderance in the area surrounding the binding pocket, preventing the benzyl group from coming into contact with the CYS145 acceptor residue.

G156C was again identified in a screen for drug-resistant mutations in MGMT induced by treatment of a human rhabdomyosarcoma cell line with BCNU (carmustine), a chloroethylating agent used in the treatment of gliomas and lymphomas [35,43]. It is worth noting that the human colorectal cell line in which the G156C mutant was initially identified had not been treated with drugs [21]. MGMT is of particular interest in brain tumors due to the extensive use of alkylating agents in their treatment and the consistent linkage of MGMT silencing via promoter methylation to a positive prognosis for chemotherapy [40]. The rhabdomyosarcoma line with the newly induced G156C mutation was resistant to BCNU but only mildly resistant to O⁶BG [43], suggesting that these cells may have additional alterations that permit repair of BCNU-induced lesions and O⁶BG resistance.

The severity of the binding deficiency (40×) for the G156C mutant presents a greater problem for repair than the modest drop in reaction rate (2×), particularly as MGMT does not turn over for reuse after accepting alkylation damage. The severe reduction in DNA binding ability is surprising given that it is not in the DNA binding domain for MGMT [44]. A mutation at G156 to cysteine yields a bulkier side chain, which may interfere with a nearby alpha helix spanning residues 163–165 that has been implicated in protein–protein interactions necessary for the preferred cooperative binding of four MGMT molecules around a DNA strand [45,46].

The Presence of MGMT Mutations Effects Cellular Responses to Treatment With O⁶BG

A significant body of literature has shown that variants of MGMT have an important effect on the function of the protein and clinical outcome for patients treated with chemotherapeutic alkylating agents. Here, we have demonstrated that two tumor-associated mutants, G132R and G156C, have functional and biochemical differences from wild type MGMT that manifest in diminished repair capacity. G132R appears to be modestly resistant to O⁶BG but our earlier experiments show that it is reasonably well functional overall. G156C is entirely resistant to O⁶BG but, as shown earlier, has greatly impaired binding to damaged DNA. These variants exhibit different levels of resistance to O⁶BG, as measured by the O⁶BG binding assay. Our results suggest that the molecular status of MGMT is important regarding responses of the cells to chemotherapeutic agents. Examining tumor sequences for somatic mutations, such as those that give rise to MGMT G132R and G156C, may provide valuable guidance for treatment of human tumors.

Acknowledgments

This work was supported by NCI P01 CA129186. K.L. was supported in part by the Cell and Molecular Biology Training Grant at Yale University, NIH National Research Service Award T32 GM007223.

Abbreviations

MGMT	O ⁶ -methylguanine DNA methyltransferase
BCNU	carmustine
TMZ	temozolomide
O⁶BG	O ⁶ -benzyl-guanine
O⁶MeG	O ⁶ -methylguanine

MNNG *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
WT wild type

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Figure 1.

MGMT G132R and G156C are near the active site of MGMT. MGMT bound to the minor groove of DNA (black, bottom center), with one DNA base flipped into the MGMT active site (acceptor residue at CYS145) [24]. Dotted lines represent measured distances, numbers are distances measured in Ångströms (Å). Mutations to cysteine and arginine could result in residues larger than glycine and may distort the structure. Generated in MacPyMol using the 1T38 crystal structure [24].

Figure 2. MGMT G132R and G156C do not fully rescue methyl-transferase deficient *E. coli*. (A) Western blot for MGMT expression in *GWR111 E. coli* shows the ratio of WT:G132R:G156C expression to be 1:11.0:11.2. There is no discernable MGMT expression in empty vector cells. (B) Survival curves for *E. coli*. Log-phase *GWR111* cultures expressing wild type (■) or variant (● G132R, ▽ G156C) human MGMT and the empty pBAD expression vector (✕) were incubated with graded doses of MNNG for 30 min then plated in serial dilutions. Surviving colonies were counted the next day. Representative survival curves are shown.

Figure 3. G132R and G156C do not change the overall structure of MGMT. Multiple circular dichroism spectra for WT, G132R, and G156C purified human MGMT were measured, averaged, and normalized. The overall shapes of the spectra, with peaks of intensity at 208 and 217 nm, were the same for all three proteins.

Figure 4.

MGMT variants have decreased affinity for substrate. (A) Schematic of substrates and the cutting ability *PvuII* on those substrates. (B) Gel showing separation of repaired and unrepaired products of time course for 33 nM of G132R with 5.3 nM substrate. Time is given in seconds. U is undamaged control substrate. (C) Determination of pre-steady state k_{obs} for G132R. Fraction of substrate repaired is plotted against time. The scale of the X-axis changes at 100 s to allow visualization of points in the early timepoints of the curve, before saturation. 5.3 nM substrate was reacted with varying concentrations (○ 500 nM, ▲ 333 nM, * 33 nM, ◆ 25 nM, + 5 nM, ■ 2 nM) of G132R MGMT in separate time courses. Repair was evaluated via gel electrophoresis followed by quantification on a phosphorimager and normalized to a no damage control. A single exponential equation was fit to the plot to yield a k_{obs} for each concentration. (D) Single hyperbolic plot to determine K_d and k_r for MGMT G132R. The k_{obs} values as calculated in (A) were plotted against nM concentration of MGMT and fit to a single hyperbolic equation to determine the reaction rate (k_r) and the binding efficiency (K_d).

Figure 5. MGMT G132R and G156C cannot fully rescue MGMT-deficient mammalian cells. (A) Expression of the variants in EMT6 cells. The ratio of WT:G132R:G156C expressed in these pools is 1:1.3:0.7. (B) Survival of EMT6 cells after treatment with MNNG. EMT6 mouse mammary carcinoma cells transfected with empty pRVY expression vector ($\times$), pRVY containing WT MGMT ($\blacksquare$), G132R MGMT ($\bullet$), or G156C MGMT (∇) were treated with graded doses of MNNG and plated over a range of dilutions. Colonies were stained 10 d later and counted. Points are geometric means from three separate experiments on pools

within a range of three passages and error bars represent the standard error of the mean. * $P = 0.03$ and $P = 0.04$ for WT versus G132R and G156C, respectively, paired t -test.

Figure 6.

G156C does not react with O⁶BG. 2×10^6 EMT6 mouse mammary carcinoma cells were incubated with tritiated benzylguanine. Free benzylguanine was removed by washing with methanol and the amount of tritium transferred to cells was measured by scintillation counting. Averages and standard deviations for triplicate assays are shown. ** $P < 0.001$; $P < 0.0001$.

Table 1

MGMT G132R and G156C Bind DNA Poorly and Remove Damage Slowly

	k_r (s ⁻¹)	K_d (nM)
MGMT WT	0.49 ± 0.01	6.2 ± 0.7
G132R	0.41 ± 0.02	14 ± 3
G156C	0.15 ± 0.01	240 ± 60