Published in final edited form as:

Biochem Pharmacol. 2008 February 1; 75(3): 618–626.

Differential inactivation of polymorphic variants of human O^6 -alkylguanine-DNA alkyltransferase

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Abstract

The human DNA repair protein O^6 -alkylguanine-DNA alkyltransferase (hAGT) is an important source of resistance to some therapeutic alkylating agents and attempts to circumvent this resistance by the use of hAGT inhibitors have reached clinical trials. Several human polymorphisms in the *MGMT* gene that encodes hAGT have been described including L84F and the linked double alteration I143V/K178R. We have investigated the inactivation of these variants and the much rarer variant W65C by O^6 -benzylguanine, which is currently in clinical trials, and a number of other second generation hAGT inhibitors that contain folate derivatives (O^4 -benzylfolate, the 3' and 5' folate esters of O^6 -benzyl-2'-deoxyguanosine and the folate γ ester of O^6 -(p-hydroxymethyl) benzylguanine). The I143V/K178R variant was resistant to all of these compounds. The resistance was due solely to the I143V change. These results suggest that the frequency of the I143V/K178R variant among patients in the clinical trials with hAGT inhibitors and the correlation with response should be considered.

Keywords

Alkyltransferase; O^6 -benzylguanine; polymorphisms; cancer chemotherapy; O^4 -benzylfolate; DNA repair

1. Introduction

DNA adducts at the O^6 -position of guanine are important for the antitumor action of a number of drugs including the methylating agents, temozolomide, procarbazine and dacarbazine, and the chloroethylating agents, BCNU and CCNU [1-4]. These adducts are repaired rapidly by the action of human O^6 -alkylguanine-DNA alkyltransferase (hAGT). This protein is therefore an important source of resistance to these agents. Compounds that inactivate hAGT and overcome this resistance in cultured cells and xenografts are currently in clinical trials.

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The inhibitors currently undergoing trials are *O*⁶-benzylguanine (BG) [4-6] and *O*⁶-(4-bromophenyl)guanine (PaTrin2) [7,8]. Despite some responses in the trials with BG, the lack of selectivity of the drug towards the tumor hAGT is likely to limit its effectiveness. Another concern is that alterations in hAGT causing resistance to BG (and presumably PaTrin2) arise very readily in laboratory studies [9-12]. These results have led to efforts to synthesize second-generation hAGT inhibitors that would be more potent, tumor specific and able to inactivate BG-resistant variants at doses achievable in the clinic. One promising approach has been to make folate derivatives of BG that would selectively be taken up into tumors by the folate carrier, a transport system known to be more active in many tumors than in normal cells. These efforts have led to the synthesis of *O*⁴-benzylfolic acid (BF), the 3' and 5' folate esters of *O*⁶-benzyl-2'-deoxyguanosine (3FBDG and 5FBDG, respectively) and the folic acid γ ester of *O*⁶-(p-hydroxymethyl)benzylguanine (FHMBG) [13,14]. The structures of these compounds are shown in Fig. 1.

Several variants in the *MGMT* gene that encodes the hAGT protein, which affect the primary sequence of the protein, have been described. Variants W65C [15,16], L84F [15-28], I143V/K178R [17-24,27,29-31] and G160R [29,32] have been reported by multiple laboratories. These reports raise the possibility that there may be individual variation in response to hAGT inactivators. This possibility was strongly supported by the finding that a G160R variant was substantially resistant to BG [33]. However, subsequent studies have shown that the frequency of the G160R variant is very low (>1%) [17,29,30,34,35]. Recently, it was reported that the I143V/K178R variant, which is much more common with a frequency of c. 24%, may be resistant to PaTrin-2 [24].

We therefore examined variants W65C, L84F and I143V/K178R for inactivation by BG and its folate derivatives described above.

2. Materials and Methods

2.1. Materials

Primers were synthesized and purified by the Macromolecular Core Facility, Hershey Medical Center. *E. coli* XL1-blue bacterial strain, Pfu Turbo hot-start DNA polymerase, Pfu polymerase enzyme and Quick Change Site-directed Mutagenesis Kit were purchased from Stratagene (La Jolla, CA). DNA isolation kits and the pQE-30 plasmid were obtained from Qiagen (Chatsworth, CA). Restriction enzymes *Bam*HI and *Kpn*I were obtained from Promega (Madison, WI). Ampicillin, isopropyl β -D-thiogalactopyranoside (IPTG), hemocyanin, calf thymus DNA, and most other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Talon Metal Affinity IMAC Resin was obtained from BD Bioscience Clontech (Palo Alto, CA), the Rapid DNA Ligation Kit and urea were purchased from Roche Diagnostic Corporation (Indianapolis, IN). Nitrocellulose filters (0.45 μ m) were obtained from Millipore (USA). Tobacco etch virus (Tev) protease was provided by Dr. J. M. Flanagan (Dept. of Biochemistry & Molecular Biology, Pennsylvania State University College of Medicine). BG [36], BF [13] and 3FBDG, 5FBDG and FHMBG [14] were synthesized as previously described.

2.2. Construction of pQE plasmids for expression of different hAGT variants

Plasmids for the production of C-terminal His₆-tagged hAGT [37] and for the production of N-terminal His₆-tagged hAGT [38], and the mutants L84F, I143V and I143V/K178R were prepared as described previously [39,40]. Plasmids for the production of N-terminal His₆-tagged W65C and K178R of hAGT were constructed from the template plasmids of pQE30-hAGT [38] with Quick Change Site-directed Mutagenesis Kit used according to the manufacturer's instructions. Primers W65C-P1: 5'-d (CAGTGCACAGCCTGCCTGAATGCCTATTTC)-3' and W65C-P2: 5'-d

(GAAATAGGCATTCAG**G**CAGGCTGTGCACTG)-3' and primers K178R-P1: 5'-d (CACCGGTTGGGGAG**G**CCAGGCTTGGGAGGG)-3' and K178R-P2: 5'-d (CCCTCCCAAGCCTGGC**C**TCCCCAACCGGTG)-3' were used to construct the W65C and K178R variants with N-terminal His₆-tags. The mutated sites are shown in bold and underlined. All of the N-terminal His₆-tagged hAGT proteins differ from the wild type by the addition of a terminal MRGS(H)₆GS- to the protein [38]. The C-terminal His₆-tagged hAGT have a (His)₆ sequence replacing residues 202–207 [37].

Plasmids for the expression of wild type and I143V/K178R hAGT with a cleavable N-terminal His₆-tagged hAGT in the pQE30 vector were constructed as follows by PCR using the pQE30-hAGT and pQE30-I143V/K178R as templates. The hAGT sequence was modified to form a tobacco etch virus (Tev) protease recognition site [41] using primer- 1 5'-d (GCACAGATGGATCC**GAGAACCTGTACTTCCAATCC**ATGGACAAGGATTGTG)-3' as the sense primer and primer 2: 5'-d(GGATCTATCAACAGGAGTCC)-3' as anti-sense primer. The *Bam*HI restriction site in primer 1 is underlined and Tev protease recognition site is shown in bold and italic. The PCR was carried out using Pfu polymerase under the conditions described previously [42]. The PCR product and plasmid vector were digested with *Bam*HI and *Kpn*I enzymes, the digested products were purified with Eppendorf Perfectprep Gel Cleanup kit of Brinkmann company (Westbury, NY), and the fragments containing the desired sequences were ligated into a pQE30 vector plasmid digested with the same enzymes to form pQE30-TevhAGT and pQE30-TevI143V/K178R. All constructed plasmids were verified by DNA sequencing carried out by the Macromolecular Core Facility, Hershey Medical Center. The resulting verified plasmids were used for protein expression and purification after transformation of XL-1 blue cells. The hAGT proteins produced from this vector after cleavage with Tev protease differ from the wild type by the addition of an N-terminal Ser residue.

2.3. Protein purification

The XL-1 blue cells containing pQE30-hAGT and variants or pQE30-TevhAGT and variants were cultured for protein expression and purification using Talon IMAC resin as previously described [42]. For pQE30-TevhAGT and pQE30-TevI143V/K178R, after the protein was dialyzed in 50 mM Tris-HCl, pH 7.6, 1 mM DTT and 0.1 mM EDTA, the solution containing hAGT was concentrated with Centrion Ultracel YM-10 of Millipore corporation (Bedford, MA). His₆-tagged Tev protease was then added (1:20 ratio) and digestion was carried out at 4°C for 12–16h. The digested protein mixture was then loaded into Talon IMAC resin and the flow-through collected [41]. The purified protein was analyzed by SDS-PAGE on 12% gels.

2. 4. Inactivation of alkyltransferase activity by BG and other compounds

Appropriate amounts of hAGT and variants with or without His₆-tag were incubated with different concentrations of hAGT inhibitors in 0.5 ml hAGT assay buffer of 50 mM Tris-HCl pH7.6, 5 mM DTT, 0.1 mM EDTA and 50 µg hemocyanin for 30 min at 37°C. For assay in the presence of DNA, 10 µg calf thymus DNA was added to the buffer in place of the hemocyanin. The residual hAGT activity was then determined by method described previously [13]. A graph of the percentage of the hAGT activity remaining against inhibitor concentration was then plotted and the ED₅₀ value representing the amount of inhibitor needed to produce a 50% loss of activity was calculated from the equation fitting the best fit curve using an exponential curve fitting program (KaleidaGraph, Synergy Software, Reading, PA 19606).

3. Results

The effects of known polymorphic changes in hAGT sequence on the inactivation by BG and BF were initially studied using hAGT with an N-terminal His₆-tag since the variants had previously been prepared using this construct. All of these proteins were purified to

homogeneity and all of the variants had similar activity to wild type hAGT in the repair of methylated DNA *in vitro* (results not shown). As previously reported by others [43], the W65C protein was relatively unstable and was obtained in a lower yield.

As shown in Fig. 2a and 2e, and summarized in Table 1, there was a small increase in the ED₅₀ for BG with W65C and I143V/K178R but no change from wild type and L84F. This was seen in assays conducted in the absence (Fig. 2a) and presence of DNA (Fig. 2e) when, as previously reported [44], BG was a more potent inactivator.

The I143V/K178R variant was clearly more resistant to inactivation by BF than wild type (Fig. 2b and 2f). This difference was also seen in the both the presence and the absence of DNA but BF, as previously reported [13], was much less active in the presence of DNA. The L84F and W65C variants were also slightly more resistant to BF but this difference was not statistically significant.

The ability of 3FBDG, 5FBDG and FHMBG to inactivate the polymorphic forms of hAGT was also studied (Fig. 2c, d, g and h) and Table 2. The I143V/K178R variant was significantly more resistant to inactivation by all of these compounds. The inactivation by 3FBDG and 5FBDG was less when DNA was present (Fig. 2g and 2h) but the difference between wild type and I143V/K178R was still highly significant.

The ED₅₀ value of 2.0 μ M for the inactivation of N-terminal His₆-tag wild type hAGT by BF in the presence of DNA found in this study was higher than that previously reported (ED₅₀ of 0.47 μ M) [13] but the previous study was carried out using hAGT with a C-terminal His₆-tag. We therefore prepared both the wild type and the I143V/K178R variant, which showed the most extensive change in inactivation by BF, using a construct that contained a cleavable His₆-tag and removed the additional sequence using Tev protease. The purity of resulting proteins was confirmed by SDS-PAGE analysis (Fig. 3). There was no difference between the wild type and the I143V/K178R variant in activity for the repair of methylated DNA when the protein with either N- or C-terminal His₆-tag or no tag was tested (results not shown). The difference in the inactivation by BG, BF, 3FBDG and 5FBDG was still seen with the protein from which the tag had been removed (Fig. 4 and Tables 1 and 2). The only major alteration in the results was that the ED₅₀ values for BF, 3FBDG and 5FBDG in the presence of DNA were lower for both wild type and the I143V/K178R variant when the tag was removed. Thus, it appears that the addition of the amino terminal His₆-tag alters the ability of hAGT to interact with folate derivatives in the presence of DNA but does not influence the ability of the I143V/K178R variant to reduce the sensitivity to inhibition. We re-examined the wild type hAGT with a C-terminal His₆-tag and observed that the inactivation by BF resembled that seen with the protein with no tag (Table 1 bottom line).

The SNPS leading to the I143V and K178R changes are in almost perfect disequilibrium [17,18,24,30] and it is highly likely that both changes occur in the protein derived from this gene. In order to examine the extent to which two linked alterations contribute to the resistance to inhibitors observed, N-terminal His₆-tagged hAGT proteins with the individual mutations were produced separately and tested (Table 3). With all of the compounds examined, the ED₅₀ value for the I143V mutant was higher than for the K178R mutant and the latter resembled wild type whereas the I143V gave result similar to the I143V/K178R variant. Thus, it appears that the resistance to these compounds is due to the presence of the Val residue replacing Ile.

4. Discussion

Polymorphisms in human DNA repair genes may have important implications in individual sensitivity to carcinogens and response to antitumor agents. Since hAGT activity is well established as a major factor in the response to alkylating agents, which are widely distributed

environmental carcinogens and also common antitumor drugs, it is highly conceivable that variants of hAGT may have important effects. However, at present, these are not clearly established. There are a substantial number of epidemiological and biochemical reports on the properties of these variants (reviewed in [48]) but these do not provide a consistent picture with some studies showing increased risk of tumor development and others not [19,20,22,23,25,26,29,31,45-47]. This may be a consequence of the small sample size in most of these studies. Also, the W65C alteration is very rare and there are even fewer reports of G160R alteration. The two common alterations are L84F and I143V/K178R. Neither of these alterations appears to affect the ability of hAGT to repair *O*⁶-methylguanine adducts in DNA.

The purified L84F recombinant protein does not differ in activity from wild type hAGT in the repair of *O*⁶-methylguanine *in vivo* or *in vitro* or in the relative repair *in vitro* of *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine, an adduct formed by the tobacco specific nitrosamines [40,43]. The lack of alteration in DNA repair ability for L84F is consistent with the fact that this is a conservative change of one hydrophobic amino acid side-chain for another at a position in the N-domain of hAGT, a significant distance away from the active site. The findings reported here that the L84F variant had little if any effect on the interaction with inhibitors is also consistent with the lack of change in the active site pocket.

The I143V/K178R form of hAGT appears to be potentially the most interesting polymorphic variant. The Ile143 is located in the active site pocket and is very close to the Cys145 acceptor site. However, this protein appears not to differ from wild type in the repair of methylated DNA [19,24,39]. This is not surprising since the position equivalent to Ile143 in hAGT is variable when sequences from a wide range of organisms are compared with the most common alteration being the presence of Val rather than Ile. This alteration occurs in the *S. cerevisiae* and *B. subtilis* AGTs that are known to be active. However, it is well established that there are striking species differences in the ability of AGTs to repair more bulky adducts [2,49,50]. The I143V/K178R hAGT was active in the repair of *O*⁶-*n*-butylguanine, *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine or BG when these were contained in oligodeoxynucleotides [39]. However, the repair of *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine by this variant was less sensitive to sequence context than wild type or the L84F form [40]. These results indicate that the I143V substitution alters the geometry of the hAGT substrate-binding pocket to permit efficient repair of bulky *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine even when it is in conformations that may be poorly repaired by wild type hAGT.

Our results showing that the I143V/K178R form of hAGT is more resistant to inactivation by BG and benzyl folate derivatives can also be explained by steric alterations in the active site pocket region where these inhibitors must bind. The extent to which the alteration changes the sensitivity is greatest with the more potent inhibitors (3–4 fold). Models based on the crystal structure of hAGT and the S-benzyl form of the protein formed by reaction with BG show that the binding of BG is strongly influenced by a stacking interaction with Pro140 and forms hydrogen bonds with residues Tyr144, Val148 and Ser159 [51,52]. The greater potency of BF and 3FBDG is likely to be due to additional interactions via the folate moiety. The alteration of Val for Ile143 in the binding pocket may cause subtle changes that affect these interactions. Our results are in agreement with a recent report that there was a small difference in the sensitivity to inactivation by PaTrin-2 when I143V/K178R and I143V variants were compared to wild type [24]. Although the increase in ED₅₀ values were not calculated in these experiments there was 76% loss of activity with wild type and only 67% loss with the I143V alteration with 10 μM PatrIn-2 suggesting that the alteration is similar in magnitude to that seen with BG.

Our results show that all of the alterations in response to inhibitors of the I143V/K178R variant was due to the I143V change which affects the binding pocket. This is consistent with the report of resistance of I143V to PaTrin-2 and with known structural data. hAGT can be truncated to

end at position Leu176 without loss of activity [53] and the position of the K178R change is not in a conserved part of the protein and has no interaction with the active site in the crystal structure [51].

Our studies with the very rare W65C mutant suggest that this protein may also be slightly resistant to some of the inhibitors tested but not to 3FBDG or to BF. A more critical issue with this variant is likely to be the instability of the protein. The purified recombinant W65C protein was obtained in a much lower yield and was less stable when incubated *in vitro*. Previous studies have also observed a lowered activity and ability to protect from methylation damage compared to wild type when W65C was expressed in either bacteria or a mammalian cell line [43]. Thus, it is unlikely that W65C would provide resistance to therapeutic alkylating agents similar to that imparted by wild type hAGT. In contrast, previous studies with mutant G160R have shown that this alteration provides substantial resistance to BG *in vitro* (ED₅₀ of 9 μ M without DNA and 4 μ M with DNA) and to the killing of cells by BCNU plus BG in culture [54]. However, numerous follow up studies have failed to confirm the frequency of about 15% that was reported for this G160R variant [32], and many studies failed to find any cases [17, 29,30,34,35]. Thus, it is unlikely that either W65C or G160R will prove to be important in response to therapy in clinical trials.

In contrast, the I143V/K178R variant is quite common with a frequency of c. 24% (11–28%) in various studies [17-24,27,29-31], and it is active in protecting cells from alkylation damage. Even in individuals with one allele, the very strong selection pressure that is provided under conditions involving treatment with temozolomide or BCNU plus a hAGT inhibitor would select for cells in which a hAGT form resistant to an inhibitor was present. Therefore, determination of the frequency of the I143V/K178R variant and correlation with response in patient populations treated with such drugs would be advisable. It may also prove useful to design and examine potential new hAGT inactivators for improved ability to react with this hAGT variant.

Acknowledgements

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. Work in AEP's laboratory was supported by grants CA-018137 and CA-071976 from the National Cancer Institute, National Institutes of Health, USA.

Abbreviations

AGT, *O*⁶-alkylguanine-DNA alkyltransferase; hAGT, human AGT; BG, *O*⁶-benzylguanine; PaTrin-2, *O*⁶-(4-bromophenyl)guanine; BF, *O*⁴-benzylfolic acid; 3FBDG, *O*⁶-benzyl-3'-*O*-(γ -folyl)-2'-deoxyguanosine; 5FBDG, *O*⁶-benzyl-5'-*O*-(γ -folyl)-2'-deoxyguanosine; FHMBG, *O*⁶-[4-[(γ -folyl)-oxymethyl]benzyl]guanine; IPTG, isopropyl β -D-thiogalactopyranoside; Tev, tobacco etch virus..

REFERENCES

1. Dolan ME, Pegg AE. *O*⁶-Benzylguanine and its role in chemotherapy. *Clinical Cancer Res* 1997;3:837–47. [PubMed: 9815757]
2. Pegg AE. Repair of *O*⁶-alkylguanine by alkyltransferases. *Mutation Res* 2000;462:83–100. [PubMed: 10767620]
3. Margison GP, Santibáñez-Koref MF. *O*⁶-Alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. *BioEssays* 2002;24:255–66. [PubMed: 11891762]
4. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer* 2004;4:296–307. [PubMed: 15057289]

5. Quinn JA, Desjardins A, Weingart J, Brem H, Dolan ME, Delaney SM, et al. Phase I trial of temozolomide plus *O*⁶-benzylguanine for patients with recurrent or progressive malignant glioma. *J Clin Oncol* 2005;23:7178–87. [PubMed: 16192602]
6. Warren KE, Aikin AA, Libucha M, Widemann BC, Fox E, Packer RJ, et al. Phase I study of *O*⁶-benzylguanine and temozolomide administered daily for 5 days to pediatric patients with solid tumors. *J Clin Oncol* 2005;23:7646–53. [PubMed: 16234526]
7. Middleton MR, Margison GP. Improvement of chemotherapy efficacy by inactivation of a DNA-repair pathway. *Lancet Oncol* 2003;4:37–44. [PubMed: 12517538]
8. Ranson M, Middleton MR, Bridgewater J, Lee SM, Dawson M, Jowle D, et al. Lomeguatrib, a potent inhibitor of *O*⁶-alkylguanine-DNA-alkyltransferase: phase I safety, pharmacodynamic, and pharmacokinetic trial and evaluation in combination with temozolomide in patients with advanced solid tumors. *Clin Cancer Res* 2006;12:1577–84. [PubMed: 16533784]
9. Bacolod MD, Johnson SP, Pegg AE, Dolan ME, Moschel RC, Bullock NS, et al. Brain tumor cell lines resistant to *O*⁶-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have *O*⁶-alkylguanine-DNA alkyltransferase mutations. *Mol Cancer Ther* 2004;3:1127–35. [PubMed: 15367707]
10. Xu-Welliver M, Kanugula S, Pegg AE. Isolation of human *O*⁶-alkylguanine-DNA alkyltransferase mutants highly resistant to inactivation by *O*⁶-benzylguanine. *Cancer Res* 1998;58:1936–45. [PubMed: 9581836]
11. Xu-Welliver M, Pegg AE. Point mutations at multiple sites including highly conserved amino acids maintain activity but render *O*⁶-alkylguanine-DNA alkyltransferase insensitive to *O*⁶-benzylguanine. *Biochem J* 2000;347:519–26. [PubMed: 10749682]
12. Fontes AM, Davis B, Encell L, Lingas K, Comas DT, Zago MA, et al. Differential competitive resistance to methylating versus chloroethylating agents among five *O*⁶-alkylguanine DNA alkyltransferases in human hematopoietic cells. *Mol Cancer Therap* 2006;6:121–9. [PubMed: 16432170]
13. Nelson ME, Loktionova NA, Pegg AE, Moschel RC. 2-Amino-*O*⁴-benzylpteridine derivatives: potent inactivators of *O*⁶-alkylguanine-DNA alkyltransferase. *J Med Chem* 2004;47:3887–91. [PubMed: 15239666]
14. Savanmard S, Loktionova NA, Fang Q, Pauly GT, Pegg AE, Moschel RC. Inactivation of *O*⁶-alkylguanine-DNA alkyltransferase by folate esters of *O*⁶-benzyl-2'-deoxyguanosine and of *O*⁶-[4-(hydroxymethyl)benzyl]guanine. *J Med Chem*. 2007
15. Otsuka M, Abe M, Nakabeppu Y, Sekiguchi M, Suzuki T. Polymorphism in the human *O*⁶-methylguanine-DNA methyltransferase gene detected by PCR-SSCP analysis. *Pharmacogenetics* 1996;6:361–3. [PubMed: 8873223]
16. Abe M, Inoue R, Suzuki T. A convenient method for genotyping of human *O*⁶-methylguanine-DNA methyltransferase polymorphism. *Jpn J Human Genet* 1997;42:425–8. [PubMed: 12503189]
17. Deng C, Capasso H, Zhao Y, Wang L-D, Hong J-Y. Genetic polymorphism of human *O*⁶-alkylguanine-DNA alkyltransferase: identification of a missense variation in the active site region. *Pharmacogenetics* 1999;9:81–7. [PubMed: 10208646]
18. Egyházi S, Platz A, Smoczynski K, Ringborg U. Novel *O*⁶-methylguanine DNA methyltransferase SNPs: a frequency comparison of patients with familial melanoma and healthy individuals. *Hum Mutat* 2002;20:408–9.
19. Ma S, Egyhazi S, Ueno T, Lindholm C, Kreklau EL, Stierner U, et al. *O*⁶-Methylguanine-DNA-methyltransferase expression and gene polymorphisms in relation to chemotherapeutic response in metastatic melanoma. *Br J Cancer* 2003;89:1517–23. [PubMed: 14562026]
20. Krzesniak M, Butkiewicz D, Samojedny A, Chorazy M, Rusin M. Polymorphisms in TDG and MGMT genes - epidemiological and functional study in lung cancer patients from Poland. *Ann Hum Genet* 2004;68:300–12. [PubMed: 15225156]
21. Huang WY, Olshan AF, Schwartz SM, Berndt SI, Chen C, Llaça V, et al. Selected genetic polymorphisms in MGMT, XRCC1, XPD, and XRCC3 and risk of head and neck cancer: a pooled analysis. *Cancer Epidemiol Biomarkers Prev* 2005;14:1747–53. [PubMed: 16030112]

22. Ritchey JD, Huang WY, Chokkalingam AP, Gao YT, Deng J, Levine P, et al. Genetic variants of DNA repair genes and prostate cancer: a population-based study. *Cancer Epidemiol Biomarkers Prev* 2005;14:1703–9. [PubMed: 16030105]
23. Shen J, Terry MB, Gammon MD, Gaudet MM, Teitelbaum SL, Eng SM, et al. MGMT genotype modulates the associations between cigarette smoking, dietary antioxidants and breast cancer risk. *Carcinogenesis* 2005;26:2131–7. [PubMed: 16014702]
24. Margison GP, Heighway J, Pearson S, McGown G, Thorncroft MR, Watson AJ, et al. Quantitative trait locus analysis reveals two intragenic sites that influence *O*⁶-alkylguanine-DNA alkyltransferase activity in peripheral blood mononuclear cells. *Carcinogenesis* 2005;26:1473–80. [PubMed: 15831531]
25. Wiencke JK, Aldape K, McMillan A, Wiemels J, Moghadassi M, Miike R, et al. Molecular features of adult glioma associated with patient race/ethnicity, age, and a polymorphism in *O*⁶-methylguanine-DNA-methyltransferase. *Cancer Epidemiol Biomarkers Prev* 2005;14:1774–83. [PubMed: 16030116]
26. Li C, Liu J, Li A, Qian L, Wang X, Wei Q, et al. Exon 3 polymorphisms and haplotypes of *O*⁶-methylguanine-DNA methyltransferase and risk of bladder cancer in southern China: a case-control analysis. *Cancer Lett* 2005;227:49–57. [PubMed: 15885889]
27. Hill CE, Wickliffe JK, Wolfe KJ, Kinslow CJ, Lopez MS, Abdel-Rahman SZ. The L84F and the I143V polymorphisms in the *O*⁶-methylguanine-DNA-methyltransferase (MGMT) gene increase human sensitivity to the genotoxic effects of the tobacco-specific nitrosamine carcinogen NNK. *Pharmacogenet Genomics* 2005;15:571–8. [PubMed: 16007001]
28. Chae MH, Jang JS, Kang HG, Park JH, Park JM, Lee WK, et al. *O*⁶-Alkylguanine-DNA alkyltransferase gene polymorphisms and the risk of primary lung cancer. *Mol Carcinog* 2006;45:239–49. [PubMed: 16385589]
29. Kaur TB, Travaline JM, Gaughan JP, Richie JP, Stellman SD, Lazarus P. Role of polymorphisms in codons 143 and 160 of the *O*⁶-alkylguanine-DNA alkyltransferase gene in lung cancer risk. *Cancer Epidemiol Biomarkers & Prevention* 2000;9:339–42.
30. Boffetta P, Nyberg F, Mukeria A, Benhamou S, Constantinescu V, Batura-Gabryel H, et al. *O*⁶-Alkylguanine-DNA-alkyltransferase activity in peripheral leukocytes, smoking and risk of lung cancer. *Cancer Lett* 2002;180:33–9. [PubMed: 11911967]
31. Yang M, Coles BF, Caporaso NE, Choi Y, Lang NP, Kadlubar FF. Lack of association between Caucasian lung cancer risk and *O*⁶-methylguanine-DNA methyltransferase-codon 178 genetic polymorphism. *Lung Cancer* 2004;44:281–6. [PubMed: 15140540]
32. Imai Y, Oda H, Nakatsuru Y, Ishikawa T. A polymorphism at codon 160 of human *O*⁶-methylguanine-DNA methyltransferase gene in young patients with adult type cancers and functional assay. *Carcinogenesis* 1995;16:2441–5. [PubMed: 7586149]
33. Xu-Welliver M, Leitao J, Kanugula S, Meehan WJ, Pegg AE. The role of codon 160 in sensitivity of human *O*⁶-alkylguanine-DNA alkyltransferase to *O*⁶-benzylguanine. *Biochem Pharmacol* 1999;58:1279–85. [PubMed: 10487529]
34. Gerson SL, Schupp J, Liu L, Pegg AE, Srinivasan S. Leukocyte *O*⁶-alkylguanine-DNA alkyltransferase from human donors is uniformly sensitive to *O*⁶-benzylguanine. *Clin Cancer Res* 1999;5:521–4. [PubMed: 10100702]
35. Wu MH, Lohrbach KE, Olopade OI, Kokkinakis DM, Friedman HS, Dolan ME. Lack of evidence for a polymorphism at codon 160 of human *O*⁶-alkylguanine-DNA alkyltransferase gene in normal tissue and cancer. *Clin Cancer Res* 1999;55:209–13. [PubMed: 9918221]
36. Dolan ME, Moschel RC, Pegg AE. Depletion of mammalian *O*⁶-alkylguanine-DNA alkyltransferase activity by *O*⁶-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc Natl Acad Sci U S A* 1990;87:5368–72. [PubMed: 2164681]
37. Liu L, Xu-Welliver M, Kanugula S, Pegg AE. Inactivation and degradation of *O*⁶-alkylguanine-DNA alkyltransferase after reaction with nitric oxide. *Cancer Res* 2002;62:3037–43. [PubMed: 12036910]
38. Edara S, Kanugula S, Goodtzova K, Pegg AE. Resistance of the human *O*⁶-alkylguanine-DNA alkyltransferase containing arginine at codon 160 to inactivation by *O*⁶-benzylguanine. *Cancer Res* 1996;56:5571–5. [PubMed: 8971155]

39. Mijal RS, Thomson NM, Moschel RC, Kanugula S, Fang Q, Pegg AE, et al. The repair of the tobacco-specific nitrosamine derived adduct, O^6 -[4-oxo-4-(3-pyridyl)butyl]guanine by O^6 -alkylguanine-DNA alkyltransferase variants. *Chem Res Toxicol* 2004;17:424–34. [PubMed: 15025514]
40. Mijal RS, Kanugula S, Vu CC, Fang Q, Pegg AE, Peterson LA. DNA sequence context affects repair of the tobacco-specific adduct O^6 -[4-oxo-4-(3-pyridyl)butyl]guanine by human O^6 -alkylguanine-DNA alkyltransferases. *Cancer Res* 2006;66:4968–74. [PubMed: 16651455]
41. Nallamsetty S, Kapust RB, Tozser J, Cherry S, Tropea JE, Copeland TD, et al. Efficient site-specific processing of fusion proteins by tobacco vein mottling virus protease in vivo and in vitro. *Protein Expr Purif* 2004;38:108–15. [PubMed: 15477088]
42. Fang Q, Kanugula S, Pegg AE. Function of domains of human O^6 -alkylguanine-DNA alkyltransferase. *Biochemistry* 2005;44:15396–405. [PubMed: 16285744]
43. Inoue R, Abe M, Nakabeppu Y, Sekiguchi M, Mori T, Suzuki T. Characterization of human polymorphic DNA repair methyltransferases. *Pharmacogenetics* 2000;10:59–66. [PubMed: 10739173]
44. Pegg AE, Chung L, Moschel RC. Effect of DNA on the inactivation of O^6 -alkylguanine-DNA alkyltransferase by 9-substituted O^6 -benzylguanine derivatives. *Biochem Pharmacol* 1997;53:1559–64. [PubMed: 9260884]
45. Inoue R, Isono M, Abe M, Abe T, Kobayashi H. A genotype of the polymorphic DNA repair gene MGMT is associated with de novo glioblastoma. *Neurol Res* 2003;25:875–9. [PubMed: 14669534]
46. Huang WY, Chow WH, Rothman N, Lissowska J, Llaca V, Yeager M, et al. Selected DNA repair polymorphisms and gastric cancer in Poland. *Carcinogenesis* 2005;26:1354–9. [PubMed: 15802298]
47. Cohet C, Borel S, Nyberg F, Mukeria A, Bruske-Hohlfeld I, Constantinescu V, et al. Exon 5 polymorphisms in the O^6 -alkylguanine-DNA alkyltransferase gene and lung cancer risk in non-smokers exposed to second-hand smoke. *Cancer Epidemiol Biomarkers Prev* 2004;13:320–3. [PubMed: 14973087]
48. Pegg AE, Fang Q, Loktionova NA. Human variants of O^6 -alkylguanine-DNA alkyltransferase. *DNA Repair* 2007;6:1071–8. [PubMed: 17482892]
49. Goodtzova K, Kanugula S, Edara S, Pauly GT, Moschel RC, Pegg AE. Repair of O^6 -benzylguanine by the *Escherichia coli* Ada and Ogt and the human O^6 -alkylguanine-DNA alkyltransferase. *J Biol Chem* 1997;272:8332–9. [PubMed: 9079656]
50. Kanugula S, Pauly GT, Moschel RC, Pegg AE. A bifunctional DNA repair protein from *Ferroplasma acidarmanus* exhibits O^6 -alkylguanine-DNA alkyltransferase and endonuclease V activities. *Proc Natl Acad Sci US,A* 2005;102:3617–22.
51. Daniels DS, Mol CD, Arvai AS, Kanugula S, Pegg AE, Tainer JA. Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical binding. DNA damage reversal revealed by mutants and structures of active and alkylated human AGT. *EMBO J* 2000;19:1719–30. [PubMed: 10747039]
52. Daniels DS, Woo TT, Luu KX, Noll DM, Clarke ND, Pegg AE, et al. Novel modes of DNA binding and nucleotide flipping by the human DNA repair protein AGT. *Nat Struct Mol Biol* 2004;11:714–20. [PubMed: 15221026]
53. Hazra TK, Roy R, Biswas T, Grabowski DT, Pegg AE, Mitra S. Specific recognition of O^6 -methylguanine in DNA by active site mutants of human O^6 -methylguanine-DNA methyltransferase. *Biochemistry* 1997;36:5769–76. [PubMed: 9153417]
54. Loktionova NA, Xu-Welliver M, Crone T, Kanugula S, Pegg AE. Mutant forms of O^6 -alkylguanine-DNA alkyltransferase protect CHO cells from killing by BCNU plus O^6 -benzylguanine or O^6 -8-oxo-benzylguanine. *Biochem Pharmacol* 1999;58:237–44. [PubMed: 10423163]

Fig. 1.
Structures of inhibitors used

Fig. 2.

Inactivation of N-(His)₆-tagged hAGT and variants in the presence or absence of calf thymus DNA. The upper panels show the inhibition graphs in the absence of DNA. Results are shown for hAGT (filled circles), L84F (open circles), I143V/K178R (filled squares), and W65C (open squares) inactivated by: a, BG; b, BF; c, 3FBDG; d, 5FBDG. The lower panels show the inhibition graphs in the presence of DNA. Results are shown for hAGT + DNA (filled circles), L84F + DNA (open circles), I143V/K178R + DNA (filled squares), and W65C + DNA (open squares) inactivated by: e, BG; f, BZ; g, 3FBDG; h, 5FBDG.

Fig. 3. Purity of hAGT. N-Tev-hAGT and hAGT were isolated and analyzed by SDS-PAGE as described under Experimental procedures, the gels were stained with Coomassie brilliant blue. Lane 1: N-Tev-hAGT (with N-His₆-tag and Tev recognition site). lane 2: hAGT (after removal of N-His₆-Tag). Lane 3: Eluted proteins with elution buffer. The positions of pure proteins are indicated on the right side (I, Tev protease; II, hAGT; III, cut N-Tev His₆-tag). Molecular markers shown on left side.

Fig. 4.

Inactivation of hAGT and I143V/K178R variant in the presence or absence of calf thymus DNA. The upper panels show the inhibition graphs in the absence of DNA. Results are shown for hAGT (filled circles), and I143V/K178R (open circles) inactivated by: a, BG; b, BF; c, 3FBDG; d, 5FBDG. The lower panels show the inhibition graphs in the presence of DNA. Results are shown for hAGT + DNA (filled circles) and I143V/K178R+DNA (open circles) inactivated by e: BG; f, BF, g, 3FBDG; h, 5FBDG.

Table 1
Inactivation of wild type hAGT and variants by BG and BF

AGT used	ED ₅₀ (μM) for inhibitor shown			
	+DNA	BG -DNA	+DNA	BF -DNA
N-tag-hAGT	0.10 ± 0.01	0.40 ± 0.05	2.0 ± 0.1	0.012 ± 0.002
N-tag-I143V/K178R	0.18 ± 0.01 ^a	0.55 ± 0.04 ^b	3.5 ± 0.2 ^a	0.034 ± 0.002 ^a
N-tag-L84F	0.09 ± 0.01	0.36 ± 0.05	2.6 ± 0.1	0.018 ± 0.003
N-tag-W65C	0.27	0.74	2.3 ± 0.2	0.020 ± 0.004
hAGT	0.09 ± 0.01	0.29 ± 0.01	0.65 ± 0.05	0.012 ± 0.002
I143V/K178R	0.17 ± 0.02 ^a	0.68 ± 0.03 ^a	0.94 ± 0.03 ^a	0.053 ± 0.07 ^a
C-tag-hAGT	0.1 ± 0.01	0.40 ± 0.06	0.75 ± 0.08	0.008 ± 0.006

ED₅₀ values were calculated from graphs of the percentage of remaining hAGT activity against inhibitor concentration shown in Figs. 1 and 3. Experiments where an S. D. is shown were repeated 3–5 times and the mean shown. Other values are the mean of two experiments.

^a Significantly different from wild type hAGT, p<0.001

^b Significantly different from wild type hAGT, p<0.01

Table 2

Inactivation of wild type hAGT and variants by 3FBDG, 5FBDG and FHMBG

AGT used	3FBDG		ED ₅₀ (μM) for inhibitor shown		FHMBG	
	+DNA	-DNA	+DNA	-DNA	+DNA	-DNA
N-tag-hAGT	2.90 ± 0.05	0.009 ± 0.004	5.35 ± 0.13	0.56 ± 0.04	0.23 ± 0.02	0.54
N-tag-I143V/K178R	3.82 ± 0.22 ^b	0.032 ± 0.008 ^a	8.62 ± 0.94 ^a	1.19 ± 0.06 ^a	0.48 ± 0.01 ^a	1.10
N-tag-L84F	3.35	0.011	5.44	0.54	0.21	0.45
N-tag-W65C	2.77	0.008	8.42	1.24	0.46	0.94
hAGT	0.68 ± 0.02	0.016 ± 0.002	3.06 ± 0.18	0.64 ± 0.05	0.24	0.68
I143V/K178R	0.95 ± 0.02 ^a	0.048 ± 0.008 ^a	7.34 ± 0.62 ^a	1.25 ± 0.12 ^a	0.56	1.30

ED₅₀ values were calculated as described in Table 1. Experiments where an S. D. is shown were repeated 3–5 times and the mean shown. Other values are the mean of two experiments.

^a Significantly different from wild type hAGT, p<0.001

^b Significantly different from wild type hAGT, p<0.01.

Table 3

Inactivation of I143V and K178R hAGT mutants

Drugs	+DNA		ED ₅₀ (μ M)		-DNA	
	N-tag-I143V	N-tag-K178R	N-tag-I143V	N-tag-K178R	N-tag-I143V	N-tag-K178R
BG	0.16	0.08	0.48	0.28		
BF	3.20	1.80	0.038	0.013		
3FBDG	4.18	2.98	0.028	0.009		
5FBDG	8.86	6.04	1.08	0.42		
FHMBG	0.46	0.28	0.98	0.46		

The I143V and K178R mutant proteins were prepared with an N-terminal His₆-tag from the pQE30 vector. The ED₅₀ value was calculated as described in Table 1. Each experiment was repeated twice.