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Differential inactivation of polymorphic variants of human O⁶-alkylguanine-DNA alkyltransferase

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ABSTRACT

The human DNA repair protein O⁶-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 O⁶-benzylguanine, which is currently in clinical trials, and a number of other second generation hAGT inhibitors that contain folate derivatives (O⁴-benzylfolic acid, the 3' and 5' folate esters of O⁶-benzyl-2'-deoxyguanosine and the folic acid γ ester of O⁶-(*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.

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1. Introduction

DNA adducts at the O⁶-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 O⁶-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.

The inhibitors currently undergoing trials are O⁶-benzylguanine (BG) [4–6] and O⁶-(4-bromothienyl)guanine (PaTrin-2) [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 PaTrin-2) 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

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Abbreviations: AGT, O⁶-alkylguanine-DNA alkyltransferase; hAGT, human AGT; BG, O⁶-benzylguanine; PaTrin-2, O⁶-(4-bromothienyl)guanine; BF, O⁴-benzylfolic acid; 3FBDG, O⁶-benzyl-3'-O-(γ -folyl)-2'-deoxyguanosine; 5FBDG, O⁶-benzyl-5'-O-(γ -folyl)-2'-deoxyguanosine; FHMBG, O⁶-[4-[(γ -folyl)-oxymethyl]benzyl]guanine; IPTG, isopropyl β -D-thiogalactopyranoside; Tev, tobacco etch virus.

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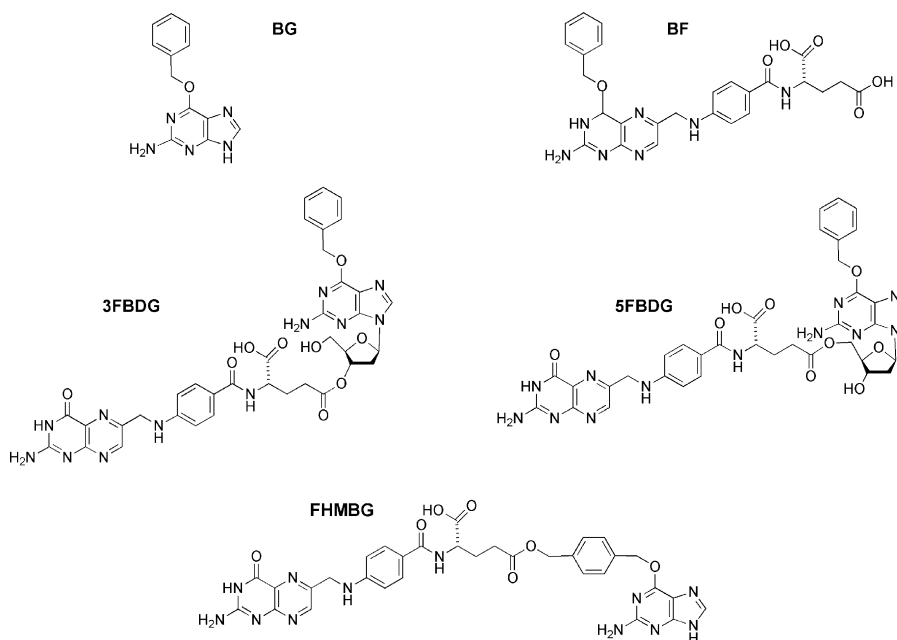


Fig. 1 – Structures of inhibitors used.

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 ca. 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. *Escherichia 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 BamHI and KpnI 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 (Department 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(GAAATAGGCATTTCAGGCAGGCTGTGACTG)-3' and primers K178R-P1: 5'-d(CACCGTTGGGGAGGCCAGGCT-TGGGAGGG)-3' and K178R-P2: 5'-d(CCCTCCCAAGCCTG-GCCTCCCCAACCGGTG)-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(GCACAGATGGATCCGAGAACCTGTACTTCCAATCCATGGACAAGGATTGTG)-3' as the sense primer and primer 2: 5'-d(GGATCTATCAACAGGAGTCC)-3' as anti-sense primer. The BamHI 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 BamHI and KpnI 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 12–16 h. 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 pH 7.6, 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 e, 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 f). 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 h) but the difference between wild type and I143V/K178R was still highly significant.

The ED₅₀ value of 2.0 µM 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 µM) [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

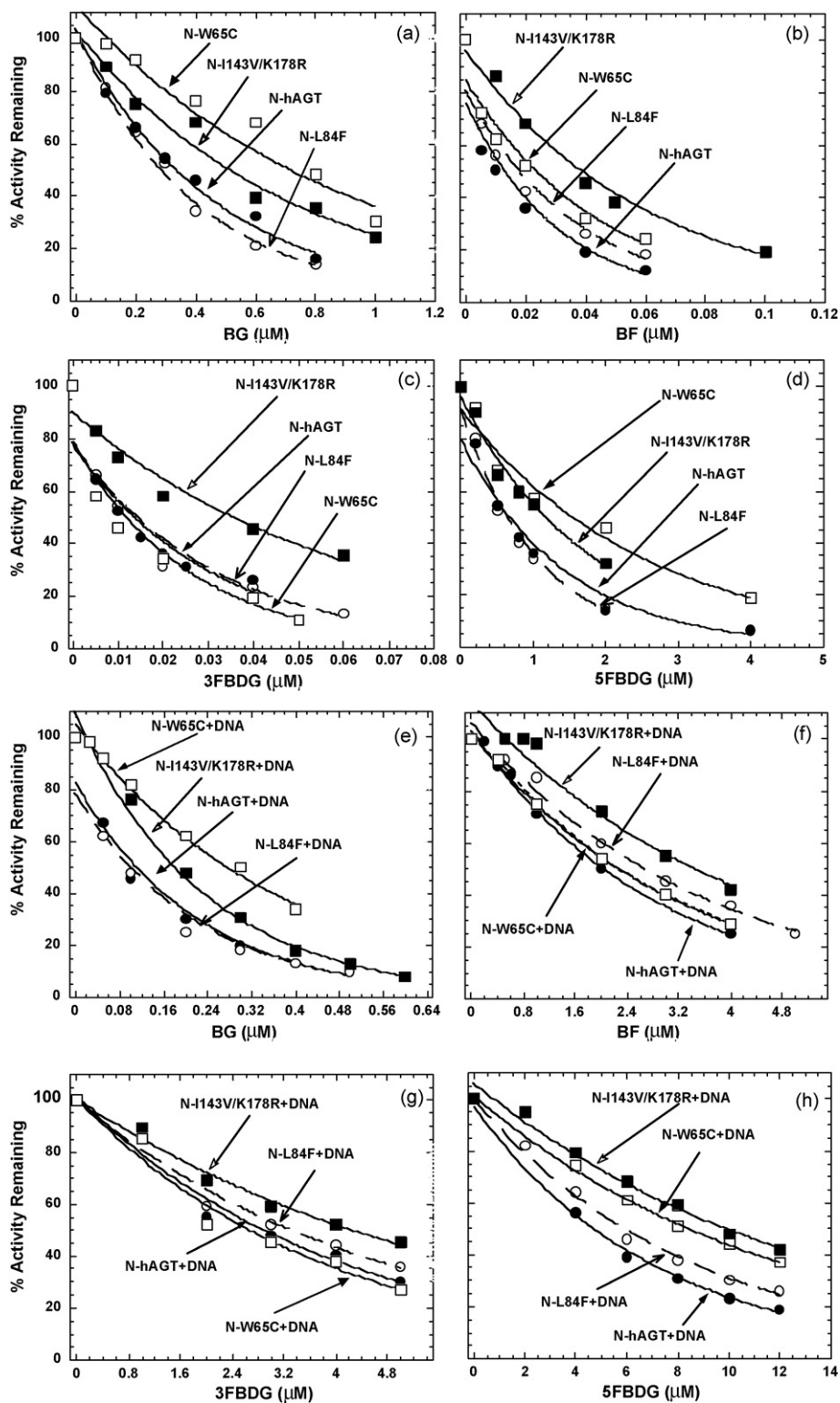


Fig. 2 – Inactivation of N-(His)₆-tagged hAGT and variants in the presence or absence of calf thymus DNA. The upper panels show the inhibition graphs in the absence of DNA. Results are shown for hAGT (filled circles), L84F (open circles), I143V/K178R (filled squares), and W65C (open squares) inactivated 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.

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

AGT used	ED ₅₀ (μM) for inhibitor shown			
	BG		BF	
	+DNA	–DNA	+DNA	–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 ^{***}	0.55 ± 0.04 ^{**}	3.5 ± 0.2 ^{***}	0.034 ± 0.002 ^{***}
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 ^{***}	0.68 ± 0.03 ^{***}	0.94 ± 0.03 ^{***}	0.053 ± 0.07 ^{***}
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.

^{**} Significantly different from wild type hAGT, $p < 0.01$.

^{***} Significantly different from wild type hAGT, $p < 0.001$.

Table 2 – Inactivation of wild type hAGT and variants by 3FBDG, 5FBDG and FHMBG

AGT used	ED ₅₀ (μM) for inhibitor shown					
	3FBDG		5FBDG		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 ^{**}	0.032 ± 0.008 ^{***}	8.62 ± 0.94 ^{***}	1.19 ± 0.06 ^{***}	0.48 ± 0.01 ^{***}	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 ^{***}	0.048 ± 0.008 ^{***}	7.34 ± 0.62 ^{***}	1.25 ± 0.12 ^{***}	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.

^{**} Significantly different from wild type hAGT, $p < 0.01$.

^{***} Significantly different from wild type hAGT, $p < 0.001$.

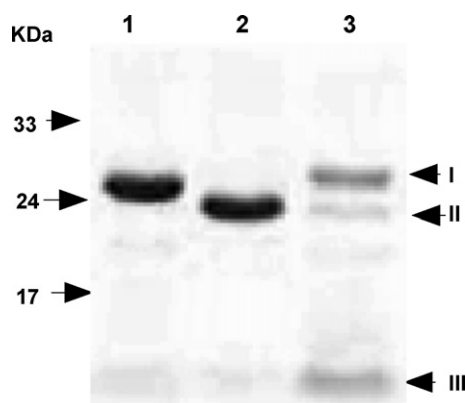


Fig. 3 – Purity of hAGT. N-Tev-hAGT and hAGT were isolated and analyzed by SDS-PAGE as described under Experimental procedures, the gels were stained with Coomassie brilliant blue. Lane 1: N-Tev-hAGT (with N-His₆-tag and Tev recognition site). Lane 2: hAGT (after removal of N-His₆-Tag). Lane 3: eluted 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.

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 carcino-

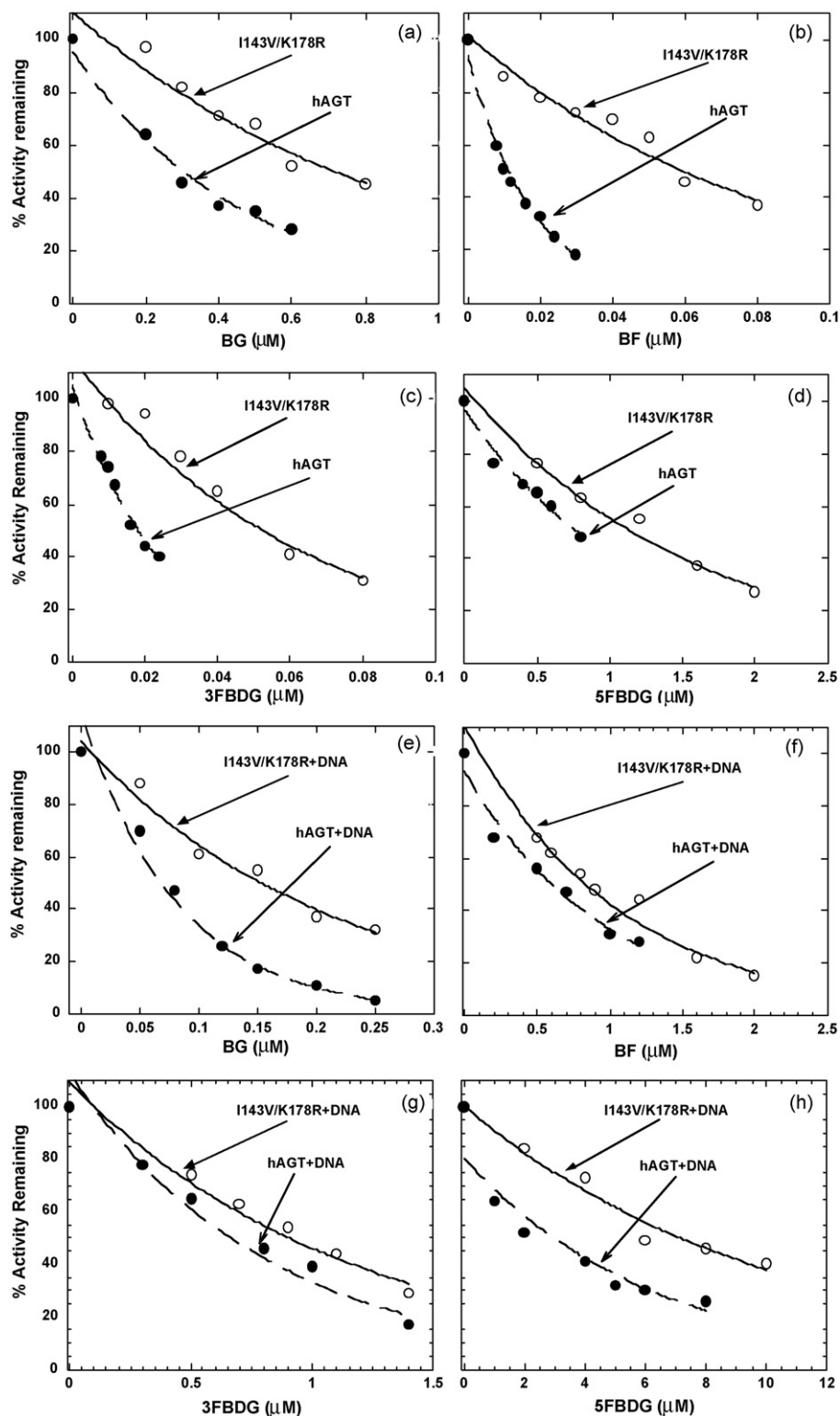


Fig. 4 – Inactivation of hAGT and I143V/K178R variant in the presence or absence of calf thymus DNA. The upper panels show the inhibition graphs in the absence of DNA. Results are shown for hAGT (filled circles), and I143V/K178R (open circles) inactivated by: a, BG; b, BF; c, 3FBDG; d, 5FBDG. The lower 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 3 – Inactivation of I143V and K178R hAGT mutants

Drugs	ED ₅₀ (μM)			
	+DNA		–DNA	
	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.

gens 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 O⁶-methylguanine adducts in DNA.

The purified L84F recombinant protein does not differ in activity from wild type hAGT in the repair of O⁶-methylguanine *in vivo* or *in vitro* or in the relative repair *in vitro* of O⁶-[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 O⁶-n-butylguanine, O⁶-[4-oxo-4-(3-pyridyl)butyl]guanine or BG when these were contained in oligodeoxynucleotides [39]. However, the repair of O⁶-[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 O⁶-[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 (three to four 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 Tyr114, 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 ca. 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.

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