Repair of Oligodeoxyribonucleotides by O^6-Alkylguanine-DNA Alkyltransferase†

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ABSTRACT: Activity of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) is an important source of tumor cell resistance to alkylating agents. AGT inhibitors may prove useful in enhancing chemotherapy. AGT is inactivated by reacting stoichiometrically with O^6-benzylguanine (b^6G), which is currently in clinical trials for this purpose. Short oligodeoxyribonucleotides containing a central b^6G are more potent inactivators of AGT than b^6G. We examined whether human AGT could react with oligodeoxyribonucleotides containing multiple b^6G residues. The single-stranded 7-mer 5′-d[T(b^6G)$_5$G]-3′ was an excellent AGT substrate with all five b^6G adducts repaired although one adduct was repaired much more slowly. The highly b^6G-resistant Y158H and P140K AGT mutants were also inactivated by 5′-d[T(b^6G)$_5$G]-3′. Studies with 7-mers containing a single b^6G adduct showed that 5′-d[TGGG(b^6G)G]-3′ was more poorly repaired by wild-type AGT than 5′-d[T(b^6G)GGGGG]-3′ and 5′-d[TGG(b^6G)GGG]-3′ and was even less repairable by mutants Y158H and P140K. This positional effect was unaffected by interchanging the terminal 5′- or 3′-nucleotides and was also observed with single-stranded 16-mer oligodeoxyribonucleotides containing O^6-methylguanine, where a minimum of four nucleotides 3′ to the lesion was required for the most efficient repair. Annealing with the reverse complementary strands to produce double-stranded substrates increased the ability of AGT to repair adducts at all positions except at positions 2 and 15. Our results suggest that AGT recognizes the polarity of single-stranded DNA, with the best substrates having an adduct adjacent to the 5′-terminal residue. These findings will aid in designing novel AGT inhibitors that incorporate O^6-alkylguanine adducts in oligodeoxyribonucleotide contexts.

The repair of DNA adducts formed at the O^6-position of guanine is an important means of defense against the mutagenic and toxic effects of certain alkylating agents (*1*−*3*). The predominant pathway of such repair is via the activity of O^6-alkylguanine-DNA alkyltransferase (AGT)[1] (*4*−*6*). Repair is accomplished in a direct single reaction in which the alkyl group from the adduct is transferred to a cysteine in the AGT active site. Studies of the crystal structure of AGT suggest that the O^6-alkylguanine is flipped out of the DNA helix and into a binding pocket which contains this active site (*7*, *8*). Although no crystal structures of the protein complexed with a DNA substrate have yet been obtained, these models suggest that the DNA is bound via a winged helix−turn−helix motif, and studies of AGT proteins with mutations in the residues making up this DNA binding domain are consistent with these models. The *S*-alkylcysteine that is formed in the AGT protein via the repair reaction is not regenerated so the protein can therefore act only once. In mammalian cells, the alkylated form of the protein is ubiquitinated and rapidly degraded (*9*, *10*). Thus, substrates of the AGT protein are also inactivators.

Several methylating and chloroethylating agents are used for cancer chemotherapy, and the formation of O^6 adducts in DNA by these agents is a major factor in their ability to kill tumor cells (*11*−*13*). Repair of O^6-methylguanine (m^6G) or O^6-(2-chloroethyl)guanine by AGT protects cells from the cytotoxicity of such methylating and chloroethylating agents, respectively (*4*, *6*). Therefore, attempts are being made to use inactivators of the AGT protein to increase the sensitivity of tumor cells to these agents. The most widely studied compound that is being used for this purpose is O^6-benzylguanine (b^6G) (*11*, *14*). This free base is a substrate for human AGT and is "repaired", forming guanine and *S*-benzylcysteine in the AGT (*15*). Clinical trials are currently in progress to use b^6G and a similar compound, O^6-(5-bromothenyl)guanine, as chemotherapy adjuvants (*16*−*19*). However, the use of these compounds may be limited by their low solubility, relatively low potency, and the inability to inactivate mutant forms of AGT that contain single point mutations that prevent binding to such purine bases.

The reaction of AGT with b^6G is much slower than the reaction with DNA containing m^6G or b^6G residues. This is due to the weak binding of the free base, which cannot interact with the DNA binding domain of AGT (*15*). However, relatively short oligodeoxyribonucleotides that contain a b^6G residue are much better substrates for AGT (*20*−*24*). Previous studies have shown that an 11-mer oligodeoxyribonucleotide containing a single central b^6G and modified with terminal methylphosphonate linkages to protect it from nucleases was a good substrate for AGT with

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; b^6G, O^6-benzylguanine; m^6G, O^6-methylguanine; ss, single-stranded; ds, double-stranded.

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an ED_{50} value more than 100 times lower than b^6G (*25*). When added to the cell culture medium, this oligodeoxyribonucleotide was able to cause a prolonged inactivation of cellular AGT in HT29 cells for at least 72 h and greatly sensitized the cells to killing by a chloroethylating agent (*25*).

These studies provided useful preliminary information that suggested it may be possible to use oligodeoxyribonucleotides as AGT substrates/inhibitors in place of b^6G, but there is only a limited amount of data available on the ability of the AGT protein to act on such compounds. One potential advantage of the oligodeoxyribonucleotide approach is that multiple b^6G adducts could be placed in the same molecule. This could enable a greater number of AGT molecules to be inactivated by a single oligomer. Additionally, properly positioned b^6G residues might limit degradation of oligodeoxyribonucleotides by nucleases while the hydrophobic benzyl residues might improve uptake into cells. Therefore, we have studied the ability of wild-type AGT and the mutants Y158H and P140K to react with a single-stranded (ss) 7-mer 5′-d[T(b^6G)$_5$G]-3′. It was found that this was an excellent substrate for AGT and that all five b^6G adducts could be repaired but that one of the adducts was repaired much more slowly. A detailed investigation of this finding using ss 7-mers containing a single b^6G adduct and ss 16-mers containing a single m^6G adduct at different positions indicated that adducts nearest the 3′-end were poorly repaired. Such adducts were even less repairable with mutants Y158H and P140K. When using double-stranded (ds) oligodeoxyribonucleotides, only the adduct on the penultimate 3′-position of the substrate strand was poorly repaired by wild-type and mutant AGTs. These findings provide new information on the interaction of AGT with substrates and will aid in the synthesis of potent AGT inhibitors that can inactivate mutant forms of AGT resistant to b^6G.

MATERIALS AND METHODS

Materials. Snake venom phosphodiesterase (type II) from *Crotalus adamanteus* and bacterial alkaline phosphatase (type III) from *Escherichia coli* were from Sigma Chemical Co. (St. Louis, MO). Silica gel for column chromatography was Davisil, grade 633, 200−425 mesh, from Aldrich Chemical Co., Inc. (Milwaukee, WI). Other reagents and solvents were from Aldrich or Glen Research (Sterling, VA). O^6-Methylguanine-containing 16-mer oligodeoxyribonucleotides and the corresponding reverse complement strands were purchased from Qiagen Operon (Alameda, CA).

General Methods. ^{1}H NMR spectra were determined on a Varian INOVA 300 or 400 MHz spectrometer. Chemical shifts are reported as δ values in parts per million relative to TMS as internal standard. Positive ion (+ve) fast atom bombardment (FAB) mass spectra (MS) were recorded on a Finnigan Mat 95 spectrometer. UV absorption spectra were measured on a Beckman Coulter DU7400 diode array spectrophotometer. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems, Inc., Model 380B DNA synthesizer on a 10 μmol scale. Oligodeoxyribonucleotides were purified by high-pressure liquid chromatography (HPLC) on a semipreparative 10 mm × 25 cm Hamilton PRP-1 10 μm column from Phenomenex (Torrance, CA) using two Waters 6000A pumps, a Model 660 solvent programmer, a Model 450 variable-wavelength detector, and a Model U6K sample injector. Enzymatic digests of the oligodeoxyribonucleotides were analyzed by chromatography on a 4.6 mm × 25 cm Phenomenex Luna 5 μm C-18(2) column using a Hewlett-Packard 1090 series II liquid chromatography system. Elemental analyses were performed by Atlantic Microlabs, Inc. (Norcross, GA).

O^6-Benzyl-N^2-phenoxyacetyl-2′-deoxyguanosine. This compound was prepared through adaptations of methods described previously (*26*, *27*). O^6-Benzyl-2′-deoxyguanosine (4.1 g, 11.5 mmol) was evaporated twice to dryness from anhydrous pyridine and was dissolved in 40 mL of anhydrous pyridine. Chlorotrimethylsilane (6.3 g, 58 mmol) was added, and the mixture was stirred at room temperature for 30 min. This reaction was then cooled on ice. Separately, 1-hydroxybenzotriazole (3.4 g, 25 mmol) that was twice evaporated to dryness from anhydrous acetonitrile was suspended in 7 mL of dry acetonitrile, and phenoxyacetyl (PAc) chloride (3.2 mL, 23 mmol) was added. A precipitate began to form shortly thereafter. Additional dry pyridine was added to aid transfer of the suspension that was then added to the O^6-benzyl-2′-deoxyguanosine solution. The resulting mixture was stirred overnight at room temperature and was then cooled on ice and treated with 10 mL of H_2O. After 10 min on ice, 20 mL of concentrated ammonium hydroxide was added, and the mixture was allowed to stir on ice for several minutes to remove the trimethylsilyl protecting groups. The solvents were removed by rotary evaporation. The residue was suspended in 250 mL of H_2O and was extracted twice with 150 mL portions of chloroform. The resulting aqueous emulsion was allowed to stand at 4 °C overnight, and the white flocculent material that formed was filtered off, washed with water, and dried at room temperature under vacuum. The dried material was crystallized from ethanol to give 4.4 g (78%) of O^6-benzyl-N^2-phenoxyacetyl-2′-deoxyguanosine: UV [MeOH/10 mM Tris, pH 7.5 (1/9)] λ_{max} 268 nm; ^{1}H NMR (Me_2SO-d_6/TMS) δ 2.30 (m, 1H, H-2′), 2.70 (m, 1H, H-2′), 3.55 (m, 2H, H-5′), 3.86 (m, 1H, H-4′), 4.41 (m, 1H, H-3′), 4.90 (t, 1H, 5′-O*H*, exchanges with D_2O), 5.06 (s, 2H, Bn-CH_2), 5.31 (d, 1H, 3′-O*H*, exchanges with D_2O), 5.63 (s, 2H, PAc-CH_2), 6.35 (t, 1H, H-1′), 6.95−7.55 (m, 10H, PAc-Ar + Bn-Ar), 8.46 (s, 1H, H-8), 10.67 (s, 1H, N*H* exchanges with D_2O); +ve FAB MS 492 ([M + H]$^+$), 514 ([M + Na]$^+$). Anal. Calcd for $C_{25}H_{25}N_5O_6$: C, 61.09; H, 5.13; N, 14.25. Found: C, 61.24; H, 5.21; N, 14.40.

O^6-Benzyl-5′-O-(4,4′-dimethoxytrityl)-N^2-phenoxyacetyl-2′-deoxyguanosine. O^6-Benzyl-N^2-phenoxyacetyl-2′-deoxyguanosine (4.4 g, 9 mmol) was dried three times by evaporation from anhydrous pyridine. The material was then dissolved in 45 mL of anhydrous pyridine under argon. 4-(Dimethylamino)pyridine (122 mg, 1.0 mmol), anhydrous triethylamine (1.4 g, 13.6 mmol), and 4,4′-dimethoxytrityl chloride (4.6 g, 13.6 mmol) were added, and the reaction was stirred at room temperature for 2 h. The reaction was then poured into 200 mL of H_2O and was extracted three times with 200 mL portions of ethyl acetate. The combined ethyl acetate extract was dried with sodium sulfate and evaporated to an orange solid foam. This material was twice chromatographed on a 4 cm × 35 cm silica gel column eluted with ethyl acetate/dichloromethane/triethylamine (4.5:4.5:1). The product-containing fractions were pooled and evaporated to afford 5.9 g (82%) of O^6-benzyl-5′-*O*-(4,4′-dimethoxytrityl)-N^2-phenoxyacetyl-2′-deoxyguanosine as a white solid foam: UV [MeOH/10 mM Tris, pH 7.5 (1/9)] λ_{max} 274 nm; ^{1}H NMR

Table 1: Chromatographic and Physical Properties of Oligodeoxyribonucleotides

sequence	HPLC retention time (min)		nucleoside composition found/(expected)			
	DMT[a] on	DMT[b] off	dGuo	dThd	b^{6}dGuo[c]	ϵ ($\times 10^{-4}$)
5′-d[T(b^{6}G)$_5$G]-3′	60	49	1.15/(1)	1.05/(1)	4.81/(5)	6.8 at 250 nm
5′-d[T(b^{6}G)GGGGG]-3′	39	29, 39[d]	4.93/(5)	1.10/(1)	0.97/(1)	10.9 at 254 nm
5′-d[TGG(b^{6}G)GGG]-3′	36, 39	24	5.06/(5)	1.02/(1)	0.92/(1)	9.5 at 254 nm
5′-d[TGGGG(b^{6}G)G]-3′	37, 45, 51	25	5.05/(5)	0.93/(1)	1.03/(1)	11.0 at 254 nm
5′-d[G(b^{6}G)GGGGT]-3′	38	26	4.98/(5)	1.16/(1)	0.87/(1)	9.2 at 254 nm
5′-d[GGGGG(b^{6}G)T]-3′	35	26	4.90/(5)	1.10/(1)	1.00/(1)	10.0 at 254 nm

[a] Oligodeoxyribonucleotide containing a 4,4′-dimethoxytrityl group on the terminal 5′-nucleotide. [b] Oligodeoxyribonucleotide without a 4,4′-dimethoxytrityl group. [c] b^{6}dGuo = O^6-benzyl-2′-deoxyguanosine. [d] Where significant multiple peaks were observed, the retention time for the major peak is underlined.

(Me_2SO-d_6/TMS) δ 2.35 (m, 1H, H-2′), 2.87 (m, 1H, H-2′), 3.09 (m, 1H, H-5′), 3.27 (m, 1H, H-5′), 3.69 (s, 3H, DMT-O-CH_3), 3.70 (s, 3H, DMT-O-CH_3), 3.98 (m, 1H, H-4′), 4.48 (m, 1H, H-3′), 5.02 (s, 2H, Bn-CH_2), 5.33 (s, 1H, 3′-O*H*, exchanges with D_2O), 5.63 (s, 2H, PAc-CH_2), 6.39 (t, 1H, H-1′), 6.73−7.56 (m, 23H, PAc-Ar, Bn-Ar, and DMT-Ar), 8.35 (s, 1H, H-8), 10.65 (s, 1H, N*H* exchanges with D_2O); +ve FAB MS 816 ([M + Na]$^+$). Anal. Calcd for $C_{46}H_{43}N_5O_8$: C, 69.60; H, 5.46; N, 8.82. Found: C, 69.59; H, 5.63; N, 8.77.

Oligodeoxyribonucleotide Synthesis and Purification. O^6-Benzyl-5′-*O*-(4,4′-dimethoxytrityl)-N^2-phenoxyacetyl-2′-deoxyguanosine was converted to the 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite derivative immediately before use in oligodeoxyribonucleotide synthesis as previously described (*27*). The standard Applied Biosystems, Inc., 10 μmol scale synthesis cycle was used except the O^6-benzyl-2′-deoxyguanosine phosphoramidite derivative was allowed to couple for an additional 15 min. At the end of the synthesis the 4,4′-dimethoxytrityl group was not removed from the last nucleotide in the sequence. The oligodeoxyribonucleotides were cleaved from the solid support by the standard 10 μmol end procedure, and the resulting ammonium hydroxide solution was heated at 55 °C overnight to complete the removal of protecting groups. The ammonium hydroxide was then removed by evaporation.

Each of the dimethoxytrityl-containing oligodeoxyribonucleotides was purified in three batches on a semipreparative 10 mm × 25 cm Hamilton PRP-1 10 μm column. Samples were dissolved in 200 μL of 80% formamide/water and were heated to 70 °C for several minutes. Six hundred microliters of water was added to each sample immediately prior to injection into the column. The solvents for chromatography were aqueous 0.1 M triethylammonium acetate (A) and acetonitrile (B). A gradient of 10−50% solvent B over 60 min at a flow rate of 3 mL/min was run, and absorbance at 270 nm was monitored. Many of the oligodeoxyribonucleotides eluted in more than one major peak despite efforts to minimize aggregate formation by loading in formamide solution (*28*). When this occurred, the multiple peaks were isolated and subsequently treated separately. Retention times for oligodeoxyribonucleotides containing a 4,4′-dimethoxytrityl group on the terminal 5′-nucleotide (DMT-on) are shown in Table 1. Following evaporation to dryness the oligodeoxyribonucleotides were detritylated by treatment with acetic acid/H_2O (8:2 v/v) for 15 min, followed by coevaporation with ethanol under vacuum. The detritylated oligodeoxyribonucleotides were further purified by a second round of chromatography on the system described above using the same formamide loading procedure. Chromatographs were run with a gradient of 5−50% solvent B over 60 min at 3 mL/min. Once again, some of the oligodeoxyribonucleotides gave multiple peaks that were handled separately for digestion and analysis. Retention times for the detritylated samples (DMT off) are also shown in Table 1.

Samples of the oligonucleotides were digested to nucleosides with snake venom phosphodiesterase and alkaline phosphatase. The nucleoside composition was determined in a manner similar to that described previously (*27*), and the extinction coefficients (ϵ) were determined by measuring the number of moles of deoxyribonucleoside liberated by enzymatic digestion of a known number of OD units of the oligodeoxyribonucleotide. These results are summarized in Table 1.

HPLC Analysis. Four hundred picomoles of 5′-d[T(b^{6}G)$_5$G]-3′ was incubated with various amounts of wild-type or mutant AGTs (15−75 μg, His-tagged purified) in 50 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA for 20 min at 37 °C. The reaction was stopped by addition of 6 M urea (final concentration) and was kept at 37 °C for 20 min before separation on a Beckman reverse-phase C-18 Ultrasphere ODS 5 μm column (4.6 × 250 mm) equipped with a Brownlee RP-300 Aquapore 7 μm precolumn (0.46 × 30 mm). Separation was performed at 40 °C using a flow rate of 1 mL/min and a linear gradient of 10−65% methanol in 25 mM sodium phosphate buffer, pH 6.3, over 60 min, and maintaining the eluent at 65% methanol for an additional 15 min. For oligodeoxyribonucleotides with a single b^{6}G adduct, 600 pmol was preheated at 50 °C for 20 min and allowed to cool for 2 min at 25 °C before addition of 50 μg of wild-type or mutant AGTs in 50 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA for 20 min at 37 °C. The reaction was stopped as above, and separation was performed using a linear gradient of 10−65% methanol in 25 mM sodium phosphate buffer, pH 6.3, over 60 min.

Inactivation Assay. Oligos were dissolved in 10 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA. The concentration of b^{6}G-containing oligodeoxyribonucleotides was determined using the measured extinction coefficient (ϵ) at 250 or 254 nm (Table 1). The extinction coefficients for m^{6}G-containing oligodeoxyribonucleotides at 260 nm were provided by the manufacturer (Qiagen Operon). Purified wild-type (50 ng) or mutant AGT proteins (80−100 ng) were incubated with various concentrations of oligodeoxyribonucleotides (0.1−1000 nM) in a 125 μL reaction buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM DTT) containing 50 μg of hemocyanin for 30 min at 37 °C. AGT activity was assayed by addition of ^{3}H-methylated calf thymus DNA substrate

for 30 min at 37 °C as described previously (*29*, *30*). The reaction was stopped by addition of 6 M urea (final) and was filtered through a 25 mm MF-Millipore 0.45 μm pore nitrocellulose membrane. The amount of [^{3}H]methyl transferred from substrate DNA to AGT was quantitated by scintillation counting, and the results were expressed as the percentage of AGT activity remaining as a function of increasing inhibitor concentration. The ED_{50} value, defined as the concentration of inhibitor needed to reduce the activity of AGT by 50%, was determined from graphs of inactivation against inhibitor concentration (*31*).

For the inactivation assay, ds 16-mer oligodeoxyribonucleotide mixtures were annealed by mixing the reverse complement strand with the m^{6}G-containing strand (with C pairing opposite m^{6}G) in a 1:1 molar ratio. This mixture was incubated at 65 °C for 30 min and allowed to cool to 22 °C in a beaker filled with 300 mL water preequilibrated at 65 °C.

Binding Studies. Single-stranded 7-mer oligodeoxyribonucleotides were 5′-end labeled in a 60 μL reaction using [γ-^{35}S]ATPγS and T4 polynucleotide kinase followed by passage through a Sephadex MicroSpin G-25 column (Amersham Pharmacia Biotech) to remove unincorporated [^{35}S]-ATP. Binding reactions were carried out on ice in 10 mM Tris (pH 7.6), 5 mM DTT, and 0.1 mM EDTA using the His-tagged purified C145S AGT mutant protein. Briefly, in a 65 μL stock reaction containing 1 μM ^{35}S-labeled oligos, 10 μg of C145S AGT was added and mixed, and aliquots were taken for serial dilution (0.66/step) from the stock solution using the buffer above. The mixtures were allowed to equilibrate on ice for 30 min. Samples were then loaded onto a prerun, preequilibrated (4 °C) 8% (75:1 acrylamide/bisacrylamide) polyacrylamide gel (15 × 18 cm) and electrophoresed in 10 mM Tris−acetate and 0.25 mM EDTA, pH 7.6, buffer at 175 V for 5 min and then at 100 V for the remaining 30 min. Gels were fixed, vacuum dried, and quantified on a PhosphorImager using the program ImageQuant (Molecular Dynamics). Duplicate runs were done for each oligo. The dissociation constant (K_d) and binding stoichiometry were estimated from the gel mobility shift assay as detailed (*32*). Briefly, if the binding between *n* molecules of AGT protein (P) to 7-mer oligodeoxyribonucleotide (D) is expressed as $nP + D \rightleftharpoons P_nD$, the slope of the plot of ln [PD]/[D] as a function of ln [P] equals the stoichiometry *n*, while at half-saturation, ln [PD]/[D] = 0 and the K_d can be determined from the expression ln $K_n = -n$ ln [P].

RESULTS

Synthesis and Characterization of 5′-d[T(b^{6}G)$_5$G]-3′. A ss 7-mer oligodeoxyribonucleotide with five b^{6}G residues, 5′-d[T(b^{6}G)$_5$G]-3′, was synthesized and characterized. Synthesis was facilitated by incorporating a more base-labile phenoxyacetyl protecting group (*26*, *27*) at the N^2-position of the *O*^{6}-benzyl-2′-deoxyguanosine intermediates used in automated DNA synthesis. This made it possible to deprotect all of the amino groups in the final DNA segments (Table 1) using aqueous ammonia solutions rather than more complicated mixtures of amines (*25*) or sodium hydroxide solutions.

Preparative HPLC purification of the guanine-rich sequences (Table 1) proved cumbersome since several sequences eluted as more than one significant peak due to aggregate or complex formation. To minimize this aggregation, formamide was added to the samples immediately before the samples were loaded onto the HPLC system (*28*). Although this usually led to elution of sequences in one major peak, other peaks of significance were still observed in some cases (Table 1). When this occurred, the various peaks were collected separately. However, they all exhibited the same nucleoside composition following enzymatic digestion to their component deoxyribonucleosides (Table 1). Analytical HPLC runs were carried out after urea was added to the prewarmed oligodeoxyribonucleotide samples immediately before the samples were loaded on the HPLC system (see Materials and Methods). This caused each oligodeoxyribonucleotide to elute as a single significant peak (see below).

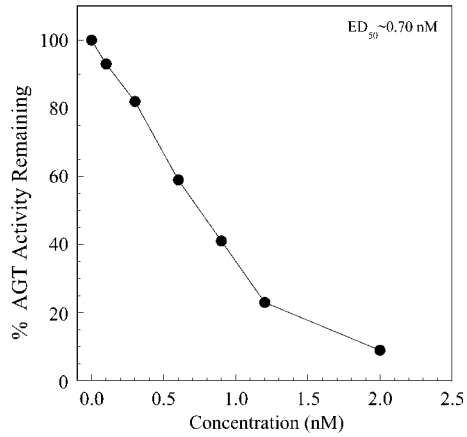


FIGURE 1: Inactivation of wild-type AGT by 5′-d[T(b^{6}G)$_5$G]-3′. Purified AGT was incubated with various concentrations of 5′-d[T(b^{6}G)$_5$G]-3′ for 30 min at 37 °C in 125 μL of solution containing 50 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1 mM EDTA, and 50 μg of hemocyanin. The remaining alkyltransferase activity was measured by a filter-binding assay as described under Materials and Methods.

Reaction of AGT with 5′-d[T(b^{6}G)$_5$G]-3′. The ability of 5′-d[T(b^{6}G)$_5$G]-3′ to inhibit AGT activity was examined by incubating various concentrations with a small amount of AGT (50−100 ng) for 30 min and then measuring the AGT activity remaining (Figure 1). It was found that 5′-d[T(b^{6}G)$_5$G]-3′ was a potent inactivator of wild-type AGT with an ED_{50} value of 0.7 nM (Figure 1), which is 2 orders of magnitude less than the ED_{50} value for b^{6}G (*14*). The oligo 5′-d[T(b^{6}G)$_5$G]-3′ was also effective in inhibiting AGT mutants Y158H and P140K that are highly resistant to b^{6}G, although the ED_{50} values for these mutants were considerably greater than for wild-type AGT. These values for Y158H and P140K AGT mutants, which were 73 and 440 nM, respectively, are at least 4 orders of magnitude less than those for b^{6}G (Table 2).

The products of the reaction of 5′-d[T(b^{6}G)$_5$G]-3′ with wild-type, Y158H, or P140K AGT were analyzed by HPLC (Figure 2). Two major products were seen when wild-type AGT was used. One of these was the completely debenzylated 7-mer product 5′-d[T(G)$_6$]-3′, and based on the elution position and the UV absorbance spectrum, the other was a 7-mer containing a single b^{6}G residue. Other intermediate repair products of 5′-d[T(b^{6}G)$_5$G]-3′ could be detected, especially when 5′-d[T(b^{6}G)$_5$G]-3′ was incubated with only 15 or 30 μg of Y158H or P140K. These intermediate groups of peaks are numbered 1−5 in Figure 2 (panel F). The peaks

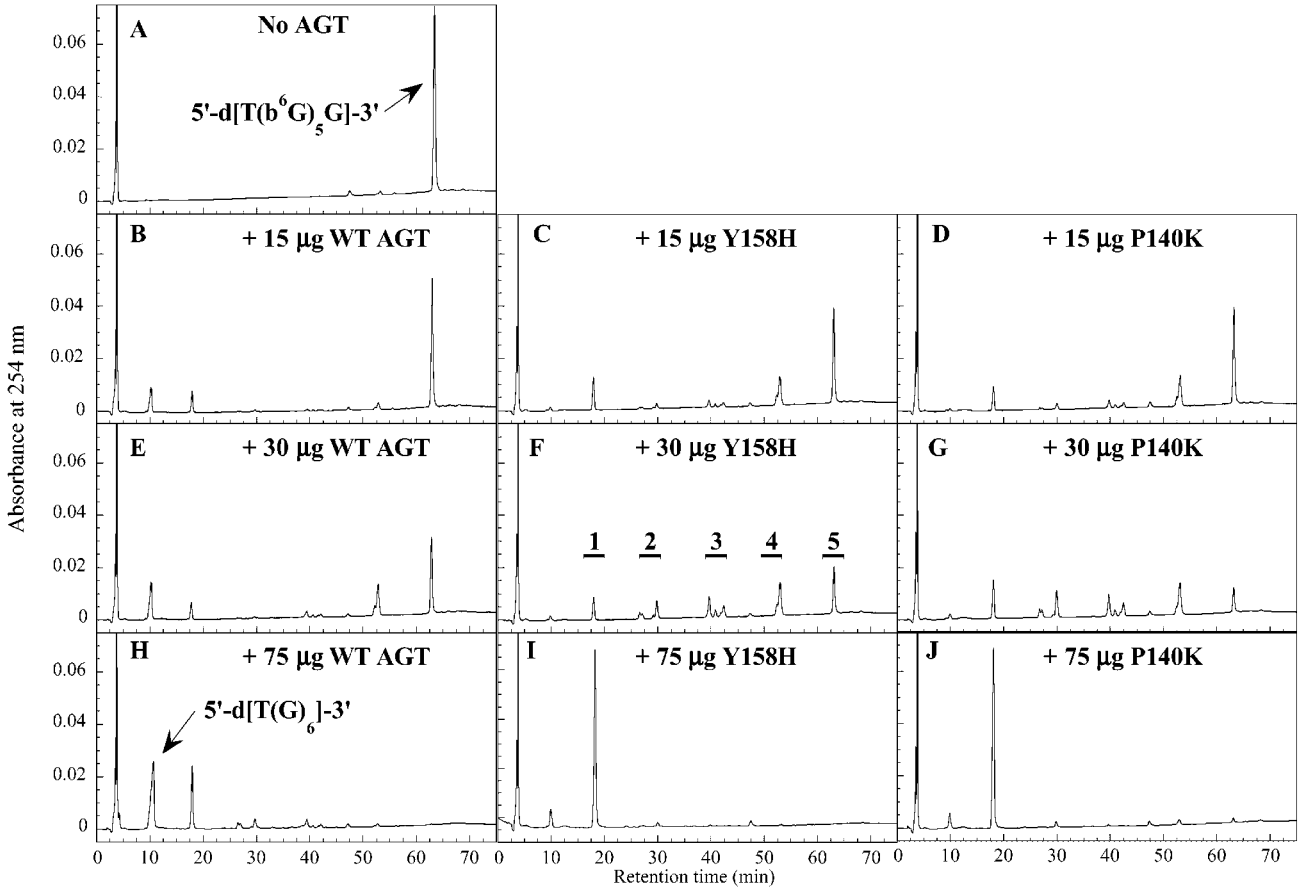


FIGURE 2: HPLC profile for the repair of 5′-d[T(b^6G)$_5$G]-3′ by wild-type, Y158H, and P140K AGT. After 400 pmol of 5′-d[T(b^6G)$_5$G]-3′ was incubated with the amount of AGT protein shown for 20 min at 37 °C, the products were separated by HPLC on a reverse-phase C-18 Ultrasphere ODS column as detailed in the text. Panel A shows the results for 5′-d[T(b^6G)$_5$G]-3′ with no AGT added. Groups of peaks representing intermediate repair products of 5′-d[T(b^6G)$_5$G]-3′ are indicated in panel F. A peak corresponding to an authentic marker of the repaired product 5′-d[T(G)$_6$]-3′ is indicated in panel H.

Table 2: Inactivation of Wild-Type AGT and b^6G-Resistant AGT Mutants by ss 7-mer Oligodeoxyribonucleotides Containing b^6G Residue(s)

	ED_{50} value (nM)		
compound	wild type	Y158H	P140K
b^6G	200	630000	>1200000
5′-d[T(b^6G)$_5$G]-3′	0.7	73	440
5′-d[T(b^6G)GGGGG]-3′	5.0	38	330
5′-d[TGG(b^6G)GGG]-3′	3.7	28	11700
5′-d[TGGGG(b^6G)G]-3′	104	165000	>200000
5′-d[G(b^6G)GGGGT]-3′	3.2	41	650
5′-d[GGGGG(b^6G)T]-3′	82	2080	>200000

likely represent unrepaired b^6G residues remaining in the 7-mer, as the retention times seem to decrease with the removal of each hydrophobic b^6G residue. The UV absorbance spectra for these peaks are consistent with this analysis (data not shown). Groups 2−4 contained multiple peaks (Figure 2, panel F), and this could indicate partial resolution of 7-mers in which the remaining b^6Gs are at different positions.

Since about half of the substrate 5′-d[T(b^6G)$_5$G]-3′ was converted to 5′-d[T(G)$_6$]-3′ by 75 μg of wild-type AGT during a 20 min incubation (Figure 2, panel H), it seemed that all five b^6G residues of 5′-d[T(b^6G)$_5$G]-3′ were repaired. Indeed, complete repair of 5′-d[T(b^6G)$_5$G]-3′ to 5′-d[T(G)$_6$]-3′ could be achieved with excess (>85 μg) wild-type protein (results not shown). However, with 75 μg of the Y158H and P140K AGT mutants, the final reaction product was a 7-mer other than 5′-d[T(G)$_6$]-3′ (Figure 2, panels I and J), showing that excess amounts of Y158H and P140K could not convert 5′-d[T(b^6G)$_5$G]-3′ to the completely debenzylated product within the time periods used. As described above, the product formed was likely to be an oligodeoxyribonucleotide containing at least one b^6G residue.

Reaction of AGT with 7-mers Containing a Single b^6G Residue. To determine which of the five b^6G residues in 5′-d[T(b^6G)$_5$G]-3′ might be less efficiently repaired, three ss 7-mers containing a single b^6G residue at either the second, fourth, or sixth nucleotide position were synthesized. HPLC analysis showed that although a 20 min incubation with 50 μg of wild-type, Y158H, or P140K AGT could repair all three oligodeoxyribonucleotides, the Y158H and P140K AGT mutants did not repair the majority of 5′-d[TGGGG(b^6G)G]-3′ under the assay conditions (Figure 3, panels J and M). This suggests that the residual product (peak with retention time ∼18 min) in the experiment with 5′-d[T(b^6G)$_5$G]-3′ shown in Figure 2 (panels H−J) is 5′-d[TGGGG(b^6G)G]-3′. This conclusion is consistent with the HPLC separation of the three 7-mer oligodeoxyribonucleotides in Figure 3. The retention time for 5′-d[TGGGG(b^6G)G]-3′ is 18 min (Figure 3, panel D) and corresponded exactly to the unrepaired products seen in Figure 2 (panels H−J). The retention times for 5′-d[T(b^6G)GGGGG]-3′ (Figure 3, panel B) and 5′-d[TGG(b^6G)GGG]-3′ (Figure 3, panel C) were 21 and 22 min, respectively.

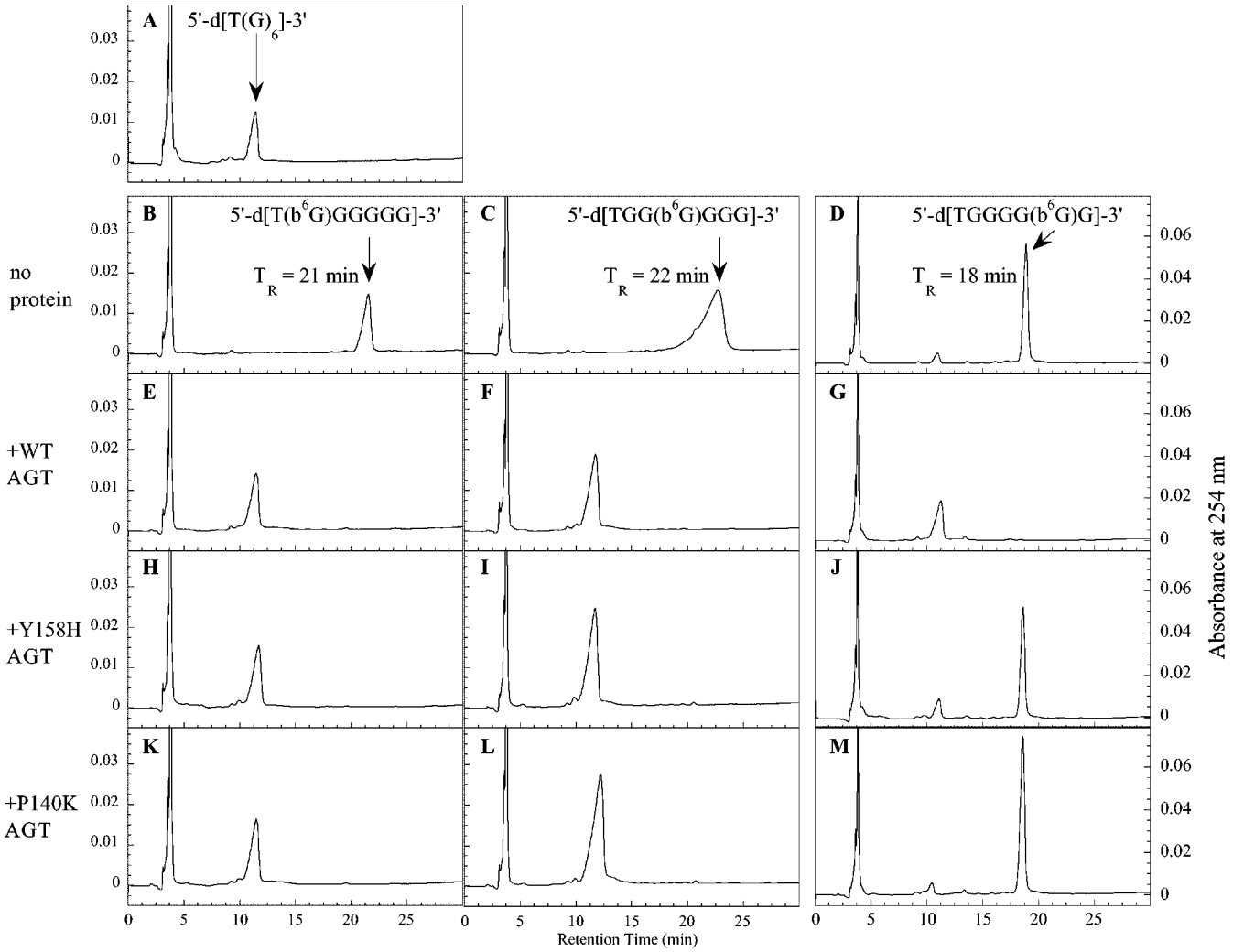


FIGURE 3: HPLC profile for the repair of 5′-d[T(b^{6}G)GGGGG]-3′, 5′-d[TGG(b^{6}G)GGG]-3′, and 5′-d[TGGGG(b^{6}G)G]-3′ by wild-type, Y158H, and P140K AGT. After 600 pmol of the indicated oligodeoxyribonucleotides was incubated with 50 μg of AGT for 20 min at 37 °C, the products were separated by HPLC on a reverse-phase C-18 Ultrasphere ODS column as detailed in the text. The peak corresponding to the repair product 5′-d[T(G)$_6$]-3′ is indicated in panel A. Panels B−D show the results for the oligodeoxyribonucleotides with no AGT added. T_R = retention time.

Studies of the inactivation of AGT when it was incubated with an excess of these 7-mers containing a single b^{6}G residue are also in agreement with the above observation that 5′-d[TGGGG(b^{6}G)G]-3′ seemed to be a poor substrate for AGT. 5′-d[TGGGG(b^{6}G)G]-3′ was a poor inactivator of wild-type AGT with an ED_{50} value of about 100 nM, which is about 20 times greater than the ED_{50} values for 5′-d[T(b^{6}G)GGGGG]-3′ and 5′-d[TGG(b^{6}G)GGG]-3′, i.e., 3.7 and 5 nM, respectively (Table 2). This difference was even more striking when the Y158H and P140K mutant AGTs were used. The ED_{50} values for inactivation of these mutants by 5′-d[TGGGG(b^{6}G)G]-3′ were 165 and >200 μM, respectively. With the P140K mutant, the most effective inhibitor was 5′-d[T(b^{6}G)GGGGG]-3′ (ED_{50} of 330 nM), whereas for the Y158H mutant, 5′-d[T(b^{6}G)GGGGG]-3′ and 5′-d[TGG(b^{6}G)GGG]-3′ were equally effective (ED_{50} of 28 and 38 nM, respectively).

This positional effect in the repair reaction by wild-type AGT was not due to the nature of the 5′- or 3′-terminal nucleotide adjacent to the b^{6}G residue. Similar ED_{50} values (5 and 3.2 nM, respectively) were seen when wild-type AGT was inactivated with either 5′-d[T(b^{6}G)GGGGG]-3′ or 5′-d[G(b^{6}G)GGGGT]-3′. The corresponding ED_{50} values for 5′-d[TGGGG(b^{6}G)G]-3′ and 5′-d[GGGGG(b^{6}G)T]-3′ were 104 and 82 nM, respectively, for wild-type AGT (Table 2). The nature of the 5′-nucleotide also had no effect on the inactivation of P140K and Y158H AGTs by 5′-d[T(b^{6}G)-GGGGG]-3′ and 5′-d[G(b^{6}G)GGGGT]-3′. There was an increase in the ED_{50} value for Y158H when 5′-d[TGGGG-(b^{6}G)G]-3′ was used instead of 5′-d[GGGGG(b^{6}G)T]-3′.

Reaction of AGT with 16-mers Containing a Single m^{6}G Residue. The positional effect was not specific for b^{6}G residues and was not limited to short oligodeoxyribonucleotides. Single-stranded 16-mer oligodeoxyribonucleotides containing *O*^{6}-methylguanine (m^{6}G) at different positions were synthesized and tested for their ability to inactivate AGT. As shown in Figure 4, inactivation of AGT required much higher concentrations of the oligodeoxyribonucleotide when the m^{6}G was located near the 3′-end. The ED_{50} values for the inactivation of wild-type AGT with these ss 16-mers with m^{6}G at positions 2, 6, 11, and 12 from the 5′-end were between 9 and 21 nM for wild-type AGT (Table 3). The ED_{50} values for the 16-mers with m^{6}G at positions 13 and 14 from the 5′-end were 54.5 and 57 nM, respectively. When

Table 3: Inactivation of Wild-Type AGT and b^{6}G-Resistant AGT Mutants by ss and ds 16-mer Oligodeoxyribonucleotides Containing m^{6}G at Different Positions

		ED_{50} (nM)					
		WT		Y158H		P140K	
position	O^6-methylated oligos	ss	ds	ss	ds	ss	ds
2	5′-d[T(m^{6}G)CGTGAAGTGAGTGA]-3′	9.5	7.3	75	71	105	161
6	5′-d[TGCGT(m^{6}G)AAGTGAGTGA]-3′	13.0	5.3	137	13	374	13
11	5′-d[TGCGTGAAGT(m^{6}G)AGTGA]-3′	11.5	4.2	81	12	240	13
12	5′-d[TGCGTGAAGTG(m^{6}G)ATGA]-3′	20.8	9.5	244	17	341	18
13	5′-d[TGCGTGAAGTGA(m^{6}G)TGA]-3′	54.5	7.4	382	25	513	29
14	5′-d[TGCGTGAAGTGAG(m^{6}G)TA]-3′	57.0	9.2	890	29	964	33
15	5′-d[TGCGTGAAGTGAGT(m^{6}G)A]-3′	>50000	>50000	>50000	nd	>50000	nd

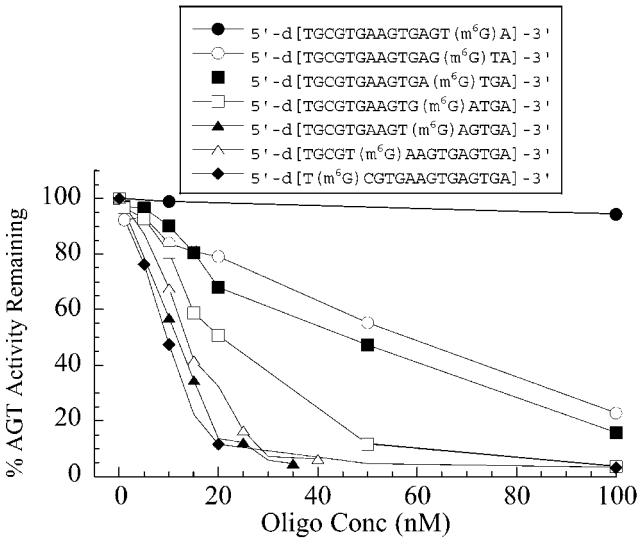


FIGURE 4: Inactivation of wild-type, Y158H, and P140K AGT by ss 16-mer oligodeoxyribonucleotides containing m^{6}G residue at different positions. Purified His-tagged AGT was incubated with various concentrations of 16-mer oligodeoxyribonucleotides, and the remaining alkyltransferase activity was determined as outlined in the legend for Figure 1 and in the text.

the m^{6}G was at position 15 (i.e., adjacent to the 3′-terminal residue), there was a huge reduction in the ability to interact with AGT, leading to an ED_{50} value of >50 μM.

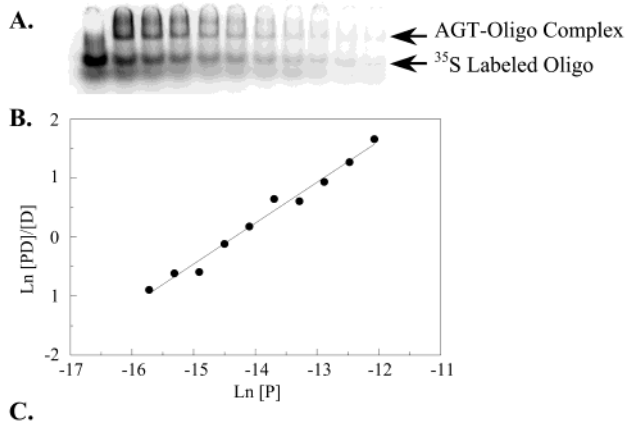
The corresponding ED_{50} values of these ss 16-mers for the Y158H and P140K AGT mutants were at least 7−9-fold (respectively) higher than for the wild-type protein when an m^{6}G residue was at positions 2−14 from the 5′-end. The two mutants showed a similar trend in that higher concentrations of the 16-mers were required for inactivation as the m^{6}G was located nearer to the 3′-end. It is particularly noteworthy that, with both WT and the mutant AGTs, the ss 16-mer that reacted most readily was that which had the m^{6}G closest to the 5′-end in position 2 (Table 3).

For all positions of the m^{6}G lesion, except those at positions 2 and 15 which are adjacent to the termini, annealing with the reverse complement 16-mer (with C pairing opposite m^{6}G) to generate a ds substrate increased the ability to react with AGT (Table 3). The ED_{50} for 16-mers containing m^{6}G located at positions 3−14 from the 5′-end for wild-type AGT ranged from 4.2 to 10 nM for ds compared to 11.5−57 nM for ss. This difference in the ED_{50} between ss and ds 16-mers was even more apparent with the Y158H and P140K mutants. For these mutants, the ED_{50} values for ds 16-mers with m^{6}G located at these positions ranged from 12 to 29 nM compared to 81−964 nM with ss substrates. The largest differences in the ED_{50} were observed when ss and ds 16-mers were compared with an m^{6}G adduct located closer to the 3′-end. For wild-type AGT, there was about a 6-fold difference between the ED_{50} for ss and ds 16-mers with m^{6}G located at position 14 (57 vs 9 nM). For the Y158H and P140K mutants, there was about a 30-fold difference between the ED_{50} for ss and ds 16-mers with m^{6}G located at position 14 (890 vs 29 nM and 964 vs 33 nM, respectively). For ss 16-mers with m^{6}G residues at different positions, the ED_{50} began to increase gradually, starting with the substrate with m^{6}G at position 12. The ED_{50} progressively increased as the m^{6}G residue was positioned closer to the 3′-end, culminating in a poorly repaired substrate with m^{6}G at position 15 (ED_{50} > 50 μM). In contrast, ds 16-mer oligodeoxyribonucleotides with m^{6}G at different positions had ED_{50} values that fell into a relatively narrow range and did not increase as the m^{6}G residue was located closer to the 3′-end until an abrupt change at position 15. Only when the m^{6}G residue is at position 15 did the ED_{50} for the ds 16-mer increase dramatically to >50 μM. This value is similar to that of the ss 16-mer.

Binding of AGT to 7-mers Containing b^{6}G. A gel mobility shift assay was used to determine if a difference in the affinity of AGT for the 7-mer oligodeoxyribonucleotides could account for the less efficient repair and higher ED_{50} of 5′-d[TGGGG(b^{6}G)G]-3′. This experiment was carried out with the inactive AGT mutant C145S since repair of the oligodeoxyribonucleotide by the active wild-type AGT would prevent this analysis. A representative serial dilution gel mobility shift assay is shown in Figure 5A. The plot of ln [PD]/[D] as a function of ln [P] (Figure 5B) was used to estimate the stoichiometry n and the K_d as detailed elsewhere (*32*). The value of n for the ss 7-mer oligodeoxyribonucleotides ranged from 0.6 to 0.8. Except for the ss 7-mer with a b^{6}G residue in the central position (5′-d[TGG(b^{6}G)GGG]-3′), which surprisingly was bound less tightly, the K_d values for the oligodeoxyribonucleotides were not significantly different from each other or from that for the oligodeoxyribonucleotide 5′-d[T(G)$_6$]-3′, which lacks adducts (Figure 5C), and were in the expected range on the basis of previous studies (*32*). Thus, there is no correlation between binding and the ability to serve as a substrate.

DISCUSSION

Previous studies have shown that short 5−9-mer ss oligodeoxyribonucleotides containing a b^{6}G residue were more effective than b^{6}G itself in inhibiting wild-type AGT and b^{6}G-resistant AGT mutants (*24*, *25*). The first objective of our studies was to determine whether AGT was able to recognize and react with a substrate that contains multiple adjacent b^{6}G residues. Such a substrate would have a



C.

Oligo	K_d (μM)
5'-d(TGGGGGG)-3'	3.9
5'-d[T(b^6G)$_5$G]-3'	3.7
5'-d[T(b^6G)GGGGG]-3'	2.9
5'-d[TGG(b^6G)GGG]-3'	25
5'-d[TGGGG(b^6G)G]-3'	4.4

FIGURE 5: Gel mobility shift assay analysis of AGT binding to ss 7-mer oligodeoxyribonucleotides. (A) Representative serial dilution (0.66/step) of C145S AGT and the ^{35}S-labeled oligodeoxyribonucleotide mixture in 10 mM Tris (pH 7.6), 5 mM DTT, and 0.1 mM EDTA. (B) Representative plot of ln [PD]/[D] as a function of ln [P] for the estimation of the binding affinity (K_d) for the AGT−oligodeoxyribonucleotide interaction. The solid line is a linear least-squares fit of the data. [PD] = protein−DNA concentration; [D] = DNA concentration; [P] = protein concentration. (C) Binding affinity of AGT mutant C145S to ss 7-mer oligodeoxyribonucleotides.

significant advantage as a therapeutic inactivator of AGT since multiple molecules of AGT could be inactivated by the same oligodeoxyribonucleotide. Our results show clearly that, despite the unusual nature of 5′-d[T(b^6G)$_5$G]-3′, it is a very good substrate and inactivator of wild-type AGT and is the first compound reported to have an ED_{50} value of <1 nM. Furthermore, the inactivation of the mutants Y158H and P140K with ED_{50} values of less than 0.5 μM represents a significant advance since these mutant AGTs cannot be effectively inactivated by single purine base inactivators (*29*, *33*, *34*). However, although 5′-d[T(b^6G)$_5$G]-3′ was the most active compound tested on wild-type AGT, the inactivation of Y158H and P140K by 5′-d[T(b^6G)GGGGG]-3′ was as effective as that by 5′-d[T(b^6G)$_5$G]-3′ when tested under conditions where the oligodeoxyribonucleotide was in excess.

This difference is likely to be related to the second significant finding of our studies, which is that AGT requires several bases on the 3′-side of the lesion when acting on ss oligodeoxyribonucleotides but works very effectively on adducts that are close to the 5′-end. This effect was seen with wild-type AGT but is even more striking when the Y158H and P140K mutants were examined. Rapid reaction with AGT therefore probably requires the protein to bind the oligodeoxyribonucleotide substrate in such a way that there are additional contacts with the DNA on the 3′-side of the lesion.

Previous studies in which oligodeoxyribonucleotide substrates have been used with AGT have placed the adduct to be repaired in a central position (*22*, *24*, *25*, *35*−*39*), but our studies show that this is not necessary so long as positions near the 3′-end are avoided. The presence of one or more b^6G at the 5′-end of a short ss oligodeoxyribonucleotide may also be useful in preventing its degradation by 5′-endonucleases (*40*−*42*).

Although we first observed this difference with oligodeoxyribonucleotides containing b^6G residues, it also applies to ss 16-mer oligodeoxyribonucleotides with m^6G residues, as the ED_{50} values for the inactivation of wild-type and mutant AGTs increase as the residue with the adduct is positioned closer to the 3′-end. The oligodeoxyribonucleotide in which m^6G is at position 15 was a very poor substrate (Table 3). The results with m^6G at positions 12−14 suggest that AGT somehow recognizes the polarity of ss DNA and requires a minimum of four nucleotides 3′ to an m^6G residue for efficient repair of m^6G residues.

Except when the m^6G residue was at position 2 or 15 in a 16-mer, wild-type AGT and the Y158H and P140K mutants were more efficient at repairing the lesion in the context of ds compared to ss oligodeoxyribonucleotide. This finding of an enhanced efficiency of wild-type and mutant AGTs to repair m^6G residues in a ds oligodeoxyribonucleotide context irrespective of the position of the lesion (except at the penultimate 3′- and 5′-positions) is consistent with biological studies showing that ds DNA is the natural substrate for AGTs in vivo. It is also consistent with in vitro work showing that AGT has a higher affinity for ds oligodeoxyribonucleotides (*23*) than ss and that the protein prefers ds B DNA over Z DNA (*43*), DNA·RNA hybrids (*44*), and ss DNA (*6*, *36*, *45*). It has been reported that the inactivation of AGT by 25-mer oligodeoxyribonucleotides containing an O^6-alkylguanine adduct at position 7 was not greater when ds rather than ss substrates were tested (*22*). Our results are not in agreement with this report, and there is no obvious reason for this discrepancy. However, the differences with lesions at such positions are quite small with wild-type AGT (see Table 3) and may not have been obvious with the relatively small number of observations made in the previous study.

In the case of the m^6G present at position 15, it is possible that the apparent lack of effect when the complementary 16-mer was added is due to the fact that the ends of these substrates are not annealed well enough to be truly double stranded since there is only one adjacent residue to form hydrogen bond base pairing. This interaction may not be stable enough to maintain the duplex structure at the ends. This possibility is supported by the absence of any difference between the ds and ss substrates when the m^6G was in either position 2 (which is a good substrate) or position 15 (which is a very poor substrate) (Table 3).

The oligodeoxyribonucleotides containing a single b^6G used in this study contain a large percentage of guanine residues because they were made to follow up the observations on 5′-d[T(b^6G)$_5$G]-3′. It is known that guanine-rich sequences can form complex secondary structures such as quadruplexes and that these structures may have profound effects on protein binding (*46*−*48*). Such secondary structure may contribute to some of the anomalous behavior on chromatography described in the text. It is also quite possible that the formation of secondary structures affects the ability

of AGT to repair the 7-mers with a single b^{6}G residue and the ability of the C145S mutant to bind to them. This may account for the reduced binding of the C145S mutant to 5′-d[TGG(b^{6}G)GGG]-3′ since the structure may depend on the location of the adduct. However, it is unlikely that it plays any major role in the preference for adducts located away from the 3′-end seen in these studies since this preference was also found in studies with the 16-mers containing a single m^{6}G residue. Only a slight sequence specificity (at most a factor of 4 with a guanine residue 5′ to the lesion slightly reducing the rate of reaction) was seen in the repair of *O*^{6}-methylguanine or *O*^{6}-ethylguanine by AGT in oligodeoxyribonucleotides with a central adduct (*49*−*51*). As shown in Table 2, altering the 5′ or 3′ base did not affect the results for the repair of b^{6}G in the 7-mers with b^{6}G at positions 2 or 6, and the results with the 16-mers containing m^{6}G which do not have contiguous guanine residues also show that repair occurs much more readily with the adduct at position 2 (Table 3).

Our results show clearly that oligodeoxyribonucleotides containing multiple adducts repairable by AGT can be used as inhibitory "pseudosubstrates" for the protein at very low concentrations and can inactivate multiple molecules of the protein. They therefore may provide a useful alternative to the use of free bases such as b^{6}G itself (*11*), *O*^{6}-cyclopentenylmethylguanine (*34*), or *O*^{6}-(5-bromothenyl)guanine (*52*) to enhance the chemotherapeutic activity of alkylating agents if potential problems of delivery and stability can be solved.

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