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Kinetics of O^6-Pyridyloxobutyl-2′-deoxyguanosine Repair by Human O^6-alkylguanine DNA Alkyltransferase

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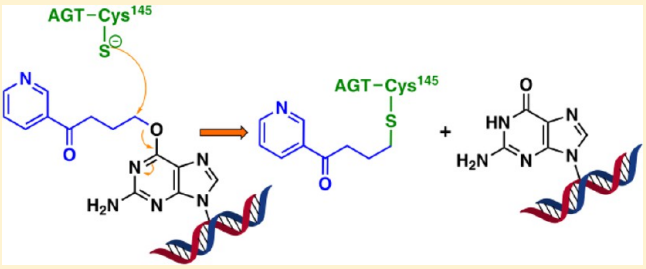
Supporting Information

ABSTRACT: Tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonicotine (NNN) are potent carcinogens believed to contribute to the development of lung tumors in smokers. NNK and NNN are metabolized to DNA-reactive species that form a range of nucleobase adducts, including bulky O^6-[4-oxo-4-(3-pyridyl)-but-1-yl]deoxyguanosine (O^6-POB-dG) lesions. If not repaired, O^6-POB-dG adducts induce large numbers of G → A and G → T mutations. Previous studies have shown that O^6-POB-dG can be directly repaired by O^6-alkylguanine-DNA alkyltransferase (AGT), which transfers the pyridyloxobutyl group from O^6-alkylguanines in DNA to an active site cysteine residue within the protein. In the present study, we investigated the influence of DNA sequence context and endogenous cytosine methylation on the kinetics of AGT-dependent repair of O^6-POB-dG in duplex DNA. Synthetic oligodeoxynucleotide duplexes containing site-specific O^6-POB-dG adducts within *K-ras* and *p53* gene-derived DNA sequences were incubated with recombinant human AGT protein, and the kinetics of POB group transfer was monitored by isotope dilution HPLC-ESI$^+$-MS/MS analysis of O^6-POB-dG remaining in DNA over time. We found that the second-order rates of AGT-mediated repair were influenced by DNA sequence context (10-fold differences) but were only weakly affected by the methylation status of neighboring cytosines. Overall, AGT-mediated repair of O^6-POB-dG was 2−7 times slower than that of O^6-Me-dG adducts. To evaluate the contribution of AGT to O^6-POB-dG repair in human lung, normal human bronchial epithelial cells (HBEC) were treated with model pyridyloxobutylating agent, and O^6-POB-dG adduct repair over time was monitored by HPLC-ESI$^+$-MS/MS. We found that HBEC cells were capable of removing O^6-POB-dG lesions, and the repair rates were significantly reduced in the presence of an AGT inhibitor (O^6-benzylguanine). Taken together, our results suggest that AGT plays an important role in protecting human lung against tobacco nitrosamine-mediated DNA damage and that inefficient AGT repair of O^6-POB-dG at a specific sequences contributes to mutational spectra observed in smoking-induced lung cancer.



Nicotine-derived nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonicotine (NNN)[1] are among the most potent lung carcinogens present in cigarette smoke. Cytochrome P450-mediated hydroxylation of the α-methylene position of NNK produces methyl diazonium ions, while hydroxylation of the methyl group of NNK generates pyridyloxobutyl diazonium ions (Scheme 1).[2−5] Pyridyloxobutyl diazonium ions are also formed upon metabolic activation of NNN.[2,6] Methyl diazonium and pyridyloxobutyl diazonium ions are strongly electrophilic species that modify multiple positions of DNA to give a range of nucleobase adducts, including N7-methyl-deoxyguanosine (N7-Me-dG), O^6-methyl-deoxyguanosine (O^6-Me-dG), N7-[4-oxo-4-(3-pyridyl)-but-1-yl]deoxyguanosine (N7-POB-dG), O^6-[4-oxo-4-(3-pyridyl)but-1-yl]deoxyguanosine (O^6-POB-dG), O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O^2-POB-T), O^2-[4-(3-pyridyl)-4-oxobut-1-yl]-deoxycytidine (O^2-POB-dC), and O^4-methyl-deoxythymidine (O^4-Me-dT).[7] Among these, O^6-methyl-deoxyguanosine (O^6-Me-dG) and O^6-pyridyloxobutyl-deoxyguanosine (O^6-POB-dG) (Scheme 1) are known to be strongly miscoding lesions that are thought to contribute to NNK-mediated lung tumor formation in A/J mice.[6,8−11]

O^6-Alkylguanine-DNA alkyltransferase (AGT) protein can directly remove the O^6-alkyl group from O^6-alkylguanines in

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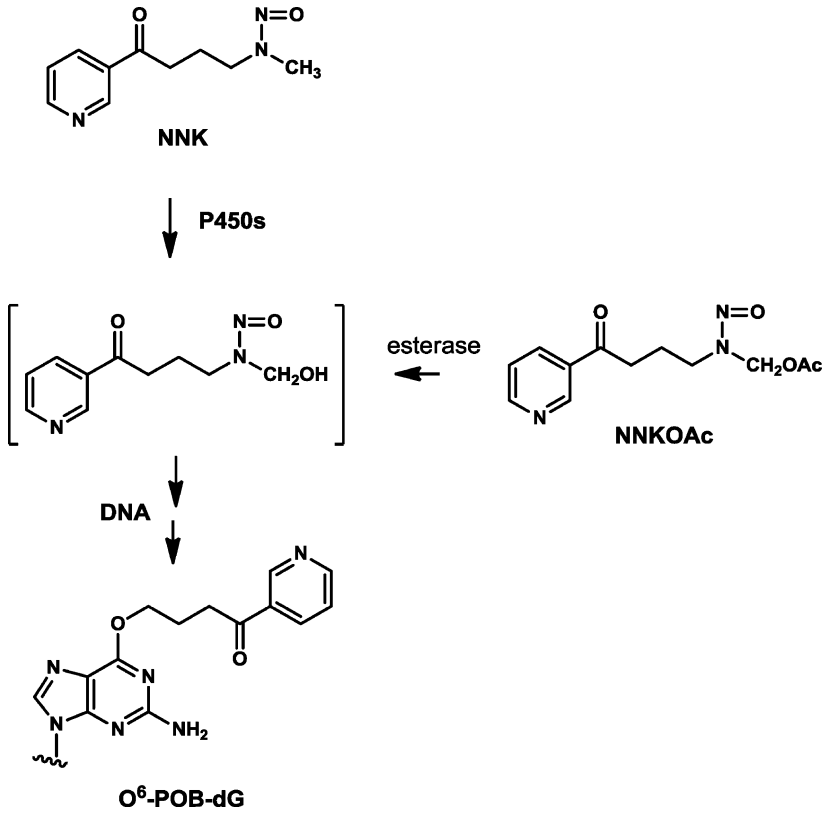
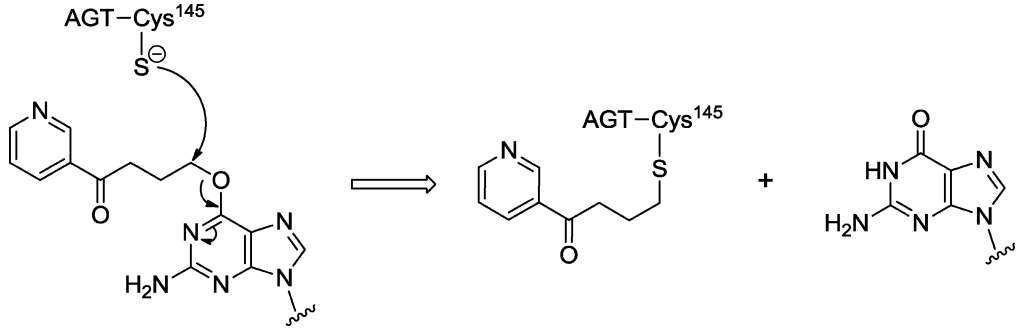
Scheme 1. Metabolic Activation of NNK and the Formation of O^6-Pyridyloxobutyl-dG Adducts



Scheme 2. AGT Repair of O^6-Pyridyloxobutyl-dG Adducts



DNA, restoring normal guanine (Scheme 2).[12−14] AGT protein binds to the minor groove of DNA via the helix−turn−helix motif, inducing flipping of O^6-Alk-dG out of the DNA helix to enter the protein active site.[12,15,16] Cysteine-145 thiol within the active site of the AGT protein is deprotonated via interactions with other active site residues, and the resulting Cys-145 thiolate anion undergoes nucleophilic attack at the α-carbon of the O^6-alkyl group, leading to its transfer from DNA to the protein (Scheme 2).[12,15] Alkylation of Cys145 inactivates the AGT protein and destabilizes its structure, signaling for protein ubiquitination and proteasomal degradation.[17,18] Since the protein is not regenerated following repair reaction, AGT acts nonenzymatically but rather as a stoichiometric reactant, with one molecule of protein used up per each adduct repaired.[14] Recent studies suggest that AGT binds DNA to form cooperative clusters; this cooperative DNA binding appears to be important for lesion search and/or repair.[19,20]

Previous studies by several laboratories, including ours, have revealed that the efficiency of AGT-mediated repair of O^6-alkylguanine adducts can be influenced by the local DNA sequence context.[21,22] For example, the Spratt laboratory employed first-order kinetics[23] to examine the rates of repair of alkylguanine lesions placed within *H-ras* codon 12 (G_1G_2A).[21] The relative rates of AGT-mediated alkyl transfer were dependent on the alkyl group identity, e.g., benzyl > methyl > ethyl > 2-hydroxyethyl > 4-(3-pyridyl)-4-oxobutyl (POB), and dealkylation rates were greater for adducts present at G_1 as compared to those on G_2.[21] It has been suggested that in some sequence contexts, bulky alkylguanine lesions may bind to the AGT protein in an inactive conformation that is not conductive to alkyl transfer.[21] However, the published kinetic data for O^6-POB-dG adduct repair is limited to *H-ras* codon 12, while other DNA sequences have not been previously examined.

AGT repair rates can also be affected by neighboring 5-methylcytosine (^{Me}C), an epigenetic nucleobase modification that is present at all CpG dinuclotides within the coding sequence of the human *p53* tumor suppressor gene.[24] The

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majority of *p53* mutations associated with smoking are found at guanine bases within endogenously methylated ^{Me}CpG dinucleotides, e.g., codons 157, 158, 245, 248, and 273.[25−28] One possible mechanism for the increased mutagenesis at these sites involves inefficient repair of tobacco carcinogen-induced DNA adducts such as O^6-Me-dG and O^6-POB-dG, leading to their accumulation at methylated CpG sequences. Indeed, inactivation of the AGT gene by hypermethylation of the promoter region results in a significant increase in G → A *p53* gene mutations in nonsmall cell lung cancer.[29] Our earlier study has demonstrated that AGT binding and repair of O^6-Me-dG was only weakly affected by C-5 methylation of neighboring cytosine bases.[30] However, similar experiments have not been conducted for O^6-POB-dG.

In the present study, we employed a mass spectrometry-based methodology developed in our laboratory[31] to investigate AGT repair of O^6-POB-dG adducts placed within the context of the *K-ras* gene (5′-G_1TA G_2TT G_3G_4A G_5CT G_6G_7T G_8G_9C G_{10}T-3′, where G_3, G_4, G_5, G_6, or G_7 = O^6-POB-dG). We also examined the influence of cytosine methylation on AGT-mediated repair of O^6-POB-dG adduct present within frequently mutated *p53* codons 157, 158, 245, 248, 249, and 273.[32] Finally, we evaluated the kinetics of O^6-POB-dG repair in human bronchial epithelial cells (HBEC) in the absence and in the presence of AGT inhibitor.

EXPERIMENTAL PROCEDURES

Materials. 1,3-Dithiane protected O^6-POB-dG (O^6-[3-[2-(3-pyridyl)-1,3-dithyan-2-yl]propyl]-deoxyguanosine) was synthesized according to a previously published method[33] and converted to its corresponding phosphoramidite using standard phosphoramidite chemistry.[34] Standard nucleoside phosphoramidites, solvents, and solid supports for the solid phase synthesis of oligodeoxynucleotides were purchased from Glen Research Corporation (Sterling, VA). γ-^{32}P-ATP was obtained from Perkin-Elmer Life Sciences (Waltham, MA), while T4-PNK enzyme and buffer were procured from New England Biolabs (Ipswich, MA). MicroSpin Illustra G25 columns were obtained from GE Healthcare (Buckinghamshire, UK). 29% Acrylamide solution was purchased from BioRad (Hercules, CA). Tetramethylethylenediamine (TEMED) and ammonium persulfate were obtained from Sigma-Aldrich (Milwaukee, WI). 4-(Acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) was purchased from Toronto research Chemicals (Toronto, Canada). The rest of the chemicals employed in the study were from Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Fairlawn, NJ). Human recombinant AGT protein with a C-terminal histidine tail (WT hAGT) and its C145A variant were expressed in *Escherichia coli* and isolated as reported elsewhere.[35,36] The activity of the AGT protein was determined by titrating the recombinant protein with DNA duplexes containing site-specific O^6-MeG, followed by HPLC-ESI$^+$-MS/MS analysis as described previously.[31,37] D_4-O^6-POB-dG was a gift from Professor Stephen Hecht (University of Minnesota Masonic Cancer Center). HBEC cells were grown in Keratinocyte SFM media (Life Technologies, NY) supplemented with human recombinant epidermal growth factor (EGF 1−53, Life Technologies, NY) and Bovine Pituitary Extract media (Life Technologies, NY).

Preparation of O^6-POB-dG Containing DNA. Synthetic oligodeoxynucleotides containing O^6-POB-dG adducts within the context of *K-ras* and *p53* genes were prepared by solid phase DNA synthesis using 5′-*O*-(4,4′-dimethoxytrityl)-*N*,*N*-dimethyl-formamidine-O^6-{3-[2-(3-pyridyl)-1,3-dithyan-2-yl]-propyl}-2′-deoxyguanosine-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite synthesized as described elsewhere.[34] The oligonucleotides were cleaved from the solid support and deprotected using concentrated ammonia (55 °C, 18 h) and dried, followed by deprotection of the dithiane group with 10 equivalents of fresh 10 mg/mL *N*-chlorosuccinimide solution in 80% acetonitrile (30 min at room temperature in the dark).[34] Synthetic oligodeoxynucleotides were purified by reverse phase HPLC using an Agilent 1100 HPLC system and a Supelcosil LC-18DB column (10 mm × 250 mm, 5 μm) maintained at 40 °C. HPLC solvents were 100 mM TEAAc (buffer A, pH 7) and acetonitrile containing 50% A (buffer B). A linear gradient of 16.8−26% B in 21 min and further to 38% B over the next 14 min was used.[37−39] All oligodeoxynucleotides used in this work were >99% purity (see HPLC trace in Supporting Information, S-6). The molecular weights of all synthetic DNA oligomers were confirmed by capillary HPLC-ESI$^-$ MS (Table 1), and their concentrations were determined by dG quantitation in enzymatic digests.[40]

To prepare double-stranded DNA substrates for repair experiments, complementary DNA strands (6 nmol each) were combined in 10 mM Tris-HCl buffer (pH 8) containing 50 mM NaCl (30 μL). The mixture was heated at 90 °C for 5 min and slowly cooled to room temperature to produce double-stranded DNA.[38]

Table 1. Sequences and ESI$^-$ MS Analysis Results for Synthetic DNA Oligomers Employed in This Work

adduct location	sequence	MW calculated	MW observed
(+) *K-ras*-G3-POB	GTA GTT [O^6-POB-G]GA GCT GGT GGC GT	6407.3	6406.1
(+) *K-ras*-G4-POB	GTA GTT G[O^6-POB-G]A GCT GGT GGC GT	6407.3	6406.1
(+) *K-ras*-G5-POB	GTA GTT GGA [O^6-POB-G]CT GGT GGC GT	6407.3	6406.1
(+) *K-ras*- G6-POB	GTA GTT GGA GCT [O^6-POB-G] GT GGC GT	6407.3	6406.1
(+) *K-ras*-G7-POB	GTA GTT GGA GCT G[O^6-POB-G]T GGC GT	6407.3	6406.1
(−) *K-ras*-POB	ACG CCA CCA GCT CCA ACT AC	5975.9	5974.6
p53 codon 248	CATGAACC[O^6-POB-G] GAGGCCCATC	5930.0	5930.4
	CATGAAC^{Me}C[O^6-POB-G] GAGGCCCATC	5944.0	5944.8
	GATGGGCCT CCG GTTCATG	5835.8	5835.6
	GATGGGCCT C ^{Me}C G GTTCATG	5849.9	5849.4
p53 codon 245	GCATGGGC[O^6-POB-G] GCATGAACCG	6476.3	6477.1
	GCATGGG^{Me}C[O^6-POB-G] GCATGAACCG	6490.3	6490.4
	CGGTTCAT GCC GCCCATGC	5740.8	5740.8
	CGGTTCAT GC ^{Me}C GCCCATGC	5754.8	5754.8
p53 codon 158	ACCCGCGTCC[O^6-POB-G] CGCCATGGCC	6026.0	6026.9
	ACCCGCGTC^{Me}C[O^6-POB-G] CGCCATGGCC	6040.1	6040.4
	GGCCATGGC GCG GACGCGGGT	6529.3	6529.4
	GGCCATGGC G^{Me}CG GACGCGGGT	6543.3	6543.2

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Table 2. UV Melting Points of O^6-POB-G Containing DNA Duplexes

Oligonucleotide ID	Sequence	T_m(°C) Calculated[a,b]	T_m(°C) Observed[c]
ds *p53* exon 5 codon 158, O^6-POB-G	ACCCGCGTC TGGGCGCAG C[O^6-POB-G] G C CGCCATGGCC GCGGTACCGG	75.0	74.89 ± 0.62
ds *p53* exon 5 codon 158, 5′-^{Me}C, O^6-POB-G	ACCCGCGTC TGGGCGCAG ^{Me}C[O^6-POB-G] G C CGCCATGGCC GCGGTACCGG		75.31 ± 1.37
ds *p53* exon 5 codon 158, BP-^{Me}C, O^6-POB-G	ACCCGCGTC TGGGCGCAG C[O^6-POB-G] G ^{Me}C CGCCATGGCC GCGGTACCGG		74.86 ± 0.78
ds *p53* exon 5 codon 158, Both-^{Me}C, O^6-POB-G	ACCCGCGTC TGGGCGCAG ^{Me}C[O^6-POB-G] G ^{Me}C CGCCATGGCC GCGGTACCGG		75.08 ± 1.15
ds *p53* exon 7 codon 245, O^6-POB-G	GCATGGG CGTACCC C[O^6-POB-G] G C GCATGAACCG CGTACTTGGC	66.0	64.71 ± 0.93
ds *p53* exon 7 codon 245, 5′-^{Me}C, O^6-POB-G	GCATGGG CGTACCC ^{Me}C[O^6-POB-G] G C GCATGAACCG CGTACTTGGC		65.50 ± 1.28
ds *p53* exon 7 codon 245, BP-^{Me}C, O^6-POB-G	GCATGGG CGTACCC C[O^6-POB-G] G ^{Me}C GCATGAACCG CGTACTTGGC		65.45 ± 1.22
ds *p53* exon 7 codon 245, Both-^{Me}C, O^6-POB-G	GCATGGG CGTACCC ^{Me}C[O^6-POB-G] G ^{Me}C GCATGAACCG CGTACTTGGC		65.70 ± 0.70
ds *p53* exon 7 codon 248, O^6-POB-G	CATGAAC GTACTTG C[O^6-POB-G] G C GAGGCCCATC CTCCGGGTAG	64.0	61.01 ± 0.61
ds *p53* exon 7 codon 248, 5′-^{Me}C, O^6-POB-G	CATGAAC GTACTTG ^{Me}C[O^6-POB-G] G C GAGGCCCATC CTCCGGGTAG		62.96 ± 0.94
ds *p53* exon 7 codon 248, BP-^{Me}C, O^6-POB-G	CATGAAC GTACTTG C[O^6-POB-G] G ^{Me}C GAGGCCCATC CTCCGGGTAG		62.96 ± 0.61
ds *p53* exon 7 codon 248, Both-^{Me}C, O^6-POB-G	CATGAAC GTACTTG ^{Me}C[O^6-POB-G] G ^{Me}C GAGGCCCATC CTCCGGGTAG		62.87 ± 1.17

[a]Calculated T_m does not include the presence of O^6-POB-G, instead a G was used http://www.basic.northwestern.edu/biotools/oligocalc.html. [b]9.7 μM dsDNA with 66 mM salt (Na^+). [c]T_m obtained from 4 to 6 hyperbolic curves.

Determination of DNA Melting Temperatures. DNA duplexes containing site-specific O^6-POB-dG adducts (3 nmol) were dissolved in sodium phosphate buffer (10 mM, pH 7.0) containing 50 mM sodium chloride (9.7 μM DNA). DNA melting temperatures were obtained with a Varian Cary-100 Bio UV−visible spectrophotometer using a temperature gradient between 30 and 90 °C. Temperature increments or decrements of 0.5 °C/min were used. The experiment was repeated 4−6 times to determine DNA melting temperatures using Cary WinUV Thermal software (Varian, Palo Alto, CA) (Table 2).

Single Time Point AGT Repair Experiments. DNA duplexes containing O^6-POB-G at a specified site (500 fmol) were mixed with recombinant human AGT protein (400 fmol) in 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 0.5 mg/mL BSA, and 0.5 mM DTT (final volume, 90 μL) and incubated at room temperature for 15 s. The repair reactions were quenched with 0.1 N HCl, fortified with D_4-O^6-POB-dG (500 fmol, internal standard for mass spectrometry), and subjected to acidic hydrolysis (70 °C, 1 h) to release O^6-POB-G and D_4-O^6-POB-G. The reaction mixtures were neutralized with NH_4OH. O^6-POB-G and D_4-O^6-POB-G were purified by solid phase extraction (SPE) on Strata X cartridges.[31] O^6-POB-G was quantified by capillary HPLC-ESI$^+$-MS/MS using D_4-O^6-POB-G internal standard as described elsewhere.[31] Data reported are an average of 9−14 individual experiments.

Time Course AGT Repair Experiments. Human recombinant AGT protein (400 fmol) was combined with O^6-POB-dG-containing DNA duplexes (500 fmol) in 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 0.5 mg/mL BSA, and 0.5 mM DTT (final volume, 90 μL). The resulting mixtures were incubated at room temperature for specified time periods (0−50 s) and manually quenched with 0.2 N HCl (90 μL, 0.1 N final concentration). Following the addition of D_4-O^6-POB-dG internal standard (250 fmol), the DNA was hydrolyzed by heating at 70 °C for 1 h and neutralized with 0.2 N ammonium hydroxide. O^6-POB-G and D_4-O^6-POB-G were purified by solid phase extraction (SPE) and quantified by capillary HPLC-ESI$^+$-MS/MS as described previously.[31]

The concentrations of O^6-POB-G repaired at time t were plotted as a function of time, and the resulting data were fitted to the second-order kinetic equation (eq 1)[30] using the KaleidaGraph software program (Synergy Software, Reading, PA):

$$kt = \frac{1}{B_0 - A_0} \ln \frac{A_0(B_0 - C_t)}{B_0(A_0 - C_t)} \quad (1)$$

where A_0 is the concentration of AGT protein used, B_0 is the initial concentration of O^6-POB-dG containing DNA, C_t is the concentration of O^6-POB-G repaired at time t, and k is the second order rate of O^6-POB-G repair.

Electrophoretic Mobility (Gel Shift) Assay. Electrophoretic mobility (gel shift) experiments were conducted according to the previously published protocols.[30,41] In brief, O^6-POB-dG-containing double-stranded DNA was end-labeled in the presence of γ-^{32}P-ATP and polynucleotide kinase. Excess γ-^{32}P-ATP was removed with MicroSpin Illustra G25 columns (GE Healthcare). The resulting 5′-^{32}P-end-labeled DNA duplexes (2 pmol, 0.1 μM) were spiked with the corresponding unlabeled DNA (0.8 μM) and calf thymus (CT) DNA (1 μg) and dissolved in 50 mM Tris-HCl, pH 7.8, buffer containing 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mg/mL BSA. Human recombinant C145A AGT protein was added (0−6 μM), and the solutions were incubated at room temperature for 45 min. DNA−protein complexes were detected using 10% PAGE (acrylamide: *N*,*N*′-methylene bisacrylamide = 29:1 cast in 100 mM TAE buffer, pH 7.6). The gels were run in 10 mM Tris-

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acetate buffer (pH 7.6) at 100 V for 2.5 h and imaged using a Molecular Dynamics STORM 840 phosphorimager (Amersham Biosciences Corporation, Piscataway, NJ). The radiolabeled DNA bands were quantified by densitometry using the ImageQuant software. Dissociation constants (K_d) were determined by plotting the $[D]/[D]_T$ ratios vs $[P]_T$, where $[D]$, $[D]_T$, and $[P]_T$ are the molar concentrations of free DNA, total DNA, and AGT protein, respectively. The data were fitted to eq 2:[30,42]

$$\frac{[D]}{[D]_T} = \frac{K_d}{K_d + [P]_T} \quad (2)$$

The values of $[D]$ and $[D]_T$ were calculated from the density of radioactive bands on the gel.

Cell Cytotoxicity Assays Used To Determine the Concentration of NNKOAC To Use in the O^6-POB-G Repair. Human bronchial epithelial cells (HBEC, 5×10^3) were seeded into a 96-well plate and allowed to grow overnight in 150 μL of Keratinocyte SFM media (Life Technologies, NY) supplemented with human recombinant epidermal growth factor (EGF 1−53) and bovine pituitary extract (37 °C, 5% CO_2). Cells (in triplicate) were treated with 0−400 μM NNKOAC for 1 h at 37 °C and 5% CO_2. Following treatment, carcinogen-containing media was removed, and the cells were washed with PBS buffer and allowed to grow in Keratinocyte SFM media (150 μL) for 48 h. To determine cell viability, they were treated with MTT reagent (37 °C, 5% CO_2, 2 h), followed by cell density measurements with a Biotek LLX808 microplate reader (BioTek, VT).

O^6-POB-G Adduct Formation and Repair in Human Bronchial Epithelial Cells (HBEC). HBECs were grown in Keratinocyte SFM media (Life Technologies, NY) supplemented with human recombinant epidermal growth factor (EGF 1−53) and bovine pituitary extract (37 °C, 5% CO_2) until fully confluent. Cells (in triplicate, 15 cm dishes) were treated with 150 μM NNKOAc or DMSO control for 1 h. Following treatment, carcinogen-containing media was removed, and the cells were washed three times with PBS buffer (Life Technologies, NY). Following the addition of normal growth media, cells were incubated at 37 °C in 5% CO_2 for specified periods of time (0, 1, 2, 4, 8, and 12 h) to allow for adduct repair. At the end of incubation, the cells were washed, harvested, and stored at −80 °C until DNA extraction.

For experiments including AGT inhibitor (O^6-Bz-G), fully confluent HBEC cells were pretreated with 10 μM O^6-Bz-G for 10 min (37 °C, 5% CO_2), followed by 150 μM NNKOAc/10 μM O^6-Bz-G treatment for 1 h at 37 °C under 5% CO_2 atmosphere as described above. Control cells were treated with O^6-Bz-G only. Carcinogen-containing media was replaced with fresh media containing 5 μM O^6-Bz-G, and the cells were incubated at 37 °C in 5% CO_2 for the specified lengths of time (0, 1, 2, 4, 8, or 12 h). At the end of the repair period, the cells were washed, harvested, and stored at −80 °C until DNA extraction.

DNA Isolation from HBEC Cells. NNKOAc-treated HBEC cells ($4-4.5 \times 10^6$) were lysed with 2× cell lysis buffer (20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 2% (v/v) Triton-X, 650 mM sucrose, 30 min on ice) and pelleted at 4000 rpm for 15 min. The nuclei were suspended in 0.5 mL of saline−EDTA solution (75 mM NaCl, 24 mM EDTA, pH 8) and digested with RNase A (3 mg at 37 °C for 1 h) and proteinase K (5 mg, 18 h at 37 °C). DNA was isolated by phenol/chloroform extraction and precipitated with ethanol/sodium acetate. DNA amounts were estimated by UV and accurately quantified followed by HPLC analysis of dG in enzymatic digests.[40]

Quantitation of O^6-POB-G Adducts in HBEC DNA Treated with NNKOAc. DNA isolated from HBEC cells (32−36 μg) was dissolved in 100 μL of water and spiked with D_4-O^6-pob-dG (3 pmol, internal standard for mass spectrometry). O^6-POB-G was quantified by capillary HPLC-ESI$^+$-MS/MS following acid hydrolysis and SPE purification as described elsewhere.[31]

Statistical Analysis. All statistical analyses were carried out by the University of Minnesota Masonic Cancer Center Biostatistics Core. Time course repair experiments were analyzed using a linear regression model, which included a linear term for time and a factor for methylation condition or O^6-POB-G position, depending on the experiments. All pairwise comparisons were considered, and a Bonferroni adjustment was used to control for multiple comparisons. A similar analysis was completed for the association between AGT protein concentration and % unbound DNA (EMSA assays) with the only difference being that a quadratic relationship between AGT protein concentration and % unbound DNA was considered instead of a linear relationship. A one-way analysis of variance (ANOVA) was used to compare the extent of AGT-mediated repair for O^6-POB-G placed at different positions within *K-ras* gene derived DNA sequence. All pairwise comparisons were again considered, and the Bonferroni adjustment was used to control for multiple comparisons. Finally, *p*-values less than 0.05 were considered significant, and all analyses were completed in R version 2.15.1. Statistical results are given in the Supporting Information.

RESULTS

Selection of DNA Sequences and Characterization of Synthetic DNA Duplexes. DNA sequences selected for this study (Tables 1 and 2) were derived from codons 8−15 of the *K-ras* protooncogene and two regions of the *p53* tumor suppressor gene containing codons 158, 245, and 248. *K-ras* codon 12 and *p53* codons 158, 245, and 248 are frequently mutated in smoking-induced lung cancer, supposedly a result of preferential tobacco-carcinogen-DNA adduct formation, deficient repair, and selection processes.[43,44] Synthetic DNA oligodeoxynucleotides containing site-specific O^6-POB-G adducts were prepared by solid phase synthesis from the corresponding nucleoside phosphoramidites[34] and purified by reverse phase HPLC. Each strand was characterized by HPLC-ESI$^-$ MS (Table 1). To generate double-stranded DNA, each oligomer was annealed to the complementary strand containing cytosine opposite O^6-POB-dG. Since cytosine residues within CG dinucleotides of the *p53* gene are endogenously methylated in mammalian cells,[24] a range of *p53* codon 158, 245, and 248 sequences were investigated containing cytosine or 5-methylcytosine (^{Me}C) immediately 5′ and/or in the base paired position to O^6-POB-G (Table 2).

The thermodynamic stability of O^6-POB-dG containing DNA duplexes was characterized by UV melting. All structurally modified duplexes produced hyperbolic thermal melting curves consistent with the formation of B-form DNA (see Supporting Information S-1). For *p53* gene derived sequences, the introduction of ^{Me}C increased UV melting temperature by 0.2−2 °C, indicative of an enhanced duplex stability (Table 2). This is consistent with our previous studies,

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where 0.9−3.2 °C increases in UV melting temperatures were observed upon single C-5 cytosine methylation.[30,45−47] ^{Me}C increases DNA duplex stability due to enhanced $\pi-\pi$ stacking interactions of C-5 methylated cytosine with neighboring DNA nucleobases.[48−50] Overall, our UV melting studies confirm that O^6-POB-dG containing DNA strands (Table 1) form standard B-form duplexes, which are stabilized by the presence of ^{Me}C.

HPLC-ESI-MS/MS Approach to Follow the Kinetics of AGT-mediated Repair. The kinetics of AGT-mediated dealkylation of O^6-POB-G as a function of DNA sequence context was investigated using accurate and precise isotope dilution HPLC-ESI$^+$-MS/MS methodology recently developed in our laboratory.[31] In brief, DNA duplexes containing site-specific O^6-POB-G were incubated with human recombinant AGT protein under second-order reaction conditions (DNA/protein molar ratio = 1.25) for specified periods of time, and the reactions were quenched with hydrochloric acid (Figure 1).

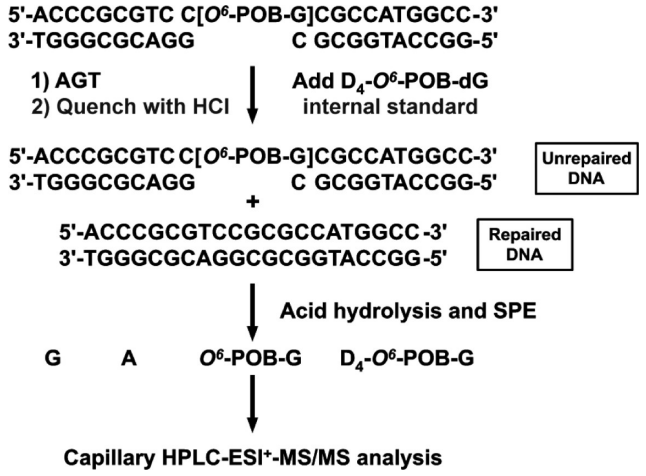

Figure 1. HPLC-ESI$^+$-MS/MS methodology employed to follow the kinetics of AGT-mediated repair of O^6-POB-G adduct. Following incubation with recombinant AGT protein for specified periods of time, DNA was spiked with D$_4$-O^6-POB-dG internal standard, subjected to acid hydrolysis to release free base adducts, and analyzed by capillary HPLC-ESI$^+$-MS/MS.

We chose to study the kinetics of repair using second-order kinetics because of the stoichiometric nature of AGT-mediated dealkylation (see above), which acts as a reactant rather than an enzyme.[14] Following spiking with D$_4$-O^6-POB-dG (internal standard for mass spectrometry), DNA was subjected to mild acid hydrolysis to release O^6-POB-G, and the amounts of O^6-POB-G remaining in DNA after repair reaction were determined by HPLC-ESI$^+$-MS/MS using D$_4$-O^6-POB-G internal standard (Figure 2).[31]

Kinetics of AGT-Mediated O^6-POB-G Repair as a Function of DNA Sequence Context. To determine whether DNA sequence context affects the efficiency of AGT-mediated repair of O^6-POB-G adducts, *K-ras* derived DNA duplexes were prepared (5′-G$_1$TA G$_2$TT G$_3$G$_4$A G$_5$CT G$_6$G$_7$T G$_8$G$_9$C G$_{10}$T-3′) where G$_3$, G$_4$, G$_5$, G$_6$, or G$_7$ were replaced with O^6-POB-dG) (Table 1). Duplexes containing site-specific adduct were incubated with human recombinant AGT protein under second-order conditions, and the unrepaired adducts were quantified by HPLC-ESI$^+$-MS/MS as described above. The extent of O^6-POB-G repair at time t (E_t) was calculated as shown in eq 3:

$$E_t = \frac{X_0 - X_t}{X_0} 100\% \quad (3)$$

where X_0 and X_t are the amounts of O^6-POB-G adducts remaining in DNA at time = 0 s and time = t s, respectively.

We found that the extent of AGT repair of O^6-POB-dG adducts located at G$_3$, G$_4$, G$_5$, G$_6$, and G$_7$ following 15 s reaction varied between 2.6% and 28.9%, depending on sequence position (Figure 3). AGT repair was most efficient at G5 (28.9%, *K-ras* codon 11, AGC context), while the lowest amount of AGT-mediated dealkylation occurred at G3 (*K-ras* codon 8, TGG context). O^6-POB-dG adducts present at G4 (codon 9), G6 (codon 12), and G7 (codon 12) exhibited intermediate AGT reactivity (8.4−13.5%) (Figure 3).

Since G6 and G7 are located within a known *K-ras* mutations "hotspot" (codon 12, GGT → GTT, GTT),[51] a more comprehensive kinetic analysis was conducted for these two sites. DNA duplexes 5′-G$_1$TA G$_2$TT G$_3$G$_4$A G$_5$CT G$_6$G$_7$T G$_8$G$_9$C G$_{10}$T-3′ containing O^6-POB-G at G6 or G7 (N = 5) were allowed to react with AGT for 5−60 s, and O^6-POB-G amounts repaired at time t were plotted versus time (Figure 4). The kinetic curves were fitted to the second order quadratic equation (eq 1 above) to obtain the second order rates of repair. On the basis of these data, the second order reaction rates for AGT repair of O^6-POB-dG adducts present at G$_6$ and G$_7$ were calculated as 8.2 ± 0.3 × 10^5 M^{-1} s^{-1} and 1.64 ± 0.1 × 10^6 M^{-1} s^{-1}, respectively. These differences in dealkylation rates were statistically significant (p < 0.001, Supporting Information S-2). Taken together, our results indicate that DNA sequence context has a considerable effect on the efficiency of AGT-mediated repair of O^6-POB-dG adducts.

Kinetics of AGT-mediated Repair of O^6-POB-G as a Function of Cytosine Methylation. Previous studies have suggested that endogenous cytosine methylation within CG dinucleotides of the *p53* gene may influence the rates of AGT repair of O^6-alkylgunaine adducts present at these sites.[30] To determine whether the rate of O^6-POB-G repair by AGT are affected by methylation status of neighboring cytosine, we conducted second-order kinetic analysis of AGT-mediated dealkylation reaction for O^6-POB-G adducts placed within unmethylated, hemimethylated, and fully methylated CG dinucleotides representing *p53* codons 158, 245, and 248 (Table 2). We found that AGT repair efficiency was only weakly affected by cytosine methylation status (Table 3 and Figure 5). For example, for O^6-POB-G adducts located within *p53* codon 248, the observed second order dealkylation rates were 3.09 ± 0.16 × 10^6 M^{-1} s^{-1} (unmethylated CG dinucleotide), 3.82 ± 0.12 × 10^6 M^{-1} s^{-1} (5′-^{Me}C), 2.65 ± 0.15 × 10^6 M^{-1} s^{-1} (base paired ^{Me}C), and 2.94 ± 0.14 × 10^6 M^{-1} s^{-1} (fully methylated CG dinucleotide) (see Figure 5C and Table 3). Similar results were obtained for O^6-POB-G adducts placed within the context of *p53* codon 158 (Table 3 and Figure 5B) and *p53* codon 245 (Table 3 and Figure 5A). Statistical results are given in Supplement S-2.

AGT Protein Binding to O^6-POB-G-Containing DNA Duplexes. To investigate AGT protein binding to O^6-POB-G-containing DNA, electrophoretic mobility shift assays were conducted with purified AGT protein and site specifically modified DNA duplexes derived from *p53* codons 248, 158, and 245 and surrounding sequences (Table 4). AGT active site mutant (C145A) was employed in these studies since the presence of C145A mutation preserves the affinity of AGT

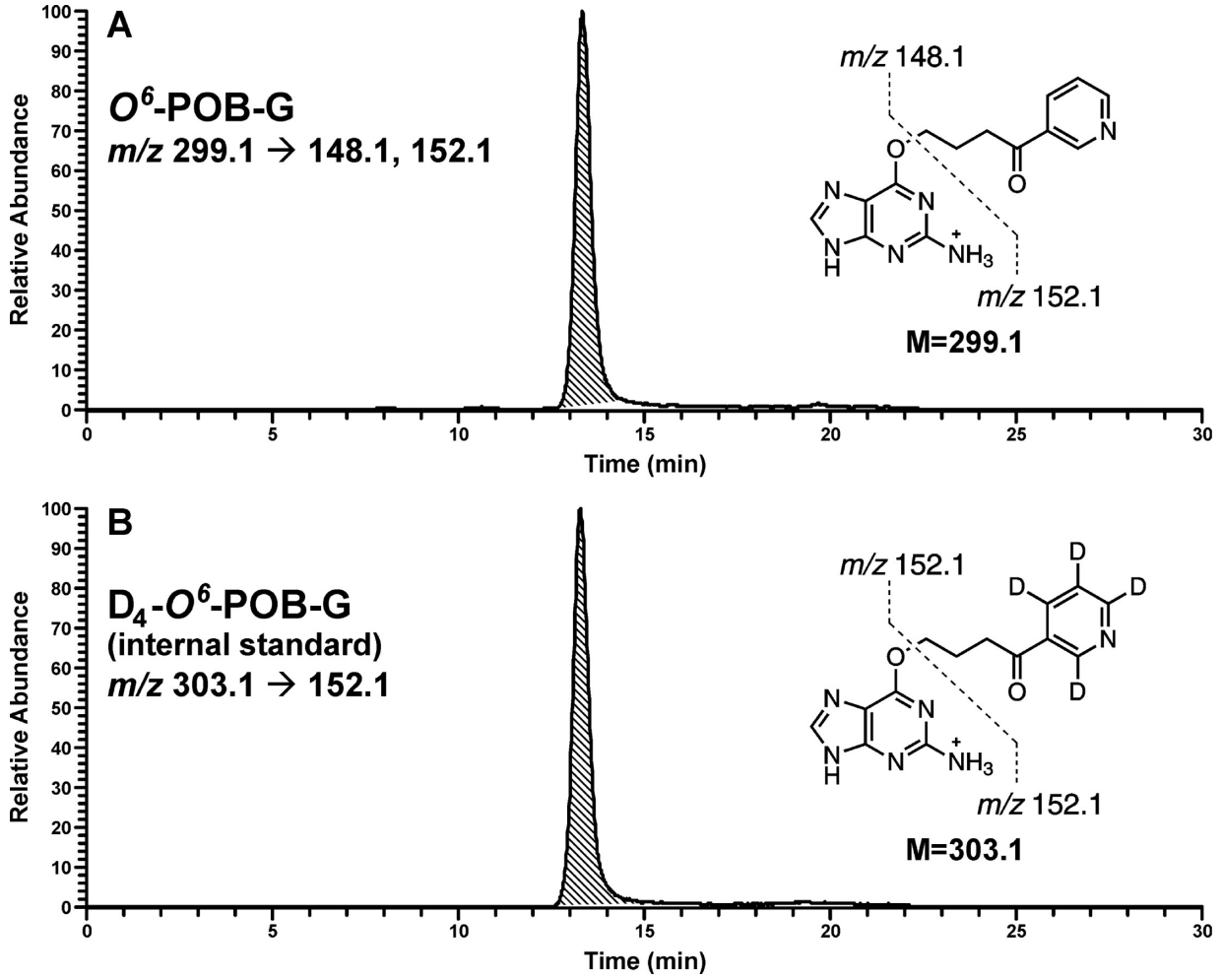

Figure 2. Representative traces for HPLC-ESI$^+$-MS/MS analyses of O^6-POB-G adducts in DNA hydrolysates. O^6-POB-G and D_4-O^6-POB-G (internal standard) were detected in the SRM mode by monitoring the transitions m/z 299.09 [M + H$^+$] → 148.1 [POB$^+$], 152.07 [Gua + H$^+$] for O^6-POB-G (A) and m/z 303.09 [M + H$^+$] → 152.07[D_4-POB$^+$], [Gua + H$^+$] for D_4-O^6-POB-G (B).

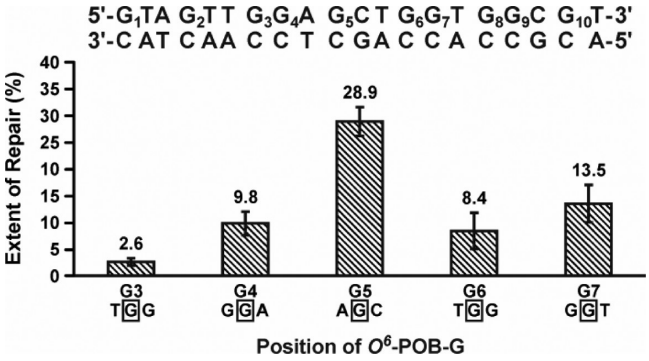

Figure 3. AGT repair of O^6-POB-dG adducts located at different positions within *K-ras* gene sequence. Synthetic DNA duplexes 5′-G_1TA G_2TT G_3G_4A G_5CT G_6G_7T G_8G_9C GT-3′ containing a single O^6-POB-dG residue at G_3, G_4, G_5, G_6, or G_7 (500 fmol) were incubated with human recombinant AGT (400 fmol) for 15 s, and O^6-POB-G adducts remaining in DNA were quantified by HPLC-ESI$^+$-MS/MS as shown in Figures 1 and 2.[31] The results were compiled from three different experiments (N = 9−14).

protein for DNA but makes the protein variant unable to participate in the alkyl transfer reaction.[52]

Following incubation of radiolabeled O^6-POB-G-containing duplexes with increasing amounts of C145A AGT protein (AGT/DNA ratios 0−7.5), AGT−DNA complexes were detected as a slowly moving band on a nondenaturing polyacrylamide gel (Figure 6). AGT−DNA dissociation constants (K_d) were obtained from the plots of $[D]/[D]_T$ vs $[P]_T$ where $[D]$, $[D]_T$, and $[P]_T$ are molar concentrations of O^6-POB-G containing DNA, bound and unbound DNA, and AGT protein, respectively.[30] Calf thymus DNA was added to the incubation mixtures to minimize any nonspecific binding interactions between the protein and O^6-POB-G containing DNA duplexes.[42]

Dissociation constant (K_d) values calculated for AGT−DNA complexes containing O^6-POB-G (Table 4, Supplement S4) were similar to AGT−DNA dissociation constants previously reported for O^6-Me-G containing DNA,[30] suggesting that O^6-alkyl group identity does not influence the dynamics of AGT−DNA interactions. The affinity of AGT protein for DNA duplexes containing O^6-POB-G in *p53* codon 248 sequence context was moderately affected by the presence of ^{Me}C, with K_d values of 2.2 ± 0.2 × 10^{-6} M, 2.0 ± 0.2 × 10^{-6} M, 1.8 ± 0.3 × 10^{-6} M, and 1.0 ± 0.1 × 10^{-6} M observed for unmethylated DNA, ^{Me}C[O^6-POB-G]sequence, O^6-POB-G: ^{Me}C base pair, and fully methylated C[O^6-POB-G] dinucleotides ($p < 0.001$), respectively (Table 4). The presence of 5′-^{Me}C decreased AGT binding affinity toward O^6-POB-G adducts within *p53* codon 158 (K_d = 4.8 ± 0.5 × 10^{-6} M) as compared to the

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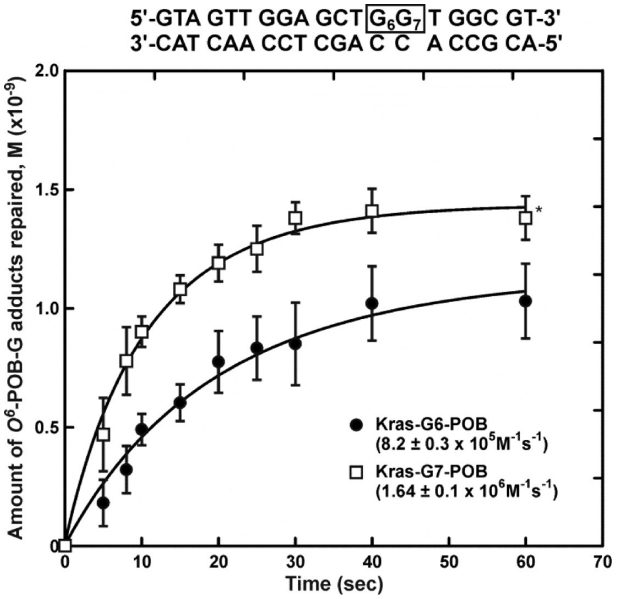

Figure 4. Time course of AGT-mediated repair of O^6-POB-dG placed at the first (G_6) and the second position of *K-ras* codon 12 (G_7). O^6-POB-dG-containing duplexes (500 fmol) were incubated with recombinant human AGT protein (400 fmol) for increasing lengths of time (0–60 s), and the reactions were quenched with HCl. The unrepaired O^6-POB-G adducts remaining in DNA were quantified by isotope dilution HPLC-ESI$^+$-MS/MS.[31] The kinetic curves (N = 5, fit between 0 and 20 s) represent the best fit to a second-order exponential equation that provides the rate of AGT-mediated dealkylation.

corresponding unmethylated DNA duplex (K_d =2.3 ± 0.2 × 10^{-6} M, p < 0.001). In contrast, cytosine methylation did not affect AGT binding to the DNA duplex derived from *p53* codon 245 (K_d = 1.3 ± 0.3 × 10^{-6} M to 1.5 ± 0.1 × 10^{-6} M, Table 4 and Supplementary Table S4).

O^6-POB-G Adduct Formation and Repair in Human Bronchial Epithelial Cells (HBEC). As described above, NNK and NNN are widely recognized as causative agents of human lung cancer.[53] Following metabolic activation to methylating and pyridyloxobutylating agents (Scheme 1), tobacco-specific nitrosamines present in tobacco smoke damage DNA within epithelial cells of the pulmonary airways, ultimately leading to mutations in critical genes and lung cancer initiation.[54] DNA repair systems can prevent malignant transformation by removing NNK-induced DNA lesions from DNA before they can be converted to heritable mutations (Scheme 2). Urban et al. conducted a comprehensive study of the formation and repair of pyridyloxobutylated DNA adducts in tissues of laboratory mice treated with a pyridyloxo-butylating agent (NNKOAc).[11] These authors reported that O^6-POB-G adducts persisted in mouse lung at significant levels for up to 96 h post-treatment, and that AGT depletion with O^6-benzylguanine led to a 2-fold increase in O^6-POB-G adduct levels.[11] However, to our knowledge, the ability of human bronchial epithelial cells to repair O^6-POB-G lesions and the respective role of AGT in their repair remains had not been elucidated. This is important given the documented species differences in POB–DNA adducts formation and repair.[11]

To study the kinetics of O^6-POB-dG repair in human lung cells, normal immortalized HBECs in culture were treated with model pyridyloxo-butylating agent, 4-acetoxynethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc). Esterase catalyzed hydrolysis of NNKOAc generates the same intermediate, pyridyloxobutyl diazohydroxide, that also forms upon metabolic activation of NNK and forms O^6-POB-dG adducts in DNA

Table 3. Second-Order Rate Constants for AGT-Mediated Repair of O^6-POB-G Adducts within Unmethylated, Hemi-Methylated, and Fully Methylated CG Dinucleotides Representing *p53* Codons 248, 158, and 245

Adduct Location	Sequence (5' → 3')	k/10^6 (M^{-1} s^{-1})
p53 exon 7 codon 248	CATGAAC C [O^6Alk-G] GAGGCCCATC GTACTTG G C CTCCGGGTAG	3.09 ± 0.16
	CATGAAC ^{Me}C [O^6Alk-G] GAGGCCCATC GTACTTG G C CTCCGGGTAG	3.82 ± 0.12
	CATGAAC C [O^6Alk-G] GAGGCCCATC GTACTTG G ^{Me}C CTCCGGGTAG	2.65 ± 0.15
	CATGAAC ^{Me}C [O^6Alk-G] GAGGCCCATC GTACTTG G ^{Me}C CTCCGGGTAG	2.94 ± 0.14
p53 exon 5 codon 158	ACCCGCGTC C [O^6Alk-G] CGCCATGGCC TGGGCGCAG G C GCGGTACCGG	2.77 ± 0.13
	ACCCGCGTC ^{Me}C [O^6Alk-G] CGCCATGGCC TGGGCGCAG G C GCGGTACCGG	3.13 ± 0.2
	ACCCGCGTC C [O^6Alk-G] CGCCATGGCC TGGGCGCAG G ^{Me}C GCGGTACCGG	2.65 ± 0.16
	ACCCGCGTC ^{Me}C [O^6Alk-G] CGCCATGGCC TGGGCGCAG G ^{Me}C GCGGTACCGG	1.94 ± 0.11*
p53 exon 7 codon 245	GCATGGG C [O^6Alk-G] GCATGAACCG CGTACCC G C CGTACTTGGC	1.79 ± 0.14
	GCATGGG ^{Me}C [O^6Alk-G] GCATGAACCG CGTACCC G C CGTACTTGGC	1.37 ± 0.07*
	GCATGGG C [O^6Alk-G] GCATGAACCG CGTACCC G ^{Me}C CGTACTTGGC	1.77 ± 0.08
	GCATGGG ^{Me}C [O^6Alk-G] GCATGAACCG CGTACCC G ^{Me}C CGTACTTGGC	1.79 ± 0.12

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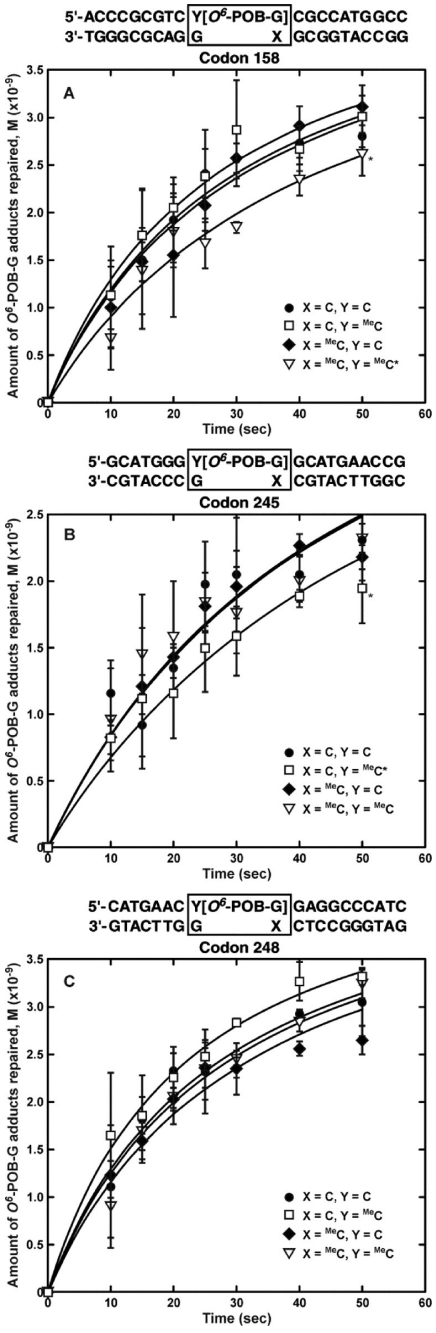

Figure 5. (A–C) Time course of AGT-mediated repair of O^6-POB-dG placed within synthetic duplexes representing unmethylated, hypomethylated, and fully methylated CG dinucleotides within *p53* codons 158, 245, 248. O^6-POB-dG-containing duplexes (500 fmol) were incubated with recombinant human AGT protein (400 fmol) for increasing lengths of time (0–50 s). The reactions were quenched with HCl, and the unrepaired O^6-POB-G adducts were quantified by isotope dilution HPLC-ESI$^+$-MS/MS.[31] The kinetic curves (N = 4) represent the best fit to a second-order exponential equation that provides the rate of AGT-mediated delakylation.

(Scheme 1).[1] Preliminary studies have shown that HBEC cell treatment with 150 μM NNKOAc for 1 h minimally affected cell viability (Supplement S-5). Following carcinogen removal, cells were allowed to recover over 1–12 h. DNA was extracted, and the amounts of O^6-POB-dG adducts remaining in DNA at each time point were determined by isotope dilution HPLC ESI$^+$-MS/MS (Figure 2). The same experiment was conducted in the presence of AGT inhibitor (O^6-benzylguanine) to evaluate potential contribution of AGT repair pathway to adduct removal in human bronchial epithelial cells.

We found that DNA of HBEC cells treated with 150 μM NNKOAc for 1 h contained 14.5 O^6-POB-dG adducts/10^7 nucleotides (Figure 7). The number of adducts was significantly greater in cells treated in the presence of AGT-inhibitor, O^6-benzylguanine (20.7 adducts/10^7 nucleotides). In the absence of O^6-bz-G, O^6-POB-dG adducts were gradually repaired, with approximately 5 adducts/10^7 nucleotides remaining after 12 h of repair incubation (Figure 7). In contrast, adduct numbers remained essentially unchanged in cells treated with AGT inhibitor O^6-benzylguanine (Figure 7), suggesting that AGT plays an important role in protecting human bronchial cells against pyridyloxobutylation damage.

DISCUSSION

O^6-POB-dG adducts induced by tobacco-specific nitrosamines NNK and NNN appear to play an important role in the etiology of smoking-induced lung cancer.[55,56] A/J mice treated with the model pyridyloxobutylating compound 4-acetoxynethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) develop lung tumors.[8,11] Furthermore, pyridyloxobutylated DNA adducts accumulate in pulmonary type II cells of rats treated with NNK and in pulmonary tissues of lung cancer patients.[57,58] Site-specific mutagenesis experiments have revealed the ability of O^6-POB to induce G to T and G to A mutations,[6,9] and the same types of mutations predominate in smoking-induced lung tumors.[32,44] G to T and G to A base substitutions in the *K-ras* protooncogene are observed in lung tumors of mice treated with NNKOAc.[8] Furthermore, O^6-POB has been shown to inhibit AGT repair of other NNK-induced DNA adducts such as O^6-methyl-dG.[59]

Direct removal of the O^6-POB group by O^6-alkylguanine DNA alkyltransferase (AGT) appears to be the main repair pathway for O^6-POB-dG adducts in cells. AGT protects cells from mutagenic effects of O^6-POB-dG.[6] Studies in Chinese hamster ovary (CHO) cell lines deficient in specific repair pathways have shown that O^6-POB-dG adducts were efficiently repaired in CHO cells expressing AGT but persisted in AGT-deficient cells.[60] In animal studies, coadministration of NNKOAc and AGT inhibitor O^6-benzylguanine significantly increased O^6-POB-dG adduct concentrations in tissues, although additional repair pathways appear to exist.[11] Unlike other bulky O^6-alkylguanine adducts, O^6-POB-dG was a poor substrate for human nucleotide excision repair pathway (NER).[60]

The rates of AGT-mediated repair of O^6-POB-dG lesions can be affected by the local DNA sequence context,[21,23,61–63] leading to adduct accumulation at specific sites within the genome. For example, Coulter et al. reported that the first-order rate for AGT-mediated repair of O^6-POB-dG placed in the first position of the *H-ras* codon 12 (5′- [O^6-POB-dG]GA-3′, 0.95×10^{-4} s^{-1}) was ~6 times higher than when the adduct was placed at the second position of codon 12 (5′ G[O^6-POB-dG]A-3′, 0.16×10^{-4} s^{-1}).[21] Similar results were obtained by Mijal et al.,[22] who measured the relative rates of AGT-mediated repair of O^6-POB-dG adducts as compared to O^6-Me-dG in *H-ras* derived sequence using a gel electrophoresis-based approach. O^6-POB-dG was repaired faster when it was placed opposite thymine rather than paired with cytosine.[22] However, to our knowledge, the kinetics of O^6-POB-dG repair in other sequence contexts has not been previously investigated.

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Table 4. Dissociation Constants for the Interaction of O^6-POB-dG Containing DNA Duplexes with Purified Human C145A AGT Protein

Adduct Location	Sequence (5′ → 3′)	K_d /10^{-6} (M)
p53 exon 7 codon 248	CATGAAC C[O^6-POB-G] GAGGCCCATC GTACTTG G C CTCCGGGTAG	2.2 ± 0.4
	CATGAAC ^{Me}C[O^6-POB-G] GAGGCCCATC GTACTTG G C CTCCGGGTAG	2.0 ± 0.2
	CATGAAC C[O^6-POB-G] GAGGCCCATC GTACTTG G ^{Me}C CTCCGGGTAG	1.8 ± 0.3
	CATGAAC ^{Me}C[O^6-POB-G] GAGGCCCATC GTACTTG G ^{Me}C CTCCGGGTAG	1.0 ± 0.1*
p53 exon 5 codon 158	ACCCGCGTC C[O^6-POB-G] CGCCATGGCC TGGGCGCAG G C GCGGTACCGG	2.3 ± 0.2
	ACCCGCGTC ^{Me}C[O^6-POB-G] CGCCATGGCC TGGGCGCAG G C GCGGTACCGG	4.8 ± 0.5*
p53 exon 7 codon 245	GCATGGG C[O^6-POB-G] GCATGAACCG CGTACCC G C CGTACTTGGC	1.5 ± 0.3
	GCATGGG ^{Me}C[O^6-POB-G] GCATGAACCG CGTACCC G C CGTACTTGGC	1.3 ± 0.1

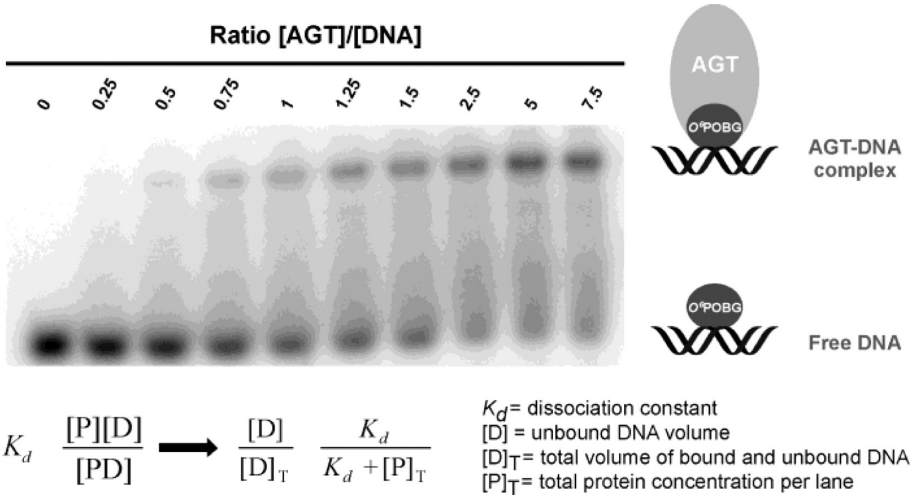

Figure 6. Representative gel shift assay result used to determine the binding affinity of human AGT protein for O^6-POB-G-containing DNA duplexes. ^{32}P endlabeled DNA duplexes (5′-CATGAAC ^{Me}C [O^6-POB-G]GAGGCCCATC-3′ and the complementary strands (0.8 μM, in triplicate) were incubated with increasing amounts of C145A AGT protein, and the resulting AGT−DNA complexes were resolved by 10% nondenaturing PAGE.

In the present work, a systematic study of sequence-dependent repair of O^6-POB-dG by AGT was undertaken. DNA duplexes representing codons 8−13 of *K-ras* proto-oncogene were selected (Table 1). *K-ras* is frequently mutated in lung tumors of smokers, specifically exhibiting G → A transitions and G → T transversions within codon 12.[64−67] The same genetic changes are observed in lung tumors of laboratory animals treated with the tobacco smoke carcinogen NNK.[8] We employed a mass spectrometry-based assay developed in our laboratory[31] to enable accurate and specific quantification of O^6-POB-dG remaining in DNA as a function of repair time (Figures 1 and 2). We found that the efficiency of AGT-mediated O^6-POB group transfer was affected by local sequence context, with ∼10-fold faster repair observed at G5 (AGC) than at the G3 (TGG) (Figure 3) Second order repair rates for O^6-POB-dG placed within the first and second positions of *K-ras* codon 12 were 8.2 and 16.4 × 10^5 M^{-1} s^{-1}, respectively (Figure 4). By comparison, the rates of repair of O^6-Me-dG adducts present at the same positions were 1.4 × 10^7 M^{-1} s^{-1} and 7.4 × 10^6 M^{-1} s^{-1}, respectively.[37] These results indicate that O^6-POB-dG repair by AGT is much slower than that of O^6-Me-dG and shows a greater dependence on local sequence environment. This can be explained by steric effects of the bulky pyridyloxobutyl group, which may interfere with correct placement of the adduct within the protein active site, inhibiting alkyl transfer.[14]

In contrast, AGT repair of O^6-POB-dG adducts was relatively unaffected by neighboring 5-methylcytosine (^{Me}C). ^{Me}C is an important endogenous DNA modification that plays an major role in many cellular processes.[68] All CG dinucleotides within the coding sequence of the *p53* tumor suppressor gene are methylated, and the same sites are frequently mutated in smoking-induced lung cancer.[24,69] Methylation of the C-5

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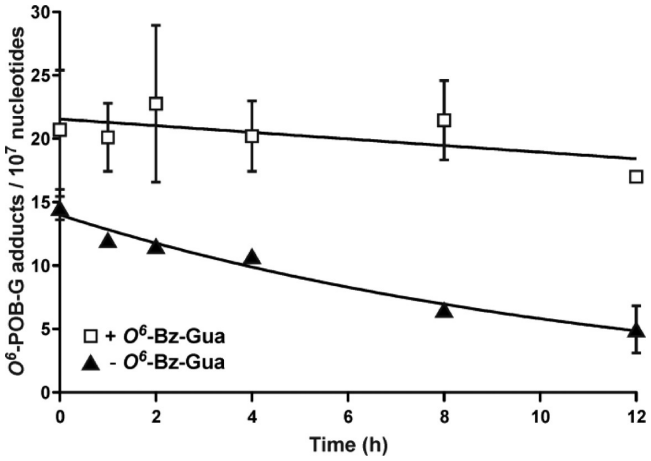

Figure 7. Time-dependent repair of O^6-POB-G adducts in normal human bronchial epithelial cells (HBEC). Cells in culture were treated with 150 μM NNKOAc for 1 h in the presence or in the absence of AGT-inhibitor, O^6-Bz-G (10 μM). Following the removal of NNKOAc, cells were grown in normal media for 0−12 h (or media containing 5 μM O^6-Bz-G), and the kinetics of O^6-POB-G repair over time were determined by HPLC-ESI$^+$-MS/MS. N = 3 experiments were averaged for each experimental condition.

position of cytosine can influence the local DNA structure and the electronic environment within ^{Me}CG dinucleotides.[70]

To investigate the potential effects of cytosine methylation on O^6-POB-G repair, site-specific adducts were placed within unmethylated, hemimethylated, and fully methylated CG dinucleotides within *p53* codons 158, 245, and 248 (Table 3). Following incubation with AGT protein, the second-order rates for AGT-mediated alkyl transfer were determined by HPLC-ESI$^+$-MS/MS. We found that the kinetics of AGT-mediated O^6-POB-dG repair was only weakly affected by neighboring ^{Me}C (Figure 5, Table 3). Gel shift experiments revealed that AGT binding to pyridyloxobutylated DNA was not influenced by cytosine methylation (Table 4). This is not unexpected since the 5-methyl group on cytosine is projected into DNA major groove,[46] while AGT binds to the minor groove of DNA.[12] Other authors previously noted that that AGT binding to O^6-alkylguanine containing DNA duplexes was not dependent on DNA sequence context or the alkyl group identity, but was increased in the presence of O^6-alkylguanine adducts as compared to unmodified DNA.[30,63,71]

We investigated the kinetics of O^6-POB-dG adduct repair in normal HBECs since these cells are targeted by nitrosamines present in tobacco smoke. Previous studies have shown that human bronchial epithelial cells in culture can be malignantly transformed following treatment with NNK (100 or 400 mg/mL) for 7 days.[72] Our results provide evidence for the ability of HBEC cells to repair O^6-POB-dG (Figure 7). Since the rate of repair was significantly reduced in the presence of AGT inhibitor (O^6-benzylguanine), we conclude that AGT repair may play an important role in preventing tobacco nitrosamine-associated lung cancer.

In conclusion, our results indicate that while local DNA sequence context influences the efficiency of O^6-POB-G repair in the context of *K-ras* protooncogene, the presence of ^{Me}C does not influence AGT protein binding to and repair of pyridyloxobutylated DNA. Since our experiments with human bronchial epithelial cells (Figure 7) indicate that direct repair by AGT plays an important role in the removal of O^6-POB-G adducts from human bronchial epithelial cells, sequence-dependent AGT repair may lead to accumulation of pyridyloxobutylating adducts and increased mutagenesis at inefficiently repaired sites.

■ ASSOCIATED CONTENT

Supporting Information

DNA melting curves (S-1), results of statistical analyses (Tables S-2, S-3, and S-4), cytotoxicity data for HBEC cells treated with NNKOAC (S-5), a sample HPLC-UV trace showing purity of (−) *K-ras*-POB oligonucleotide strand (S-6) and HPLC ESI$^-$ MS spectra for the synthetic oligodeoxynucleotides used in this work (S-7). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AGT, O^6-alkylguanine-DNA-alkyltransferase; BSA, bovine serum albumin; O^6-Bz-dG, O^6-benzyl-deoxyguanosine; O^6-Bz-G, O^6-benzylguanine; DTT, dithiothreitol; DNase I, deoxyribonuclease I; EDTA, ethylenediamine tetraacetic acid; HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry; HBEC, human bronchial epithelial cells; N7-MeG, N7-methylguanine; O^6-Me-dG, O^6-methyl-2′-deoxyguanosine; O^6-Me-G, O^6-methylguanine; PAGE, polyacrylamide gel electrophoresis; N7-POB-G, N7-[4-oxo-4-(3-pyridyl)-but-1-yl]guanine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosonicotine; O^6-POB-dG, O^6-[4-oxo-4-(3-pyridyl)but-1-yl]-deoxyguanosine; D_4-O^6-POB-dG, D_4-O^6-[4-oxo-4-(3-pyridyl)-but-1-yl]deoxyguanosine; O^6-POB-G, O^6-[4-oxo-4-(3-pyridyl)but-1-yl]guanine; PDE I, phosphodiesterase I; PDE II, phosphodiesterase II; POB, pyridyloxobutyl; SPE, solid phase extraction; TEAOAc, triethylamonium acetate; PNK, polynucleotide kinase

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