Cytosine Methylation Effects on the Repair of O\textsuperscript{6}-Methylguanines within CG Dinucleotides\textsuperscript{a}\textsuperscript{5}

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O\textsuperscript{6}-Alkyldeoxyguanine adducts induced by tobacco-specific nitrosamines are repaired by O\textsuperscript{6}-alkylguanine DNA alkyltransferase (AGT), which transfers the O\textsuperscript{6}-alkyl group from the damaged base to a cysteine residue within the protein. In the present study, a mass spectrometry-based approach was used to analyze the effects of cytosine methylation on the kinetics of AGT repair of O\textsuperscript{6}-methylguanosine (O\textsuperscript{6}-Me-dG) adducts placed within frequently mutated 5′-CG-3′ dinucleotides of the p53 tumor suppressor gene. O\textsuperscript{6}-Me-dG-containing DNA duplexes were incubated with human recombinant AGT protein, followed by rapid quenching, acid hydrolysis, and isotope dilution high pressure liquid chromatography-electrospray ionization tandem mass spectrometry analysis of unrepaired O\textsuperscript{6}-methylguanine. Second-order rate constants were calculated in the absence or presence of the C-5 methyl group at neighboring cytosine residues. We found that the kinetics of AGT-mediated repair of O\textsuperscript{6}-Me-dG were affected by neighboring 5-methylcytosine (\textsuperscript{Me}C) in a sequence-dependent manner. AGT repair of O\textsuperscript{6}-Me-dG adducts placed within 5′-CG-3′ dinucleotides of p53 codons 245 and 248 was hindered when \textsuperscript{Me}C was present in both DNA strands. In contrast, cytosine methylation within p53 codon 158 slightly increased the rate of O\textsuperscript{6}-Me-dG repair by AGT. The effects of \textsuperscript{Me}C located immediately 5′ and in the base paired position to O\textsuperscript{6}-Me-dG were not additive as revealed by experiments with hypomethylated sequences. Furthermore, differences in dealkylation rates did not correlate with AGT protein affinity for cytosine-methylated and unmethylated DNA duplexes or with the rates of AGT-mediated nucleotide flipping, suggesting that \textsuperscript{Me}C influences other kinetic steps involved in repair, e.g. the rate of alkyl transfer from DNA to AGT.

Metabolic activation of the tobacco carcinogen 4-(methylnitrosoamo)-1-(3-pyridyl)-1-butaneone (NNK)\textsuperscript{3} produces methylyl- and pyridoxobutyldiazonium ions that can react with DNA to give O\textsuperscript{6}-methyldeoxyguanosine (O\textsuperscript{6}-Me-dG) and O\textsuperscript{6}-pyridoxobutyl deoxyguanosine (O\textsuperscript{6}-POB-dG) lesions (1). Both adducts are strongly mutagenic because DNA polymerases preferentially misinsert thymine opposite O\textsuperscript{6}-alkylguanines, resulting in G → A transitions (2). Studies in laboratory animals have provided evidence for a direct involvement of O\textsuperscript{6}-Me-dG in NNK-mediated carcinogenesis (3).

A specialized repair protein, O\textsuperscript{6}-alkylguanine DNA alkyltransferase (AGT), removes the alkyl group from the O\textsuperscript{6} position of modified guanine bases, such as O\textsuperscript{6}-Me-dG and O\textsuperscript{6}-POB-dG, restoring normal guanine bases and preventing mutagenesis. AGT preferentially binds double-stranded DNA through a helix-turn-helix motif (4). In the resulting AGT-DNA complex, one recognition helix of the protein is found within the minor groove of the DNA, whereas the other one interacts with the phosphodiester backbone (4). The adducted nucleotide is flipped into a binding pocket within the protein, whereas Arg-128 takes its place in the double helix (4). A hydrogen bonding network around the active site involving His-148, Glu-172, and a water molecule promotes the deprotonation of the active site cysteine (Cys-145) (4, 5). The resulting thiolate anion acts as a nucleophile, displacing the O\textsuperscript{6} substituent of O\textsuperscript{6}-alk-G and regenerating normal guanine (Fig. 1) (4, 5). The alkylated protein is inactive and is rapidly degraded by the ubiquitin proteolytic pathway (6–8).

AGT-mediated repair of O\textsuperscript{6}-Me-dG lesions includes multiple kinetic steps (9). First, the AGT protein must bind adducted DNA in a reactive conformation. The alkylated nucleotide is flipped out of the DNA base stack to enter the hydrophobic pocket within AGT, and the methyl group is transferred from DNA to the protein. Finally, alkylated AGT protein dissociates from the repaired DNA. Zang et al. (9) reported that the chemical step of alkyl transfer is rate-limiting in the case of O\textsuperscript{6}-Me-dG, but not O\textsuperscript{6}-benzyl-dG. Furthermore, previous studies have shown that the repair of O\textsuperscript{6}-Me-dG by mammalian AGT is influenced by the nature of the O\textsuperscript{6}-alkyl group, the length of oligonucleotide duplex, the placement of the adduct, and the identities of neighboring nucleotides (10–14).

Removing the alkyl group from O\textsuperscript{6}-Me-dG by AGT regenerates normal guanine and protects the genome from G → A
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FIGURE 1. Direct repair of O^6^-alkyl-guanine adducts by AGT.

among the major 35% of mutations at the CG dinucleotides (15, 19). Given the (18). Of all produced lung cancer, p53 reduced lung cancer tumors (16). methylated ing protein expression (15). In contrast, 33% of tumors with a transition mutations in the p53 gene when the promoter region of the gene coding for AGT was not methylated, thereby allowing protein expression (15). In contrast, 33% of tumors with a methylated AGT promoter had G → A mutations within the p53 gene (15). The p53 gene is mutated in over 50% of non-small cell lung cancer tumors (16).

All CpG sites within the coding sequence of the p53 gene are endogenously methylated (17). Importantly, the same sites are among the major p53 mutational “hotspots” in smoking-induced lung cancer, e.g. codons 157, 158, 245, 248, 249, and 273 (18). Of all p53 mutations, G → A transitions account for 18–24% of genetic changes observed in lung cancer, including 35% of mutations at the CG dinucleotides (15, 19). Given the established role of NNK-induced O^6^-alkylguanine lesions in tobacco carcinogenesis and mutagenesis (20), they are likely to be involved in the induction of smoking-associated G → A transitions in the p53 gene.

The presence of MeC residues may hinder the repair of O^6^-Me-dG lesions within endogenously methylated CG dinucleotides (14). For example, Bentivenga and Bresnick (14) showed that the repair of O^6^-Me-dG by recombinant AGT in the context of codon 248 of the p53 gene was reduced by 75% when MeC was placed immediately 5′ to the O^6^-Me-dG lesion. However, the effects of cytosine methylation on AGT repair of O^6^-Me-dG in other sequence contexts have not been previously investigated, and it is not known which individual steps in the repair of O^6^-methyl group are affected by neighboring MeC.

Cytosine methylation leads to small structural changes of DNA duplex, including an increase in the base pair rise, roll, and local curvature angles, narrowing of the DNA minor groove, and decreased depth of the major groove (21–23). These structural alterations may influence the affinity of the AGT protein for alkylated DNA. Furthermore, MeC enhances base stacking (24) and stabilizes the DNA duplex by increasing the molecular polarizability of the cytosine base (25), which can have an effect on the rate of AGT-mediated nucleotide flipping. The alkyl transfer step itself may be meditated by the presence of MeC through its effects on transition state geometry.

The goal of the present study was to systematically examine the effects of cytosine methylation on AGT-mediated repair of O^6^-Me-dG lesions placed within 5′-CG-3′ dinucleotides representing major p53 mutational hotspots observed in lung cancer. The kinetics of alkyl transfer were analyzed using rapid-quench methods coupled with quantitative analyses of O^6^-Me-dG by isotope dilution-high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) (26). Furthermore, we examined the effects of cytosine methylation on AGT binding to O^6^-Me-dG-containing DNA and its influence on the rate of

EXPERIMENTAL PROCEDURES

Materials—DNA oligodeoxynucleotides used in this study were prepared by standard phosphoramidite chemistry at the University of Minnesota Microchemical Facility. 5′-Dimethoxytrityl-N-isobutyl-2′-deoxyguanosine, 3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (O6-Me-dG-CE phosphoramidite), 5′-dimethoxytrityl-N-benzoyl-5-methyl-2′-deoxyctydine,3′-[(2-cyanoethyl)-(N,N-diisopro- pyl)]-phosphoramidite (5-Me-dC-CE phosphoramidite), and 5′-dimethoxytrityl-[6-methyl-pyrrrolo-[2,3-d]-pyrimidine-2(3H)-one]-2′-deoxyribonucleoside, and 3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (pyrrrolo-dC-CE phosphoramidite) were obtained from Glen Research Corp. (Sterling, VA). Human recombinant AGT proteins (C-terminal histidine-tagged wild-type hAGT and N-terminal histidine-tagged C145A hAGT mutant) were prepared as described previously (27, 28). The activity of the AGT protein was determined as previously described (26). All HPLC and liquid chromatography/mass spectrometry grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Centricron YM-10 filters (0.5 ml) were procured from Phenomenex Corp. (Torrance, CA). [γ-^32P]ATP was obtained from PerkinElmer Life Sciences, and T4 polynucleotide kinase was from New England Biolabs (Beverly, MA).

O^6^-Trideuteriomethyl-deoxyguanosine (O^6^-CD3-dG) was synthesized as previously described (29). O^6^-Trideuteriomethyl-guanine (O^6^-CD3-G) was prepared by acid hydrolysis of O^6^-CD3-dG (0.1 N HCl, 70 °C for 1 h). O^6^-CD3-G was purified by HPLC and identified by UV and MS/MS as previously described (26).

Preparation of O^6^-Me-dG Containing DNA Strands—DNA sequences were derived from p53 exon 5 (codons 155–161) and p53 exon 7 (codons 242–247 and 246–251) containing the major p53 lung cancer mutational hotspots at codons 158, 245, and 248. Synthetic oligodeoxynucleotides containing O^6^-Me-dG at a defined site were prepared at the University of Minnesota Microchemical facility. Nucleoside phosphorimidates were purchased from Glen Research Corporation. Synthetic oligodeoxynucleotides containing O^6^-Me-dG were deprotected in the presence of 10% 1,8-diazabicyclo[5,4,0]undec-7-ene/anhydrous methanol (room temperature, 5 days in the dark); all oligodeoxynucleotides were purified by reverse-phase HPLC as described elsewhere (26, 29, 30). The identities of HPLC-puriﬁed DNA strands were conﬁrmed by HPLC-ESI−MS using previously published methods (31) (supplemental Table S1). To obtain double-stranded DNA, equimolar amounts of the complementary strands were combined in a buffer containing 10 mM Tris–HCl, pH 8.0, and 50 mM sodium chloride, followed by
heating to 90 °C and slowly cooling to room temperature. In all double-stranded DNA substrates, cytosine or MeC were introduced opposite O6-Me-dG.

**Determination of UV Melting Temperatures**—DNA duplexes (Table 1) were dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 50 mM sodium chloride, to give a 16.1 μM solution of DNA. UV melting temperatures ($T_m$) were determined using a Varian Cary 100 Bio UV-visible spectrophotometer. Two temperature ramps were performed on each sample (in triplicate): (i) 30–90 °C at a rate of 0.5 °C/min and (ii) 90 to 30 °C at a rate of 0.5 °C/min; data were collected at 0.5 °C intervals. The $T_m$ of each duplex was determined using the Cary WinUV Thermal software (Varian, Palo Alto, CA) (supplemental Table S2).

**Circular Dichroism (CD) Spectroscopy of Oligonucleotides**—DNA duplexes were dissolved in 50 mM sodium chloride, 10 mM sodium phosphate, pH 7.0, buffer to give a 16.1 μM solution. CD spectra were obtained on a Jasco J-710 spectropolarimeter from 350 to 200 nm at a wavelength step of 0.5 nm using a 1-mm quartz cuvette. Spectra were generated from an average of three scans (supplemental Fig. S1).

**HPLC-ESI-MS/MS Analysis**—Capillary HPLC-ESI-MS/MS analyses were performed with an Agilent 1100 Capillary HPLC system interfaced to a Finnigan Quantum Ultra triple quadrupole (TSQ) mass spectrometer. Capillary HPLC separations were performed on a Synergi 4 μm-Hydro-RP 80-Å column (250 mm × 0.5 mm, 4 μm) obtained from Phenomenex (Torrance, CA). The column was eluted isocratically with 25 mM ammonium acetate buffer containing 13.5% methanol and 4.5% acetonitrile. The temperature was maintained at 40 °C, and the flow rate was 12 μl/min. Under these conditions, O6-methylguanine (O6-Me-G) and O6-CD3-G (internal standard) eluted at 8.5 min. Quantitative analysis was performed in the selected reaction monitoring mode using the MS/MS transitions corresponding to the neutral loss of NH3 from O6-Me-G and O6-CD3-G (m/z 166.1 → 149.1) and O6-CD3-G (m/z 169.1 → 152.1) (26).

**AGT Reactions with O6-Me-dG-containing DNA Duplexes**—An RQF-3 Rapid Quench instrument (KinTek Corp., Austin, TX) was used in the AGT repair experiments with double-stranded substrates. All experiments were performed in triplicate. DNA duplexes containing a single O6-Me-dG residue (11.52 nM, 20.4 μM) were mixed with AGT protein (8.58 nM active protein, 21.2 μM) in 50 mM Tris-HCl buffer, pH 7.8, containing 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, and 0.5 mM DTT. A constant quench (92 μl/min). Under these conditions, O6-Me-G and O6-CD3-G (internal standard for MS) was eluted (26) from 350 to 200 nm at a wavelength step of 0.5 nm using a 1-mm quartz cuvette. Spectra were generated from an average of three scans (supplemental Fig. S1).

The extent of O6-Me-dG dealkylation was calculated from the ratios of the HPLC-ESI-MS/MS peak areas corresponding to O6-Me-G ($A_{O6}$) and O6-CD3-G internal standard ($A_{D3}$) in control samples ($R_o$) and in samples incubated with AGT for fixed periods of time ($R_t$). The difference in the ratio for the control sample and the ratio for the incubated sample was divided by the ratio for the control sample and then multiplied by 100% to obtain percent repair (Equations 1 and 2).

$$\% \text{ Repair}(t) = \frac{R_o - R_t}{R_o} \quad \text{(Eq. 1)}$$

where

$$R_o = \frac{A_{D3} - A_{O6}}{A_o - A_{D3}} \quad R_t = \frac{A_{D3} - A_{O6}}{A_t - A_{D3}} \quad \text{(Eq. 2)}$$

Control samples were incubated in the absence of AGT and represent 0% repair.

Second-order rate constants for AGT-mediated repair of O6-Me-G in the context of codons 158, 245, and 248 with and without neighboring MeC were determined by plotting the concentration of demethylated O6-Me-G versus time and fitting the data to Equation 3 using the KaleidaGraph software from Synergy Software (Reading, PA).

$$kt = \frac{1}{B_0 - A_0} \times \ln \frac{A_0(B_0 - C_t)}{B_0(A_0 - C_t)} \quad \text{(Eq. 3)}$$

In Equation 3, A and B are the concentrations of AGT and O6-Me-G, respectively, and C is the concentration of O6-Me-G repaired at time t. The subscripts 0 and t refer to the concentrations of these species at time 0 and time t.

**AGT Reactions with Single-stranded DNA Containing O6-Me-dG**—Single-stranded oligodeoxynucleotides containing a 6-Me-dG residue at a specific site neighboring C or MeC (500 fmol) were dissolved in 50 mM Tris-HCl, pH 7.8, buffer containing 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, and 0.5 mM DTT. Samples were incubated with AGT (400 fmol), and the reactions were manually quenched at specified times (0–3600 s) by the addition of HCl to obtain a final concentration of 0.1 M HCl. Following the addition of the internal standard (O6-CD3-G, 267.2 fmol), samples were processed and analyzed as described above.

**Electrophoretic Mobility Shift Assay**—A synthetic DNA duplex (0.8 μM) spiked with the corresponding 5'-32P-end-labeled duplex (1 μCi) was incubated with increasing amounts of mutant human recombinant AGT protein (C145A; 0–6 μM) in 50 mM Tris-HCl, pH 7.8, buffer containing 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, and 0.5 mM DTT for 30 min at 20 °C. Electrophoresis was performed using 10% polyacrylamide gels (acrylamide:N,N'-methylene bisacrylamide = 75:1). Images were obtained by exposing the gel to a Storage Phosphor Screen (Molecular Dynamics), and then imaged using a Bio-Rad Molecular Imager FX or a Molecular Dynamics Storm 840 system and quantified by densitometry using the ImageJ software.

The dissociation constants for the AGT-DNA complex ($K_d$) were determined as described in the literature (32). The ratios
of the concentration of free DNA ([D]) to the total DNA ([D]T) were calculated from the density of the corresponding signals on the non-denaturing gel. Equation 4 can be converted to Equation 5, where [P] is the protein concentration and [PD] is the AGT-DNA complex concentration.

\[
K_d = \frac{[P][D]}{[PD]} \quad \text{(Eq. 4)}
\]

\[
\frac{[D]}{[D]_T} = \frac{K_d}{K_d + [P]_T} \quad \text{(Eq. 5)}
\]

Estimates of \(K_d\) were obtained by fitting Equation 5 to the experimentally determined values of \([D]/[D]_T\) versus \([P]_T\) plots using the KaleidaGraph software from Synergy Software (Reading, PA), where \([P]_T\) is the total protein concentration in the reaction mixture.

**Steady-state Fluorescence Experiments**—Fluorescence experiments were carried out with a Varian Cary Eclipse fluorescence spectrophotometer following the experimental procedures of Zang et al. (9). The samples containing a final concentration of 200 nM double-stranded DNA were mixed with 0, 0.16, 1.6, 3.2, or 4.8 μM C145A-AGT in 50 mM Tris-HCl, pH 7.8, buffer containing 0.1 mM EDTA and 0.5 mM DTT. Samples were excited at 360 nm and emission was monitored from 400–500 nm. The bandpass was 5 nm for both the excitation and emission slits.

**Stopped-flow Fluorescence Experiments**—Stopped-flow fluorescence experiments were carried out using an Applied Photophysics SX-17MV stopped-flow spectrophotometer (Letherhead, UK) following the experimental procedures of Zang et al. (9). Equal volumes of double-stranded DNA containing O6-Me-dG paired with the fluorescent dC analog, 6-methylpyrrolo[2,3-d]pyrimidine-2(3H)-one (pyrrolo-dC, 400 nM) and C145-AGT (3.2 μM) in 50 mM Tris-HCl, pH 7.8, buffer containing 0.1 mM EDTA and 0.5 mM DTT were mixed. The solution was excited at 347 nm, and the emission was monitored using a 400-nm cutoff filter. Data were collected and analyzed using the Applied Photophysics software. All reactions were done at 5 °C and the data from each reaction was fit to a single-exponential equation. The average rate and standard deviation from at least 6 reactions is reported (Table 4).

**Statistical Analysis of the Data**—All statistical analyses were carried out at the University of Minnesota Biostatistics Core.
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FIGURE 2. Effects of cytosine methylation on the kinetics of AGT-mediated repair of O\textsuperscript{6}-Me-G adducts within double-stranded p53 codon 248 (A), p53 codon 245 (B), and p53 codon 158 (C). Synthetic DNA duplexes containing O\textsuperscript{6}-Me-G in the context of unmethylated, hemimethylated, or fully methylated CG dinucleotides (5.65 nM, final concentration) were incubated with human recombinant AGT protein (4.37 nM, final concentration) and the reactions were terminated at specified time points using a Rapid Quench instrument. The samples were acid-hydrolyzed to release O\textsuperscript{6}-Me-G, which was quantified by isotope dilution HPLC-ESI-MS/MS. The solid lines represent the best fit to a second-order rate equation (Equation 3). * statistically significant difference as compared to unmethylated CG dinucleotide (p < 0.036). Error bars represent S.D.

using SAS 9.1 (SAS Institute Inc., Cary, NC) software. Two-way analysis of variance with factors of treatment and time was used to compare the time course curves for O\textsuperscript{6}-Me-G repair in different sequences as a function of cytosine methylation. p values of pairwise comparisons between treatments were adjusted by the Tukey method.

RESULTS

Selection and Characterization of DNA Sequences—To investigate the effects of endogenous cytosine methylation on AGT-mediated repair of O\textsuperscript{6}-Me-dG lesions, a series of synthetic oligodeoxynucleotide duplexes (19–21 bp in length) containing centrally positioned p53 codons 158, 245, and 248 and surrounding sequences were prepared (Table 1). In each duplex, O\textsuperscript{6}-Me-G was placed within p53 codons 158, 245, or 248, and MeC was introduced immediately 5’ to O\textsuperscript{6}-Me-G, opposite to O\textsuperscript{6}-Me-G, or at both positions as observed physiologically. All DNA strands were purified by HPLC and characterized by ESI-MS (supplemental Table S1).

The thermodynamic stability of O\textsuperscript{6}-Me-G-containing DNA duplexes with different cytosine methylation status was determined by UV thermal denaturation. Hyperbolic curves were observed for each strand, indicating that all p53-derived oligodeoxynucleotides formed stable duplexes. UV melting temperatures of the duplexes containing MeC were greater than for the corresponding duplexes lacking MeC (supplemental Table S2), consistent with the known ability of MeC to enhance base stacking and DNA duplex stability (25, 33). However, CD spectra obtained for each set of oligodeoxynucleotides of the same sequence were identical regardless of cytosine methylation status (supplemental Fig. S1), consistent with previous reports that the presence of a single MeC per strand does not significantly affect the overall secondary structure of DNA (23).

Kinetics of AGT-mediated Repair of O\textsuperscript{6}-Me-G as a Function of Cytosine Methylation in Duplex DNA—Our approach for analyzing the kinetics of AGT-mediated dealkylation involved incubating O\textsuperscript{6}-Me-G-containing DNA with purified human recombinant AGT protein for fixed periods of time (1–25 s), followed by rapid quenching, acid hydrolysis, and quantitative analysis of O\textsuperscript{6}-Me-G nucleobases remaining in DNA by isotope dilution HPLC-ESI-MS/MS (26). AGT repair rates for O\textsuperscript{6}-Me-G adducts placed within p53 codons 158, 245, and 248 were determined. To evaluate the effects of 5’-neighboring and base paired MeC independently, AGT repair experiments were conducted for C[O\textsuperscript{6}-Me-G] dinucleotides with different cytosine methylation status (unmethylated, hemimethylated, and fully methylated) (Fig. 2, Table 1).

For all three p53-derived DNA duplexes, the extent of repair of O\textsuperscript{6}-Me-G by AGT increased over time (Fig. 2), but the consequences of cytosine methylation were different depending on the sequence. For O\textsuperscript{6}-Me-dG present within p53 codon 248, the most pronounced effect (2.7-fold decrease in repair rate) was observed in strands containing 5’-neighboring MeC (codon 248 5’-MeC, k = 7.3 ± 0.7 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) as compared with unmethylated duplex (codon 248, no MeC k = 19.7 ± 1.0 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) (Fig. 2A). A smaller reduction (∼25–40%) was seen for duplexes containing base-paired MeC (codon 248 base paired MeC, k = 14.5 ± 1.6 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) and for fully methylated C[O\textsuperscript{6}-Me-G] dinucleotides (codon 248 both MeC, k = 11.3 ± 0.6 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) (Fig. 2A). These results are consistent with previous findings of Bentivegna and Bresnick (14) and suggest that O\textsuperscript{6}-Me-dG adducts formed at p53 codon 248 are less efficiently repaired upon endogenous cytokine methylation, potentially contributing to mutagenesis at this site.

For the duplexes containing O\textsuperscript{6}-Me-dG in the context of p53 codon 245, AGT-mediated repair of O\textsuperscript{6}-Me-dG was faster in oligodeoxynucleotides that lacked cytosine methylation (no MeC, k = 7.3 ± 0.6 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) than for the duplexes containing fully methylated dinucleotides (both MeC, k = 4.5 ± 0.3 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}). However, the rates of O\textsuperscript{6}-Me-dG repair were increased in hemimethylated strands (codon 245 5’-MeC, k = 12.6 ± 1.1 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}; codon 245 base paired MeC, k = 19.2 ± 1.0 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}), despite the overall rate reduction in
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fully methylated CG dinucleotides (Table 1 and Fig. 2B). It is possible that the presence of a single MeC residue in this sequence context (GC(O⁶-Me-G)G) leads to changes of DNA-AGT complex geometry that are more favorable for alkyl transfer than that observed for fully methylated CG dinucleotides.

In contrast to our results for p53 codons 245 and 248, the rate of repair of O⁶-Me-dG placed in the physiologically methylated p53 codon 158 was slightly increased in the presence of MeC (codon 158 both MeC, \( k = 9.6 \pm 0.5 \times 10^6 \text{M}^{-1} \text{s}^{-1} \)) as compared with duplex lacking MeC (codon 158 no MeC, \( k = 8.8 \pm 0.8 \times 10^6 \text{M}^{-1} \text{s}^{-1} \), see Table 1 and Fig. 2C), although this difference was not statistically significant (\( p = 0.52 \)). Small rate increases were also noted for hemimethylated strands (codon 158 5’-MeC, \( k = 10.2 \pm 0.8 \times 10^6 \text{M}^{-1} \text{s}^{-1} \); codon 158 base paired MeC, \( k = 11.3 \pm 0.8 \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (Table 1, Fig. 2C).

Effects of MeC on AGT-mediated Repair of O⁶-Me-G in Single-stranded DNA—Because AGT may repair O⁶-Me-G lesions present in single-stranded regions of DNA that exist during replication and transcription, the kinetics of AGT-mediated O⁶-Me-G repair were also evaluated in single-stranded oligodeoxynucleotides. Second-order AGT repair rates were determined for single-stranded DNA substrates containing O⁶-Me-G in the context of p53 codons 158, 245, or 248 in the absence and presence of 5’ neighboring MeC (Table 2 and Fig. 3). Overall, the rates of AGT repair of O⁶-Me-G were much slower in single-stranded DNA as compared with the corresponding double-stranded DNA (compare Tables 1 and 2), consistent with previous studies (11, 12, 34). Furthermore, the demethylation rate was particularly low for O⁶-Me-G located within p53 codon 248 (Table 2), possibly due to the formation of secondary structures in the p53 codon 158 and 245 containing sequences (35). In the context of codon 248, a 2-fold decrease in the rate of O⁶-Me-G repair by AGT was observed upon cytosine methylation (\( (+) \) codon 248, no MeC, \( k = 0.08 \pm 0.006 \times 10^6 \text{M}^{-1} \text{s}^{-1} \); (++) codon 248, 5’MeC, \( k = 0.04 \pm 0.002 \times 10^6 \text{M}^{-1} \text{s}^{-1} \)), which is consistent with the effect of 5’-neighboring MeC in the corresponding DNA duplex (Table 1). AGT repair of O⁶-Me-G in the context of single-stranded p53 codon 245 was not affected by the presence of MeC (\( (+) \) codon 245, no MeC, \( k = 2.4 \pm 0.4 \times 10^6 \text{M}^{-1} \text{s}^{-1} \); (++) codon 245, 5’MeC, \( k = 2.4 \pm 0.2 \times 10^6 \text{M}^{-1} \text{s}^{-1} \)) (Fig. 3B). In the context of codon 158, the rate of O⁶-Me-G by AGT was increased in the presence of MeC (\( (+) \) codon 158, no MeC, \( k = 0.7 \pm 0.05 \times 10^6 \text{M}^{-1} \text{s}^{-1} \); (++) codon 158, 5’MeC, \( k = 1.2 \pm 0.1 \times 10^6 \text{M}^{-1} \text{s}^{-1} \)) (Fig. 3C), which is similar to the effect of 5’-MeC in double-stranded codon 158 (Table 1).

Binding of AGT to O⁶-Me-G-containing Oligodeoxynucleotides—To determine whether cytosine methylation influences the binding affinity of AGT for O⁶-Me-G containing DNA duplexes, electrophoretic mobility shift assays were conducted. These experiments utilized DNA duplexes containing p53 codons 158, 245, and 248 previously employed for AGT repair kinetics studies (Table 3). AGT active site mutant C145A was employed, which is unable to repair O⁶-alk-G lesions, but retains DNA binding properties of the native protein (36). DNA duplexes were incubated with increasing concentrations of C145A-AGT (AGT/DNA ratio = 0–7.5) and the formation of DNA-protein complexes was evaluated by non-denaturing polyacrylamide gel electrophoresis (see an example in supplemental Fig. S2).

We found that the affinity of the AGT protein for O⁶-Me-G-containing DNA duplexes was only weakly affected by the presence of MeC (Fig. 4, Table 3). \( K_d \) values increased in the presence of competitor calf thymus DNA, which was added to limit non-

**TABLE 2**

| Oligonucleotide ID | Sequence (5’-3’)) | \( k/10^6 \text{M}^{-1} \text{s}^{-1} \) |
|-------------------|-----------------|------------------|
| (+) codon 158, no MeC | ACCGGGCTTC (O⁶-Me-G) GCCCATGGCC | 0.7 \pm 0.05 |
| (+) codon 158, 5’MeC | ACCGGGCTTC (O⁶-Me-G) GCCCATGGCC | 1.2 \pm 0.1 |
| (+) codon 245, no MeC | GCGTGGG C (O⁶-Me-G) GCCATGGCCG | 2.4 \pm 0.4 |
| (+) codon 245, 5’MeC | GCGTGGG (O⁶-Me-G) GCCATGGCCG | 2.4 \pm 0.2 |
| (+) codon 248, no MeC | CATGACC CG (O⁶-Me-G) GAGGCCCATC | 0.08 \pm 0.006 |
| (+) codon 248, 5’MeC | CATGACC (O⁶-Me-G) GAGGCCCATC | 0.04 \pm 0.002 |

**FIGURE 3.** Effects of cytosine methylation on the kinetics of AGT-mediated repair of O⁶-Me-G adducts within single-stranded p53 codon 248 (A), p53 codon 245 (B), and p53 codon 158 (C). Synthetic DNA oligomers containing O⁶-Me-G in the context of unmethylated or methylated CG dinucleotides (5.56 mM, final concentration) were incubated with human recombinant AGT protein (4.44 nM, final concentration) and the reactions were terminated at specified time points. The samples were acid-hydrolyzed to release O⁶-Me-G, which was quantified by isotope dilution HPLC-ESI-MS/MS. The solid lines represent the best fit to a second-order rate equation (Equation 3). * indicates statistically significant difference as compared with unmethylated CG dinucleotide (\( p < 0.0004 \)). Error bars are S.D.
specific binding (Table 3). Taken together, our results suggest that AGT-DNA binding is not significantly affected by cytosine methylation, consistent with previous studies (37, 38).

**Effects of Cytosine Methylation on AGT-mediated Nucleotide Flipping**—The AGT repair reaction requires that the O\(^6\)-alkylguanine nucleotide is flipped out of the DNA base stack to enter the alkyl acceptor site within the protein, whereas Arg-128 takes its place inside the duplex (4). We conducted pre-steady state stopped-flow fluorescence experiments (9) to investigate the rate of AGT-mediated O\(^6\)-Me-G flipping in the presence or absence of a neighboring MeC residue. DNA duplexes derived from the p53 gene containing O\(^6\)-Me-G base paired to a fluorescent analog of cytosine, 6-methylpyrrolo[2,3-d]pyrimidine-2(3H)-one (pyrrolo-dC), with or without a MeC immediately 5’ to the O\(^6\)-Me-G adduct, were used (Table 4). Nucleotide flipping experiments were conducted with the C145A-AGT mutant that was unable to repair O\(^6\)-Me-G but binds O\(^6\)-Me-G containing DNA in the same manner as the native protein.

First, steady state fluorescence experiments were carried out to determine the concentration of AGT-DNA complexes. In the context of p53 codons 248 and 245 only fully methylated and unmethylated CG dinucleotides containing strands were analyzed. Each plot represents the average of three experiments and the error bars are the S.D. The solid line is the fit of the data to Equation 5.

![Figure 4](image-url)

**FIGURE 4.** Effects of cytosine methylation on binding affinity of C145A-AGT for O\(^6\)-Me-G-containing double-stranded p53 codon 248 (A), p53 codon 245 (B), and p53 codon 158 (C). Plot of the ratio of free oligonucleotide to total oligonucleotide density versus total AGT concentration. The plots were utilized to determine the dissociation constants for AGT-DNA complexes. In the context of p53 codons 248 and 245 only fully methylated and unmethylated CG dinucleotides containing strands were analyzed. Each plot represents the average of three experiments and the error bars are the S.D. The solid line is the fit of the data to Equation 5.

**TABLE 3**

| Oligonucleotide ID | Sequence | \(K_d/10^6\) (M) | \(K_d/10^6\) (M) + CT-DNA |
|-------------------|----------|-----------------|--------------------------|
| codon 158, no MeC | ACCCGGT Ac [O\(^6\)-Me-G] CGCATGGCC G | 1.3 ± 0.09 | 3.2 ± 0.5 |
| codon 158, 5’-MeC | ACCCGGT Ac [O\(^6\)-Me-G] CGCATGGCC G | 1.2 ± 0.04 | 3.2 ± 0.5 |
| codon 158, base paired MeC | ACCCGGT Ac [O\(^6\)-Me-G] CGCATGGCC G | 1.1 ± 0.09 | 3.2 ± 0.5 |
| codon 158, both MeC | ACCCGGT Ac [O\(^6\)-Me-G] CGCATGGCC G | 1.2 ± 0.04 | 4.3 ± 0.5 |
| codon 245, no MeC | GCATGGG C [O\(^6\)-Me-G] GCATGAACCG G | 0.9 ± 0.06 | 3.2 ± 0.5 |
| codon 245, both MeC | GCATGGG MeC [O\(^6\)-Me-G] GCATGAACCG G | 0.9 ± 0.08 | 3.2 ± 0.5 |
| codon 248, no MeC | CATGAA C [O\(^6\)-Me-G] GAGGCCATCG G | 0.6 ± 0.06 | 3.2 ± 0.5 |
| codon 248, both MeC | CATGAA MeC [O\(^6\)-Me-G] GAGGCCATCG G | 0.8 ± 0.05 | 3.2 ± 0.5 |
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>400 nm was recorded from 0 to 0.5 s (Fig. 5 and supplemental Fig. 4). We found that the rates of nucleotide flipping for duplexes containing O^6-Me-G within the context of p53 codons 248 and 245 were between 80 and 182 s^{-1} (Table 4). The introduction of MeC immediately 5' to the O^6-Me-G residue did not significantly influence the rate of AGT-mediated nucleotide flipping (p > 0.05). A second kinetic event was observed, most clearly, in the fluorescence experiment for DNA duplexes containing p53 codon 158. The second event has a k of ~4 s^{-1} and may correspond to the dissociation of the protein from DNA.

DISCUSSION

Direct repair of O^6-alkylguanine lesions by AGT represents the first line of cellular defense against DNA damage induced by alkylating agents such as metabolites of tobacco carcinogen NNK. If not repaired, O^6-alkylguanine pairs with thymine instead of cytosine during DNA replication, resulting in G → A transition mutations (39, 40). Smoking-induced adenocarcinomas of the lung frequently contain G → A transitions at 5'-MeCG-3' dinucleotides of the p53 tumor suppressor gene, especially when the AGT gene is transcriptionally inactivated (15). This mutational specificity is not a result of targeted NNK adduct formation, because the presence of MeC residues at 5'-CG-3' dinucleotides appears to protect them against O^6-guanine alkylation by NNK diazohydroxides (29, 30). It also cannot be caused by sequence effects on polymerase fidelity, because replicative DNA polymerases place T opposite the lesion irrespective of the local sequence environment (41).

Because only DNA adducts that escape repair can be converted to heritable mutations, differential AGT repair of O^6-alkylguanine lesions may play a role in shaping the mutational spectra along the p53 gene (13). C-5 cytosine methylation increases the molecular polarizability of dC, facilitating base stacking and stabilizing the DNA duplex (24, 42). It also strengthens hydrogen bonding within the MeC:G base pair and leads to narrowing of the DNA minor groove (22). These changes can potentially influence DNA-protein interactions and mediate DNA repair kinetics. For example, Muheim et al. (43) found that endogenous methylation of cytosine at CG sites affects nucleotide excision repair of N^2-BPDE-dG lesions, probably a result of conformational changes induced by MeC (44). We therefore hypothesized that neighboring MeC reduces the rates of AGT repair of O^6-alkylguanines located at MeCG dinucleotides, leading to adduct accumulation and increased mutagenesis at these sites.

We chose to analyze the effects of cytosine methylation on AGT repair of O^6-Me-G residues placed within the context of p53 codons 158, 245, and 248 because these guanines are within endogenously methylated MeCG sequences and are frequently mutated in lung cancer (45). A mass spectrometry based assay developed in our laboratory (26) was used to determine second-order rates for AGT-mediated repair of O^6-Me-G in the pres-

![FIGURE 5. Fluorescence changes attributed to AGT-induced O^6-Me-G nucleotide flipping opposite of the dC analog, pyrrolo-dC. C145A-AGT (final concentration 1.6 μM) was mixed with p53-derived oligodeoxynucleotide duplexes containing O^6-Me-G base paired to pyrrolo-dC (final concentration 200 nM). The changes in fluorescence were monitored using an excitation wavelength of 347 nm and collecting emission >400 nm from 0 to 0.5 s. The rates of nucleotide flipping were estimated by fitting the data from 0 to 0.1 s to a single exponential (solid black line). Each plot is an average of at least 6 runs. The residual error is plotted on the same scale as the data and are shown below each graph. A, p53 codon 245, no MeC; B, p53 codon 245, MeC 5' to O^6-Me-G.](image)
ence and absence of neighboring MeC. Furthermore, the effects of cytosine methylation on AGT protein binding to O\textsuperscript{6}-Me-G-containing DNA and on the rates of AGT-mediated nucleotide flipping were evaluated.

Our results indicate that in double-stranded DNA in the context of p53 codons 245 and 248, AGT repair of O\textsuperscript{6}-Me-G was inhibited by the presence of MeC (Table 1, Figs. 2, A and B), confirming a previous report of Bentivegna and Bresnick (14) for codon 248. Deficient repair of NNK-induced O\textsuperscript{6}-Me-G adducts can potentially contribute to mutagenesis at these sites. In contrast, AGT repair of O\textsuperscript{6}-Me-G in the context of codon 158 was somewhat facilitated in the presence of MeC (Table 1, Fig. 2C). This discrepancy may be explained by next to neighbor effects on AGT repair kinetics (22, 46).

Repair of single-stranded DNA during replication may be the last opportunity to prevent promutagenic O\textsuperscript{6}-Me-G lesions from inducing mutations. We found that the rates of AGT repair of O\textsuperscript{6}-Me-G containing single-stranded DNA are much lower than the corresponding rates for double-stranded DNA (Tables 1 and 2). AGT binding to double-stranded DNA is known to induce bending of the duplex away from the protein, promoting the flipping of the adducted O\textsuperscript{6}-alk-dG nucleotide out of the base stack (7, 8). This event may be different in single-stranded DNA, accounting for the decreased rate of repair (6, 47). Furthermore, we found that AGT repair of O\textsuperscript{6}-Me-G placed in the context of single-stranded p53 codon 158 is facilitated by the presence of 5\textsuperscript{\textprime}MeC (Table 2, Fig. 3C). In contrast, AGT repair of single-stranded substrates representing p53 codons 248 and 245 is inhibited and unaffected, respectively, when 5-methylcytosine is introduced next to O\textsuperscript{6}-Me-G (Table 2, Fig. 3, A and B).

Binding interactions between AGT and O\textsuperscript{6}-Me-G containing DNA are not affected by cytosine methylation as indicated by the results of our gel shift assay (Table 3). This observation is consistent with previous structural and kinetic studies of AGT-DNA interactions. AGT binding to DNA appears to be diffusion limited, regardless of sequence (9). Furthermore, AGT binds the minor groove of DNA (4), whereas the C-5 methyl group of MeC projects into the major groove (22) and does not directly interfere with initial AGT-DNA binding. Unfortunately, the gel shift assay cannot distinguish between AGT binding to DNA in a reactive or an unreactive conformation.

Flipping the O\textsuperscript{6}-Me-G nucleotide out of the DNA base stack to enter the binding pocket of AGT constitutes a key step in the dealkylation mechanism (4). Because MeC increases base stacking and enhances DNA duplex stability, we explored the possibility that cytosine methylation slows down the rate of AGT-mediated nucleotide flipping. However, only a small change in the rate of nucleotide flipping was observed when MeC was introduced immediately 5\textsuperscript{\textprime} to the adduct (Table 4). These results are consistent with previous findings of Zang et al. (9) showing that the rate of nucleotide flipping is more rapid than alkyl transfer and is not significantly dependent on the sequence surrounding the O\textsuperscript{6}-Me-G lesion.

Taken together, our results indicate that cytosine methylation has a relatively small, sequence-dependent effect on the rate of AGT repair of O\textsuperscript{6}-Me-G residues within the context of p53 codons 158, 245, and 248. Furthermore, the presence of MeC does not influence the affinity of AGT for DNA or the rate of nucleotide flipping, suggesting that a later step in the repair reaction, such as alkyl transfer, must be affected by cytosine methylation.

Recently, Coulter et al. (37) proposed that the local sequence context affects the orientation of the O\textsuperscript{6}-alk-dG lesions in the AGT binding pocket. Crystal structures have shown that correct positioning of the adducted nucleotide in the AGT active site is required for in-line displacement of the O\textsuperscript{6}-substituent by the active site cysteine (4, 48). It is possible that the differences in AGT repair kinetics observed in the present study are the consequence of altered placement of the O\textsuperscript{6}-substituent in the AGT active site resulting when the neighboring cytosine base is methylated. Future studies are needed to determine whether the presence of MeC influences the geometry of the AGT-DNA complex.

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REFERENCES

1. Hoffmann, D., Brunemann, K. D., Prokopczyk, B., and Djordjevic, M. V. (1994) J. Toxicol. Environ. Health 41, 1–52
2. Warren, J. J., Forsberg, L. J., and Reese, L. S. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 19701–19706
3. Peterson, L. A., and Hecht, S. S. (1991) Cancer Res. 51, 5557–5564
4. Daniels, D. S., Woo, T. T., Luu, K. X., Noll, D. M., Clarke, N. D., Pegg, A. E., and Tainer, J. A. (2004) Nat. Struct. Mol. Biol. 11, 714–720
5. Daniels, D. S., Mol, C. D., Arvai, A. S., Kanugula, S., Pegg, A. E., and Tainer, J. A. (2000) EMBO J. 19, 1719–1730
6. Fried, M. G., Kanugula, S., Bromberg, J. L., and Pegg, A. E. (1996) Biochemistry 35, 15295–15301
7. Major, G. N., Brady, M., Notarianni, G. B., Collier, J. D., and Douglas, M. S. (1997) Biochem. Soc. Trans. 25, 3595
8. Xu-Welliver, M., and Pegg, A. E. (2002) Carcinogenesis 23, 823–830
9. Zang, H., Fang, Q., Pegg, A. E., and Guengerich, F. P. (2005) J. Biol. Chem. 280, 30873–30881
10. Liem, L. K., Wong, C. W., Lim, A., and Li, B. F. L. (1993) J. Mol. Biol. 231, 950–959
11. Luu, K. X., Kanugula, S., Pegg, A. E., Pauly, G. T., and Moschel, R. C. (2002) Biochemistry 41, 8689–8697
12. Bhattacharyya, D., Foote, R. S., Boulden, A. M., and Mitra, S. (1990) Eur. J. Biochem. 193, 337–343
13. Dolan, M. E., Olinger, M., and Pegg, A. E. (1988) Carcinogenesis 9, 2139–2143
14. Bentivegna, S. S., and Bresnick, E. (1994) Cancer Res. 54, 327–329
15. Wolf, P., Hu, Y. C., Dufek, K., Sidransky, D., and Ahrendt, S. A. (2001) Cancer Res. 61, 8113–8117
16. Hussain, S. P., and Harris, C. C. (2000) Mutat. Res. 462, 311–322
17. Tornaletti, S., and Pfeifer, G. P. (1995) Oncogene 10, 1493–1499
18. Hussain, S. P., and Harris, C. C. (1999) Mutat. Res. 428, 23–32
19. Ahrendt, S. A., Chow, J. T., Yang, S. C., Wu, L., Zhang, M. J., Jen, J., and Sidransky, D. (2000) Cancer Res. 60, 3155–3159
20. Hecht, S. S. (2003) Nat. Rev. Cancer 3, 733–744
21. Marcourt, L., Cordier, C., Couesnon, T., and Dodin, G. (1999) Eur. J. Biochem. 265, 1032–1042
22. Lefebvre, A., Mauffret, O., el Antri, S., Monnot, M., Lescot, E., and Ferrandjian, S. (1995) Eur. J. Biochem. 229, 445–454
23. Heinemann, U., and Hahn, M. (1992) J. Biol. Chem. 267, 7332–7341
24. Norberg, I., and Vihinen, M. (2001) J. Mol. Struct. Theochem. 546, 51–62
25. Sowers, L. C., Shaw, B. R., and Sedwick, W. D. (1987) Biochem. Biophys. Res. Commun. 148, 790–794
Effects of Cytosine Methylation on Repair of O6-Alkyl-dG

26. Guza, R., Rajesh, M., Fang, Q., Pegg, A. E., and Tretyakova, N. (2006) *Chem. Res. Toxicol.* **19**, 531–538
27. Liu, L., Xu-Welliver, M., Kanugula, S., and Pegg, A. E. (2002) *Cancer Res.* **62**, 3037–3043
28. Edara, S., Kanugula, S., Goodtzova, K., and Pegg, A. E. (1996) *Cancer Res.* **56**, 5571–5575
29. Ziegel, R., Shallop, A., Upadhyaya, P., Jones, R., and Tretyakova, N. (2004) *Biochemistry* **43**, 540–549
30. Matter, B., Wang, G., Jones, R., and Tretyakova, N. (2004) *Chem. Res. Toxicol.* **17**, 731–741
31. Matter, B., Wang, G., Jones, R., and Tretyakova, N. (2004) *Chem. Res. Toxicol.* **17**, 731–741
32. Spratt, T. E., Wu, J. D., Levy, D. E., Kanugula, S., and Pegg, A. E. (1999) *Biochemistry* **38**, 6801–6806
33. Frederick, C. A., Saal, D., van der Marel, G. A., van Boom, J. H., Wang, A. H., and Rich, A. (1987) *Biopolymers* **26**, (suppl.) S145–S160
34. Rasimas, J. J., Pegg, A. E., and Fried, M. G. (2003) *J. Biol. Chem.* **278**, 7973–7980
35. Mijal, R. S., Kanugula, S., Vu, C. C., Fang, Q., Pegg, A. E., and Peterson, L. A. (2006) *Cancer Res.* **66**, 4968–4974
36. Hazra, T. K., Roy, R., Biswas, T., Grabowski, D. T., Pegg, A. E., and Mitra, S. (1997) *Biochemistry* **36**, 5769–5776
37. Coulter, R., Blandino, M., Tomlinson, J. M., Pauly, G. T., Krajewska, M., Moschel, R. C., Peterson, L. A., Pegg, A. E., and Spratt, T. E. (2007) *Chem. Res. Toxicol.* **20**, 1966–1971
38. Mijal, R. S., Thomson, N. M., Fleischer, N. L., Pauly, G. T., Moschel, R. C., Kanugula, S., Fang, Q., Pegg, A. E., and Peterson, L. A. (2004) *Chem. Res. Toxicol.* **17**, 424–434
39. Essigmann, J. M., Loechler, E. L., and Green, C. L. (1986) *Prog. Clin. Biol. Res.* **209A**, 433–440
40. Basu, A. K., and Essigmann, J. M. (1990) *Mutat. Res.* **233**, 189–201
41. Delaney, J. C., and Essigmann, J. M. (1999) *Chem. Biol.* **6**, 743–753
42. Zacharias, W., O’Connor, T. R., and Larson, J. E. (1988) *Biochemistry* **27**, 2970–2978
43. Muheim, R., Buterin, T., Colgate, K. C., Kolbanovskiy, A., Geacintov, N. E., and Naegeli, H. (2003) *Biochemistry* **42**, 3247–3254
44. Huang, X., Colgate, K. C., Kolbanovskiy, A., Amin, S., and Geacintov, N. E. (2002) *Chem. Res. Toxicol.* **15**, 438–444
45. Hernandez-Boussard, T., Rodriguez-Tome, P., Montesano, R., and Hainaut, P. (1999) *Hum. Mutat.* **14**, 1–8
46. Georgiadis, P., Smith, C. A., and Swann, P. F. (1991) *Cancer Res.* **51**, 5843–5850
47. Rasimas, J. J., Kar, S. R., Pegg, A. E., and Fried, M. G. (2007) *J. Biol. Chem.* **282**, 3357–3366
48. Duguid, E. M., Rice, P. A., and He, C. (2005) *J. Mol. Biol.* **350**, 657–666