Cytotoxic and mutagenic properties of \(O^6\)-alkyl-2′-deoxyguanosine lesions in *Escherichia coli* cells

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Environmental exposure and cellular metabolism can give rise to DNA alkylation, which can occur on the nitrogen and oxygen atoms of nucleobases, as well as on the phosphate backbone. Although \(O^6\)-alkyl-2′-deoxyguanosine (\(O^6\)-alkyl-dG) lesions are known to be associated with cancer, not much is known about how the alkyl group structures in these lesions affect their repair and replicative bypass in *vivo* or how translesion synthesis DNA polymerases influence the latter process. To answer these questions, here we synthesized oligodeoxyribonucleotides harboring seven \(O^6\)-alkyl-dG lesions, with the alkyl group being Me, Et, \(n\)Pr, \(i\)Pr, \(n\)Bu, \(i\)Bu, or \(s\)Bu, and examined the impact of these lesions on DNA replication in *Escherichia coli* cells. We found that replication past all the \(O^6\)-alkyl-dG lesions was highly efficient and that SOS-induced DNA polymerases play redundant roles in bypassing these lesions. Moreover, these lesions directed exclusively the G → A mutation, the frequency of which increased with the size of the alkyl group on the DNA. This could be attributed to the varied repair efficiencies of these lesions by \(O^6\)-alkylguanine DNA alkyltransferase (MGMT) in cells, which involve the MGMT Ogt and, to a lesser extent, Ada. In conclusion, our study provides important new knowledge about the repair of the \(O^6\)-alkyl-dG lesions and their recognition by the *E. coli* DNA replication machinery. Our results suggest that the lesions’ carcinogenic potentials may be attributed, at least in part, to their strong mutagenic potential and their efficient bypass by the DNA replication machinery.

The integrity of genomic DNA is constantly challenged by environmental and endogenous damaging agents; because of the ubiquitous presence of alkylating agents in the environment and in cells, alkylation constitutes a major type of DNA damage (1, 2). DNA alkylation products, because of their cytotoxic and mutagenic properties, may pose a significant threat to human health (2, 3). Unrepaired DNA lesions can compromise DNA replication and transcription and elicit mutations. Unrepaired DNA lesions can compromise DNA replication and transcription and elicit mutations. Environmental exposure and cellular metabolism can give rise to DNA alkylation, which can occur on the nitrogen and oxygen atoms of nucleobases, as well as on the phosphate backbone. Although \(O^6\)-alkyl-2′-deoxyguanosine (\(O^6\)-alkyl-dG) lesions are known to be associated with cancer, not much is known about how the alkyl group structures in these lesions affect their repair and replicative bypass in *vivo* or how translesion synthesis DNA polymerases influence the latter process. To answer these questions, here we synthesized oligodeoxyribonucleotides harboring seven \(O^6\)-alkyl-dG lesions, with the alkyl group being Me, Et, \(n\)Pr, \(i\)Pr, \(n\)Bu, \(i\)Bu, or \(s\)Bu, and examined the impact of these lesions on DNA replication in *Escherichia coli* cells. We found that replication past all the \(O^6\)-alkyl-dG lesions was highly efficient and that SOS-induced DNA polymerases play redundant roles in bypassing these lesions. Moreover, these lesions directed exclusively the G → A mutation, the frequency of which increased with the size of the alkyl group on the DNA. This could be attributed to the varied repair efficiencies of these lesions by \(O^6\)-alkylguanine DNA alkyltransferase (MGMT) in cells, which involve the MGMT Ogt and, to a lesser extent, Ada. In conclusion, our study provides important new knowledge about the repair of the \(O^6\)-alkyl-dG lesions and their recognition by the *E. coli* DNA replication machinery. Our results suggest that the lesions’ carcinogenic potentials may be attributed, at least in part, to their strong mutagenic potential and their efficient bypass by the DNA replication machinery.

Among all the DNA alkylation adducts, \(O^6\)-alkyl-2′-deoxyguanosine (\(O^6\)-alkyl-dG)2 lesions are known to be associated with cancer initiation and progression, because these lesions alter the hydrogen-bonding property of the guanine base (3–5). In addition, among the four exocyclic oxygen atoms of nucleobases, the \(O^6\) position of guanine is the most facile site to be alkylated, where \(O^6\)-methyl-dG (\(O^6\)-Me-dG) accounts for ~6% of total adducts induced by some methylating agents (6, 7). Thus, it is important to understand how the \(O^6\)-alkyl-dG lesions perturb the efficiency and accuracy of DNA replication and how they are repaired.

Several shuttle vector studies with the use of site-specifically incorporated DNA lesions have been conducted for assessing how the \(O^6\)-alkyl-dG lesions compromise DNA replication in *Escherichia coli* and mammalian cells (8–12). Moderately high frequencies of the G → A mutation were observed for \(O^6\)-Me-dG (~19%) and \(O^6\)-ethyl-dG (\(O^6\)-Et-dG, ~11%) in alkyltransferase-deficient Chinese hamster ovary cells, although no mutation was detected in repair-proficient cells (8). Moreover, \(O^6\)-alkyl-dG was substantially more mutagenic when located in the single-stranded region of gapped plasmids than in double-stranded plasmids, because of forced replication in the gapped plasmid and/or more efficient repair of the \(O^6\)-alkyl-dG lesions in double-stranded than in single-stranded DNA (13). For instance, \(O^6\)-Me-dG, \(O^6\)-Et-dG, and \(O^6\)-benzyl-dG induced ~5% mutation in a double-stranded plasmid; by contrast, the mutation frequencies of these lesions in gapped plasmid were increased to ~60, ~95, and ~70%, respectively (11). However, it remains elusive about the roles of translesion synthesis polymerases in bypassing this family of lesions and in modulating their mutagenic properties.

\(O^6\)-Alkylguanine DNA alkyltransferase (MGMT) is known to be crucial in repairing \(O^6\)-alkyl-dG lesions (3, 14, 15), as manifested by the finding that the mutation frequencies of \(O^6\)-alkyl-dG lesions are significantly higher in the alkyltransferase-deficient cells (8, 11). For instance, Delaney and Essigmann (16) showed a drastic increase in mutation frequency for \(O^6\)-Me-dG from 10% in repair-proficient cells to 100% in repair-deficient cells. The *E. coli* genome encodes two MGMTs, which may lead to genomic instability (1). On the other hand, the cytotoxic properties of alkylating agents also render them an effective and widely prescribed class of cancer chemotherapeutic agents (2).

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The abbreviations used are: dG, deoxyguanosine; Me, methyl; Et, ethyl; nPr, \(n\)-propyl; \(i\)Pr, iso-propyl; \(n\)Bu, \(n\)-butyl; \(i\)Bu, iso-butyl; \(s\)Bu, sec-butyl; MGMT, \(O^6\)-alkylguanine DNA alkyltransferase; ESI, electrospray ionization; ODN, oligodeoxyribonucleotide; Pol, polymerase; SAP, shrimp alkaline phosphatase; PNK, polynucleotide kinase.

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Ogt and Ada, which are constitutively expressed and inducible, respectively (3). It was found that the efficiencies for the MGMT-mediated repair of O\(^6\)-alkyl-dG lesions decrease with the increase in size of the alkyl groups (14). For instance, purified Ada protein repairs O\(^6\)-Me-dG 1000 times more efficiently than O\(^6\)-Et-dG; nevertheless, it is not able to repair O\(^6\)-isopropyl-dG (O\(^6\)-iPr-dG) (17). Ogt is more efficient than Ada in repairing those O\(^6\)-alkyl-dG lesions carrying larger alkyl groups (3); whereas the two methyltransferases exhibit similar repair rates toward O\(^6\)-Me-dG, Ogt could repair O\(^6\)-Et-dG at an efficiency that is ~170-fold higher than Ada (18). This effect of alkyl group size on repair efficiency also holds true for mammalian MGMT in vitro (19, 20); however, cellular repair experiments have so far only been conducted for O\(^6\)-Me-dG. Thus, it is important to investigate systematically the MGMT-mediated repair of O\(^6\)-alkyl-dG lesions in cells and the modulation of this repair by the structures of the alkyl groups in vivo.

Although site-specific mutagenesis studies have been carried out for several O\(^6\)-alkyl-dG lesions, these studies were conducted with the use of different shuttle vector systems, and most of these experiments rely on colony picking/counting and Sanger sequencing to estimate the degrees to which these lesions impede DNA replication and induce mutations, respectively. In the present study, we set out to assess comprehensively, by using a highly quantitative and robust shuttle vector-based assay (21), how the size and shape of the alkyl groups installed on the major-groove O\(^6\) position of guanine affect the fidelity and efficiency of DNA replication. We also examined how replication past these lesions is affected by MGMT proteins and SOS-induced DNA polymerases.

Results

The major goal of this project was to elucidate comprehensively the impact of O\(^6\)-alkylguanine lesions on the efficiency and fidelity of DNA replication in E. coli cells. To this end, we first synthesized a series of ODNs harboring site-specifically incorporated O\(^6\)-alkylguanine lesions (Fig. 1) and confirmed their identities by using electrospray ionization (ESI)–MS and MS/MS analyses (Figs. S30–S36). After ligating these oligodeoxynucleotides (ODNs) into single-stranded M13 plasmid (Fig. 2), we employed a modified competitive replication and adduct bypass assay, as described elsewhere (22, 23), to investigate the degrees to which the O\(^6\)-alkyl-dG lesions inhibit DNA replication and elicit mutations in this process. In addition, we assessed how the replicative bypass of these lesions is influenced by translesion synthesis DNA polymerases and two DNA repair proteins, i.e. Ada and Ogt.

Because of the variations in transfection efficiencies among different experiments, a nonlesion competitor genome was employed as an internal standard. In this respect, by premixing the O\(^6\)-alkyl-dG-bearing genome or the control dG-containing genome with the competitor genome at defined molar ratios prior to transfection, the blockage to DNA replication can be accurately quantified after normalizing the signal ratio for the lesion/competitor genome to that for the control/competitor genome. After replication, the region of interest in the isolated progeny M13 genomes was amplified by PCR. The resulting PCR products were digested with two restriction enzymes, i.e. BbsI and MluCI, and subjected to LC–MS/MS and PAGE analyses (Figs. 3 and 4 and Figs. S37–S43).

Impacts of O\(^6\)-alkyl-dG lesions on the efficiency of DNA replication in E. coli cells

We were able to observe only the 10-mer radiolabeled restriction fragments for the PCR products from the progenies of the lesion- and the control dG-containing M13 genomes (Fig. 3B and Figs. S39–S43). This result indicates the absence of insertion or deletion mutations, which are also supported by the results obtained from LC–MS/MS analysis (Fig. 4 and Fig. S37). Therefore, the bypass efficiencies for the O\(^6\)-alkyl-dG lesions can be calculated from the ratio of 10-mer over 13-mer products as described under “Materials and methods,” with the consideration of the molar ratios of the lesion and control over competitor genomes employed in the initial transfection. It turned out that the size and shape of the alkyl groups at the O\(^6\) position of guanine exert negligible impact on replication efficiency, because all lesions were bypassed with high efficiency in WT AB1157 E. coli cells (Fig. 5A).

We also investigated the roles of SOS-induced DNA polymerases in bypassing the O\(^6\)-alkyl-dG lesions by conducting the assay in E. coli strains deficient in these DNA polymerases, i.e. Pol II, Pol IV, and Pol V. Our results revealed no significant differences in bypass efficiencies for all O\(^6\)-alkyl-dG lesions in AB1157 cells and the isogenic cells with single depletion of any of the three polymerases (Fig. 5A). However, for all the O\(^6\)-alkyl-dG lesions except O\(^6\)-Me-dG and O\(^6\)-Et-dG, a substantial reduction in bypass efficiency was observed when all three polymerases were simultaneously depleted (Fig. 5A), indicating that the three SOS-induced DNA polymerases play somewhat redundant roles in bypassing the O\(^6\)-alkyl-dG lesions.
Effects of O<sup>6</sup>-alkyl-dG lesions on the fidelity of DNA replication in E. coli cells

The result from native PAGE analysis also allows us to quantify the mutation frequencies of the O<sup>6</sup>-alkyl-dG lesions during their replicative bypass in E. coli cells. In keeping with the previously reported results, only G → A mutation was observed for all O<sup>6</sup>-alkyl-dG lesions in all E. coli strains studied (Fig. 5B). Interestingly, the G → A mutation frequencies increase with the size of the alkyl group, from ~20% for O<sup>6</sup>-Me-dG to ~80% for O<sup>6</sup>-nBu-dG (Figs. 5 and 6B). In addition, the G → A mutation frequencies were nearly 100% for all three lesions carrying a branched-chain alkyl group.

We also found that the frequencies of G → A mutation for the O<sup>6</sup>-alkyl-dG lesions were not influenced by depletion of any of the three SOS-induced DNA polymerases, alone or all three in combination. This result suggests that the observed mutation arises from the intrinsic chemical properties of the O<sup>6</sup>-alkyl-dG lesions.

Involvement of repair proteins in the replicative bypass of the O<sup>6</sup>-alkyl-dG lesions

Two alkyltransferases, Ada and Ogt, are known to be involved in the direct repair of O<sup>6</sup>-alkyl-dG lesions in E. coli cells (3, 14, 15), and the above size-dependent G → A mutation frequencies elicited by the O<sup>6</sup>-alkyl-dG lesions may be attributed to their different repair rates. To explore this possibility, we performed the replication experiments with the use of M13 genomes containing an O<sup>6</sup>-Me-dG, O<sup>6</sup>-Et-dG, O<sup>6</sup>-nPr-dG, or O<sup>6</sup>-nBu-dG in isogenic E. coli cells that are proficient or deficient in Ogt, Ada, or both. Our results revealed that the bypass efficiencies of the four O<sup>6</sup>-alkyl-dG lesions were not substantially impacted upon the depletion of these DNA repair proteins (Fig. 6A). However, depletion of Ogt, alone or in conjunction with Ada and AlkB, results in elevated G → A mutation frequencies, where even O<sup>6</sup>-Et-dG induces an almost 100% G → A mutation. Dual knockout of Ada and AlkB, however, only slightly increased G → A mutation frequencies for these four O<sup>6</sup>-alkyl-dG lesions (Fig. 6B). Intriguingly, G → A muta-
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![Figure 3. Native PAGE (30%) for monitoring the bypass efficiencies and mutation frequencies of $O^6$-alkyl-dG lesions in SOS-induced WT AB1157 E. coli cells.](image)

A, sequential restriction enzyme digestion and selective radiolabeling of the strand initially bearing the lesion. SAP and PNK designate shrimp alkaline phosphatase and T4 polynucleotide kinase, respectively. B, gel image showing the 13- and 10-mer radiolabeled restriction fragments released from the original lesion-containing strand of the PCR products of the progeny of the competitor genome and the control or lesion-carrying genome, where the original lesion-containing strand of the PCR products of the progeny showing the 13- and 10-mer radiolabeled restriction fragments released from alkaline phosphatase and T4 polynucleotide kinase, respectively.

Discussion

The $O^6$ of guanine is only one of many positions in DNA that can be modified upon reaction with alkylating agents; nevertheless, $O^6$-alkyl-dG lesions are of great importance in conferring the mutagenic and cytotoxic effects of these alkylating agents (3). Previous studies on several $O^6$-alkyl-dG lesions revealed some important findings about their mutagenic properties and repair; however, these experiments were conducted with disparate plasmid-based systems, and the mutation frequencies in most of these studies were determined by using the conventional colony-picking and Sanger sequencing methods (8–12). In addition, the involvement of translesion synthesis DNA polymerases in bypassing these lesions was not investigated. Here, we examined systematically the cytotoxic and mutagenic properties of a series of $O^6$-alkyl-dG lesions with varying sizes and structures of the alkyl group in E. coli cells. The results from these experiments offered several important observations.

First, we observed that none of the $O^6$-alkyl-dG lesions impede DNA replication strongly in E. coli cells, which differs from the findings made from in vitro experiments using purified DNA polymerases (25–28). This difference could be attributed to the cooperation of different polymerases and/or the presence of accessory protein factors in cellular DNA replication (29, 30).

Second, our results demonstrated that the nucleotide insertion opposite the $O^6$-alkyl-dG lesions is specific, where only G → A mutation was observed for the seven lesions. In addition, the mutation frequencies for the $O^6$-alkyl-dG lesions were not affected by SOS-induced DNA polymerases, where ablation of Pol II, Pol IV, and Pol V, alone or all three in conjunction, did not result in any appreciable changes in G → A mutation frequency. Furthermore, the mutation frequencies are not altered by SOS induction in WT AB1157 cells, suggesting that the mutagenic properties of the $O^6$-alkyl-dG lesions arise from the intrinsic perturbations of Watson–Crick hydrogen-bonding property of the nucleobase emanating from $O^6$-alkylation and is independent of the size or shape of the alkyl groups.

Third, our results revealed that the three SOS-induced DNA polymerases play somewhat redundant roles in bypassing the $O^6$-alkyl-dG lesions, because the bypass efficiencies for all these lesions, except $O^6$-Me-dG and $O^6$-Et-dG, were decreased substantially only when all three polymerases were simultaneously depleted. This result underscores that the $O^6$-alkyl-dG lesions are well-tolerated by replicative polymerase in E. coli cells, particularly for those lesions carrying small alkyl groups.

Fourth, we found that Ogt and, to a much lower extent, Ada, are responsible for the removal of alkyl groups from the $O^6$ position of guanine in E. coli cells, which is in line with previous findings made from in vitro biochemical assays (16–18). The basal level of Ogt protein was estimated to be 10-fold higher than that of Ada (3). However, after full induction of adaptive response by alkylating agents, the level of Ada protein can increase by ~3000-fold (3). Because Ogt has a substantially higher repair efficiency toward $O^6$-alkyl-dG lesions with larger alkyl groups, depletion of Ogt alone results in nearly 100% G → A mutation for $O^6$-Et-dG, $O^6$-Pr-dG, and $O^6$-Bu-dG. Ada and Ogt, nonetheless, exhibit similar repair efficiencies toward $O^6$-Me-dG (17, 18). In addition, the C-terminal component of Ada, after methylation of Cys-321 emanating from repair of $O^6$-Me-dG, can enhance the interaction between the protein and RNA polymerase, resulting in elevated transcription of Ada target genes, including Ada itself (31, 32). Hence, $O^6$-Me-dG can only induce 100% G → A mutation when both alkyltransferases are simultaneously depleted.

It is worth comparing the results obtained from this study with what we observed previously for other O-alkylated thyminidine lesions, i.e. the $O^4$- and $O^2$-alkyl-thymidine ($O^4$- and $O^2$-alkyl-dT) lesions (22, 23). In this vein, the findings made for the major-groove $O^6$-alkyl-dG lesions resemble what were observed previously for the major-groove $O^4$-alkyl-dT lesions, i.e. they do not block DNA replication in E. coli cells and direct exclusively one type of mutation (G → A and T → C transitions for $O^6$-alkyl-dG and $O^4$-alkyl-dT, respectively) (22, 23). In addition, the mutagenic properties of $O^6$ lesions are not modulated by SOS-induced DNA polymerases, and both are better substrates for Ogt than Ada protein, although the repair efficiency of $O^4$-alkyl-dT is lower than that of $O^6$-alkyl-dG. It is worth noting that, unlike $O^4$-Bu-dT, $O^6$-Bu-dG does not exhibit any blockage to DNA replication even it is not repaired by MGMT, indicating that the $O^6$-alkyl-dG lesions are better
tolerated by DNA polymerases relative to the O₆-alkyl-dT lesions (23). The observations made for these major-groove lesions, however, differ markedly from the minor-groove O₂-alkyl-dT lesions, which are highly blocking to DNA replication, direct promiscuous nucleotide misincorporations, and whose mutation frequencies are modulated by Pol IV and Pol V (22).

In summary, our systematic shuttle vector-based study of a group of structurally defined O₆-alkyl-dG lesions provided important novel insights into the impact of this group of DNA lesions on the efficiency and fidelity of DNA replication and the repair of these lesions in vivo. Our results also indicated that the carcinogenic potentials of these lesions

Figure 4. Higher-resolution “zoom” scan ESI–MS for monitoring the [M – 3H]⁺ ions of the lesion-containing strand of the restriction fragments of the PCR products from the replication of control dG- (A), O₆-Me-dG- (B), O₆-Et-dG- (C), O₆-nPr-dG- (D), O₆-nBu-dG- (E), O₆-iPr-dG- (F), O₆-iBu-dG- (G), and O₆-sBu-dG-bearing (H) single-stranded M13 genomes in SOS-induced WT AB1157 cells. The G → A mutation products were further confirmed by MS/MS analyses, and representative MS/MS results for O₆-nPr-dG are shown in Fig. S37. +Na and +K designate the Na⁺ and K⁺ ion adducts, respectively.

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may be attributed, at least in part, to their strong mutagenic potential and their efficient bypass by DNA replication machinery. It will be important to assess how the $O^6$-alkyl-dG lesions compromise DNA replication and transcription in mammalian cells in the future.

**Materials and methods**

All chemicals, unless otherwise specified, were from Sigma–Aldrich or Thermo Fisher Scientific. 1,1,1,3,3,3-Hexafluoro-2-propanol was obtained from Oakwood Products, Inc. (West Columbia, SC). Reagents for solid-phase DNA synthesis
were obtained from Glen Research Co. (Sterling, VA), and unmodified ODNs were from Integrated DNA Technologies (Coralville, IA). All enzymes were obtained from New England Biolabs (Ipswich, MA), and \( ^{32} \text{P} \)-labeled ATP was procured from PerkinElmer Life Sciences.

M13mp7(L2), WT AB1157 and C215 E. coli strains, and Ada- and Ogt-deficient E. coli strains (as FC215 derivatives), including C216 (\( \Delta \text{ogt}::\text{kan} \)), C217 (\( \Delta \text{ada} \Delta \text{alkB}::\text{cam} \)) and C218 (\( \Delta \text{ogt}::\text{kan} \Delta \text{ada} \Delta \text{alkB}::\text{cam} \)) were kindly provided by Prof. John M. Essigmann (16). Polymerase-deficient AB1157 strains (\( \Delta \text{pol B}1::\text{spec} \) (Pol II-deficient), \( \Delta \text{dinB} \) (Pol IV-deficient), \( \Delta \text{umuC}::\text{kan} \) (Pol V-deficient), \( \Delta \text{umuC}::\text{kan} \Delta \text{dinB} \) (Pol IV, Pol V-double knockout), and \( \Delta \text{pol B}1::\text{spec} \Delta \text{dinB} \Delta \text{umuC}::\text{kan} \) (Pol II, Pol IV, Pol V-triple knockout)) were generously provided by Prof. Graham C. Walker (33).

**MS and NMR**

ESI–MS and MS/MS experiments were carried out on an LCQ Deca XP ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A mixture of acetonitrile and water (50:50, v/v) was used as solvent for electrospray. The spray voltage was 3.0 kV, and the temperature of the ion transport tube was maintained at 275 °C. High-resolution mass spectra were acquired on an Agilent 6510 quantitative time-of-flight LC/MS instrument (Agilent Technologies, Palo Alto, CA) equipped with an ESI source. \(^1\)H NMR spectra were recorded at 400 MHz on a Varian Inova 400 NMR spectrometer (Varian Inc., Palo Alto, CA), and \(^31\)P NMR spectra were acquired at 120 MHz on a Varian Inova 300 NMR or at 160 MHz on a Varian Inova 400 NMR spectrometer at 25 °C.

**Chemical synthesis**

ODNs containing seven \( O^6 \)-alkyl-dG lesions at a specific site were synthesized (Fig. 1). The synthetic route for the phosphoramidite building blocks is described below. A 1:1 solution of acetonitrile and water was used as the solvent for electrospray. The spray voltage was 3.0 kV, and the temperature of the ion transport tube was maintained at 275 °C. High-resolution mass spectra were acquired on an Agilent 6510 quantitative time-of-flight LC/MS instrument (Agilent Technologies, Palo Alto, CA) equipped with an ESI source. \(^1\)H NMR spectra were recorded at 400 MHz on a Varian Inova 400 NMR spectrometer (Varian Inc., Palo Alto, CA), and \(^31\)P NMR spectra were acquired at 120 MHz on a Varian Inova 300 NMR or at 160 MHz on a Varian Inova 400 NMR spectrometer at 25 °C.
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phosphoramidite building blocks of the O^6-alkyl-dG lesions was adapted from published procedures (Scheme S1) (34, 35).

General procedures for nucleophilic substitution

Compound 1 (400 mg, 0.65 mmol), prepared with an overall yield of 68% following published procedures (34, 36), was dissolved in 10 ml of anhydrous methylene chloride. Triethylamine (0.774 ml, 2-mesitylenesulfonyl chloride (0.61 g, 2.78 mmol), and 4-dimethylaminopyridine (7 mg) were added to the solution. The resulting mixture was stirred at room temperature for 40 min prior to being cooled to 0 °C. N-Methylpyrroliidine (1.4 ml) was subsequently added, and the solution was then stirred at 0 °C. After 30 min, appropriate alcohol (10 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.61 ml, 4.16 mmol) were added to the reaction mixture. The resulting mixture was then allowed to warm to room temperature and stirred for 6 h. The reaction mixture was then poured into a 15-ml solution of 1 M aqueous KH_2PO_4 (pH 6.5), and the organic layer was isolated, dried over anhydrous Na_2SO_4, and filtered. The filtrate was concentrated, and the resulting residue was subjected to purification using silica gel column chromatography with a step gradient of ethyl acetate (0–50%) in hexane to afford the desired product 2a–g.

General procedures for desilylation reaction

Compound 2a–g (100 mg) was dissolved in 4 ml of THF, followed by addition of t-butylammonium fluoride in THF (1.0 M, 5 eq). After stirring at room temperature for 2 h, the reaction mixture was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography with methanol (0–10%) in methylene chloride to afford the desired product 3a–g.

General procedures for tritylation reaction

Compound 3a–g (100 mg) was dissolved in anhydrous pyridine (10 ml), and the solution was cooled in an ice bath. 4-Dimethylaminopyridine (0.5% mol) and 4,4'-dimethoxytrityl chloride (1.2 eq) were added, and the resulting solution was stirred at room temperature for 10 h. The reaction was then quenched with methanol (0.5 ml), and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography with ethyl acetate as the mobile phase to give the desired product 4a–g.

General procedures for phosphorylation reaction

To a chilled solution (0 °C) of compound 4 (60 mg) in anhydrous methylene chloride (3.0 ml) was added N,N-diisopropylamine (2.2 eq), which was followed by dropwise addition of 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite (1.2 eq). The formed mixture was then stirred at room temperature for 1 h under an inert atmosphere. The reaction was diluted with 8.0 ml of ethyl acetate in an ice bath. The organic layer was washed with saturated aqueous NaHCO_3 and brine, and dried over anhydrous Na_2SO_4. The solvent was subsequently evaporated under vacuum to afford 5a–g in a foam that was used directly for ODN synthesis. The reaction yields and spectroscopic characterizations of the above-synthesized products are provided in the supporting information, and the NMR spectra for these compounds are displayed in Figs. S1–S28.

Preparation of the lesion-carrying ODNs

The 12-mer lesion-containing ODNs, 5’-ATGGCGXGC-TAT-3’ (where X indicates O^6-alkyl-dG), were synthesized via automated solid-phase DNA synthesis on a Beckman Oligo 1000M DNA synthesizer (Fullerton, CA). Commercially available ultramild phosphoramidites were employed for the incorporation of the unmodified nucleotides (Glen Research Inc., Sterling, VA) following the standard ODN assembly protocol.

The synthesized 12-mer ODNs were cleaved from the controlled pore glass support and deprotected with 0.25 M NaOH at room temperature overnight. The solution was subsequently neutralized with 0.5 M HCl, and solvents were removed under vacuum. The solid residues were redissolved in water and purified via reversed-phase HPLC, with the use of a Kinetex XB-C18 column (4.6 × 150 mm, 5 μm in particle size, and 100 Å in pore size; Phenomenex Inc., Torrance, CA), as described elsewhere (22, 23). The HPLC traces for the purification of the 12-mer lesion-carrying ODNs are displayed in Fig. S29, and the mass spectrometric characterizations of the purified lesion-containing ODNs are shown in Figs. S30–S36. The purified 12-mer O^6-alkyl-dG-containing ODNs were then ligated individually with a 10-mer ODN (5’-AGTGGAAGAC-3’) with T4 DNA ligase in the presence of a template ODN, and the desired 22-mer lesion-bearing ODNs were purified using denaturing PAGE, as described previously (22, 23).

Construction of single-stranded lesion-containing and lesion-free competitor M13 genomes

The lesion-containing and lesion-free M13mp7(L2) genomes were prepared according to the previously reported procedures (Fig. 2) (37). Briefly, 20 pmol of single-stranded M13 genome was first linearized via digestion with 40 units EcoRI at 23 °C for 8 h. The resulting linearized vector was then annealed with two scaffolds: 5’-CTTCCACTCTCAATGAT-CATGGCTAGCTTC-3’ and 5’-AAACGACGGCA-GTGAATATACG-3’ (25 pmol). To the resulting mixture was subsequently added 30 pmol of the 5’-phosphorylated 22-mer O^6-alkyl-dG-bearing ODN or the competitor ODN (25-mer, 5’-ATGGGAAGACATGGCAGATAGCTTACG-3’). The mixture was then treated with T4 DNA ligase at 16 °C for 8 h. After the ligation, excess scaffolds and the unligated vector were degraded by incubating with T4 DNA polymerase (22.5 U) at 37 °C for 4 h, and the resultant mixture was purified using Cycle Pure Kit (Omega) to afford the lesion-containing and the lesion-free M13 genomes. The constructed lesion-containing or control genomes were normalized against the lesion-free competitor genome following published procedures to determine accurately the relative concentrations of the constructed genomes (37).

Transfection of lesion-containing and competitor M13 genomes into E. coli cells

The control lesion-free M13 genome was mixed with the competitor genome at a molar ratio of 1:1. The O^6-alkyl-dG-
containing M13 genomes were digested individually with the competitor genome at a molar ratio of 2:1. The mixtures were transfected into SOS-induced, electrocompetent WT AB1157 E. coli cells and the isogenic E. coli cells that are deficient in Pol II, Pol IV, Pol V, or all three polymerases, as well as WT E. coli cells and isogenic strains that are deficient in Ogt, Ada, or both, following the previously published procedures (37). In this vein, the SOS response was induced by irradiating the E. coli cells with 254-nm light at a dose of 45 J/m², as previously described (33). The E. coli cells were subsequently grown in lysogenic broth culture medium at 37 °C for 6 h. The phage was recovered from the supernatant by centrifugation at 13,000 rpm for 5 min and further amplified in SCS110 E. coli cells to increase the progeny/lesion-genome ratio. The amplified phage was finally purified using the QIAprep Spin M13 kit (Qiagen) to obtain the single-stranded M13 DNA template for PCR amplification.

Quantification of bypass efficiencies and mutation frequencies

A modified version of the competitive replication and adduct bypass assay was employed to assess the bypass efficiencies of the O⁶-alkyl-dG lesions in vivo (21, 37). The region of interest in the purified single-stranded M13 DNA template was amplified by PCR with Phusion high-fidelity DNA polymerase (New England Biolabs) using two primers: 5’-YCACTATGACATGATTAGTGATGGA-3’ and 5’-YTCGGTGCGGGGCTCTTCGCTATTAC-3’ (where Y is an amino group). The PCR amplification was conducted for 30 cycles, each of which consisted of 10 s at 98 °C, 30 s at 59–70 °C, and 15 s at 72 °C, with a final extension at 72 °C for 5 min.

The PCR products were then subjected to sequential restriction endonuclease digestion and native PAGE analysis for determination of bypass efficiency and mutation frequency (Fig. 3). Briefly, 80 ng of the PCR products were treated with BbsI (10 units) restriction endonuclease and shrimp alkaline phosphatase (SAP, 1 unit) in a 10-µl CutSmart buffer (New England Biolabs) at 37 °C for 30 min. The SAP was deactivated by heating at 80 °C for 10 min, and the mixture was incubated with T4 polynucleotide kinase (PNK, 10 units), DTT (5 mM), and [γ-32P]ATP at 37 °C for 30 min to radiolabel the newly released 5'-termini of the restriction fragments. After deactivation of T4 PNK via heating at 80 °C for 10 min, the resultant mixture was digested with MluCI (10 µl) at 37 °C for 30 min. Under these conditions, the 10-mer ODNs emanating from initial lesion-situated strand were radiolabeled (Fig. 3A) as d(p*GGCGMGCTAT), with “M” being A, T, G, and C, and “p*” designating the 5’-32P-labeled phosphate.

The digestion was subsequently quenched with 15 µl of formamide gel loading buffer (2×), and the labeled fragments were separated using 30% native PAGE, as previously described (22, 23). The intensities of the radiolabeled DNA bands were measured using a Typhoon 9410 variable mode imager, and the bypass efficiency was derived using the following equation:

\[
\text{bypass efficiency (%) = (lesion signal/competitor signal)/(control signal/competitor signal)} \times 100% ,
\]

where the competitor signal was employed as the internal standard.

The mixture frequencies were quantified following our previously published procedures (22, 23). As shown in Fig. 3B, the two products with M being an A or G could be well-resolved by native PAGE analysis; hence, native PAGE analysis of the original lesion-situated strand was sufficient for quantifying the G → A mutation frequency.

Identification of mutagenic products by LC–MS/MS

The PCR products were digested with 50 units BbsI restriction endonuclease and 20 units shrimp alkaline phosphatase in a 250-µl NEB CutSmart buffer at 37 °C for 2 h, followed by deactivation of the enzymes by heating at 80 °C for 20 min. To the mixture was added 50 U of MluCI, and the solution was incubated at 37 °C for 1 h. The resulting solution was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The aqueous layer was subsequently dried in a SpeedVac, desalted with HPLC, and dried again. The resultant solid residues were reconstituted in 20 µl of water, and a 10-µl aliquot was injected for LC–MS/MS analysis using an Agilent Zorbax SB-C18 column (0.5 × 250 mm, 5 µm in particle size). The gradient for LC–MS/MS analysis was 5 min of 5–20% methanol followed by 35 min of 20–50% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol. The temperature for the ion-transport tube was maintained at 300 °C. The LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) was set up for monitoring the fragmentation of the [M-3H]3⁻ ions of the 10-mer d(GGCGMGCTAT), where M represents A, T, C or G. The fragment ions detected in the MS/MS were manually assigned.

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