Cytotoxic and mutagenic properties of minor-groove $O^2$-alkylthymidine lesions in human cells

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Running Title: Replication studies of $O^2$-alkylthymidine lesions

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Abstract

Endogenous metabolism, environmental exposure, and cancer chemotherapy can lead to alkylation of DNA. It has been well documented that, among the different DNA alkylation products, minor-groove $O^2$-alkylthymidine ($O^2$-alkyldT) lesions are inefficiently repaired. In the present study, we examined how seven $O^2$-alkyldT lesions, with the alkyl group being a Me, Et, nPr, iPr, nBu, iBu or sBu, are recognized by the DNA replication machinery in human cells. We found that the replication bypass efficiencies of these lesions decrease with increasing length of the alkyl chain, and that these lesions induce substantial frequencies of T→A and T→G mutations. Replication experiments using isogenic cells deficient in specific translesion synthesis (TLS) DNA polymerases revealed that the absence of polymerase η or polymerase ζ, but not polymerase κ or polymerase λ, significantly decreased both the bypass efficiencies and the mutation frequencies for those $O^2$-alkyldT lesions carrying a straight-chain alkyl group. Moreover, the mutagenic properties of the $O^2$-alkyldT lesions were influenced by the length and topology of the alkyl chain and by TLS polymerases. Together, our results provide important new knowledge about the cytotoxic and mutagenic properties of $O^2$-alkyldT lesions, and illustrated the roles of TLS polymerases in replicative bypass of these lesions in human cells.

Introduction

DNA is intrinsically unstable, where it may undergo spontaneous deamination or depurination under physiological conditions (1). In addition, endogenous metabolic processes and environmental exposure can give rise to covalent modifications of DNA (2). The resulting DNA lesions, if left unrepaired, may compromise genomic integrity by impeding DNA replication and transcription, and eliciting mutations in these processes, which may ultimately lead to the development of cancer and other human diseases (3).

Alkylation is a common type of DNA damage, and it also constitutes the major mechanism of action for some widely prescribed anti-cancer
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drugs (4-6). Alkylating agents can react with DNA directly or following metabolic activation, which result in the conjugation of DNA with varying sizes of alkyl groups. For instance, a number of anticancer drugs, including dacarbazine, procarbazine, streptozotocin and temozolomide, can give rise to methylation of DNA (7). In addition, metabolites of some tobacco-derived N-nitrosamines can lead to the conjugation of bulky pyridyloxybutyl (POB) and pyridylhydroxybutyl (PHB) groups with nucleobases and the phosphate backbone of DNA (8-10).

Among the plethora of alkylated DNA damage products, minor-groove O\textsuperscript{2}-alkyldT lesions are known to be poorly repaired. In this respect, the POB and PHB derivatives of guanine and thymine could be detected in various tissues of rats exposed with tobacco-derived N-nitrosamines (11-14). Nevertheless, O\textsuperscript{2}-POBdT could accumulate at markedly higher levels than O\textsuperscript{6}-POBdG, and substantial frequencies of mutations at A:T base pairs could be detected in genomic DNA of Chinese hamster ovary cells treated with NNK (11,14). Likewise, tissues of rats treated with DNA ethylating agents displayed much higher levels of O\textsuperscript{2}-EtdT than O\textsuperscript{6}-EtdG (15-17), and lymphocyte DNA of smokers exhibited elevated levels of O\textsuperscript{2}-EtdT relative to that of non-smokers (18). Together, these findings suggest that the minor-groove O\textsuperscript{2}-alkyldT lesions are recalcitrant to repair (15-17); hence, it is important to examine how these lesions are recognized by DNA replication machinery.

Some replication experiments have been conducted for the O\textsuperscript{2}-alkyldT lesions. Zhai et al. (19,20) showed that O\textsuperscript{2}-alkyldT lesions strongly compromised the efficiency and fidelity of DNA replication in Escherichia coli cells. Additionally, the three SOS-induced DNA polymerases exert distinct effects on replicative bypass of these lesions, where depletion of Pol V, but not Pol II or Pol IV, elicit significant diminutions in bypass efficiencies of these lesions in E. coli cells. Moreover, both Pol IV and Pol V are essential for the misinsertion of dCMP opposite these lesions, whereas misincorporation of dTMP opposite the lesions requires only Pol V. A recent study by Basu and coworkers (21) showed that O\textsuperscript{2}-MedT and O\textsuperscript{2}-POBdT were strong impediments to DNA replication and were highly mutagenic in HEK293T human embryonic kidney cells. Replication experiments were also performed in the same cells following knockdown of individual translesion synthesis (TLS) DNA polymerases (21). However, sometimes unequivocal conclusions about the involvement of a specific TLS polymerase in bypassing a given lesion could not be reached owing to the inability of the siRNA technology in depleting completely these polymerases (21,22).

In the present study, we aim to achieve a comprehensive understanding about the recognition of minor-groove O\textsuperscript{2}-alkyldT lesions by DNA replication machinery in human cells. We introduced, into double-stranded plasmids, seven O\textsuperscript{2}-alkyldT lesions with varying sizes and structures of the alkyl group (Figure 1) and investigated how these lesions impede DNA replication and induce mutations in HEK293T cells. We also assessed the roles of TLS DNA polymerases in bypassing these lesions by conducting replication experiments in the isogenic HEK293T cells where individual TLS polymerases were genetically ablated by the CRISPR/Cas9 method (23).

**Results**

The primary objectives of the present study were to assess comprehensively the extents to which the O\textsuperscript{2}-alkyldT lesions with varying sizes and structures of the alkyl group perturb the efficiencies and fidelities of DNA replication in cultured human cells, and to establish the roles of
TLS DNA polymerases in replication past these lesions.

We first constructed double-stranded shuttle vectors containing a site-specifically inserted O2-alkyldT lesion, as well as the corresponding non-lesion control vector with an unmodified dT at the lesion site. The lesion-carrying or the undamaged control vectors were mixed individually with the damage-free competitor vector at fixed molar ratios and co-transfected into human cells. The competitor vector, which contains three additional nucleotides relative to the control or lesion-harbor vector, serves as the internal reference for determining the degrees to which the O2-alkyldT lesions block DNA replication in human cells. The progeny genomes were extracted from human cells at 24 h following the transfection, and the residual unreplicated plasmids were removed by treatment with DpnI and exonuclease III. The progeny plasmids were then amplified by PCR using a pair of primers spanning the original lesion site. In this respect, one of the primers (P1) contains a G at the 3'-terminus corresponding to the C/C mismatch site (Figure 2b), which permits the selective amplification of the progeny genomes arising from the replication of the bottom, lesion-situated strand of the plasmids under suitable conditions (24). The P1 primer also carries a C/A mismatch three bases from its 3'-end, which improves the specificity of strand-specific PCR, as described elsewhere (25). The ensuing PCR products were digested with appropriate restriction enzymes, *i.e.* NcoI and SfaNI (Figure 3a), and the digestion products were subjected to LC-MS/MS and PAGE analyses (Figure 3b&c and Figures S1-S5).

Our results showed that, in wild-type HEK293T cells, the bypass efficiencies were 60%, 43%, 37% and 29% for O2-MedT, O2-EtdT, O2-nPrdT and O2-nBudT, respectively. Hence, the bypass efficiencies decrease with the chain length for those O2-alkyldT lesions bearing a straight-chain alkyl group. The bypass efficiencies were lower for those O2-alkyldT lesions harboring a branched-chain alkyl group, as reflected by the bypass efficiencies of 34%, 14% and 14% for O2-PrdT, O2-iPrdT and O2-sBudT, respectively (Figure 4a). These results are reminiscent of previous findings made for the replicative bypass of these lesions in *E. coli* cells (20).

We next asked how the replicative bypass of the O2-alkyldT lesions in mammalian cells is affected by depletion of translesion synthesis (TLS) DNA polymerases by conducting replication experiments with the use of isogenic HEK293T cells depleted of individual TLS polymerases. Our results showed that in general the depletion of Pol ι or Pol κ did not appreciably impact the bypass efficiencies of the O2-alkyldT lesions except O2-iPrdT and O2-sBudT, for which loss of Pol ι led to significant decreases in bypass efficiencies (Figure 4a). On the other hand, individual depletion of Pol η or Pol ζ resulted in significant decreases (by ~16-30%) in bypass efficiencies for all the O2-alkyldT lesions carrying a straight-chain alkyl group (Figure 4a), supporting that Pol η and Pol ζ play crucial roles in bypassing these O2-alkyldT lesions. In contrast, individual depletion of Pol η or Pol ζ did not appreciably perturb the replicative bypass for the three O2-alkyldT lesions possessing a branched-chain alkyl functionality (Figure 4a).

To determine the mutagenic properties of the O2-alkyldT lesions, we identified the mutant products by employing LC-MS/MS and PAGE analyses of restriction fragments of PCR products from progeny genomes. In the LC-MS/MS method, we monitored the fragmentations of the [M – 3H]− ions of d(AATTACAGCMCGC), with ‘M’ designating the nucleotides inserted opposite the initial damage site. Our LC-MS/MS data revealed that all seven O2-alkyldT lesions exhibit similar miscoding properties, where *T*→*A* and *T*→*G*.
transversions, but not T→C transition, were observed (representative LC-MS and MS/MS data are shown in Supplementary Figures S3-S5). With the use of 30% native PAGE, we were able to resolve [5-32P]-labeled d(p*CATGGCGTGCTGT) (non-mutagenic product, 13mer-T) from the corresponding products carrying a T→A or T→G mutation, i.e. d(p*CATGGCGAGCTGT) (13mer-A) and d(p*CATGGCGGGGCTGT) (13mer-G, Figure 3b&c and Figures S1&S2). However, the non-mutagenic product co-migrated with the corresponding product with a T→C mutation at the lesion site, i.e. d(p*CATGGCGAGCTGT) (13mer-G, Figure 3b&c and Figures S1&S2). Owing to the lack of T→C mutation arising from replication past the O2-alkyldT lesions, we determined the frequencies of the lesion-induced mutations by using native PAGE analysis.

Similar to what were observed previously in E. coli cells (20), the major type of mutation induced by the O2-alkyldT lesions in HEK293T cells was T→A transversion, which was detected at frequencies of 1-13% and was accompanied with lower frequencies (0.5-6%) of T→G substitution (Figure 4b&c). In addition, the frequencies for T→A and T→G mutations for the O2-alkyldT lesions with straight-chain alkyl group rise with the increasing length of the alkyl group (Figure 4b&c). These elevations in mutation frequencies were associated with concomitant decreases in bypass efficiencies for the lesions (vide supra). Thus, the increases in chain length of the alkyl group adducted to the O2 position of thymine led to greater diminutions in both the efficiency and fidelity of replication across the O2-alkyldT lesions in human cells.

We next examined the mutagenic properties of the O2-alkyldT lesions in HEK293T cells that are deficient in Pol η, Pol ι, Pol κ or Pol ζ and compared the results with what we obtained for the parental HEK293T cells. Our results revealed that Pol η and Pol ζ played an important role in the misincorporation of dTMP opposite the O2-alkyldT lesions regardless of the alkyl chain being straight or branched, as reflected by the finding that the depletion of either polymerase resulted in a significant drop in T→A mutation (Figure 4b). The only exception was that the loss of Pol ζ did not alter the frequency of T→A mutation for O2-sBudT (Figure 4b). Likewise, genetic ablation of Pol η led to substantial diminutions in T→G mutation for all O2-alkyldT lesions except O2-βBudT and O2-sBudT (Figure 4c), indicating a role of Pol η in the misincorporation of dCMP opposite these O2-alkyldT lesions. Moreover, for those lesions bearing straight-chain alkyl groups, depletion of Pol κ led to marked reductions in T→G mutation (Figure 4c), whereas Pol ζ functions in dCMP misincorporation opposite those O2-alkyldT lesions with branched-chain alkyl groups (Figure 4c). On the other hand, Pol ι played an important role in dTMP misinsertion opposite the sites of those O2-alkyldT lesions carrying a large alkyl group (i.e. O2-βBudT, O2-γBudT, and O2-sBudT, Figure 4b). Cumulatively, these results demonstrated that the effects of depletion of TLS DNA polymerases on the mutagenic properties of the O2-alkyldT lesions are highly dependent on the chemical structures (i.e. chain length and chain topology) of the alkyl group being conjugated with the O2 position of thymidine, and are further modulated by TLS DNA polymerases.

Discussion

In this study, we assessed comprehensively the cytotoxic and mutagenic properties of O2-alkyldT lesions in HEK293T cells and the isogenic cells where the individual TLS polymerases were depleted by the CRISPR/Cas9 genome editing method. Our results revealed that, similar to what we observed in E. coli cells (20), the O2-alkyldT lesions inhibit strongly DNA replication in
mammalian cells, with the blockage effect increasing with the size and branching of the alkyl groups (Figure 4a). This result underscores the increased difficulty experienced by the DNA polymerases in accommodating those $O^2$-alkyldT lesions with longer and bulkier alkyl chain into their active sites, which confers reduced efficiencies in nucleotide incorporation at or near the lesion site. In this context, it is worth noting that the differential rate of repair of the $O^2$-alkyldT lesions may also contribute in part to the differences in the observed replication bypass efficiencies. In addition, we found that the bypass efficiencies for all the $O^2$-alkyldT lesions carrying a straight chain alkyl group were significantly reduced in cells depleted of Pol $\eta$ or Pol $\zeta$ (Figure 4a), suggesting that these two TLS polymerases play important roles in bypassing these lesions in vivo. This is reminiscent of our previous finding that Pol V, the E. coli ortholog of human Pol $\eta$, constitutes the major TLS polymerase for bypassing these lesions (20), and is consistent with results obtained from in-vitro replication experiments showing that human Pol $\eta$ was capable of bypassing readily these lesions in template DNA (26). In addition, Pol $\zeta$ is known to participate in extension step of the TLS after nucleotide incorporation opposite the lesion site (27). Together, our results suggest that Pol $\eta$ and Pol $\zeta$ may cooperate in bypassing the $O^2$-alkyldT lesions with a straight-chain alkyl group, with Pol $\eta$ and Pol $\zeta$ being involved in the insertion and extension steps of the lesion bypass, respectively. It will be important to assess, in the future, how replication across these lesions in human cells are affected by simultaneous depletion of both Pol $\eta$ and Pol $\zeta$, and biochemically how Pol $\eta$ and Pol $\zeta$ may function together in bypassing the $O^2$-alkyldT lesions in vitro.

Our results demonstrated that the $O^2$-alkyldT lesions primarily directed the misincorporations of pyrimidine nucleotides (i.e. dTMP and dCMP), and replicative bypass of these lesions yields T$\rightarrow$A and T$\rightarrow$G mutations. This finding is in line with the notion that the incorporation of an alkyl group to the $O^2$-position of thymine may render the nucleobase unfavorable in pairing with any of the four canonical nucleobases (20). This observation is also in agreement with the finding that T$\rightarrow$A and T$\rightarrow$G transversions constitute the two main types of mutations induced by these lesions in E. coli (20). Different from what we found in E. coli cells, we did not observe T$\rightarrow$C mutation for any of the $O^2$-alkyldT lesions in wild-type HEK293T cells or the isogenic cells depleted of any of the TLS polymerases (Figure S3-S5), which could be attributed to the differential recognition of these lesions by replication machineries of human and E. coli cells.

By conducting replication experiments in cells depleted of TLS polymerases, we showed that misincorporation of dTMP opposite $O^2$-alkyldT and perhaps the subsequent extension beyond the lesion site require both Pol $\eta$ and Pol $\zeta$ (Figure 4b&c), suggesting that Pol $\eta$ and Pol $\zeta$ are the major polymerases responsible for dTMP misincorporation and subsequent extension beyond the $O^2$-alkyldT lesions in human cells. In keeping with the previous finding that both Pol IV and Pol V were required for inducing T$\rightarrow$G mutation (20), their orthologues in mammalian cells, i.e. Pol $\kappa$ and Pol $\eta$, were the major polymerases contributing to the induction of T$\rightarrow$G mutation for the straight-chain $O^2$-alkyldT lesions (Figure 4c). On the other hand, Pol $\zeta$ was the only TLS polymerase found to be required for the T$\rightarrow$G mutation induced by the three branched-chain $O^2$-alkyldT lesions (Figure 4c), though it remains unclear which polymerase might be involved in the dCMP misincorporation opposite these lesions.

Taken together, our systematic shuttle vector-based study on a group of structurally defined $O^2$-
alkyldT lesions provided important new insights into the impact of this under-investigated group of DNA lesions on the efficiency and accuracy of DNA replication in mammalian cells. The findings made in the present study unveiled that, in mammalian cells, the cytotoxic and mutagenic properties of these DNA lesions depend on the size and branching of the alkyl group and are further modulated by TLS DNA polymerases. Moreover, the significant inhibitory effects of the $O^2$-alkyldT lesions on DNA replication, their strong mutagenic potentials, and their resistance to repair suggest that the $O^2$-alkyldT lesions constitute a family of biologically important DNA lesions.

**Experimental Procedures**

All chemicals, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO) or EMD Millipore. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was obtained from TCI America (Portland, OR). [γ-32P]ATP was purchased from Perkin Elmer (Piscataway, NJ), and all other enzymes were obtained from New England Biolabs (Ipswich, MA). All unmodified oligodeoxyribonucleotides (ODNs) were obtained from Integrated DNA Technologies (Coralville, IA). The 12-mer ODNs harboring a site-specifically incorporated $O^2$-alkyldT were synthesized previously (20). The identities and purities of all the lesion-harboring ODNs were confirmed by liquid chromatography-mass spectrometry (LC-MS) and tandem MS (MS/MS) analyses prior to their insertion into double-stranded plasmids. HEK293T cells with POLH, POLI, POLK and POLZ genes, which encode DNA polymerases η, ι, κ and ζ, respectively, being individually depleted by the CRISPR-Cas9 genomic editing method were previously described (23).

**Construction of lesion-containing and lesion-free plasmids**

The lesion-containing and lesion-free genomes were prepared according to the previously reported procedures (22,23). The parent vector was subsequently digested with Nt.BstNBI, followed with removing the 25-mer ODN by annealing with a 25-mer complementary ODN in large excess to generate a gapped vector. The gapped vector was subsequently purified from the mixture by centrifugation using 100 kDa-cutoff ultracentrifugal filter units (Millipore). The gap in the vector was filled with a 5'-phosphorylated 13-mer lesion-free ODN (5'-AATGAGTCGATG-3') and a 5'-phosphorylated 12-mer lesion-containing or lesion-free ODN (5'-ATGGCGXGCTAT-3', where X = $O^2$-alkyldT or dT) by using T4 DNA ligase in the presence of ATP (Figure 2a). The successfully ligated, supercoiled plasmid was isolated from the ligation mixture by using agarose gel electrophoresis. The amounts of constructed lesion-containing vectors were normalized against that of the lesion-free competitor vector following published procedures (22,23).

**Cellular DNA replication and plasmid isolation**

The lesion-bearing and the corresponding non-lesion control plasmids were individually premixed with the competitor genome at a molar ratio of 19:1 for all replication experiments. The HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin and incubated at 37°C in 5% CO2 atmosphere. The HEK293T cells and CRISPR/Cas9 genome-engineered cells (1 x 10^5) were seeded in a 24-well plate and cultured for 24 h before they were transfected with the aforementioned plasmid mixtures by using Lipofectamine 2000 following the manufacturer’s recommended procedures, where a total of 300 ng of lesion/competitor or control/competitor genome mixture along with 500 ng of carrier plasmid were employed for each transfection. The cells were
harvested at 24 h following the transfection, and the progeny genomes were isolated using Qiagen Spin kit (Qiagen, Valencia, CA) with minor modifications (28). The residual unreplicated plasmids were further digested with DpnI, followed by removing the resulting linear DNA with exonuclease III, as described elsewhere (29-31). Along this line, there were 25 DpnI recognition sites in the parental plasmid; therefore, digestion at any one of these sites would result in the degradation of the entire plasmid by exonuclease III and prevent the following PCR amplification of the parental vector.

**PCR and polyacrylamide gel electrophoresis (PAGE) analyses**

The progeny genomes resulting from cellular replication were amplified by PCR with the use of GoTaq Hot Start DNA polymerase (Promega, Madison, WI). The two primers were 5'-GCTAGCGGATGCATCGACTCAATTACAG-3' and 5'-GCTGATTATGATCTAGAGTTGCGGCCGC-3', and the PCR amplifications started at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were purified using Cycle Pure Kit (Omega, Norcross, GA) and stored at -20°C until use.

For PAGE analysis, a portion of the PCR products were treated with 5 U NcoI and 1 U shrimp alkaline phosphatase (SAP) at 37°C in 10 μL of NEB buffer 3 for 1 h, and the SAP was subsequently deactivated by heating at 80°C for 20 min. The above mixture was then treated with 5 U of T4 polynucleotide kinase (T4 PNK) in 15-μL NEB buffer 3 containing 5 mM DTT and ATP (10 pmol cold, premixed with 1.66 pmol [γ-32P]ATP). The reaction was continued at 37°C for 30 min, followed by heating at 65°C for 20 min to deactivate the polynucleotide kinase. To the reaction mixture was subsequently added 2 U of SfaNI in 5 μL NEB buffer 3, and the solution was incubated at 37°C for 1.5 h, followed by quenching with 20 μL of formamide gel-loading buffer containing xylene cyanol FF and bromophenol blue dyes. The mixture was loaded onto 30% polyacrylamide gel (acylamide/bis-acylamide = 19:1), and the gel band intensities were quantified by phosphorimager analysis.

The effects of DNA lesions on replication efficiency and fidelity were characterized by bypass efficiency and mutation frequency, respectively. The bypass efficiency was calculated as: (lesion signal/competitor signal)/(non-lesion control signal/competitor signal)×100%. The mutation frequency was determined from the percentage of the amount of mutagenic product among the total amounts of products formed from replication of the lesion-containing genome (32-34).

**Identification of mutagenic products using LC-MS/MS**

The PCR products were digested with 30 U SfaNI restriction endonuclease and 15 U shrimp alkaline phosphatase in 150 μL NEB buffer 3 at 37°C for 2 h, followed by deactivation of the phosphatase at 80°C for 20 min. To the mixture was subsequently added 50 U NcoI restriction endonuclease in 5 μL NEB buffer 3, and the solution was incubated at 37°C for another 2 h. The resulting solution was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v). To the aqueous layer were subsequently added 2.5 volumes of 100% ethanol and 0.1 volume of 3.0 M sodium acetate, and the solution was incubated at -20°C overnight to precipitate the DNA. The DNA pellet was then dissolved in water for LC-MS/MS analysis. An Agilent Zorbax SB-C18 column (0.5 × 250 mm, 5 μm in particle size) was employed, and the gradient for LC-MS/MS analysis was 5 min of 5-20% methanol followed by 50 min of 20-45% methanol in 400 mM HFIP. The temperature for the
ion-transport tube was maintained at 300°C. The mass spectrometer was set up for monitoring the fragmentation of the \([M-3H]^+\) ions of the 13-mer \([d(AATTACAGCMCGC)], where ‘M’ represents A, T, C or G\]. The fragment ions detected in MS/MS were manually assigned.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Figure Legends:

Figure 1. The structures of the $O^2$-alkyldT lesions examined in the present study.

Figure 2. Schematic diagrams outlining the procedures for the preparation of the lesion-bearing plasmid (a) and the single-stranded polymerase chain reaction-competitive replication and adduct bypass (SSPCR-CRAB) assay (35) (b). ‘SV40 ORI’ and ‘X’ indicate SV40 replication origin and the site where the $O^2$-alkyldT lesions are situated, respectively. The C/C mismatch site is underlined. ‘P1’ represents one of the PCR primers, 5'-GCTAGCGGATGCATCGACTCAATTACAG-3’, which contains a G at the 3’-terminus corresponding to the C/C mismatch site of the lesion-bearing genome. The P1 primer also carries a C/A mismatch three bases away from its 3’-end to improve PCR specificity (the 3’ portion of the P1 primer is shown with the mismatched C being underlined). ‘M’ and ‘N’ designate the nucleotide incorporated at the lesion site during DNA replication and the paired nucleotide of ‘M’ in the complementary strand, respectively.

Figure 3. Restriction digestion and post-labeling method for determining the bypass efficiencies and mutation frequencies of the $O^2$-alkyldT lesions in HEK293T cells. (a) Restriction digestion with NcoI and SfaNI and post-labeling assay (p* indicates a $^{32}$P-labeled phosphate group). The recognition sequences for the restriction enzymes are highlighted in bold, and restriction cleavage sites are designated with arrows. (b-c) Representative gel images showing the NcoISfaNI-produced restriction fragments of interest. The restriction fragment arising from the competitor vector, i.e., d(CATGGCGATGCTGT), is designated as '16-mer Comp'; '13-mer C', '13-mer A', '13-mer G' and '13-mer T' indicate the standard synthetic ODNs d(CATGGCGNGCTGT), where 'N' is C, A, G and T, respectively.

Figure 4. The bypass efficiencies and mutation frequencies of the $O^2$-alkyldT lesions in HEK293T cells and the isogenic cells where the TLS polymerases were individually depleted by CRISPR/Cas9. Shown are the bypass efficiencies (a) and the frequencies for the T$\rightarrow$A (b) and T$\rightarrow$G (c) mutations observed for the $O^2$-alkyldT lesions. The data represent the mean and standard deviation of results from three independent replication experiments. ‘***’, $p < 0.01$; ‘*’, $p < 0.05$. The $p$ values were calculated using two-tailed, unpaired $t$-test, and the values referred to the comparisons between wild-type and TLS polymerase-deficient HEK293T cells.
Replication Studies of $O^2$-AlkydT Lesions

Fig. 1.

R= -CH$_3$  
  -CH$_2$CH$_3$  
  -(CH$_2$)$_2$CH$_3$  
  -CH(CH$_3$)$_2$  
  -(CH$_2$)$_3$CH$_3$  
  -CH(CH$_3$)CH$_2$CH$_3$  
  -CH$_2$CH(CH$_3$)$_2$  

$O^2$-MedT  
$O^2$-EtdT  
$O^2$-nPrdT  
$O^2$-iPrdT  
$O^2$-nBudT  
$O^2$-sBudT  
$O^2$-iBudT
Replication Studies of $O^2$-AlkydT Lesions

Fig. 2.

a

5$'$-...ATGCATCGACTCAATTATACCACGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATGGTGCGGTACCAG...-5$'$

$Nt.BstNBI$ digestion
Gapped vector purification

5$'$-...ATGCATCGACTCAATTATACCACGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATGGTGCGGTACCAG...-5$'$

GTAGCTGAGTTAAp
TATCGXGCGGTAp

5$'$-...ATGCATCGACTCAATTATACCACGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATGGTGCGGTACCAG...-5$'$

b

Lesion-bearing vector

\[ \text{SV40 ORI} \]

+ Competitor vector

\[ \text{SV40 ORI} \]

Transfection into cells
In vivo replication
Plasmid extraction

5$'$-...ATGCATCGACTCAATTATACCACGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATGGTGCGGTACCAG...-5$'$

Top-strand product

5$'$-...ATGCATCGACTCAATTATACCACGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATGGTGCGGTACCAG...-5$'$

Bottom-strand product

5$'$-...ATTACAG-3$'$
(P1)

Bottom-strand-specific PCR

5$'$-...ATGCATCGACTCAATTACAGCNCGCCATGGTC...-3$'$
3$'$-...TACGTAGCTGAGTTAATATCGMGC GGTTACCAG...-5$'$

Ncol & rSAP

\[ \gamma^{32}\text{P}]ATP, T4 PNK

SfaNI

PAGE

SfaNI & rSAP

Ncol

LC-MS/MS
### Replication Studies of $O^2$-AlkyldT Lesions

**Fig. 3.**

#### a

| 5'  | ATGCATCGACTCACATTACAGCNCG | CATGGTC...3' |
|-----|--------------------------|--------------|
| 3'  | TACGTAGCTGAGTGTACMGCCGTTACCAG...5' |

- **SfaNI**
- **NcoI**
- **NcoI & SAP**
- **$[\gamma^{32}P]$ATP, T4 PNK**
- **SfaNI**

#### b

| 16mer | 13mer A | 13mer T | 13mer G | 13mer C | Control | $O^2$-MerT | $O^2$-EldT | $O^2$-nPrdT | $O^2$-nBudT |
|-------|---------|---------|---------|---------|---------|------------|------------|------------|------------|
|       |         |         |         |         |         |            |            |            |            |

#### c

| 16mer | 13mer C | 13mer A | 13mer G | 13mer T | control | $O^2$-PrdT | $O^2$-BudT | $O^2$-sBudT |
|-------|---------|---------|---------|---------|---------|------------|------------|------------|
|       |         |         |         |         |         |            |            |            |
Figure 4

(a) Bypass Efficiency (%)

(b) T→A Mutation Frequency (%)

(c) T→G Mutation Frequency (%)
Cytotoxic and mutagenic properties of minor-groove $O^2$-alkylthymidine lesions in human cells

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