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Roles of Translesion Synthesis DNA Polymerases in the Potent Mutagenicity of Tobacco-Specific Nitrosamine-Derived O^2-Alkylthymidines in Human Cells

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Abstract

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent human carcinogen. Metabolic activation of NNK generates a number of DNA adducts including O^2-methylthymidine (O^2-Me-dT) and O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O^2-POB-dT). To investigate the biological effects of these O^2-alkylthymidines in humans, we have replicated plasmids containing a site-specifically incorporated O^2-Me-dT or O^2-POB-dT in human embryonic kidney 293T (HEK293T) cells. The bulkier O^2-POB-dT exhibited high genotoxicity and only 26% translesion synthesis (TLS) occurred, while O^2-Me-dT was less genotoxic and allowed 55% TLS. However, O^2-Me-dT was 20% more mutagenic (mutation frequency (MF) 64%) compared to O^2-POB-dT (MF 53%) in HEK293T cells. The major type of mutations in each case was targeted T→A transversions (56% and 47%, respectively, for O^2-Me-dT and O^2-POB-dT). Both lesions induced a much lower frequency of T→G, the dominant mutation in bacteria. siRNA knockdown of the TLS polymerases (pols) indicated that pol η, pol ζ, and Rev1 are involved in the lesion bypass of O^2-Me-dT and O^2-POB-dT as the TLS efficiency decreased with knockdown of each pol. In contrast, MF of O^2-Me-dT was decreased in pol ζ and Rev1 knockdown cells by 24% and 25%, respectively, while for O^2-POB-dT, it was decreased by 44% in pol ζ knockdown cells, indicating that these TLS pols are critical for mutagenesis. Additional decrease in both TLS efficiency and MF was observed in cells deficient in pol ζ plus other Y-family pols. This study provided important mechanistic details on how these lesions are bypassed in human cells in both error-free and error-prone manner.

Graphical Abstract

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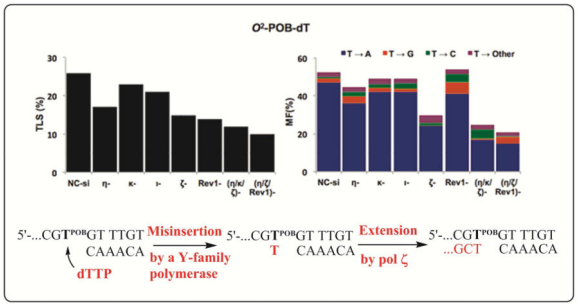
Conflict of interest statement

The authors declare that there are no conflicts of interest.



Keywords

O^2-methylthymidine; O^2-POB-thymidine; mutagenesis; TLS polymerase; HEK293T; NNK; tobacco-specific nitrosamine; siRNA knockdown

1. Introduction

Tobacco use is the single largest preventable cause of disease and premature death in the US, yet more than 45 million Americans still smoke cigarettes. Tobacco products contain over 5000 compounds and over 70 carcinogens [1]. Amongst the plethora of carcinogens present in tobacco and tobacco smoke, likely candidates for initiating cancers are the tobacco-specific nitrosamines, including (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)) and *N*'-nitrosonornicotine (NNN) [2–5]. Intermediates formed during metabolic activation of these compounds by P450 have been shown to covalently bind to DNA forming DNA adducts [6]. NNK thus far is the only carcinogen found in tobacco products that causes lung tumors in animal models, irrespective of the route of administration [7, 8].

NNK can be metabolically activated to generate methylating agents or to generate pyridyloxobutyalting agents [6, 7]. These agents react with DNA forming methyl (Me) and 4-(3-pyridyl)-4-oxobutyl (POB) adducts, respectively. The most common methylated DNA adducts identified in NNK-treated rodents formed in the methylation pathway are 7-Me-dG and O^6-Me-dG [9–11]; however, other methylation products such as O^2-Me-dC and O^2-Me-dT are also most likely formed [9]. While mutagenicity and carcinogenicity of the methylation pathway of NNK-derived O^6-Me-dG and its link to carcinogenesis is well documented [8, 10–14], the relative importance of the POB-pathway is still under investigation.

The pyridyloxobutylating pathway generates four POB adducts including O^2-POB-dT, O^2-POB-dC, O_6-POB-dG and 7-POB-dG, which were detected in NNK treated rats and mice [15–17]. A/J mice treated with a pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), showed that three POB DNA adducts, O^2-POB-dT, O_6-POB-dG and 7-POB-dG, form and persist in lung DNA at significant levels, suggesting their likely involvement in lung carcinogenesis [18]. However, O^2-POB-dT was identified as the most abundant POB adduct in lung tissues of A/J female mice [19] as well as the most persistent adduct in lung and liver of male F344 rats treated

with NNK [20]. NNKOAc-treated CHO cells produced point mutations mainly at the AT base pairs, implicating O^2-POB-dT as a candidate lesion responsible for these mutation [21].

In vivo mutagenesis study of O^2-Me-dT and O^2-POB-dT (structures shown in Chart 1) showed that these DNA lesions are highly mutagenic in *E. coli* cells [22]. Both survival and the mutagenicity were increased with SOS induction, suggesting involvement of bacterial TLS pols for their bypass and mutagenesis. Replication of O^2-alkylthymidine lesions with varying side chains in *E. coli* showed that bypass efficiency decreased as the chain length of the alkyl group increased [23] and pol V is indispensable for T→A mutations [22]. *In vitro* kinetics using exo-free Klenow fragment of *E. coli* DNA polymerase I (Kf^-), *Sulfolobus solfactaricus* DNA polymerase IV (Dpo4), human polymerase κ (pol κ) and yeast polymerase eta (pol η) established that O^2-Me-dT is a strong block to DNA synthesis and that the correct nucleotide dATP is preferentially incorporated opposite O^2-Me-dT [24, 25]. Likewise, bypass of O^2-POB-dT is inefficient by Kf^- and Dpo4, but, when bypass occurs, the wrong nucleotide dTTP is preferentially incorporated opposite O^2-POB-dT [25].

To test the hypothesis that O^2-POB-dT, the most abundant DNA adduct of the POB-pathway of NNK, is mutagenic, we have transfected a single-stranded (ss) pMS2 plasmid containing a single O^2-Me-dT or O^2-POB-dT in human embryonic kidney 293T cells (HEK293T). In addition, we investigated the involvement of TLS pols, including pol η, κ, ι, ζ and Rev1, for lesion bypass and mutagenesis by siRNA-induced knockdown of these TLS pols. We determined that both O^2-Me-dT and O^2-POB-dT are highly mutagenic in human cells, inducing predominantly T→A mutations. We also found that the lesion bypass is dependent on pol η, ζ, and Rev1.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was purchased from Du Pont New England Nuclear (Boston, MA). All enzymes, including *EcoR*V restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, uracil DNA glycosylase, and exonuclease III, were obtained from New England Bioloabs (Beverly, MA). HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA). *E. coli* DH10B cells was purchased form Life Technologies, Inc. (Grand Island, NY)

siRNAs: Synthetic siRNA duplexes against *POLH* (SI02663619), *POLK* (SI04930884), *POLI* (SI03033310), *REV1* (SI00115311), and All Stars negative control siRNA (1027280) were purchased from Qiagen (Valencia, CA). The siRNA for *REV3* was purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the siRNAs are listed in Table S1 of the Supporting Information.

2.2. Synthesis and characterization of Oligonucleotides

The modified oligonucleotides 5’-GTGCGT*GTTTGT-3’, (where T* represents O^2-Me-dT or O^2-POB-dT) containing the DNA sequence of *p53* codons 272–275 in which the lesion was located in codon 273, were synthesized and characterized as reported [22]. Oligonucleotides were analyzed by MALDI-TOF MS analysis, which gave a molecular ion

with a mass within 0.005% of theoretical. The M+1 for the O^2-Me-dT oligodeoxynucleotide is 3713 and we found an *m/z* of 3712. The M+1 for the O^2-POB-dT oligodeoxynucleotide is 3846 and we found an *m/z* of 3847. Unmodified oligonucleotides were analyzed by MALDI-TOF MS analysis, which gave a molecular ion with a mass within 0.005% of theoretical.

2.3. Construction and characterization of pMS2 vectors containing a single O^2-Me-dT or O^2-POB-dT

The single-stranded (ss) pMS2 shuttle vector, which contains its only *Eco*RV site in a hairpin region was prepared as described [26]. Synthesis and characterization of the lesion-containing and control constructs followed a protocol reported earlier [27]. The final constructs were dissolved in 1 mM Tris-HCl-0.1 mM EDTA, pH 8, and a portion was run on 1% agarose gel to assess the amount of circular DNA.

2.4. Replication and analysis in HEK 293T cells

The HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum. The cells were grown to ~90% confluency and 50 ng of each construct was transfected using 6 μL of Lipofectamine cationic lipid reagent (Invitrogen, Carlsbad, CA). After transfection with control or lesion containing pMS2, the cells were grown at 37 °C and 5% CO_2 for 24 hours and the plasmid DNA was collected and purified by the method of Hirt [28]. Each progeny DNA was then transformed *Escherichia coli* DH10B, and the transformants were analyzed by oligonucleotide hybridization followed by DNA sequence analysis as described [27].

2.5. TLS assay in human cells

The lesion-containing or control pMS2 construct was mixed in a 2:1 ratio with a single-stranded pMS2 DNA containing the same DNA sequence as the construct, except in the 12mer insert it contained a C in place of G two nucleotides 5′ to the lesion site (i.e., 5′-GTCCGTGTTTGT- 3′). The mutant DNA was used as an internal control. The mixed DNA was used to transfect HEK293T cells and processed as described above. Oligonucleotide probes for the complementary sequences for both the lesion containing plasmid and the internal control plasmid were used to analyze the progeny. The internal control gave the same number of progeny as the control construct. Typically, three independent experiments were performed to determine the extent of TLS with each pol knockdown.

2.6. Mutational analyses of TLS products from human cells with pol knockdowns

The procedure described in section **2.4** was adapted for the knockdown experiments as follows. Synthetic siRNA duplexes specific for TLS pols were transfected into HEK293T cells using Lipofectamine prior to transfection of the control and lesion containing vectors. HEK293T cells were plated in six-well plates at 50% confluence. After 24 h incubation, they were transfected with 100 pmol of the siRNA duplex mixed with Lipofectamine, diluted in Opti-MEM (Gibco), per well. One day before the transfection of the plasmid, cells were seeded in 24-well plates at 70% confluence. Cells were then co-transfected with

another aliquot of siRNA and either control or lesion containing plasmid. After 24 h incubation period, progeny plasmids were isolated as described earlier.

2.7. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the cells 72 h after the first transfection of siRNA duplexes, using the All Prep DNA/RNA/Protein Kit (Qiagen). One hundred nanograms of total RNA was used for RT-PCR analysis, performed with the One Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Primer sequences used for RT-PCR are listed in Table S2 of the Supporting Information. The conditions for RT-PCR and siRNA knockdown efficiency were determined as described elsewhere [29].

3. Results

3.1. Inhibition of replication by O^2-Me-dT and O^2-POB-dT adducts and contribution of pols η, ζ and Rev1 for lesion bypass

To investigate the mutagenic and replication blocking properties of the two O^2-alkyl-dT adducts formed by the tobacco-specific nitrosamines, we have constructed ss pMS2 plasmids containing a single O^2-Me-dT or O^2-POB-dT, and the constructs were replicated in HEK293T cells. Replication of the lesion containing ss vectors allows for the study of the lesion in the absence of repair, as ss-plasmids are inefficiently repaired by DNA repair pathways. However, the mechanism of replication is believed to be similar to lagging strand synthesis [30].

To determine the roles of individual TLS pols in the bypass of these two lesions, we employed siRNA knockdown approach to suppress their expression [31]. In agreement with previous studies, the real time PCR and Western blot analysis showed that, for each pol, the knockdown was at least 70% efficient [29]. Prior to replication of the lesion containing vectors, HEK293T cell were treated with siRNA for a specific TLS pol and allowed 48 h to reduce their expression. A non-specific siRNA was used as the negative control. After 48 h, another aliquot of siRNA was added along with the lesion containing vectors, to ensure efficient knockdown of the desired pol during translesion replication.

The relative TLS efficiency of the lesion bypass was determined by a previously reported procedure using an internal control [29]. Briefly, the lesion-containing ss-pMS2 construct was mixed in a 2:1 ratio with another modified ss-pMS2 plasmid, in which the 12mer insert contained a C instead of G two nucleotides 5' to the lesion site and the mixed DNA was transfected into siRNA treated cells. The unmodified vector was employed as an internal control to determine the TLS efficiency. After transfection, the plasmids were allowed to replicate for 24 h, the progeny plasmids were recovered, transformed to *E. coli* DH 10B cells, and the resulting colonies were analyzed by oligonucleotide hybridization [27, 29, 32]. TLS efficiency for lesion bypass was estimated as the percentage of the colonies originating from the lesion-containing plasmid relative to the same from the internal control.

As shown in Figure 1 (and in Table S3 & S4 in the Supporting Information), the TLS efficiencies of O^2-Me-dT and O^2-POB-dT in HEK293T cells transfected with negative control (NC) siRNA were 55% and 26%, respectively, relative to 100% progeny generated

from the control construct. This result is qualitatively similar to what we have observed in *E. coli* in that the bulkier O^2-POB-dT is a stronger replication block [22]. In comparison to HEK293T cells that were treated with NC-siRNA, the TLS efficiency for O^2-Me-dT decreased by 45–53% in pol η, ζ and Rev1 knockdown cells (Figure 1). Similarly, the relative %TLS of O^2-POB-dT dropped by 35–46% in pol η, pol ζ and Rev1 knockdown cells (Figure 1). However, knockdown of pol κ or pol ι did not show substantial difference in TLS of either lesion (Figure 1). These results indicate that multiple TLS pols, notably pol η, pol ζ and Rev1, play important roles in bypassing O^2-Me-dT and O^2-POB-dT, albeit none of the pols are indispensable for lesion bypass, with the caveat that there was residual activity of the enzymes even after siRNA knockdowns. We also investigated the effects of knockdown of multiple pols simultaneously. Even when pol η, pol ζ and Rev1 were simultaneously knocked down, which individually had a major effect on the TLS, the additional lowering of TLS was modest. However, it is uncertain whether the remaining low concentrations of the enzymes in the knockdown cells were sufficient to carry out part of the TLS across the lesions.

3.2. Mutational specificity of O^2-Me-dT and O^2-POB-dT in HEK293T cells

Figure 2 shows the DNA sequence analysis results of the TLS products of O^2-Me-dT and O^2-POB-dT plasmid constructs in the HEK293T cells, which showed that both O^2-Me-dT and O^2-POB-dT were highly mutagenic in human cells. For O^2-Me-dT, 64% of the progeny analyzed were mutants and only ~36% progeny yielded accurate replication across the lesion in cells transfected with NC siRNA (Figure 2). Similarly, O^2-POB-dT generated ~53% mutant progeny (Figure 2). Therefore, we conclude that, although O^2-POB-dT is a strong block to DNA replication, its replication is 20% more accurate relative to O^2-Me-dT in human cells. This result is different from the result in *E. coli*, in which O^2-POB-dT was found more mutagenic than O^2-Me-dT [22]. Most mutations in HEK293T cells were targeted one-base substitutions, while a low frequency of semi-targeted mutations (i.e., mutations near the lesion site) was also observed (Figure 3 and Tables S5–S8 in the Supporting Information). Unlike the most common T→G mutations observed in *E. coli* [22], the major type of mutations for both O^2-Me-dT and O^2-POB-dT in HEK293T cells were T→A mutations, detected in 56% and 47% frequency, respectively (Figure 3). dCMP incorporation opposite the O^2-Me-dT lesion, which leads to T→G mutations at 7% frequency, was the second most prevalent mutagenic event; however, it occurred only at ~2% frequency for O^2-POB-dT (Figure 3). A low level of T→C (1.5%) and semi-targeted (2.3%) mutations were observed for O^2-POB-dT, but T→C events were not detected in the progeny from the O^2-Me-dT construct (Figure 3). It is important to note that no mutations were detected within the 12mer sequence of the control construct in approximately 300 colonies that were analyzed (data not shown).

3.3. Contribution of pols η, ζ and Rev1 in mutagenesis of O^2-Me-dT and O^2-POB-dT

To determine the possible roles of the TLS pols in O^2-alkyl-dT mutagenesis, we analyzed the TLS products obtained from the TLS pol knockdown cells. MF for O^2-Me-dT was reduced in either pol ζ or Rev1 knockdown cells by approximately 24–25% relative to HEK293T cells treated with NC siRNA (Figure 2). A substantial 44% reduction in MF was also observed in pol ζ knockdown cells for O^2-POB-dT, but surprisingly no significant

change was observed in Rev1 knockdown cells (Figure 2). For both O^2-Me-dT and O^2-POB-dT, the decrease in MF in different pol knockdown cells was mainly due to a reduction in T→A mutations (Figure 3). For both O^2-POB-dT and O^2-Me-dT, knockdown of pol η, pol κ, or pol ι resulted in little changes in MF (Figure 2). Since knockdown of pol ζ exhibited a substantial reduction in MF for both lesions, we have explored the effect of simultaneous knockdown of this pol with other bypass pols. For O^2-Me-dT and O^2-POB-dT, simultaneous knockdown of pol η, pol ζ and Rev1 resulted in 42% and 61% decrease, respectively, in MF (Figure 2). Simultaneous knockdown of pol η, pol ζ and pol κ, on the other hand, reduced MF of O^2-Me-dT and O^2-POB-dT, respectively, by 40% and 54% (Figure 2).

4. Discussion

4.1. Involvement of pol η, ζ and Rev1 for bypass of O^2-Me-dT and O^2-POB-dT

It is well established that NNK, a tobacco specific nitrosamine, is a potent carcinogen in experimental animals and humans [4, 33, 34]. During metabolic activation of NNK, multiple reactive intermediates are generated that react with DNA, forming adducts, which are presumed to play a role in chemical carcinogenesis [8]. In this study we focused on the replication properties of O^2-Me-dT and O^2-POB-dT. O^2-POB-dT is the most abundant bulky adduct in human cells formed during metabolic activation of NNK. While O^2-Me-dT has not been identified in NNK-treated animals, small amounts are undoubtedly formed, based upon the chemistry of the methylation pathway. In our study, O^2-Me-dT serves as a model to dissect the relative importance of the altered Watson-Crick hydrogen bonding and bulkiness of O^2-POB-dT. The TLS efficiency for O^2-Me-dT and O^2-POB-dT in NC-siRNA treated HEK293T cells were 55% and 26%, respectively (Figure 1). This is an order of magnitude higher than 4.5% and 2.7%, respectively, TLS of O^2-Me-dT and O^2-POB-dT in uninduced *E. coli*, which increase only 2–4-fold upon induction of SOS [22]. Comparable magnitude of increase in TLS efficiency in human cells compared to *E. coli* has been reported in TLS of the abasic site [35, 36]. It is conceivable that TLS efficiency for most replication-blocking DNA lesions is greater in human cells than in *E. coli*, presumably due to an abundance of bypass pols in the former. Furthermore, as in *E. coli*, the bulkier O^2-POB-dT was more genotoxic and bypassed less efficiently in human cells than O^2-Me-dT. In another study in *E. coli*, it was established that the TLS efficiency decreases with longer chain lengths of O^2-alkyldT [23], which is consistent with the results of our current study.

The TLS efficiency for O^2-Me-dT in pol η, pol ζ or Rev1 knockdown cells decreased by approximately 45–53%, whereas for O^2-POB-dT it was decreased by 35–46% (Figure 1). This indicates involvement of multiple TLS pols for the lesion bypass, which supports the current hypothesis that more than one pol and multiple pol switches are involved during the TLS process [37–40]. The TLS efficiency dropped by 45% and 35%, respectively, for O^2-Me-dT and O^2-POB-dT in pol η knockdown cells (Figure 1). Pol η is considered one of the main pols that performs TLS of many lesions due to its capacious catalytic site [41–43]. Reduced TLS efficiency for both lesions in pol η knockdown cells indicates involvement of this pol in the lesion bypass. This is comparable to what we observed in *E. coli*, that is pol V, the ortholog of pol η, is essential for bypass of these lesions [22, 44].

We have established that pol ζ is critical for TLS of both O^2-Me-dT and O^2-POB-dT since the TLS efficiency was reduced in pol ζ knockdown cells by 45% and 42%, respectively (Figure 1). Pol ζ has been reported to perform extension after incorporation of a nucleotide opposite a lesion during TLS [45]. Therefore, it is conceivable that, it could function efficiently in the extension of the nucleotides that were preferentially incorporated by pol η and by pol κ or pol ι. Likewise, Rev1 is equally important for O^2-Me-dT and O^2-POB-dT bypass, as shown by 46–53% reduction in TLS in Rev1 knockdown cells. Based on experimental evidences it is accepted that Rev1 functionally interact with all other Y family pols during TLS process [46–48]. Therefore, it is likely that Rev1 acts as a structural element to form a bridge between the inserter, specifically pol η, and the extender, pol ζ, during TLS of O^2-Me-dT and O^2-POB-dT. However, when both pol η and pol ζ together with either pol κ or Rev1 were simultaneously knocked down, further reduction in TLS efficiency was modest. This suggests that, upon knockdown of these critical pols, part of TLS (~40%) may still be carried out by other TLS pols and the remaining fraction of these pols that was not knocked down.

4.2. Mutagenicity of O^2-Me-dT and O^2-POB-dT and error-prone bypass by the TLS pols

Analysis of the progeny showed that both O^2-Me-dT and O^2-POB-dT are highly mutagenic lesions in HEK293T cells with MF of 65% and 53%, respectively (Figure 2). The relative MF of these two lesions was essentially reversed from what was reported in SOS-induced *E. coli* cells, where MF was found to be 21% and 56% for O^2-Me-dT and O^2-POB-dT, respectively [22]. In HEK293T cells, although bulkier O^2-POB-dT was a stronger replication block, as reported in bacteria, it was bypassed slightly more accurately compared to O^2-Me-dT. We suspect that, owing to the small size of the alkyl chain of the O^2-Me-dT, it can be easily accommodated in the capacious catalytic site of the TLS pols, resulting in more efficient replication, albeit with low fidelity [49]. Even a relatively small methyl group at the 2 position of thymidine interferes with the base pairing modes with A and, based on thermal melting studies, G was found to be a better partner than A [50]. However, rather than T→C mutation, as suggested by the thermal melting experiments, the major type of mutations induced by both O^2-Me-dT and O^2-POB-dT was T→A transversions, at a frequency of 56% and 47%, respectively (Figure 3). The second most prevalent mutagenic event for O^2-Me-dT was T→G (7%) followed by low levels of semi-targeted mutations (0.9%). O^2-POB-dT generated approximately the same frequency of T→G, T→C and the semi-targeted mutations, which ranged from 1.5–2.3% (Figure 3). In NNK treated mice, AT→TA mutations occurred 2 to 3 times more frequently than AT→CG or AT→GC mutations [13, 51]. Furthermore, *in vitro* kinetic studies showed that dTTP incorporation opposite O^2-POB-dT was more efficient than either dCTP or dGTP incorporation by both *E. coli* Kf^- and *Sulfolobus solfataricus* DNA pol IV (Dpo4), two model DNA pols [25]. Our cellular results for O^2-POB-dT are in agreement with the mutational spectra obtained from NNK-treated rodents as well as the *in vitro* kinetic data by purified model pols. *In vitro* replication study on a template containing O^2-Me-dT, in a different sequence context, showed that it is a strong block of DNA replication by *E. coli* DNA polymerase I (Kf^-), yeast pol η, and human pol κ [24]. However, Kf- and yeast pol η preferentially incorporated the correct nucleotide, dAMP, while human pol κ preferred dCMP opposite O^2-Me-dT [24]. From our study, however, the greatest reduction in T→G mutations occurred when either

pol ι or pol ζ was knocked down (Figure 3). Yet, when pol ζ as well as two other TLS pols were knocked down simultaneously, T→G mutations remain unaffected. This suggests that a part of T→G mutations stems from dCMP incorporation opposite O^2-Me-dT by pol ι, but the mismatched pair can be extended either by pol ζ or another TLS pol.

The targeted T→A events changed in different pol knockdown cells for both O^2-Me-dT and O^2-POB-dT. Nevertheless, the pattern of the mutations remained comparable in most knockdown cells (Figure 3). Although the TLS efficiency was reduced in pol η knockdown cells, the MF was only slightly reduced for both lesions, suggesting that both error-free and erroneous TLS are catalyzed by pol η. Pol η was found to be involved in error-prone bypass of different DNA lesions [43], whereas it conducts accurate and efficient TLS of 7,8-dihydro-8-oxoguanine and *cis-syn* thymine-thymine dimer [52–54]. It is possible that it is involved in both correct nucleotide incorporation of dAMP and misincorporation of dTMP opposite both O^2-alkyl-dT lesions. But pol ι and pol κ may also be involved in misincorporation opposite these lesions. As a result, it is challenging at this time to distinguish the roles of each TLS pol, as residual 10–30% enzyme may be able to carry out TLS across the lesions in the knockdown experiments. Future experiments preferably using a combination of *in vitro* assay and knockout cell lines will be needed to distinguish the roles of individual pol in the lesion bypass.

In pol ζ knockdown cells, the MF for O^2-Me-dT and O^2-POB-dT was decreased by 25% and 44%, respectively, in addition to a reduction in TLS efficiency (Figure 2). Pol ζ is relatively tolerant of abnormal structure at the primer terminus [55, 56]. Hence, we postulate that it takes a more active role in the extension of O^2-alkyl-dT:dT mismatched base pair in comparison to the correct O^2-alkyl-dT:dA pair, leading to error-prone replication of both lesions. The extension of O^2-alkyl-dT:dT mismatch by pol ζ appeared to be more pronounced in the TLS of bulkier O^2-POB-dT, presumably because the extension of the correct O^2-POB-dT:A pair was even more inefficient (Figure 3). The MF in Rev1 knockdown cells was reduced to a similar extent as in pol ζ knockdown cells for O^2-Me-dT, indicating that Rev1 plays an indispensable structural role in pol ζ dependent bypass of O^2-Me-dT (Figure 2). It is unclear why it does not play a similar role in O^2-POB-dT bypass, since MF of O^2-POB-dT remained unaltered in Rev1 knockdown cells.

4.3. Mechanistic rationale for the error-prone TLS of O^2-alkyl-dT

The mechanistic rationale for the preferential insertion of dT and dA opposite O^2-alkyl-dT is not clear. The ability to form Watson-Crick-like structures has been proposed to explain the mutagenic potential of DNA adducts. This analysis worked well with O^6-methylguanine, in which the Watson-Crick-like structure in Figure 4a, initially proposed by Loveless in 1969 [59], explains the mutagenic incorporation of dTTP [58–60]. However, the attempt to duplicate this analysis with O^2-alkyl-dT is not as satisfying. Figure 4b shows a Watson-Crick-like structure between dA and O^2-alkyl-dT. While the structure requires protonation, the alkyl group can also make a hydrophobic interaction with the 2-position of adenine stabilizing the structure [61]. Figure 4c displays a base-pair structure between dT and O^2-alkyl-dT similar to the wobble structure that was observed in an oligodeoxynucleotide duplex containing a dT/dT mispair [62]. Evidently, this structure is not Watson-Crick-like.

There are no noticeable features of Figure 4b and 4c that would explain why dA and dT are inserted opposite O^2-alkyl-dT more often than dG in the absence of any structural studies. Figure 4d shows a potential Watson-Crick-like structure between dG and O^2-alkyl-dT. This structure is very similar to Figure 4a, except that the alkyl group is in the minor groove. Perhaps this structure explains the mutagenic incorporation of dGTP opposite O^2-alkyl-dT by pol V in *E. coli* [22, 63]. However, the preference for the incorporation of dT and dA opposite O^2-alkyl-dG in humans cells is not evident. The different specificities in different kingdoms indicate that protein-DNA interactions play a large role in determining the identity of the nucleotide inserted opposite O^2-alkyl-dTs.

5. Conclusion

In conclusion, the O^2-Me-dT and O^2-POB-dT formed by the tobacco-specific nitrosamines were found to be strong blocks of DNA replication and highly mutagenic in HEK293T cells. This result is consistent with the POB-pathway playing an important role in NNK carcinogenesis. While the bulkier O^2-POB-dT was a stronger replication block compared to O^2-Me-dT, the latter was more mutagenic. The major type of mutations induced by these lesions was T→A, but low frequencies of T→G, T→C, and semi-targeted mutations also were detected. Though pols η, ζ and Rev1 play important roles in the error-prone replication of these lesions, siRNA knockdown experiments showed that pol ζ is a key enzyme for mutagenesis induced by O^2-POB-dT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N*'-nitrosonornicotine
***O*^{2}-Me-dT**	O^2-methylthymidine
***O*^{2}-POB-dT**	O^2-[4-(3-pyridyl-4-oxobut-1-yl]thymidine
HEK293T	human embryonic kidney 293T
TLS	translesion synthesis
MF	mutation frequency
pol	DNA polymerase
Kf$^-$	exo-free Klenow fragment of DNA polymerase I
Dpo4	*Sulfolobus solfactaricus* DNA polymerase IV

Highlights

- O^2-Me-dT and O^2-POB-dT are replication blocking and highly mutagenic lesions in human cells.
- Both O^2-Me-dT and O^2-POB-dT induce T→A as the major type of mutations in human cells.
- Pols η, ζ and Rev1 play important roles in the TLS of O^2-Me-dT and O^2-POB-dT.
- Pol ζ is a key enzyme for mutagenesis induced by O^2-POB-dT.

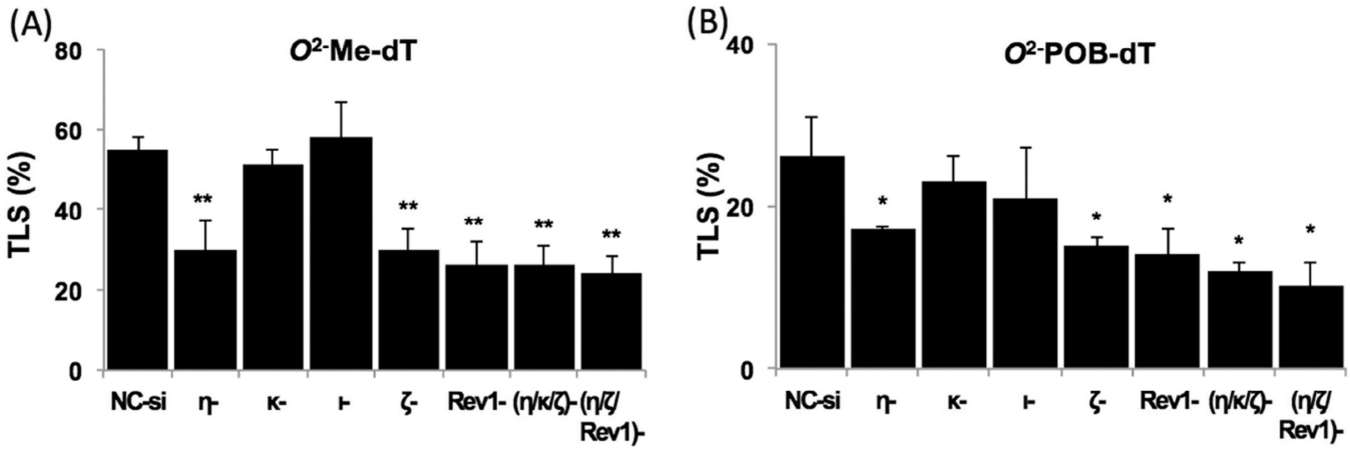


Figure 1.

Effect of siRNA-induced knockdowns of TLS pol(s) on the replicative bypass of O^2-Me-dT and O^2-POB-dT in HEK293T cells. The %TLS in various pol knockdowns was estimated using an internal control of an unmodified plasmid containing a mutation two nucleotides 5' to the lesion site. The data represent the mean and the standard deviation of results from at least 3 independent experiments (except for the triple knockdown experiments, which were performed twice). HEK293T cells were treated with negative control (NC-si) siRNA whereas the other single knockdowns are as indicated. The statistical significance between NC-siRNA treated HEK293T and TLS pols knockdowns were calculated using a two-tailed, unpaired Student's *t* test (*$p < 0.05$: **$p < 0.01$).

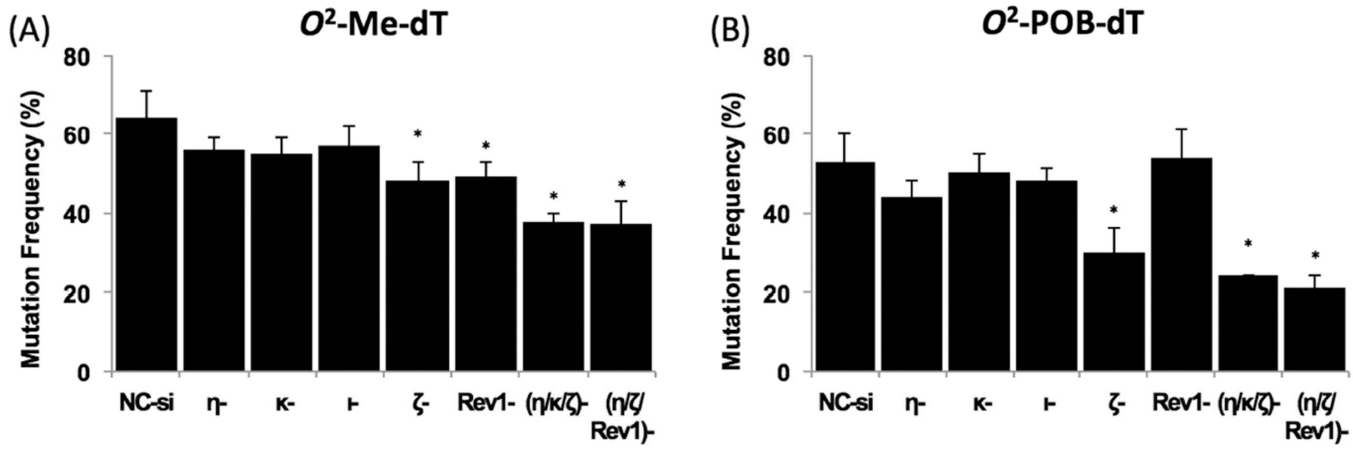


Figure 2. Total mutational frequency (MF) of O^2-Me-dT and O^2-POB-dT in HEK293T cells treated with negative control siRNA (NC-si) or siRNA for TLS pol(s)

The data represent the mean and the standard deviation of results from at least 3 independent experiments (except for pol ι and the triple knockdown experiments, which were performed twice). The statistical significance between NC-siRNA treated HEK293T and TLS pols knockdowns were calculated using two-tailed, unpaired Student's *t* test. (*$p < 0.05$).

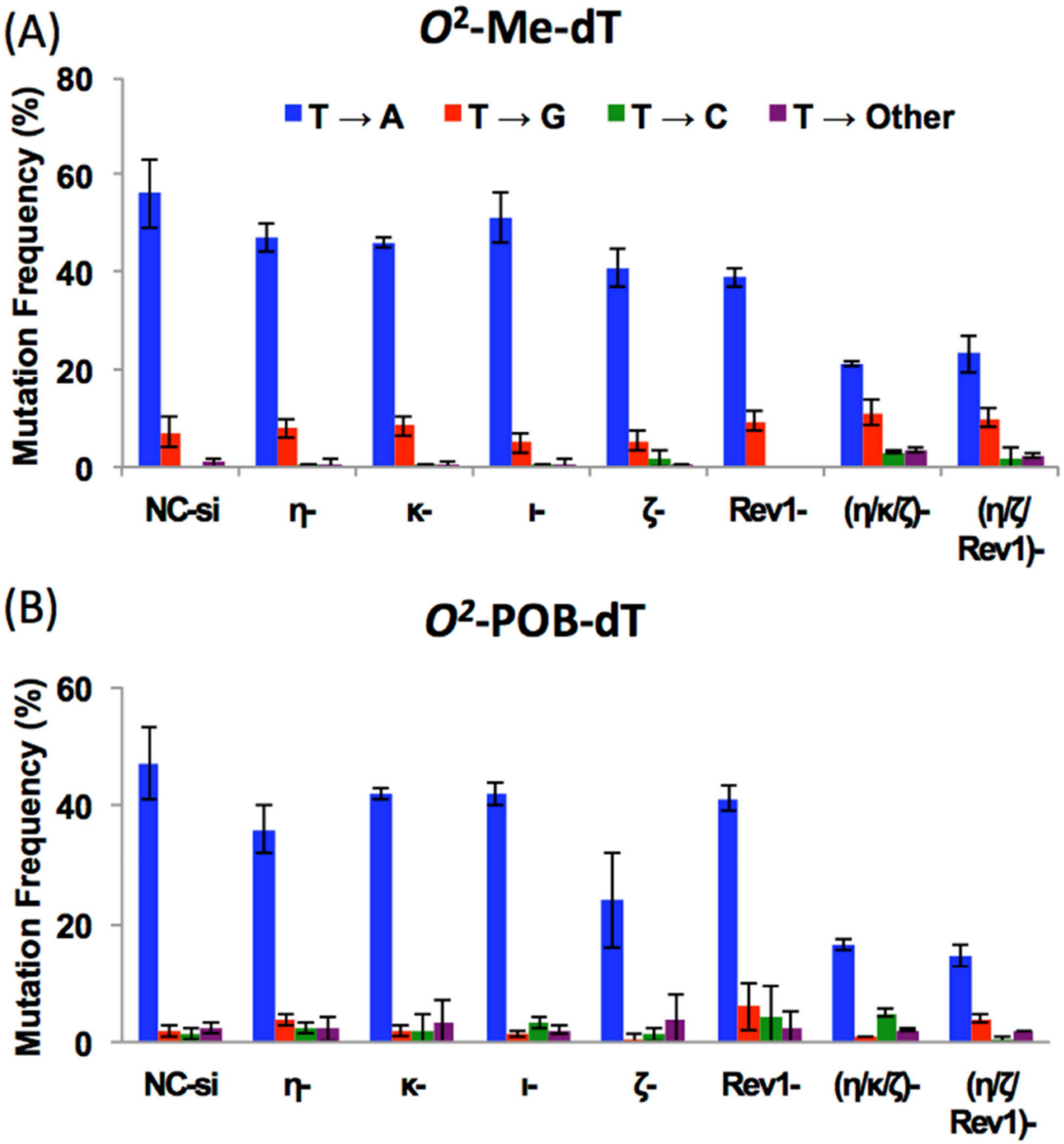


Figure 3. Types of mutations observed in HEK293T cells treated with NC siRNA (NC-si) or siRNA for individual TLS pol(s)

The data represent the mean and the standard deviation of results from at least 3 independent experiments (except for pol ι and the triple knockdown experiments, which were performed twice).

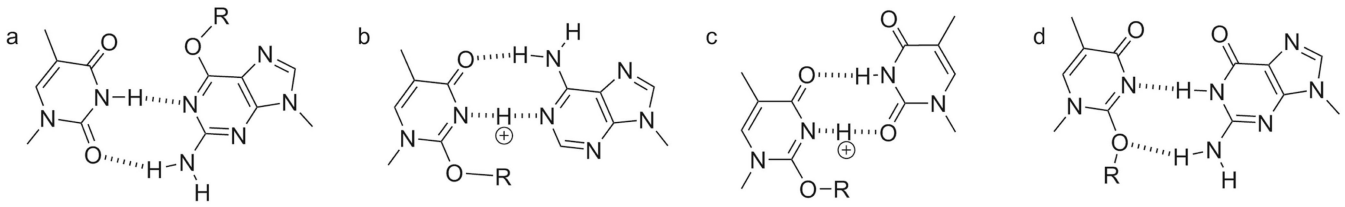
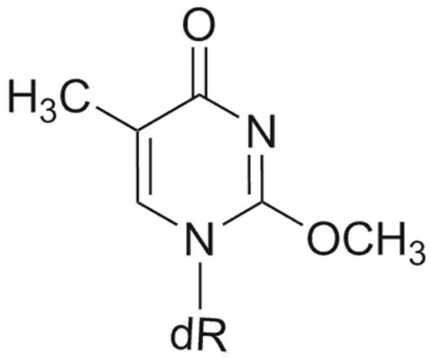
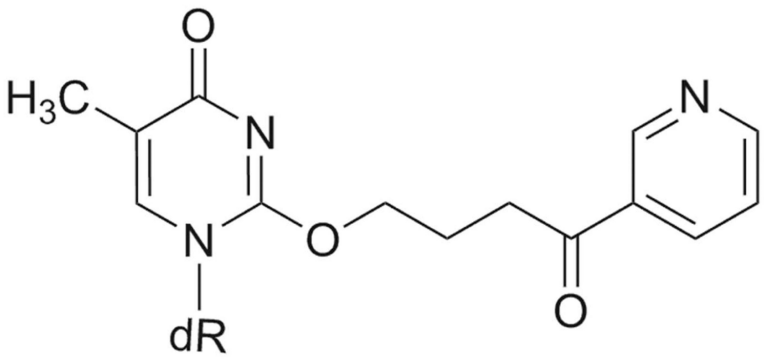


Figure 4.

Postulated base-pairing modes of (a) O_6-alkyl-dG:dT by Loveless [57], (b) protonated O^2-alkyl-dT:dA, (c) protonated O^2-alkyl-dT:dT, and (d) O^2-alkyl-dT:dG.



O^2-Me-dT



O^{2-}POB-dT

Chart 1.
Structures of O^2-Me-dT and O^2-POB-dT