Impact of tobacco-specific nitrosamine-derived DNA adducts on the efficiency and fidelity of DNA replication in human cells

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Running Title: Translesion synthesis of O-alkylated DNA adducts

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Abstract

The tobacco-derived nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) are known human carcinogens. Following metabolic activation, NNK and NNN can induce a number of DNA lesions, including several 4-(3-pyridyl)-4-oxobut-1-yl (POB) adducts. However, it remains unclear to what extent these lesions affect the efficiency and accuracy of DNA replication and how their replicative bypass is influenced by translesion synthesis (TLS) DNA polymerases. In this study, we investigated the effects of three stable POB DNA adducts (i.e. \(O^2\)-POB-dT, \(O^4\)-POB-dT and \(O^6\)-POB-dG) on the efficiency and fidelity of DNA replication in HEK293T human cells. We found that, when situated in a double-stranded plasmid, \(O^2\)-POB-dT and \(O^4\)-POB-dT moderately blocked DNA replication and induced exclusively T→A (~14.9%) and T→C (~35.2%) mutations, respectively. On the other hand, \(O^6\)-POB-dG slightly impeded DNA replication, and this lesion elicited primarily the G→A transition (~75%) together with a low frequency of the G→T transversion (~3%). By conducting replication studies in isogenic cells in which specific TLS DNA polymerases (Pols) were deleted by CRISPR-Cas9 genome editing, we observed that multiple TLS Pols, especially Pol η and Pol ζ, are involved in bypassing these lesions. Our findings reveal the cytotoxic and mutagenic properties of specific POB DNA adducts and unravel the roles of several TLS polymerases in the replicative bypass of these adducts in human cells. Together, these results provide important new knowledge about the biological consequences of POB adducts.

Introduction

Tobacco smoking leads to more than 30% of all cancer deaths in developed countries, where approximately 90% of male lung cancer deaths and 75-80% of female lung cancer deaths in the United States could be attributed to tobacco smoking (1,2). Tobacco and its smoke contain more than 70 carcinogens; among them, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN), which were shown to induce cancer in rodents, are among the most potent tobacco-derived human carcinogens (3). The carcinogenic effects of NNN and NNK are
thought to be attributed to their metabolic activation, the subsequent formation of DNA adducts, and the ensuing induction of mutations during DNA replication (4).

Both NNK and NNN can be metabolically activated by cytochrome P450 family of enzymes and the resulting metabolites can subsequently react with DNA to yield 4-(3-pyridyl)-4-oxobutyl (POB) adducts along with other DNA lesions (Figure 1) (5,6). Four POB adducts, including O2-POB-dT, O2-POB-cytosine, O6-POB-dG and N7-POB-guanine (7-POB-G), were found to accumulate at significant levels in DNA of lung tissues of NNK-treated rats (7). Three of these adducts (O2-POB-dT, O6-POB-dG and 7-POB-G) were detected in A/J mice upon exposure to another pyridyloxobutylating agent, 4-(acetoxyethylnitrosamino)-1-(3-pyridyl)-1-butane (NNKOAc) (8,9). In addition, it was found that exposure of cultured mammalian cells to NNKOAc could lead to the formation of appreciable levels of O4-POB-dT, along with the above-mentioned O2-POB-dT and O6-POB-dG (10). Several replication studies have been carried out for POB DNA adducts. In this vein, O2-POB-dT is highly blocking and miscoding during DNA replication in both *Escherichia coli* and mammalian cells (6,11). Likewise, O6-POB-dG is highly mutagenic, and it induces exclusively G→A transition in *E. coli*, and both G→A and G→T mutations in cultured human cells (12,13). The cytotoxic and mutagenic properties of O4-POB-dT, however, have not yet been examined.

The replicative DNA polymerases in eukaryotes (i.e. Pol δ and ε), despite being highly efficient and accurate in normal DNA replication, are unable to replicate across many damaged nucleosides in template DNA (14,15). To maintain genomic stability, cells are equipped with specialized translesion synthesis (TLS) DNA polymerases to bypass unrepaired DNA lesions. The Y-family polymerases Pol η, ι, κ and the B-family polymerase Pol ζ, which interact with another Y-family polymerase REV1, assume major roles in translesion synthesis (16,17). Weerasooriya et al. (6) found that both the bypass efficiency and the mutagenicity of O2-POB-dT in HEK293T cells were significantly attenuated upon siRNA-mediated knockdown of Pol η or Pol ζ, suggesting the involvement of these TLS polymerases in the mutagenic bypass of O2-POB-dT. It remains unclear how other POB lesions impede DNA replication and how their replicative bypass is influenced by TLS polymerases.

In this study, we constructed double-stranded plasmids containing site-specifically inserted O2-POB-dT, O6-POB-dT and O4-POB-dG lesions (Figure 1) and investigated how these lesions impair the efficiency and accuracy of DNA replication in HEK293T cells. We also examined the bypass efficiencies and mutation frequencies (MFs) of the three lesions in TLS polymerase-deficient cells.

**Results**

*The effects of O2-POB-dT, O6-POB-dT and O4-POB-dG on the efficiency of DNA replication and the roles of TLS polymerases in bypassing these lesions*

The main objective of the present study was to assess how the three major stable POB adducts formed on nucleobases of DNA perturb the efficiency and fidelity of DNA replication in human cells and to reveal the identities of translesion synthesis DNA polymerases in bypassing these DNA lesions.

We employed a previously described strand-specific PCR-competitive replication and adduct bypass (SSPCR-CRAB) assay for the cellular replication experiments. The method involves the construction of double-stranded plasmid carrying a single structurally defined lesion at a specific site,
co-replication of the damage-containing plasmid with a damage-free competitor plasmid in cultured human cells, isolation of the progeny genomes, selective PCR amplification of the replication products of the strand initially carrying the lesion, restriction digestion of the PCR products, and identification/quantification of restriction fragments harboring the initial damage site using native PAGE and LC-MS/MS analysis (Figures 2 and 3) (18). In particular, we first synthesized oligodeoxyribonucleotides (ODNs) harboring a site-specifically incorporated O₂-POB-dT, O⁴-POB-dT, and O⁶-POB-dG (The ESI-MS and MS/MS for these three lesion-containing ODNs are shown in Figures S1-S3). We subsequently utilized a previously established protocol to produce double-stranded, lesion-housing plasmids as well as the corresponding control (dT or dG) plasmids (Figure 2) (18). We also constructed a competitor vector, which contains three additional nucleotides in the region between the two restriction cleavage sites and serves as an internal reference for gauging the extent to which the DNA lesion impedes DNA replication. The competitor vector was co-transfected at fixed molar ratios with the lesion-harboring or control plasmids into HEK293T cells, or the isogenic cells with Pol η, Pol ι, Pol κ, Pol ζ, or both Pol η and Pol ζ being depleted by the CRISPR-Cas9 genomic editing method (Figure S4 showed the sequencing results for confirming the dual knockout of Pol η and Pol ζ). At 24 h following transfection, the progeny genomes were isolated, followed by DpnI and exonuclease III digestion to eliminate the residual unreplicated plasmids.

The progeny was subsequently amplified by SSPCR using a pair of primers spanning the site where the lesion was originally located. The forward P1 primer contains a G as the terminal 3'-nucleotide corresponding to the C/C mismatch locus (Figure 2), which facilitates the selective amplification of the progeny genomes from the replication of the lesion-containing strand. Furthermore, a C/A mismatch was incorporated at three nucleotides from its 3' terminus in the P1 primer to enhance the specificity of SSPCR, as noted previously (Figure 2) (19). The final PCR amplicon (520 bp) was digested with Ncol/SfaNI or MluCI/Cac8I (Figure 3A and 3C). The restriction fragments were subsequently analyzed using native PAGE (Figure 3B and 3D, S5 and S6) and LC-MS/MS to identify the mutagenic products induced from the cellular replication of the POB lesions (Figures S7-S9, see details in the Experimental Procedures). The quantification data from native PAGE analyses were then employed for calculating the values of relative bypass efficiency (RBE) and mutation frequency (MF).

Our PAGE analysis results revealed that O²-POB-dT significantly blocked DNA replication in HEK293T cells, with the RBE value being approximately 54% (Figure 4A and Table S1). Deletion of Pol κ did not exert any pronounced effect on the bypass efficiency of the lesion. In contrast, the bypass efficiencies decreased by 66–72% in cells deficient in Pol η, Pol ι or Pol ζ (Figure 4A and Table S1), suggesting the involvement of multiple TLS polymerases in bypassing this lesion.

The bypass efficiency of O⁴-POB-dT (26.7%) was the lowest among the three lesions in HEK293T cells (Figure 4A and Table S1). Individual knockouts of Pol η or Pol ζ led to diminished bypass efficiencies of the lesion (to ~16% and 13%, respectively, Figure 4A and Table S1), though depletion of Pol κ or Pol ι did not appreciably alter the bypass efficiency of the lesion. This result underscored the roles of Pol η and Pol ζ in bypassing the O⁴-POB-dT lesion in human cells, which is in keeping with what were observed previously for other O⁴-alkyl-dT lesions (19).

Different from O²- and O⁴-POB-dT, O⁶-POB-
dG only slightly blocked DNA replication in HEK293T cells, with the bypass efficiency being ∼82% (Figure 4A and Table S1). However, the bypass efficiencies for O₆-POB-dG were significantly decreased in cells lacking any of the four TLS polymerases, where the bypass efficiencies were approximately 22%, 60%, 37% and 51% in cells deficient in Pol η, Pol κ, Pol τ and Pol ζ, respectively (Figure 4A and Table S1).

Because both Pol η and Pol ζ are involved in bypassing all three POB lesions, we knocked out both polymerases in HEK293T cells with the CRISPR-Cas9 method (Figure S4) and conducted replication experiments for the three aforementioned lesions in these cells. Our results showed that both O₆-POB-dT and O₄-POB-dT displayed lower bypass efficiencies in the double knockout cells than in the isogenic cells depleted of Pol η or Pol ζ alone (Figure 4A and Table S1). However, further deletion of Pol ζ in Pol η-deficient background did not reduce further the bypass efficiency of O₆-POB-dG, supporting a key role of Pol η in bypassing this lesion (Figure 4A and Table S1).

The roles of TLS polymerases in modulating the mutagenicity of O²-POB-dT, O⁴-POB-dT and O⁶-POB-dG

We next employed a combination of LC-MS/MS with PAGE analyses to assess the mutagenic properties of the three POB adducts. Our data showed that O²-POB-dT was moderately mutagenic in HEK293T cells, and it gave rise to T→A transversion at a frequency of 14.9% (Figure 4B and Table S2). Furthermore, significant diminutions in mutation frequencies were observed in the isogenic background with the depletion of each of the four TLS polymerases (Figure 4B and Table S2), indicating the involvement of multiple polymerases in the mutagenic bypass of the lesion.

O⁴-POB-dT induces exclusively T→C transition mutation at a frequency of ∼35.2% in HEK293T cells (Figure 3B, 3D, 4C and Table S2). While deletion of Pol κ or Pol τ did not lead to any apparent alteration in mutation frequency for the lesion, depletion of Pol η or Pol ζ conveyed significant decreases in the frequencies of T→C mutation to ∼26.6% and ∼21.1%, respectively (Figure 4C and Table S2). A further drop in mutation frequency was observed in Pol η/Pol ζ-double knockout cells (to ∼14.2%, Figure 4C and Table S2). These findings are in agreement with the above results of bypass efficiencies and demonstrate that, among the four TLS polymerases studied, only Pol η and Pol ζ are involved in the replicative bypass of the O₄-POB-dT lesion.

O⁶-POB-dG is strongly miscoding, and it induces mainly G→A transition (73.8%), which is accompanied with a low frequency (2.9%) of G→T transversion (Figure 4D and Table S2). In addition, the mutagenic properties of the lesion were not significantly altered upon depletion of Pol η, Pol κ, Pol τ, or Pol ζ alone, or Pol η and Pol ζ in combination (Figure 4D and Table S2).

Discussion

Tobacco and its smoke contain at least 70 known carcinogens and tobacco smoking increases the risk of many types of human cancer (3). After metabolic activation, the tobacco-specific N-nitrosamines NNK and NNN, which are classified as group I carcinogens by the International Agency for Research on Cancer (IARC), could result in the formation of a variety of POB DNA adducts (Figure 1) (2). Understanding the cytotoxic and mutagenic properties of DNA adducts induced by tobacco-specific carcinogens may help fight against smoking-related diseases.

It was previously found that the minor-groove O²-POB-dT adduct is poorly repaired and moderately mutagenic (6,20,21); while the major-groove O⁶-POB-dG lesion induced substantial frequencies of G→A mutation in mammalian cells...
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In addition, replication studies in cultured human cells with individual TLS polymerases being knocked down with siRNA revealed the involvement of Pol η and Pol ζ in bypassing the $O^2$-POB-dT lesion (6). Although the siRNA approach can efficiently deplete TLS polymerases, incomplete depletion of a polymerase sometimes renders it difficult to define clearly the role of that polymerase in modulating the efficiencies and accuracies of TLS across the DNA lesion of interest (6,18). The application of the CRISPR/Cas9 genome editing method, which affords complete ablation of TLS polymerases (19), enables unambiguous delineation about the roles of different TLS polymerases in bypassing these POB lesions in human cells.

In this study, we investigated, by employing a previously established double-stranded shuttle vector and SSPCR-CRAB assay (18), the effects of three stable POB lesions (i.e. $O^2$-POB-dT, $O^4$-POB-dT, and $O^6$-POB-dG) on the efficiency and fidelity of DNA replication in HEK293T cells. Our results showed that $O^2$-POB-dT is moderately blocking to DNA replication machinery in human cells (Figure 4A and Table S1), where the bypass efficiency was higher than what was previously reported (6). The difference could be attributed in part to the use of single- versus double-stranded lesion-containing plasmids for the replication experiments (6), where lesions in double-stranded DNA are more efficiently repaired than in single-stranded DNA in mammalian cells (23,24). We observed that, in addition to the previously reported roles of Pol η and Pol ζ (6), Pol ι was also involved in bypassing the $O^2$-POB-dT lesion (Figure 4A and Table S1). Although a number of $O^2$-alkyl-dT lesions, including $O^2$-POB-dT, were found to induce all three types of single-base substitutions (T→A, T→C and T→G) in E. coli (11,25), $O^2$-POB-dT elicits exclusively T→A transversion in HEK293T cells, suggesting the differences in recognition of the lesion by DNA replication machineries in bacterial and mammalian cells. Deletion of Pol η, Pol ι or Pol ζ led to marked reduction in mutation frequencies (Figure 4B and Table S2).

We generated here, for the first time, the HEK293T cells with both Pol η and Pol ζ being knocked out by using the CRISPR-Cas9 method. We observed that the dual depletion of Pol η and Pol ζ resulted in a more pronounced decline in bypass efficiency and mutation frequency than deletion of either polymerase alone (Figure 4A, 4B, and Table S1, Table S2), suggesting that Pol η and Pol ζ, aside from functioning together as inserter and extender, respectively, in supporting the replication across the $O^2$-POB-dT lesion, may also act together with other TLS polymerases in bypassing this lesion. Interestingly, although the loss of Pol κ did not affect the bypass efficiency, it resulted in a pronounced drop in mutation frequency, indicating a potential role of this polymerase in modulating $O^2$-POB-dT-induced mutations.

To our knowledge, this is the first investigation about how $O^4$-POB-dT affects the efficiency and fidelity of DNA replication. This lesion could also be induced in mammalian cells upon treatment with NNKOAc, albeit at levels that are several times lower than $O^2$-POB-dT (10). We found that $O^4$-POB-dT constitutes a stronger blockade to DNA replication than the regioisomeric $O^2$-POB-dT and the former lesion elicits exclusively T→C mutation (Figure 4A and 4B, Table S1 and Table S2). Furthermore, the replicative bypass of this lesion requires both Pol η and Pol ζ, but not Pol κ or Pol ι (Figure 4A and Table S1). These results are in keeping with the findings made from our previous replication studies about other $O^4$-alkyl-dT lesions (19,26,27). In addition, both the bypass efficiencies and mutation frequencies were lower in Pol η/Pol ζ-double knockout cells than in the
corresponding single knockout cells, which are in line with the observations made for \(O^2\)-POB-dT (Figure 4, Table S1, and Table S2). Since mounting evidence revealed human Pol \(\zeta\) as an effective extender during lesion bypass (28-30), we reason that Pol \(\eta\) may insert one or a few nucleotides at and near the \(O^2\)-POB-dT lesion site, followed by extension of the nascent DNA strand via Pol \(\zeta\). However, an appreciable level of bypass (~8.5%) was still observed in the double-knockout cells (Figure 4A and Table S1), indicating the involvement of replicative polymerases and/or other TLS polymerases in bypassing this lesion.

Our results showed that \(O^6\)-POB-dG is not highly blocking to DNA replication machinery in HEK293T cells, but the bypass efficiencies decreased significantly after depletion of any of the four TLS polymerases (Figure 4A and Table S1). In addition, there was no significant difference in bypass efficiency for \(O^6\)-POB-dG in HEK293T cells when Pol \(\eta\) was deleted alone or in combination with Pol \(\zeta\) (Figure 4A and Table S1). Although Pol \(\zeta\) was found to be the most efficient polymerase functioning as an extender during translesion synthesis (29), it is formally possible that other TLS polymerases, e.g. Pol \(\theta\) and Pol \(\nu\) (31,32), may also participate in the extension step of TLS across the \(O^6\)-POB-dG lesion.

The major type of mutation observed for \(O^6\)-POB-dG was \(G\rightarrow A\) transition (~75%), which is associated with a low frequency of \(G\rightarrow T\) transversion (~3%, Figure 4D and Table S2). These results are in agreement with a previous replication study conducted using HEK293 cells (12). It is also worth noting that the mutation frequency of the lesion was not substantially modulated by depletion of any of the TLS polymerases (Figure 4D and Table S2), supporting that \(O^6\)-POB-dG is inherently miscoding and that it directs dTMP insertion by replicative and TLS polymerases. The high mutation frequency observed for the lesion suggests that it is not efficiently repaired prior to its encountering by the DNA replication machinery. Along this line, several recent studies have shown that \(O^6\)-POB-dG can be directly repaired by \(O^6\)-alkylguanine-DNA alkyltransferase (AGT) (33,34). Following the transfer of a methyl or other alkyl groups from \(O^6\)-alkylguanine to an internal cysteine residue (Cys145) of AGT, \(O^6\)-dG adducts are repaired and the protein becomes inactivated during the repair process (35,36). Since the “suicide” AGT protein acts only once, the number of \(O^6\)-alkylguanine adducts that can be repaired is limited to the number of AGT molecules available.

It is worth discussing our results in the context of mutations found in lung cancer patients. Previous genome-wide analysis of lung cancer patients revealed an enrichment of \(G\rightarrow T\) transversion in somatic cells of tobacco smokers, whereas \(G\rightarrow A\) transition constitutes the major type of somatic mutation in never-smokers (37,38). In addition, \(G\rightarrow C\) and \(A\rightarrow T\) mutations display higher frequencies in somatic cells than the germline (39). Our results illustrated that replication across \(O^2\)-POB-dT predominantly gives rise to \(T\rightarrow A\) transversion mutation, whereas that of \(O^6\)-POB-dG mainly leads to \(G\rightarrow A\) transition together with a low frequency of \(G\rightarrow T\) transversion (Figure 4 and Table S2). Tobacco-induced \(G\rightarrow T\) mutation in somatic cells was shown to be correlated with the \(N^2\)-dG adducts induced by metabolites of polycyclic aromatic hydrocarbons (PAHs), especially benzo[a]pyrene-7,8-diol-9,10-epoxide (40). Our results suggest that \(O^2\)-POB-dT and \(O^6\)-POB-dG may also contribute, in part, to the elevated frequencies of \(T\rightarrow A\) and \(G\rightarrow T\) mutations induced by cigarette smoking.

Taken together, we assessed the cytotoxic and mutagenic effects of three stable POB DNA adducts, which could be induced by tobacco-derived \(N\)-nitrosamines, in HEK293T cells. We
found that the replication blockage effects of these lesions follow the order of O⁶-POB-dG (with a bypass efficiency of 82%) < O²-POB-dT (54%) < O⁴-POB-dT (26.7%, Figure 4A and Table S1). Although Pol κ and Pol ε assume different roles in bypassing these three lesions, both Pol η and Pol ζ are involved in the bypass of all three lesions, indicating the importance of the “inserter” Pol η and the "extender", Pol ζ, in TLS of the bulky POB adducts in human cells. O⁶-POB-dG is highly mutagenic in HEK293T cells, where it induces mainly G→A transition along with a low frequency of G→T transversion (Figure 4D and Table S2). O²-POB-dT and O⁴-POB-dT are less mutagenic, eliciting exclusively T→A transversion and T→C transition, respectively (Figure 4 and Table S2). Hence, our study provided important new knowledge about the impact of tobacco-specific N-nitrosamine-derived POB lesions on the efficiency and accuracy of DNA replication and unveiled the roles of TLS polymerases in bypassing these lesions in human cells.

**Experimental Procedures**

All chemicals, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO), and all enzymes were from New England Biolabs (Ipswich, MA). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was obtained from Oakwood Products Inc. (West Columbia, SC) and [γ-³²P]ATP was purchased from Perkin Elmer (Piscataway, NJ). All unmodified ODNs were obtained from Integrated DNA Technologies (Coralville, IA). HEK293T cells deficient in POLH, POLI, POLK and REV3L genes, which encode DNA polymerases η, ε, κ and the catalytic subunit of polymerase ζ, were produced previously by using the CRISPR-Cas9 genome editing method (19). The Pol η, Pol ζ-double knockout HEK293T cells were obtained by deleting POLH gene from the REV3L⁻ cells using the CRISPR-Cas9 method, following previously published procedures(19). The guide sequence for human POLH gene was CTGCTCCCACGGTGAGCTGCAGG, with the PAM motif being underlined. The depletion of these two polymerases were confirmed by Sanger sequencing (Figure S4).

**Syntheses of ODNs containing a site-specifically inserted O⁶-POB-dG, O²-POB-dT, and O⁴-POB-dT**

The 12 mer ODNs containing a site-specifically incorporated POB lesion were synthesized following previously published procedures (10,41,42). The dithiane-protected ODNs were synthesized at a 1-μmol scale on a Beckman Oligo 1000M DNA synthesizer, where ultramild phosphoramidite building blocks (Glen Research, Sterling, VA) were employed for ODN assembly. The ODNs were cleaved from the controlled pore glass support and deprotected with concentrated ammonium hydroxide at room temperature for 1 h. The solvents were evaporated and the resulting solid residues redissolved in water for high-performance liquid chromatography (HPLC) purification. For the O⁶-POB-dG-containing ODNs, the N²-phenylacetyl group in the modified nucleoside was removed by treatment with 0.1 M NaOH for overnight and the solution was subsequently neutralized with 0.1 M HCl prior to HPLC purification. The dithiane protecting group was removed by incubating the aforementioned deprotected ODNs with N-chlorosuccinimide at a molar ratio of 1:5 in 50 μL of CH₃CN:H₂O (1/1, v/v) at room temperature for 10 min. The solution was chilled on ice until HPLC purification. The purified ODNs were then desalted by solid-phase extraction, where the samples were loaded onto an HLB Oasis column (Waters, Milford, MA), washed with 1.0 mL water and the ODNs were eluted with 1.0 mL of 30% methanol in H₂O. The HPLC separation was performed on an Agilent 1100 system with a Kinetex XB-C18 column (4.6×250 mm, 5 μm in particle size, CA).
purification of ODNs, a triethylammonium acetate buffer (50 mM, pH 6.8, solution A) and a mixture of solution A and acetonitrile (70/30, v/v, solution B) were employed as mobile phases. The flow rate was 0.6 ml/min, and the gradient profile in terms of solution B was 5–25% in 5 min followed by 25–60% in 60 min. The mass spectrometric characterizations of the purified lesion-containing ODNs are shown in Supplementary Figures S1-S3.

Construction of lesion-containing and control plasmids

The lesion-containing as well as the lesion-free control and competitor genomes were prepared following the previously published procedures (18,43,44). First, the parent vector was digested with Nt.BstNBI to generate a gapped plasmid, followed by removal of the resultant 25 mer single-stranded ODN through annealing with 50× excess of a 25 mer complementary ODN. The gapped plasmid was then isolated from the mixture by using 100 kDa cutoff ultracentrifugal filter units (Millipore). The purified gapped plasmid was annealed with a 5′-phosphorylated 13-mer lesion-free ODN (5′-AATTGAGTCGATG-3′) and a 5′-phosphorylated 12-mer lesion-carrying ODN, 5′-ATGGCGXGCTA T-3′ (X= O2-POB-dT, O4-POB-dT, O6-POB-dG), or the corresponding lesion-free ODN, followed by incubation with T4 DNA ligase and ATP in the ligation buffer. It is worth noting that the resulting lesion-carrying and lesion-free plasmids contain a C/C mismatch two nucleotides away from the lesion site, which facilitates the differentiation of replication products formed from the lesion-containing strand and the unmodified complementary strand (Figure 2).

Cell culture, transfection, and plasmid isolation

Replication of the above-constructed genomes was performed using the previously reported SSPCR-CRAB assay (18,45). The lesion-containing and the corresponding control plasmids were premixed individually with the competitor genome and transfected into HEK293T cells or the isogenic polymerase-deficient cells. The molar ratios of the competitor to control and lesion-bearing genome were 1:3 and 1:9, respectively. The cells (1 × 10^5) were seeded in 24-well plates and cultured overnight at 37°C in a 5% CO2 atmosphere, after which they were transfected with 300 ng of the mixed genomes by using TransIT-2020 (Mirus Bio Corporation, Madison, WI) following the manufacturer’s recommended procedures. The cells were harvested at 24 h following transfection, and the progenies of the plasmid were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, San Jose, CA). The residual unreplicated plasmid was further removed by DpnI digestion, and the resulting linear DNA was removed by exonuclease III digestion, as described elsewhere (18).

PCR amplification and polyacrylamide gel electrophoresis (PAGE) analyses

The progeny genomes emanating from cellular DNA replication were amplified by nested PCR. The primers for the first amplification were 5′-TTGGCAGTACATCAATGGGCCTGATAG-3′ and 5′-AGGGATTTGCCGATTTCGGCCTATTG-3′, and the PCR amplification started at 98°C for 2 min; then 35 cycles at 98°C for 30 s, 61°C for 30 s, and 72°C for 40 s; and a final 5-min extension at 72°C with the use of Phusion High-Fidelity DNA polymerase (New England Biolabs, Beverly, MA). The PCR products were purified using an E.Z.N.A. Cycle Pure Kit (Omega, Norcross, GA) and a nested PCR step was performed by using primers 5′-GCTAGCGGATGCATCAGACTCAATTACAG-3′ (P1 primer) and 5′-ATGATCTTTGCGTGTTAAGATCATCGGC-3′ and GoTaq Hot Start DNA polymerase (Promega, Madison, WI). In this vein, the P1 primer contains two mismatches with respect to the top-strand replication product (G/G and C/A mismatches), but...
only one mismatch with respect to the bottom-strand replication product (a C/A mismatch), thereby enabling selective amplification of the replication product(s) from the lesion-containing strand (Figure 2). The PCR products were again purified using an E.Z.N.A. Cycle Pure Kit and stored at -20°C until use.

For PAGE analysis, 150 ng of the PCR fragments were treated with 5 units of NcoI-HF and 1 unit of shrimp alkaline phosphatase (SAP) at 37°C in 10 µL of NEB buffer 3 for 1 h, followed by heating at 80°C for 20 min to deactivate the SAP. To the above mixture were then added 0.5 pmol (1.25 µCi) of [γ-32P]ATP and 5 units of T4 PNK. The reaction was continued at 37°C for 30 min, followed by heating at 75°C for 20 min to deactivate the T4 PNK. To the above mixture was subsequently added 2.0 unit SfaNI, and the solution was incubated at 37°C for 1.5 h. The digestion was subsequently terminated by addition of 20 µL formamide gel-loading buffer. The above restriction digestion yielded a 16 mer radiolabeled fragment for the competitor genome and 13 mer fragments for the control or lesion-carrying genome (Figure 3A and 3B). However, the 13 mer fragment with a T→C mutation (13mer C) cannot be resolved from the nonmutagenic 13mer T. We, therefore, employed a sequential digestion of MluCl followed by Cac8I, which yielded 10 mer radiolabeled fragments of the opposite strand that permits the differentiation of the nonmutagenic product from the product with the T→C mutation (Figure 3C and 3D). The digestion products were separated using 30% native polyacrylamide gel (acrylamide:bis-acrylamide 19:1) and quantified by phosphorimager analysis (Figure 3B and 3D). The effects of DNA lesions on replication efficiency and fidelity are represented by the relative bypass efficiency (RBE) and mutation frequency (MF), where the RBE values were calculated from the ratios of (lesion signal/competitor signal)/(non-lesion control signal/competitor signal).

Confirmation of mutagenic products by LC-MS/MS

The replication products were also confirmed by using LC-MS/MS analysis. Briefly, 2 µg of the above-described PCR products were digested with 30 units of SfaNI and 15 units of SAP in 150 µl NEB buffer 3 at 37°C for 2 h, followed by deactivating the phosphatase through heating at 80°C for 20 min. To the mixture was added 50 units of NcoI 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) and to the mixture were subsequently added 2.5 volumes of 100% ethanol and 0.1 volume of 3.0 M sodium acetate to precipitate the DNA. The DNA pellet was then reconstituted in water, and subjected to LC-MS and MS/MS analyses. An Agilent 1200 capillary HPLC system (Agilent Technologies, Santa Clara, CA) and an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) were used for all the LC-MS and MS/MS experiments. The mass spectrometer was set up for monitoring the fragmentation of the [M-3H]3- ions of the 13 mer 5′-AATTACAGCNGC-3′, where ‘N’ represents A, T, C or G (Figure S7-S9).

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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.** Metabolic activation of NNK/NNN and the ensuing formation of $O^2$-POB-dT, $O^4$-POB-dT and $O^6$-POB-dG.

**Figure 2.** A schematic diagram depicting the procedures for the construction of the lesion-containing plasmid and the SSPCR-CRAB assay. The original pTGFP-Hha10 vector was digested by Nt.BstNBI, and the resulting gapped plasmid was purified. The lesion-carrying plasmids were subsequently generated by ligating lesion-containing ODN into the gapped plasmid with T4 DNA ligase. ‘X’ indicates the location of the POB-dT lesions or the corresponding dT (or the $O^6$-POB-dG and the corresponding dG). ‘P1’ represents the forward PCR primer that contains a G as the terminal 3’-nucleotide corresponding to the C/C mismatch site of the lesion-bearing genome. A ‘C/A’ mismatch (underlined) was also introduced 2 nucleotides away from its 3’-end to improve the PCR specificity. ‘M’ designates the nucleotide incorporated at the lesion site during DNA replication, and ‘N’ denotes the paired nucleotide of ‘M’ in the complementary strand. The PCR amplicon was digested and post-labeled, and the restriction fragments were analyzed using native PAGE and LC/MS-MS.

**Figure 3.** Restriction digestion and post-labeling method for determining the bypass efficiencies and mutation frequencies of the lesions in HEK293T cells and the isogenic TLS polymerase-deficient cells. (A) Restriction digestion of PCR products using NcoI and SfaNI, and post-labeling assay. (B) The representative gel image showing the NcoI/SfaNI-produced restriction fragments of interest. The standard synthetic ODN representing the restriction fragment arising from the competitor vector (i.e. 5’-CATGGCGATATGCTGT-3’) is designated as ‘16 mer’; ‘13 mer A’, ‘13 mer G’, ‘13 mer T’ and ‘13 mer C’ represent the standard synthetic ODNs 5’-CATGGCGMGCTGT-3’, where ‘M’ is A, G, T and C, respectively. (C) Sample processing for restriction cleavage using MluCI and Cac8I, and post-labeling assay. (D) Representative gel image showing the MluCI/Cac8I-generated restriction fragments of PCR products amplified from the progenies of $O^4$-POB-dT-containing plasmid arising from replication in HEK293T cells (WT) and the isogenic polymerase-deficient cells (the five lanes on the right). ‘10 mer A’, ‘10 mer G’, ‘10 mer T’ and ‘10 mer C’ designate the standard synthetic ODNs 5’-AATTACAGCN-3’, where ‘N’ is A, G, T and C, respectively. ‘p*’ indicates a $^{32}$P-labeled phosphate group.

**Figure 4.** The bypass efficiencies (A) and mutation frequencies of $O^2$-POB-dT (B), $O^4$-POB-dG (C) and $O^6$-POB-dT (D) in HEK293T cells and the isogenic TLS polymerase-deficient cells. The bypass efficiencies and the mutation frequencies were calculated from PAGE analyses. The data represent the means and standard deviations of results from three independent replication experiments: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$. NS: not significant. The $P$-values were calculated by using unpaired two-tailed Student’s $t$-test.
Figure 1.

NNK

NNN

Cytochrome P450s

DNA

O^2-POB-dT  O^4-POB-dT  O^6-POB-dG

Translesion Synthesis of O-Alkylated DNA Adducts
Figure 2.

Transfection
Replication
Progeny extraction

Top strand product

Bottom strand product

GCTAGCGGATGCATCGACTCAATTATACCACGCGCATGGTC...

3’...TACGTAGCTGAGTTAATATCGXGCGGTACCAG...

5’...ATGCATCGACTCAATTATACCACGCGCATGGTC...

3’...TACGTAGCTGAGTTAATATCGXGCGGTACCAG...

5’...ATGCATCGACTCAATTATAGCNGCCATGGTC...

3’...TACGTAGCTGAGTTAATATCGMGCgggtACCAG...

5’...ATGCATCGACTCAATTATGATTCAG

3’...TACGTAGCTGAGTTAATATCGMGCgggtACCAG...

P1 primer

Strand-specific PCR

5’...ATGCATCGACTCAATTATACGAGCNGC|CATGGTC...

3’...TACGTAGCTGAGTTAA|TGGTMGCgggtACC...

SfaNI Ncol

Digestion

PAGE & LC-MS/MS
Figure 3.

A

\[
\begin{align*}
5' \text{ } & \text{ATGCATCGACTC} \text{ } \text{AATTACAGNCGC} \text{ } \text{CATGGTC} \text{ } \text{3'} \\
3' \text{ } & \text{TACGTAGCTGAGTTAA} \text{ } \text{TGTGCGCCGGTAC} \text{ } \text{CAG} \text{ } \text{5'} \\
\end{align*}
\]

NcoI & SAP
\[
[\gamma-^{32}P]ATP, \text{ T4 PNK} \\
\text{SfaI}
\]

\[
\begin{align*}
5' \text{ } & \text{ATGCATCGACTC} \text{ } \text{pAATTACAGNCGC} \text{ } \text{pCATGGTC} \text{ } \text{3'} \\
3' \text{ } & \text{TACGTAGCTGAGTTAAp} \text{ } \text{TGTGCGCCGGTACp} \text{ } \text{CAG} \text{ } \text{5'} \\
\end{align*}
\]

B

C

\[
\begin{align*}
5' \text{ } & \text{ATGCATCGACTC} \text{ } \text{AATTACAGNCGC} \text{ } \text{CGCCATGGTC} \text{ } \text{3'} \\
3' \text{ } & \text{TACGTAGCTGAGTTAA} \text{ } \text{TGTGCGCCGGTAC} \text{ } \text{CAGC} \text{ } \text{5'} \\
\end{align*}
\]

MluI & SAP
\[
[\gamma-^{32}P]ATP, \text{ T4 PNK} \\
\text{Cac8I}
\]

\[
\begin{align*}
5' \text{ } & \text{ATGCATCGACTC} \text{ } \text{pAATTACAGNCGC} \text{ } \text{pCGCCATGGTC} \text{ } \text{3'} \\
3' \text{ } & \text{TACGTAGCTGAGTTAAp} \text{ } \text{TGTGCGCCGGTACCAG} \text{ } \text{5'} \\
\end{align*}
\]

D

\[\text{X}
\]
Figure 4.
Impact of tobacco-specific nitrosamine-derived DNA adducts on the efficiency and fidelity of DNA replication in human cells
Hua Du, Jiapeng Leng, Pengcheng Wang, Lin Li and Yinsheng Wang

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