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RAPID REPORT

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Tobacco-Specific Nitrosamine-Derived O^2-Alkylthymidines Are Potent Mutagenic Lesions in SOS-Induced *Escherichia coli*

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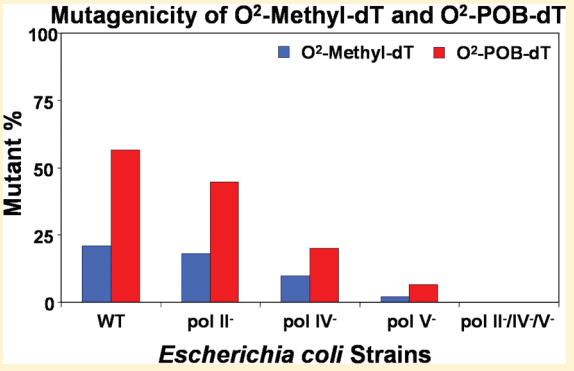
Supporting Information

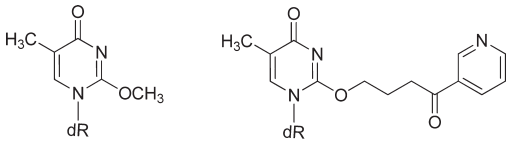
ABSTRACT: To investigate the biological effects of the O^2-alkylthymidines induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), we have replicated a plasmid containing O^2-methylthymidine (O^2-Me-dT) or O^2-[4-(3-pyridyl-4-oxobut-1-yl]thymidine (O^2-POB-dT) in *Escherichia coli* with specific DNA polymerase knockouts. High genotoxicity of the adducts was manifested in the low yield of transformants from the constructs, which was 2−5% in most strains but increased 2−4-fold with SOS. In the SOS-induced wild type *E. coli*, O^2-Me-dT and O^2-POB-dT induced 21% and 56% mutations, respectively. For O^2-POB-dT, the major type of mutation was T → G followed by T → A, whereas for O^2-Me-dT, T → G and T → A occurred in equal frequency. For both lesions, T → C also was detected in low frequency. The T → G mutation was reduced in strains with deficiency in any of the three SOS polymerases. By contrast, T → A was abolished in the pol V$^-$ strain, while its frequency in other strains remained unaltered. This suggests that pol V was responsible for the T → A mutations. The potent mutagenicity of these lesions may be related to NNK mutagenesis and carcinogenesis.



The tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are potent carcinogens in laboratory animals, inducing tumors at sites comparable to those found in smokers.[1,2] NNK is a potent lung carcinogen, but it also induces tumors in the liver, nasal cavity, and pancreas.[3,4] NNN induces tumors in the esophagus, nasal cavity, and respiratory tract.[1,5] Metabolic activation of both NNK and NNN by cytochrome P450 is required for their DNA binding, mutagenicity, and carcinogenicity.[1] NNK is metabolized to generate either a methylating agent or a pyridyloxobutylating agent, whereas NNN is metabolized only to the latter. The methylation pathway gives rise to multiple methyl (Me) adducts. 7-Me-dG and O^6-Me-dG, have been identified in NNK-treated rodents,[2,6,7] but other methylation products,[8] including O^2-Me-dC and O^2-Me-dT, are also formed. O^2-Me-pyrimidines are repaired in vitro by *E. coli* AlkA,[9,10] but otherwise, their biological properties are largely unknown. The pyridyloxobutylation pathway leads to four 4-(3-pyridyl)-4-oxobutyl (POB) adducts in vivo: O^6-POB-dG, 7-POB-dG, O^2-POB-dC, and O^2-POB-dT.[2,11−13]

It is noteworthy that O^6-POB-dG has been shown to be mutagenic in *E. coli* and mammalian cells,[14] but it is present in very low levels in NNK-treated A/J mice and rats,[15,16] In contrast, O^2-POB-dT is the most persistent POB adduct in the lung and liver of male F344 rats.[16] When the mutagenicity of a model pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), was investigated in CHO cells, it induced point mutations primarily at AT base pairs, suggesting that O^2-POB-dT might be mutagenic.[17]

Chart 1. Structures of O^2-Methylthymidine and O^2-Pyridyloxobutylthymidine



In order to determine the replication properties of the two O^2-alkyl-dT adducts formed by the methylation and pyridyloxobutylation pathway, we have constructed single-stranded pMS2 plasmids containing a single O^2-Me-dT or O^2-POB-dT (Chart 1 shows the structures), which were replicated in several isogenic strains of *E. coli* with specific DNA polymerase knockouts. The lesion repair capability of the strains remains unaltered, but single-stranded plasmids are inefficient substrates for DNA repair prior to the first round of replication. Viability was determined by

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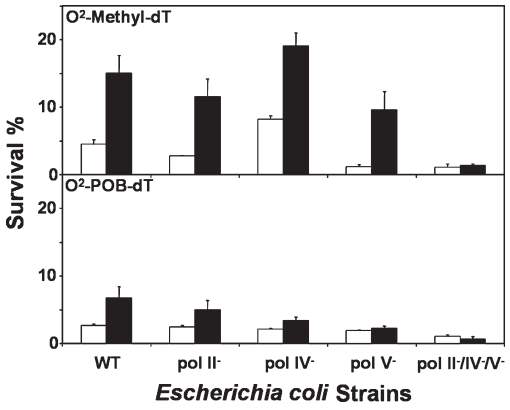

Figure 1. Viability of O^2-Me-dT and O^2-POB-dT without (open bars) and with (closed bars) SOS in different *E. coli* strains. The data represent the means and standard deviations of at least three independent experiments.

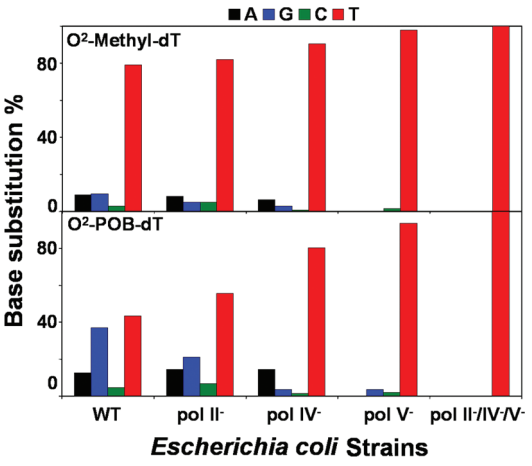

Figure 2. Progeny analysis of the replication of O^2-Me-dT and O^2-POB-dT constructs in different *E. coli* strains with SOS. The bases A (black), G (blue), C (green), and T (red) at the lesion site show the percentage of each base substitution mutant and correct base, T.

a comparison of the colony-forming units obtained per microgram of the adducted construct relative to the control, which also reflected the lesion bypass efficiency. As shown in Figure 1, the yield of transformants from each adducted construct dropped significantly, with the bulkier O^2-POB-dT being the more toxic. Upon induction of SOS, the yield of transformants increased about 2−4-fold in most *E. coli* strains. For example, in the wild type strain, transformants from the O^2-Me-dT construct were 4.5 ± 0.7 and 15.1 ± 2.6% relative to the control, without and with SOS, respectively, whereas the O^2-POB-dT construct generated 2.7 ± 0.2 and 6.7 ± 1.7% progeny, respectively, for the same.

That the SOS polymerases are responsible for survival was confirmed in the strain that lacks pol II, pol IV, and pol V. The yield of transformants from both lesion-containing constructs was approximately 1% in this strain, either with or without prior UV irradiation of the host (Figure 1). We conclude that the O^2-alkyl-dT adducts are replication blocking lesions, but increased TLS occurs with the SOS DNA polymerases. DNA alkylation products, including the extensively studied O^6-alkyl-dG adducts, have been reported to partially block DNA synthesis.[18] Several other alkylated nucleosides, including 1-Me-dA, 3-Me-dC, 3-ethyl-dC, 1-Me-dG, and 3-Me-dT, are also blocks of DNA replication.[19] However, the blockages of the first three are completely removed in strains expressing AlkB, whereas the last two exhibited the strongest blocks.[19] Although these studies used different methods of analysis, the data in the current work taken together with the earlier studies imply that the O^2-Me-dT and O^2-POB-dT adducts are two of the strongest replication blocking DNA alkylation products.

To determine the frequency of miscoding, we analyzed the progeny plasmid by oligonucleotide hybridization followed by DNA sequencing. In the wild type strain, without SOS, 96−99% progeny contained a T at the O^2-alkyl-dT site, indicating predominantly correct read-through by a DNA polymerase, most likely pol III (Supporting Information, Table S1 and S2). With SOS, mutation frequency (MF) increased to 21% and 56% for O^2-Me-dT and O^2-POB-dT, respectively, which indicates a high frequency of errors in TLS by the SOS DNA polymerases (Supporting Information, Table S1 and S2). Most mutations were targeted base substitutions, though a low frequency of targeted T deletions and semitargeted mutations also occurred (Supporting Information, Table S1 and S2). Figure 2 shows the relative population of each type of base substitution mutants relative to unaltered progeny in various SOS-induced strains, and it is apparent from this figure how each SOS DNA polymerase influences the mutational outcome. In contrast to high level of mutagenesis in the SOS-induced wild type strain, no mutants were isolated from the strain that lacks pol II, pol IV, and pol V. In the wild type strain, for O^2-Me-dT, both T → G and T → A occurred at approximately 9% frequency (Figure 2 and Supporting Information, Table S1), but T → G was the dominant mutation at 37% compared to 12% T → A induced by O^2-POB-dT (Figure 2 and Supporting Information, Table S2). For both lesions, T → A mutations were completely eliminated in the pol V-deficient strain, even though it remained approximately the same in pol II- and pol IV-deficient strains relative to the wild type (Figure 2 and Supporting Information, Table S1 and S2). The frequency of T → G, however, was reduced in each strain with a deficiency in any of the SOS polymerases, but the reduction was more pronounced in pol IV- and pol V-deficient strains. T → C mutations occurred only in the 3−5% and 5−7% frequency for O^2-Me-dT and O^2-POB-dT, respectively, but they dropped significantly in pol IV- and pol V-deficient strains. We conclude that for both lesions, T → A is induced by pol V, whereas all three SOS DNA polymerases contribute to T → G mutations. T → C mutations were likely induced by both pol IV and pol V.

To our knowledge, this is the first investigation of the replication of site-specific O^2-alkylthymidines in a cell. However, in vitro replication studies of O^2-ethyl-dT have been reported,[20,21] which showed that it blocks replication by T7 DNA polymerase and the Klenow fragment of the *E. coli* DNA polymerase I. These investigations also showed that incorporation of dA opposite O^2-ethyl-dT inhibits DNA synthesis, whereas the DNA chain is more efficiently extended when dT is incorporated opposite the lesion. The current work in *E. coli* demonstrates that in the absence of TLS polymerases, bypass of O^2-alkyl-dT is inefficient but accurate, whereas increased bypass by the TLS polymerases accompanies error-prone replication. Our study also suggests that pol V is the most error-prone of the three SOS polymerases and that only pol V incorporates dT opposite O^2-alkyl-dT. Regardless of the types of mutations, our observation that O^2-POB-dT is strongly mutagenic in *E. coli* underscores

the risk posed by NNK and NNN since it is the most persistent adduct in experimental animals. Future studies of the repair kinetics of the O^2-alkyl-dT in comparison to O^6-alkyl-dG adducts in various organs may provide deeper insight into the roles of these mutagenic adducts in the carcinogenicity of NNK and NNN.

■ ASSOCIATED CONTENT

Supporting Information. Mutation data, materials, and detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; Me, methyl; O^2-Me-dT, O^2-methylthymidine; POB, 4-(3-pyridyl)-4-oxobutyl; O^2-POB-dT, O^2-[4-(3-pyridyl-4-oxobut-1-yl]thymidine; TLS, translesion synthesis; MF, mutation frequency

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