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Replication across Regioisomeric Ethylated Thymidine Lesions by Purified DNA Polymerases

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Abstract

Causal links exist between smoking cigarettes and cancer development. Some genotoxic agents in cigarette smoke are capable of alkylating nucleobases in DNA and higher levels of ethylated DNA lesions were observed in smokers than non-smokers. In this study, we examined comprehensively how the regioisomeric *O*²-, *N*3- and *O*⁴-ethylthymidine (*O*²-, *N*3- and *O*⁴-EtdT) perturb DNA replication mediated by purified human DNA polymerases (hPol) η , κ , and ι , yeast DNA polymerase ζ (yPol ζ), and the exonuclease-free Klenow fragment (Kf⁻) of *Escherichia coli* DNA polymerase I. Our results showed that hPol η and Kf⁻ could bypass all three lesions and generate full-length replication products, whereas hPol ι stalled after inserting a single nucleotide opposite the lesions. Bypass carried out by hPol κ and yPol ζ differed markedly amongst the three lesions: Consistent with its known capability in bypassing efficiently the minor-groove *N*²-substituted 2'-deoxyguanosine lesions, hPol κ was able to bypass *O*²-EtdT, though it experienced great difficulty in bypassing *N*3-EtdT and *O*⁴-EtdT; yPol ζ was only modestly blocked by *O*⁴-EtdT, but the polymerase was highly hindered by *O*²-EtdT and *N*3-EtdT. LC-MS/MS analysis of the replication products revealed that DNA synthesis opposite *O*⁴-EtdT was highly error-prone, with dGMP being preferentially inserted, while the presence of *O*²-EtdT and *N*3-EtdT in template DNA directed substantial frequencies of misincorporation of dGMP and, for hPol ι and Kf⁻, dTMP. Thus, our results suggested that *O*²-EtdT and *N*3-EtdT may also contribute to the AT→TA and AT→GC mutations observed in cells and tissues of animals exposed to ethylating agents.

Introduction

Exposure to tobacco smoke is thought to contribute to cardiovascular and pulmonary diseases and approximately 30% of all cancer deaths in developed countries.¹ Causal links exist between smoking cigarettes and human cancers of the oral cavity, pharynx, larynx, esophagus, pancreas, urinary bladder and renal pelvis, where over 80% of all lung cancers could be attributed to tobacco smoke exposure.² Cigarette smoke contains over 6000 compounds, many of which are genotoxic.³ Carcinogens in tobacco smoke such as aromatic amines, polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines require metabolic activation by cytochrome P450 family of enzymes for the formation of reactive, electrophilic species that can react with DNA to yield covalent adducts.⁴

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Supporting Information Available. NMR spectra of synthetic compounds, HPLC trace, and LC-MS and MS/MS. This information is available free of charge via the Internet at <http://pubs.acs.org>.

Treatment with ethylating agents has been shown to induce ethylation products on backbone phosphate and all four nucleobases of DNA.⁵ For instance, ethyl methanesulfonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU) are capable of directly interacting with DNA to form ethylated nucleosides.^{6, 7} Exposure of DNA to cigarette smoke *in vitro* resulted in a dose-dependent increase of *N*7-ethylguanine (*N*7-EtG), though the identity and source of the direct-acting ethylating agent(s) in cigarette smoke has not been firmly established.⁸ It has been proposed that ethylation of biomolecules may serve as biomarkers for monitoring exposure to ethylating agents in cigarette smoke and for cancer risk assessment. Along these lines, significantly elevated levels of *N*-terminal *N*-ethylvaline were found in hemoglobin from smokers relative to non-smokers.⁹ In subsequent studies, the levels of *N*3-ethyladenine (*N*3-EtA)^{10, 11} and *N*7-EtG in urine,¹² *O*⁴-ethylthymidine (*O*⁴-EtdT) in lung tissue,¹³ and *N*7-EtG in human leukocyte DNA¹⁴ were significantly higher in smokers than non-smokers. Recently, Chen et al.¹⁵ quantified *O*²-, *N*3- and *O*⁴-ethylthymidine (*O*²-EtdT, *N*3-EtdT, and *O*⁴-EtdT) in human leukocyte DNA, where the levels of the three ethylated thymidine lesions were significantly higher in smokers than non-smokers.

Some repair and replication studies have been carried out for the alkylated thymidine derivatives. Relative to other ethylated DNA lesions such as *O*⁶-ethyl-2'-deoxyguanosine (*O*⁶-EtdG, with a half-life of ~14 hrs in rat tissue), both *O*²-EtdT and *O*⁴-EtdT are poorly repaired and thus are highly persistent in mammalian tissues (half-life of ~11–20 days in rat tissue).^{16–18} The persistence of the EtdT lesions in tissues suggests that they can be encountered by DNA replication machinery. Along this line, it was found that DNA synthesis opposite some alkylated thymidine derivatives was both blocking and mutagenic.^{19–27} Here we investigated systematically how site-specifically incorporated, regioisomeric *O*²-, *N*3- and *O*⁴-EtdT (Figure 1A) affect DNA replication mediated by purified human DNA polymerases (hPol) η , κ , and ι , yeast DNA polymerase ζ (yPol ζ), and the exonuclease-free Klenow fragment of *E. coli* DNA polymerase I (Kf[−]).

Materials and Methods

Materials

All enzymes and chemicals unless otherwise specified were purchased from New England Biolabs (Ipswich, MA) or Sigma (St. Louis, MO). Unmodified oligodeoxyribonucleotides (ODNs) used in this study were from Integrated DNA Technologies (Coralville, IA). [γ -³²P]ATP was obtained from Perkin Elmer (Piscataway, NJ). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from TCI America (Portland, OR). Phosphoramidites for unmodified nucleosides and other reagents for solid-phase DNA synthesis were obtained from Glen Research (Sterling, VA). hPol κ and yPol ζ were purchased from Enzymax (Lexington, KY), hPol ι ²⁸ and hPol η ²⁹ were kindly provided by Drs. Roger Woodgate and Wei Yang (NIH, Bethesda, MD), respectively.

Chemical synthesis of the phosphoramidite building block of *N*3-EtdT

The procedures for the synthesis of the phosphoramidite building block of *N*3-EtdT were adapted from previously described procedures for the preparation of the *N*3-carboxyethylthymidine phosphoramidite³⁰.

Synthesis of *N*3-EtdT (1, Scheme 1)—In a round bottom flask, thymidine (242 mg, 1.00 mmol) and potassium carbonate (166 mg, 1.20 mmol) were dissolved in anhydrous methanol (50 mL). To the solution was subsequently added bromoethane (129 mg, 1.20 mmol), and the resulting mixture was refluxed for 6 h. The solvent was removed under reduced pressure and the residue purified by column chromatography using 15% methanol in ethyl acetate as mobile phase to give **1** as a white foam (210 mg, 78% yield). ¹H NMR

(300 MHz, CDCl₃, Figure S1): δ 7.40 (s, 1H), 6.22 (t, J = 6.8 Hz, 1H), 4.56 (dt, J = 5.8, 3.9 Hz, 1H), 4.03-3.93 (m, 3H), 3.86 (ddd, J = 24.6, 11.8, 3.1 Hz, 2H), 2.98 (s, 2H), 2.43-2.28 (m, 2H), 1.91 (s, 3H), 1.19 (t, J = 7.0 Hz, 3H). HRMS (ESI-TOF) calcd for C₁₂H₁₉N₂O₅ [M + H]⁺ 271.1294, found 271.1314.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-N3-ethylthymidine (2, Scheme 1)—

Compound **1** was dissolved in anhydrous pyridine (10 mL) and the solution was cooled in an ice bath, to which solution were added 4-dimethylaminopyridine (DMAP, 0.5% mol) and dimethoxytrityl chloride (DMTr-Cl, 1.2 eq.). The resulting solution was stirred at room temperature for 10 hrs. The reaction was then quenched with methanol (0.5 mL) and the solvent removed under reduced pressure. The residue was purified by silica gel column chromatography with ethyl acetate as mobile phase to yield **2** as a white foam (yield 76%): ¹H NMR (300 MHz, CDCl₃, Figure S2): δ 7.55 (s, 1H), 7.43-7.21 (m, 9H), 6.83 (d, J = 8.8 Hz, 4H), 6.45 (t, J = 6.7 Hz, 1H), 4.56 (s, 1H), 4.07-3.94 (m, 3H), 3.79 (s, 6H), 3.51-3.35 (m, 3H), 2.52-2.25 (m, 3H), 1.51 (s, 3H), 1.21 (t, J = 6.9 Hz, 3H). HRMS (ESI-TOF) calcd for C₃₃H₃₆N₂O₇Na [M+Na]⁺ 595.2420, found 595.2401.

5'-O-(4,4'-dimethoxytrityl)-N3-ethylthymidine-3'-O-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite] (3, Scheme 1)—

To a round bottom flask, which was suspended in an ice bath and contained a solution of compound **2** in dry dichloromethane (3.0 mL), was added *N,N*-diisopropylethylamine (DIEA, 2.2 eq.) followed by dropwise addition of 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite (1.2 eq.). The mixture was stirred at room temperature for 1 hr under argon atmosphere. The reaction was quenched by cooling the mixture in an ice bath followed by slow addition of methanol (0.40 mL). The solution was quickly diluted with ethyl acetate (8.0 mL). The organic layer was washed with saturated NaHCO₃ (4.0 mL) and brine (4.0 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure to yield **3** in a foam that was used directly for ODN synthesis. **3**: ³¹P NMR (CDCl₃, Figure S3): δ 150.01, 149.47.

NMR

¹H NMR and ³¹P NMR spectra were acquired on a Varian Inova 300 NMR spectrometer (Varian Inc., Palo Alto, CA). Resonance assignments for *N3*-EtdT were made based on two-dimensional ¹H-¹³C heteronuclear multi-bond correlation (HMBC) experiment (Figure S4). The HMBC spectrum was acquired on a Varian Unity spectrometer operating at 500 MHz at 25°C using sweep widths of 3723.0 Hz and 30 177.3 Hz for ¹H and ¹³C, respectively. The first delay was set to match a 140 Hz coupling constant, and the second delay was set to match a long-range coupling constant of 8 Hz.

ODN synthesis

The 12-mer lesion-containing ODNs 5'-ATGGCGXGCTAT-3' ('X' represents *O*²-, *N3*-, or *O*⁴-EtdT) were synthesized on a Beckman Oligo 1000S DNA synthesizer (Fullerton, CA) at 1- μ mol scale. The phosphoramidite building blocks for *O*²-EtdT and *O*⁴-EtdT were synthesized following previously published procedures.^{31, 32} The synthesized phosphoramidite building blocks of *O*²-EtdT, *N3*-EtdT and *O*⁴-EtdT were dissolved in anhydrous acetonitrile at a concentration of 0.067 M. Conventional phosphoramidite building blocks of unmodified nucleosides were employed, and a standard ODN assembly protocol was used without any modification. The ODNs containing *O*²-EtdT and *O*⁴-EtdT were cleaved from the controlled pore glass (CPG) support with 10% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in ethanol in the dark at 23°C for five days, whereas *N3*-EtdT-containing ODN was deprotected with concentrated ammonium hydroxide at 55°C overnight. The ODNs were purified by HPLC (representative HPLC trace for the purification of *N3*-EtdT-containing ODN is shown in Figure S5) and sequences verified by

ESI-MS and MS/MS analyses (representative results for the O^4 -EtdT-containing ODN are shown in Figure S6).

HPLC

HPLC purification of synthetic ODNs was performed on an Agilent 1100 HPLC system with an Aeris XB-C18 column (4.6×150 mm, 3.6 μ m in particle size and 200 Å in pore size; Phenomenex Inc., Torrance, CA, USA). For the 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 0.8 mL/min and the gradient included 0–25% B in 5 min, 25–50% B in 40 min, 50–95% B in 10 min and at 95% B for 5 min.

Mass Spectrometry

LC-MS/MS experiments were performed using an LTQ linear ion trap mass spectrometer equipped with an electrospray ionization source (Thermo Electron, San Jose, CA). The mass spectrometer was operated in the negative-ion mode, and the spray voltage was 4.0 kV. The sheath gas flow rate was 10 arbitrary units, and the temperature for the ion transport tube was maintained at 275 °C to minimize the formation of HFIP adducts for the ODNs. An Zorbax SB-C18 column (0.5×250 mm, 5 μ m in particle size, Agilent Technologies, Santa Clara, CA) was used for the on-line separation; the flow rate was 8 μ L/min, and the gradient included a 5-min of 5–20% methanol followed by 35-min of 20–50% methanol in 400 mM HFIP (pH was adjusted to 7.0 with triethylamine).

Preparation of the lesion-carrying 22-mer ODNs

The 22mer substrates, d(ATGGCGXGCTATGAGCTCGATC) ('X' = dT, O^2 -EtdT, N^3 -EtdT or O^4 -EtdT), were obtained by ligating the above-described 12mer lesion-containing or the corresponding dT-bearing ODNs with a 5'-phosphorylated d(GAGCTCGATC) in the presence of a template ODN following previously published procedures.¹⁹ The desired lesion-containing 22mer ODNs were purified by PAGE and desalted by ethanol precipitation. The purity of the products was further confirmed by PAGE analysis.

Primer extension assays monitored by gel electrophoresis

In vitro replication experiments were performed following previously described procedures.^{27, 33} Briefly, the 22mer template, d(ATGGCGXGCTATGAGCTCGATC) ('X' = dT, O^2 -EtdT, N^3 -EtdT or O^4 -EtdT, 20 nM), was annealed with a 5'-³²P-labeled 15mer primer, d(GATCGAGCTCATAGC) (10 nM). For Kf⁻, the reactions were carried out in a buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 2 mM EDTA, 1.6 mM β -mercaptoethanol, and 5 μ g/mL BSA. For hPol η , hPol κ and hPol ι , the reactions were conducted in a buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 7.5 mM DTT. For primer extension assays, all four dNTPs and varying concentrations of a DNA polymerase, as indicated in the figures, were subsequently added to the duplex mixture and incubated for 60 min. The reactions were terminated by adding a 2-volume excess of formamide gel-loading buffer. The products were resolved on 20% (29:1) cross-linked polyacrylamide gels containing 8 M urea. Gel band intensities for the substrates and products were visualized by using a Typhoon 9410 variable-mode imager and data processed using ImageQuant 5.2 (GE Healthcare Life Sciences, Piscataway, NJ).

TLS cooperativity experiments were performed following previously described methods.³⁴ Briefly, the above-described primer/template complexes were incubated with hPol ι and all four dNTPs (100 μ M) at 37°C for 0, 10 or 30 min. hPol κ was subsequently added to the 30-min reaction mixture and incubated for an additional 30 min. These experiments were

conducted in a buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 7.5 mM DTT. The products were resolved on 20% (29:1) cross-linked polyacrylamide gels containing 8 M urea and gel bands visualized as described above (Figure S7).

Primer extension assays monitored by LC-MS/MS (Figure 1B)

The primer and lesion-containing templates (0.30 μM each) were annealed and incubated in a 50-μL solution containing hPol κ (0.10 μM), hPol η (0.10 μM), hPol ι (0.10 μM), yPol ζ (0.10 μM) or Kf⁻ (55 nM) at 37°C for 6 hrs. The reactions were conducted in the presence of all four dNTPs (0.1 μM each) in the same buffers as described above, and terminated by removing the enzymes via chloroform extraction. The aqueous layer was dried using a Speed-Vac, and the dried residues redissolved in a buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, and 1 mM DTT. SacI (40 U) and shrimp alkaline phosphatase (20 U) were subsequently added to the mixture and incubated at 37°C for 4 hrs. The proteins in the mixture were again removed by chloroform extraction, and the aqueous layer dried. The dried residues were reconstituted in 30-μL H₂O, and a 10-μL aliquot was injected for LC-MS/MS analysis.

To identify the replication products, samples were first analyzed in the data-dependent scan mode, where the most abundant ion found in MS was chosen for fragmentation in MS/MS. The fragment ions observed in MS/MS were manually assigned and the sequences of the ODNs determined (Representative MS/MS for the identification of products from hPol η-mediated reaction of the *O*²-EtdT-containing substrate is shown in Figure S8). Following identification of the ODN products, the mass spectrometer was set up for monitoring specifically the fragmentation of the precursor ions for the extended portions of the primer strand.

To correct for the varied ionization efficiencies of different ODNs, we constructed calibration curves using mixtures with varying concentrations of the standard synthetic ODNs identified in the reaction mixtures and a constant amount of d(CATCGAGCT) (9mer 5'P, which was the 5' portion of the primer liberated from SacI cleavage. Figures 1B&S9). Areas were determined for the peaks found in the selected-ion chromatograms (SICs) by monitoring the formation of 1–3 unique and abundant fragment ions for each ODN. The peak areas of individual ODNs were then normalized to that of the 9mer 5'P and plotted against the molar ratios of these ODNs over 9mer 5'P to give the calibration curves (Figure S9). The corresponding normalized ratios for the replication samples were also determined, from which we measured the molar ratios for each extended product over 9mer 5'P based on the calibration curves. The percentage of each product was then calculated from the molar ratios of all products detected in the replication mixture.

Results

The major objective of the present study was to assess systematically how the three site-specifically incorporated, regioisomeric *O*²-, *N*3- and *O*⁴-EtdT are recognized by DNA polymerases, including hPol η, hPol κ, hPol ι, yPol ζ, and Kf⁻. Kf⁻ was selected because it has been widely used as a model DNA polymerase for examining lesion bypass, and hPol η, hPol κ, hPol ι and yPol ζ were chosen because of their known abilities in bypassing a variety of DNA lesions. For example, hPol η and yPol ζ are involved in accurately bypassing *cis-syn* cyclobutane thymine dimer^{35, 36}, and both hPol κ and hPol ι are able to bypass the minor-groove *N*²-substituted dG lesions.^{37–43}

Primer extension assay monitored by PAGE

Our primer extension assay results revealed that the three regioisomeric ethylated thymidine lesions are recognized and bypassed differently by these DNA polymerases (Figure 2). While hPol η was able to bypass readily all three ethylated dT lesions and generate full-length products, O^2 -EtdT and N^3 -EtdT strongly blocked primer extension mediated by Kf⁻ and yPol ζ (Figure 2A–C). Kf⁻, for the most part, stalls after inserting one nucleotide opposite the two lesions, though full-length replication products were also observed for these two lesions. yPol ζ , on the other hand, was unable to insert any nucleotide opposite the two lesions (Figure 2A&B). hPol κ was strongly blocked by both N^3 -EtdT and O^4 -EtdT; nevertheless, the polymerase was only moderately blocked by O^2 -EtdT (Figure 2D). In this context, it is of note that blunt-end elongation products were also observed for the reactions catalyzed by Kf⁻ and hPol η (i.e., the 23-mer and 24-mer products, Figure 2B–C), as observed previously.^{44, 45} Similar as what were found previously,^{45, 46} hPol ι was strongly blocked for the template containing an unmodified dT (Figure 2E); not surprisingly, the polymerase stalls after inserting one nucleotide opposite the three EtdT lesions (Figure 2E).

Additional experiments were carried out to determine if the incomplete extension products produced by hPol ι could be extended by hPol κ . Our results showed that hPol κ indeed was able to further extend the primer to yield full-length (20-mer) products for the templates containing O^2 -EtdT, N^3 -EtdT, and O^4 -EtdT (Figures 2F and S7), even though neither polymerase alone was able to extend the primer and generate full-length products for the three EtdT lesions. These results suggest the possible cooperation between hPol ι , which inserts one nucleotide opposite the ethylated lesions, and hPol κ , which extends the primer past the lesions.

Replication products monitored by LC-MS/MS

We next employed LC-MS/MS to identify and quantify the products emanating from the *in vitro* replication reactions following previously published methods with some modifications.^{47, 48} Instead of using a uracil-containing primer, which could be subsequently treated with uracil DNA glycosylase and hot piperidine to give shorter products,^{41, 48} we employed a primer/template sequence which, after *in vitro* replication, could be cleaved with a restriction enzyme (i.e., SacI). The SacI cleavage yields shorter ODN products that are amenable to LC-MS/MS identification and quantification (Figures 1B). For the quantification, we utilized the 5' portion of the cleaved primer (9-mer 5'P) as a reference to quantify the relative amounts of replication products (See Experimental Procedures).

In vitro replication with hPol η —The LC-MS/MS quantification results revealed that hPol η is capable of extending the primer to yield full-length replication products for all four substrates. In this vein, the amounts of unextended primer (6mer) represent 28%, 23%, 26% and 26% of the total replication products for substrates containing dT, O^2 -EtdT, N^3 -EtdT and O^4 -EtdT, respectively (Table 1). This finding parallels the aforementioned PAGE analysis results showing that the three EtdT lesions are not strong blocks to DNA replication catalyzed by hPol η (Figure 2C). In addition, we observed significant amount of blunt-end elongation products where a dAMP was added to the 3' terminus of the fully extended primer (Table 1; Figures 3 and S10–S11).

As expected, no mutagenic product was detectable for the control dT-housing substrate; however, all three EtdT lesions directed substantial frequencies of nucleotide misincorporation. The full-length products with dAMP and dGMP being inserted opposite the lesion site constitute 53% and 24% of all the products detected in the replication mixture for the O^2 -EtdT-bearing substrate, 42% and 32% for the N^3 -EtdT-containing substrate, and 19% and 55% for the O^4 -EtdT-carrying substrate (Table 1; Figures 3 and S10–S11). In this

respect, the distributions of replication products obtained from three independent primer extension and LC-MS/MS experiments are highly reproducible for the hPol η -mediated replication reactions for the substrates housing the thymidine derivatives (Table 1, Figures 3 and S10–S12). Thus, the primer extension and LC-MS/MS experiments were conducted in single replicates for the other four polymerases.

In vitro replication with hPol κ —Human Pol κ was largely stalled by the three Et-dT lesions (particularly *N3*- and *O*⁴-EtdT), where the unextended primer represented 59%, 76% and 96% of the total amount of replication products for substrates containing *O*²-, *N3*- and *O*⁴-EtdT, respectively (Table 2). Although none or very small amounts (2%) of extensively extended products (i.e., + 6 nucleotides) were found for substrates harboring an *O*⁴-EtdT, the corresponding products with dAMP and dGMP being inserted opposite *O*²-EtdT occurred at frequencies of 21% and 17%, respectively (Table 2). This finding is reminiscent of the results shown in Figure 2D, where *O*²-EtdT was found to be less blocking to hPol κ -mediated primer extension than *O*⁴-EtdT and *N3*-EtdT. In addition, we found a relatively low frequency (3%, Table 2) of –1 deletion product for the *O*²-EtdT substrate, where the polymerase skipped the lesion site during primer extension.

In vitro replication with hPol ι —Similar as what was revealed from PAGE analysis, LC-MS/MS results showed that the primer extension was highly inefficient, where hPol ι largely stalled after incorporation of a single nucleotide opposite the three Et-dT lesions. For *O*²-EtdT, the insertion of dTMP (38%) dominated over those of dAMP (4%), dCMP (1%), and dGMP (5%, Table S1). While the insertion of dGMP (64%) was substantially favored over those of dAMP (2%) and dTMP (17%) for *N3*-EtdT, the insertion of the correct nucleotide (dAMP, 28%) was preferred over those of the incorrect dCMP (6%), dGMP (16%) and dTMP (15%) for *O*⁴-EtdT (Table S1).

In vitro replication with yPol ζ —*O*²-EtdT and *N3*-EtdT were extremely strong blocks to yPol ζ , as manifested by the dominance (93–96%) of the unextended primer in the replication mixtures. This is again consistent with what we observed from PAGE analysis (Figure 2A). *O*⁴-EtdT could be partially bypassed by yPol ζ , where the extended products predominantly carry the incorrect nucleotide (dGMP) opposite the lesion (Table S2).

In vitro replication with Kf[–]—The three Et-dT lesions were moderately blocking to primer extension mediated by Kf[–], where the unextended primer and +1 nucleotide extension products together occupied 21%, 45%, 24% and 22% of all the detected replication products for substrates containing a dT, *O*²-EtdT, *N3*-EtdT and *O*⁴-EtdT, respectively (Table 3). Similar to hPol η , we again observed substantial amounts of blunt-end elongation products, where the additions of a dAMP, or both a dAMP and a dTMP, to the 3' termini of the full-length products were found (Table 3).

We also detected considerable frequencies of mutations for all three EtdT derivatives. Full-length replication products with the incorporation of dAMP, dGMP, and dTMP at the lesion site represent 41%, 6% and 7%, respectively, of all the detected replication products for *O*²-EtdT-bearing substrate, while these percentages were 22%, 32% and 22% for the corresponding *N3*-EtdT substrate (Table 3). By contrast, we did not observe dTMP insertion opposite *O*⁴-EtdT, and full-length replication products with dAMP and dGMP being incorporated opposite the lesion site contributed 25% and 52% to the total amounts of replication products, respectively (Table 3).

It is of note that the LC-MS results revealed that damage-containing substrate remains largely intact during the various stages of sample handling (i.e., primer extension and restriction digestion), where less than 3% of the *O*²-EtdT-, *N3*-EtdT-, and *O*⁴-EtdT-bearing

templates were degraded to the corresponding unmodified dT-containing substrate (data not shown).

Discussion

Cigarette and its smoke contain thousands of compounds, some of which are known carcinogens. *In vitro* studies have revealed the formation of ethylated adducts in DNA exposed to cigarette smoke, though the identities of the direct-acting ethylating agents remain unknown.⁸ In addition, several studies have demonstrated elevated levels of the ethylated DNA lesions in the tissue, blood and urine samples of smokers relative to non-smokers.^{8–15, 49} In particular, a very recent study revealed that *O*²-, *N*3-, and *O*⁴-EtdT could be detected at higher levels in leukocyte DNA of smokers than non-smokers.¹⁵ Despite a substantial amount of work conducted to assess the formation of these DNA lesions, no systematic study has been carried out to interrogate how the three regioisomeric EtdT lesions are recognized by DNA polymerases.

In this study, we employed denaturing PAGE and LC-MS/MS to monitor the primer extension products of the three EtdT-containing substrates mediated by five different DNA polymerases. We found that hPol η and Kf[−] were minimally blocked by the EtdT lesions, and full-length products could be readily detected for all three lesions. With the exception of *O*²-EtdT substrate for hPol κ and *O*⁴-EtdT substrate for yPol ζ , the EtdT lesions were strong blocks to hPol κ , hPol ι and yPol ζ .

Our LC-MS/MS quantification results also underscored significant miscoding potentials of the three EtdT lesions. As observed previously for *O*⁴-alkylated thymidine derivatives,^{16, 17, 23} we found that *O*⁴-EtdT instructed the polymerases to misincorporate preferentially dGMP. Interestingly, our results showed that *O*²- and *N*3-EtdT could also direct substantial frequencies of dGMP misincorporation with multiple polymerases (Tables 1–3 and S1). In addition, these two lesions in template DNA directed hPol ι or Kf[−] to misincorporate dTMP opposite the lesions at appreciable frequencies. Exposure of rats to ENU, a known ethylating agent, was shown to give rise to a specific AT→TA point mutation which resulted in the activation of the neu oncogene in neuroblastoma cells.⁵⁰ Additionally, treatment of Chinese hamster ovary cells with ENU led to GC→AT, AT→TA and AT→GC substitutions in the coding region of the *Hprt* gene,⁵¹ where the GC→AT and AT→GC mutations were attributed to replicative bypass of *O*⁶-EtdG and *O*⁴-EtdT, respectively.⁵¹ In this respect, some previous studies suggested that *N*3-EtdT and *O*²-EtdT are capable of pairing with thymine during DNA synthesis *in vitro* carried out by Kf[−].^{20–22} Our results suggest that *O*²-EtdT and *N*3-EtdT may also lead to substantial frequencies of AT→GC and AT→TA mutations.

We also observed a better tolerance of hPol κ toward *O*²-EtdT than the other two regioisomers, which is in line with the previous observations that the polymerase can carry out efficient and error-free nucleotide incorporation opposite the minor-groove *N*²-dG lesions.^{38–43} The poor fidelity and, relative to the bypass of the *N*²-dG lesions, the reduced efficiency, observed for hPol κ -mediated nucleotide incorporation opposite *O*²-EtdT might be attributed to the inability of *O*²-EtdT to base pair favorably with any canonical nucleotides, as noted previously.²³

Taken together, this study reveals the biological consequences of the regioisomeric EtdT lesions by offering important knowledge about how these lesions compromise the efficiency and fidelity of DNA replication mediated by a number of DNA polymerases. Future structural studies about complexes formed between polymerases and lesion-containing DNA

will provide further insights about the different preferences for nucleotide incorporations opposite the regioisomeric EtdT lesions with different DNA polymerases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>O</i>²-EtdT	<i>O</i> ² -ethylthymidine
<i>N</i>3-EtdT	<i>N</i> 3-ethylthymidine
<i>O</i>⁴-EtdT	<i>O</i> ⁴ -ethylthymidine
<i>N</i>7-EtG	<i>N</i> 7-ethylguanine
ODNs	oligodeoxyribonucleotides
EMS	ethyl methanesulfonate
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
Kf[−]	Klenow fragment
hPol	human DNA polymerases
yPolζ	yeast DNA polymerase ζ
DIEA	<i>N,N</i> -diisopropylethylamine
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
DMAP	4-dimethylaminopyridine
DMTr-Cl	dimethoxytrityl chloride
HMBC	heteronuclear multi-bond correlation

References

1. American Cancer Society. Cancer Facts and Figures 2013. American Cancer Society; Atlanta, GA: 2013.
2. International Agency for Research on Cancer. Tobacco smoke and involuntary smoking. IARC Monographs Evaluation of Carcinogen Risk to Humans. 2004; 83:1–1438.
3. Tyczynski JE, Bray F, Parkin DM. Lung cancer in Europe in 2000: epidemiology, prevention, and early detection. *Lancet Oncol.* 2003; 4:45–55. [PubMed: 12517539]
4. Hecht SS. Progress and challenges in selected areas of tobacco carcinogenesis. *Chem Res Toxicol.* 2008; 21:160–171. [PubMed: 18052103]
5. Singer B. *In vivo* formation and persistence of modified nucleosides resulting from alkylating agents. *Environ Health Perspect.* 1985; 62:41–48. [PubMed: 4085444]
6. Sega GA. A review of the genetic effects of ethyl methanesulfonate. *Mutat Res.* 1984; 134:113–142. [PubMed: 6390190]
7. Nehls P, Rajewsky MF. Ethylation of nucleophilic sites in DNA by *N*-ethyl-*N*-nitrosourea depends on chromatin structure and ionic strength. *Mutat Res.* 1985; 150:13–21. [PubMed: 4000156]

8. Singh R, Kaur B, Farmer PB. Detection of DNA damage derived from a direct acting ethylating agent present in cigarette smoke by use of liquid chromatography-tandem mass spectrometry. *Chem Res Toxicol.* 2005; 18:249–256. [PubMed: 15720129]
9. Carmella SG, Chen M, Villalta PW, Gurney JG, Hatsukami DK, Hecht SS. Ethylation and methylation of hemoglobin in smokers and non-smokers. *Carcinogenesis.* 2002; 23:1903–1910. [PubMed: 12419839]
10. Prevost V, Shuker DE. Cigarette smoking and urinary 3-alkyladenine excretion in man. *Chem Res Toxicol.* 1996; 9:439–444. [PubMed: 8839047]
11. Chao MR, Wang CJ, Chang LW, Hu CW. Quantitative determination of urinary N7-ethylguanine in smokers and non-smokers using an isotope dilution liquid chromatography/tandem mass spectrometry with on-line analyte enrichment. *Carcinogenesis.* 2006; 27:146–151. [PubMed: 16000398]
12. Anna L, Kovacs K, Gyorffy E, Schoket B, Nair J. Smoking-related O^4 -ethylthymidine formation in human lung tissue and comparisons with bulky DNA adducts. *Mutagenesis.* 2011; 26:523–527. [PubMed: 21454326]
13. Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Solomon JJ. *In vitro* DNA replication implicates O^2 -ethyldeoxythymidine in transversion mutagenesis by ethylating agents. *Nucleic Acids Res.* 1992; 20:587–594. [PubMed: 1741292]
14. Balbo S, Villalta PW, Hecht SS. Quantitation of 7-ethylguanine in leukocyte DNA from smokers and nonsmokers by liquid chromatography-nanoelectrospray-high resolution tandem mass spectrometry. *Chem Res Toxicol.* 2011; 24:1729–1734. [PubMed: 21859140]
15. Chen HJ, Wang YC, Lin WP. Analysis of ethylated thymidine adducts in human leukocyte DNA by stable isotope dilution nanoflow liquid chromatography-nanospray ionization tandem mass spectrometry. *Anal Chem.* 2012; 84:2521–2527. [PubMed: 22277066]
16. Preston BD, Singer B, Loeb LA. Mutagenic potential of O^4 -methylthymine *in vivo* determined by an enzymatic approach to site-specific mutagenesis. *Proc Natl Acad Sci U S A.* 1986; 83:8501–8505. [PubMed: 3464967]
17. Singer B, Spengler SJ, Fraenkel-Conrat H, Kusmierek JT. O^4 -Methyl-, -ethyl, or -isopropyl substituents on thymidine in poly(dA-dT) all lead to transitions upon replication. *Proc Natl Acad Sci U S A.* 1986; 83:28–32. [PubMed: 3455756]
18. Jasti VP, Spratt TE, Basu AK. Tobacco-specific nitrosamine-derived O^2 -alkylthymidines are potent mutagenic lesions in SOS-induced *Escherichia coli*. *Chem Res Toxicol.* 2011; 24:1833–1835. [PubMed: 22029400]
19. Yuan B, Wang J, Cao H, Sun R, Wang Y. High-throughput analysis of the mutagenic and cytotoxic properties of DNA lesions by next-generation sequencing. *Nucleic Acids Res.* 2011; 39:5945–5954. [PubMed: 21470959]
20. Fiala KA, Brown JA, Ling H, Kshetry AK, Zhang J, Taylor JS, Yang W, Suo Z. Mechanism of template-independent nucleotide incorporation catalyzed by a templatedependent DNA polymerase. *J Mol Biol.* 2007; 365:590–602. [PubMed: 17095011]
21. Grevatt PC, Solomon JJ, Bhanot OS. *In vitro* mispairing specificity of O^2 -ethylthymidine. *Biochemistry.* 1992; 31:4181–4188. [PubMed: 1567865]
22. Huff AC, Topal MD. DNA damage at thymine N3 abolishes base-pairing capacity during DNA-synthesis. *J Biol Chem.* 1987; 262:12843–12850. [PubMed: 2442169]
23. Andersen N, Wang J, Wang P, Jiang Y, Wang Y. *In-vitro* replication studies on O^2 -methylthymidine and O^4 -methylthymidine. *Chem Res Toxicol.* 2012; 25:2523–2531. [PubMed: 23113558]
24. Gowda AS, Krishnegowda G, Suo Z, Amin S, Spratt TE. Low fidelity bypass of O^2 -(3-pyridyl)-4-oxobutylthymine, the most persistent bulky adduct produced by the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by model DNA polymerases. *Chem Res Toxicol.* 2012; 25:1195–1202. [PubMed: 22533615]
25. Bronstein SM, Skopek TR, Swenberg JA. Efficient repair of O^6 -ethylguanine, but not O^4 -ethylthymine or O^2 -ethylthymine, is dependent upon O^6 -alkylguanine-DNA alkyltransferase and nucleotide excision repair activities in human cells. *Cancer Res.* 1992; 52:2008–2011. [PubMed: 1551130]

26. Brent TP, Dolan ME, Fraenkel-Conrat H, Hall J, Karran P, Laval L, Margison GP, Montesano R, Pegg AE, Potter PM, et al. Repair of *O*-alkylpyrimidines in mammalian cells: a present consensus. *Proc Natl Acad Sci U S A*. 1988; 85:1759–1762. [PubMed: 3162305]
27. Den Engelse L, De Graaf A, De Brij RJ, Menkveld GJ. *O*²- and *O*⁴- ethylthymine and the ethylphosphotriester dTp(ET)dT are highly persistent DNA modifications in slowly dividing tissues of the ethylnitrosourea-treated rat. *Carcinogenesis*. 1987; 8:751–757. [PubMed: 3608071]
28. Tissier A, McDonald JP, Frank EG, Woodgate R. pol η , a remarkably errorprone human DNA polymerase. *Genes Dev*. 2000; 14:1642–1650. [PubMed: 10887158]
29. Biertumpfel C, Zhao Y, Kondo Y, Ramon-Maiques S, Gregory M, Lee JY, Masutani C, Lehmann AR, Hanaoka F, Yang W. Structure and mechanism of human DNA polymerase η . *Nature*. 2010; 465:1044–1048. [PubMed: 20577208]
30. Wang J, Wang Y. Chemical synthesis of oligodeoxyribonucleotides containing *N*³- and *O*⁴-carboxymethylthymidine and their formation in DNA. *Nucleic Acids Res*. 2009; 37:336–345. [PubMed: 19042973]
31. Xu YZ, Swann PF. A simple method for the solid phase synthesis of oligodeoxynucleotides containing *O*⁴-alkylthymine. *Nucleic Acids Res*. 1990; 18:4061–4065. [PubMed: 2377451]
32. Xu YZ, Swann PF. Oligodeoxynucleotides containing *O*²-alkylthymine: synthesis and characterization. *Tetrahedron Lett*. 1994; 35:303–306.
33. Choi JY, Zang H, Angel KC, Kozekov ID, Goodenough AK, Rizzo CJ, Guengerich FP. Translesion synthesis across 1,*N*²-ethenoguanine by human DNA polymerases. *Chem Res Toxicol*. 2006; 19:879–886. [PubMed: 16780368]
34. Kotapati S, Maddukuri L, Wickramaratne S, Seneviratne U, Goggin M, Pence MG, Villalta P, Guengerich FP, Marnett L, Tretyakova N. Translesion synthesis across 1,*N*⁶-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine (1,*N*⁶- γ -HMHP-dA) adducts by human and archbacterial DNA polymerases. *J Biol Chem*. 2012; 287:38800–38811. [PubMed: 22977231]
35. Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η . *Nature*. 1999; 399:700–704. [PubMed: 10385124]
36. Nelson JR, Lawrence CW, Hinkle DC. Thymine-thymine dimer bypass by yeast DNA polymerase ζ . *Science*. 1996; 272:1646–1649. [PubMed: 8658138]
37. Pence MG, Blans P, Zink CN, Hollis T, Fishbein JC, Perrino FW. Lesion bypass of *N*²-ethylguanine by human DNA polymerase ι . *J Biol Chem*. 2009; 284:1732–1740. [PubMed: 18984581]
38. Jarosz DF, Godoy VG, Delaney JC, Essigmann JM, Walker GC. A single amino acid governs enhanced activity of DinB DNA polymerases on damaged templates. *Nature*. 2006; 439:225–228. [PubMed: 16407906]
39. Yuan B, Cao H, Jiang Y, Hong H, Wang Y. Efficient and accurate bypass of *N*²-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase *in vitro* and *in vivo*. *Proc Natl Acad Sci USA*. 2008; 105:8679–8684. [PubMed: 18562283]
40. Yuan B, You C, Andersen N, Jiang Y, Moriya M, O'Connor TR, Wang Y. The roles of DNA polymerases κ and ι in the error-free bypass of *N*²-carboxyalkyl-dG lesions in mammalian cells. *J Biol Chem*. 2011; 286:17503–17511. [PubMed: 21454642]
41. Choi JY, Angel KC, Guengerich FP. Translesion synthesis across bulky *N*²-alkyl guanine DNA adducts by human DNA polymerase κ . *J Biol Chem*. 2006; 281:21062–21072. [PubMed: 16751196]
42. Suzuki N, Ohashi E, Kolbanovskiy A, Geacintov NE, Grollman AP, Ohmori H, Shibutani S. Translesion synthesis by human DNA polymerase κ on a DNA template containing a single stereoisomer of dG-(+)- or dG-(-)-*anti-N*²-BPDE (7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene). *Biochemistry*. 2002; 41:6100–6106. [PubMed: 11994005]
43. Zhang Y, Yuan F, Wu X, Wang M, Rechkoblit O, Taylor JS, Geacintov NE, Wang Z. Error-free and error-prone lesion bypass by human DNA polymerase κ *in vitro*. *Nucleic Acids Res*. 2000; 28:4138–4146. [PubMed: 11058110]

44. Frank EG, Tissier A, McDonald JP, Rapic-Otrin V, Zeng X, Gearhart PJ, Woodgate R. Altered nucleotide misinsertion fidelity associated with pol η -dependent replication at the end of a DNA template. *EMBO J.* 2001; 20:2914–2922. [PubMed: 11387224]
45. Vaisman A, Takasawa K, Iwai S, Woodgate R. DNA polymerase ϵ -dependent translesion replication of uracil containing cyclobutane pyrimidine dimers. *DNA Repair (Amst).* 2006; 5:210–218. [PubMed: 16263340]
46. Vaisman A, Woodgate R. Unique misinsertion specificity of pol η may decrease the mutagenic potential of deaminated cytosines. *EMBO J.* 2001; 20:6520–6529. [PubMed: 11707422]
47. Chowdhury G, Guengerich FP. Liquid chromatography-mass spectrometry analysis of DNA polymerase reaction products. *Curr Protoc Nucleic Acid Chem.* 2011; Chapter 7(Unit 7):16, 11. [PubMed: 22147421]
48. Gu C, Wang Y. *In vitro* replication and thermodynamic studies of methylation and oxidation modifications of 6-thioguanine. *Nucleic Acids Res.* 2007; 35:3693–3704. [PubMed: 17517786]
49. Godschalk R, Nair J, van Schooten FJ, Risch A, Drings P, Kayser K, Dienemann H, Bartsch H. Comparison of multiple DNA adduct types in tumor adjacent human lung tissue: effect of cigarette smoking. *Carcinogenesis.* 2002; 23:2081–2086. [PubMed: 12507931]
50. Perantoni AO, Rice JM, Reed CD, Watatani M, Wenk ML. Activated neu oncogene sequences in primary tumors of the peripheral nervous system induced in rats by transplacental exposure to ethylnitrosourea. *Proc Natl Acad Sci U S A.* 1987; 84:6317–6321. [PubMed: 3476947]
51. Op het Veld CW, van Hees-Stuivenberg S, van Zeeland AA, Jansen JG. Effect of nucleotide excision repair on hprt gene mutations in rodent cells exposed to DNA ethylating agents. *Mutagenesis.* 1997; 12:417–424. [PubMed: 9412994]

Figure 1.

(A) Structures of the regioisomeric ethylated thymidines. “dR” denotes 2-deoxyribose. (B) Experimental procedures for monitoring primer extension products using SacI cleavage and LC-MS/MS. ‘X’ represents dT, O²-EtdT, N³-EtdT or O⁴-EtdT, and ‘N’ designates the nucleotide incorporated opposite X during *in vitro* replication.

Figure 2.

Primer extension assays for *O*²-EtdT-, *N*³-EtdT- and *O*⁴-EtdT-bearing substrates and the control undamaged substrate with yPol ζ (A), Kf⁻ (B), hPol η (C), hPol κ (D), and hPol ι (E). (F) Cooperativity of human DNA polymerases ι and κ in the bypass of the *O*²-EtdT lesion. Primer-template complex containing *O*²-EtdT (50 nM) was incubated with hPol ι (10 nM) for 0, 10 or 30 min. To the 30 min reaction mixture was added 5 nM of hPol κ to determine whether the primer extension initiated by hPol ι and stalled by the lesion could be completed by hPol κ. The sequences for the templates are d(ATGGCGXGCTATGAGCTCGATC) ('X' represents dT, *O*²-EtdT, *N*³-EtdT or *O*⁴-EtdT), and a 5'-[³²P]-labeled d(GATCGAGCTCATAGC) was used as the primer (see Figure 1B). "22mer" refers to a standard ODN for the fully extended primer strand.

Figure 3.

Selected-ion chromatograms obtained from the LC-MS and MS/MS analysis of the hPol η -induced replication products for O^2 -EtdT-bearing substrate. The replication products were treated with restriction enzyme SacI and shrimp alkaline phosphatase, and 10-pmol of the replication mixture was injected for the analysis.

Scheme 1. Chemical synthesis of N3-EtdT phosphoramidite building block^a

^aReagents and conditions: a) bromoethane, K₂CO₃, MeOH, reflux, 6 h; b) DMTr-Cl/DMAP, pyridine, r.t., 10 h; c) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, DIEA, CH₂Cl₂, r.t., 2 h.

A summary of the percentages of products formed from hPol η -mediated replication of dT-, *O*²-EtdT-, *N*³-EtdT- and *O*⁴-EtdT-containing substrates as determined by LC-MS/MS. The bases highlighted in bold represent those incorporated during primer extension. Listed under sequences are the SacI cleavage products bearing the extended portion of the primer. The data represent the means and standard deviations of results from three independent primer extension and LC-MS/MS experiments.

Table 1

Name	Sequence	dT	<i>O</i> ² -EtdT	<i>N</i> ³ -EtdT	<i>O</i> ⁴ -EtdT
6mer unextended primer	5'-CATAGC-3'	28 ± 3	23 ± 3	26 ± 2	26 ± 1
13A +7	5'-CATAGC ACGCC AT-3'	46 ± 3	12 ± 3		2 ± 0
13G +7	5'-CATAGC GGGCC AT-3'		5 ± 1		4 ± 1
14AA (blunt end elongation) +8	5'-CATAGC ACGCC CATA-3'	26 ± 1	41 ± 0	42 ± 1	17 ± 2
14GA (blunt end elongation) +8	5'-CATAGC GGGCC CATA-3'		19 ± 1	32 ± 2	51 ± 2

A summary of the percentages of products formed from hPol κ -mediated replication of dT-, *O*²-EtdT-, *N*3-EtdT- and *O*⁴-EtdT-containing substrates as determined by LC-MS/MS. The bases highlighted in bold represent those incorporated during primer extension. Listed under sequences are the SacI cleavage products bearing the extended portion of the primer.

Table 2

Name	Sequence	dT	<i>O</i> ² -EtdT	<i>N</i> 3-EtdT	<i>O</i> ⁴ -EtdT
6mer unextended primer	5'-CATAGC-3'	4	59	76	96
7A +1	5'-CATAGCA-3'				2
7G +1	5'-CATAGCG-3'				1
9C +3	5'-CATAGCCCCG-3'			11	
9A +3	5'-CATAGCA CG -3'			1	
9G +3	5'-CATAGCG CG -3'			6	
10Del (at lesion site) +4	5'-CATAGCCGCC-3'			3	
10ADel (+1 from lesion site)+4	5'-CATAGCA GCC -3'			3	
11 Del (at lesion site) +5	5'-CATAGCCGCCA-3'		3		
12A +6	5'-CATAGCA CGCCA -3'	96	21		1
12G +6	5'-CATAGCG CGCCA -3'		17		1

A summary of the percentages of products formed from Kf⁻-mediated replication of dT-, O²-EtdT-, N3-EtdT- and O⁴-EtdT-containing substrates as determined by LC-MS/MS. The bases highlighted in bold represent those incorporated during primer extension. Listed under sequences are the SacI cleavage products bearing the extended portion of the primer.

Table 3

Name	Sequence	dT	O ² -EtdT	N3-EtdT	O ⁴ -EtdT
6mer unextended primer	5'-CATAGC-3'	16	9	2	22
7T +1	5'-CATAGCT-3'			2	
7A +1	5'-CATAGCA-3'	5	36	12	
7G +1	5'-CATAGCG-3'			8	
8A +2	5'-CATAGCAC-3'	5			
13A +7	5'-CATAGCACGCCAT-3'	10			
14TA (blunt end elongation) +8	5'-CATAGCTCGCCATA-3'		4	15	
14AA (blunt end elongation) +8	5'-CATAGCACGCCATA-3'	11	21	17	17
14GA (blunt end elongation) +8	5'-CATAGCGGCCATA-3'		4	21	35
15TAT (blunt end elongation) +9	5'-CATAGCTCGCCATAT-3'		3	7	
15AAT (blunt end elongation) +9	5'-CATAGCACGCCATAT-3'	53	20	5	8
15GAT (blunt end elongation) +9	5'-CATAGCGGCCATAT-3'		2	11	17