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Replication across Regioisomeric Ethylated Thymidine Lesions by Purified DNA Polymerases

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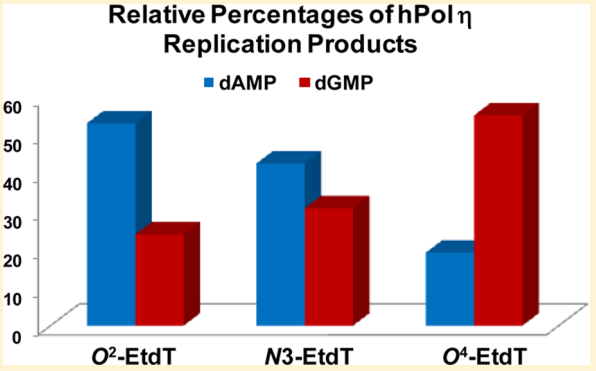
Supporting Information

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 in nonsmokers. 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, respectively) perturb DNA replication mediated by purified human DNA polymerases (hPols) 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 conducted by hPol κ and yPol ζ differed markedly among the three lesions. Consistent with its known ability to efficiently bypass 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 strongly 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 more than 80% of all lung cancers could be attributed to tobacco smoke exposure.[2] Cigarette smoke contains more than 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 the cytochrome P450 family of enzymes for the formation of reactive, electrophilic species that can react with DNA to yield covalent adducts.[4]

Treatment with ethylating agents has been shown to induce ethylation products on the 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 in the level of *N*7-ethylguanine (*N*7-EtG), though the identity and source of the direct-acting ethylating agent(s) in cigarette smoke have 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 the levels in hemoglobin from nonsmokers.[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 DNA[14] were significantly higher in smokers than in nonsmokers. Recently, Chen et al.[15] quantified O^2-, *N*3-, and O^4-ethylthymidine (O^2-, *N*3-, and O^4-EtdT, respectively) in human leukocyte DNA, where the levels of the three ethylated thymidine lesions were signicantly higher in smokers than in nonsmokers.

Some repair and replication studies have been conducted 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 h in rat tissue), both O^2-EtdT and O^4-EtdT are poorly repaired and thus are highly persistent in

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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

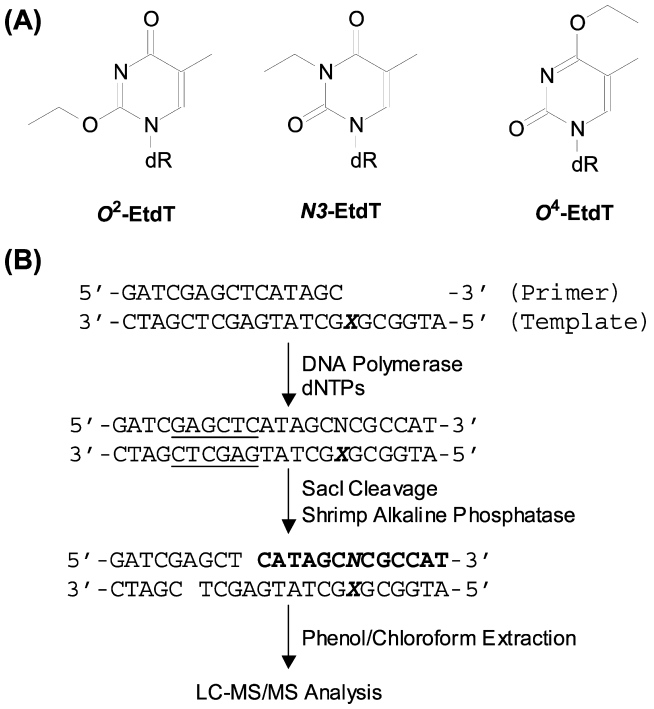


Figure 1. (A) Structures of the regioisomeric ethylated thymidines. "dR" denotes 2-deoxyribose. (B) Experimental procedures for monitoring primer extension products using SacI cleavage and LC−MS/MS. X represents dT, O^2-EtdT, *N*3-EtdT, or O^4-EtdT, and N represents the nucleotide incorporated opposite X during *in vitro* replication.

polymerases (hPols) *η*, *κ*, and *ι*, yeast DNA polymerase *ζ* (yPol *ζ*), and the exonuclease-free Klenow fragment of *Escherichia 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-^{32}P]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), and hPol *ι*[28] and hPol *η*[29] were kindly provided by R. Woodgate and W. Yang (National Institutes of Health, Bethesda, MD), respectively.

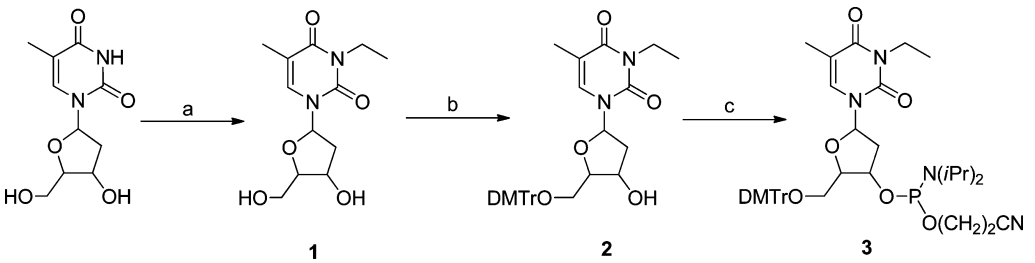
Chemical Synthesis of the Phosphoramidite Building Block of *N*3-EtdT. The procedures for the synthesis of the phosphoramidite building block of *N*3-EtdT were adapted from previously described procedures for the preparation of the *N*3-carboxyethylthymidine phosphoramidite.[30]

*Synthesis of N3-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): ^{1}H NMR [300 MHz, $CDCl_3$ (Figure S1 of the Supporting Information)]: 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 $C_{12}H_{19}N_2O_5$ $[M + H]^+$ 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 were added 4-dimethylaminopyridine (DMAP, 0.5% mol) and dimethoxytrityl chloride (DMTr-Cl, 1.2 equiv). The resulting solution was stirred at room temperature for 10 h. 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 the mobile phase to yield **2** as a white foam (yield 76%): ^{1}H NMR [300 MHz, $CDCl_3$ (Figure S2 of the Supporting Information)]: 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 $C_{33}H_{36}N_2O_7Na$ $[M + Na]^+$ 595.2420, found 595.2401.

*5′-O-(4,4′-Dimethoxytrityl)-N3-ethylthymidine-3′-O-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] [**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 equiv) followed by dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.2 equiv). The mixture was stirred at room temperature for 1 h under an 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 $NaHCO_3$ (4.0 mL) and brine (4.0 mL) and dried over anhydrous Na_2SO_4. The solvent was

Scheme 1. Chemical Synthesis of the *N*3-EtdT Phosphoramidite Building Block[α]



[α]Reagents and conditions: (a) bromoethane, K_2CO_3, MeOH, reflux, 6 h; (b) DMTr-Cl/DMAP, pyridine, room temperature, 10 h; (c) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIEA, CH_2Cl_2, room temperature, 2 h.

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evaporated under reduced pressure to yield **3** in a foam that was used directly for ODN synthesis. **3**: ^{31}P NMR [$CDCl_3$ (Figure S3 of the Supporting Information)]: δ 150.01, 149.47.

NMR. ^{1}H NMR and ^{31}P NMR spectra were acquired on a Varian Inova 300 NMR spectrometer (Varian Inc., Palo Alto, CA). Resonance assignments for *N*3-EtdT were made on the basis of a two-dimensional ^{1}H−^{13}C heteronuclear multibond correlation (HMBC) experiment (Figure S4 of the Supporting Information). The HMBC spectrum was acquired on a Varian Unity spectrometer operating at 500 MHz at 25 °C using sweep widths of 3723.0 and 30 177.3 Hz for ^{1}H and ^{13}C, 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′-ATGG-CGXGCTAT-3′ (X represents O^2-, *N*3-, or O^4-EtdT) were synthesized on a Beckman (Fullerton, CA) Oligo 1000S DNA synthesizer at a 1 μmol 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, *N*3-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 5 days, whereas the *N*3-EtdT-containing ODN was deprotected with concentrated ammonium hydroxide at 55 °C overnight. The ODNs were purified by HPLC (a representative HPLC trace for the purification of the *N*3-EtdT-containing ODN is shown in Figure S5 of the Supporting Information) and sequences verified by ESI-MS and MS/MS analyses (representative results for the O^4-EtdT-containing ODN are shown in Figure S6 of the Supporting Information).

HPLC. HPLC purification of synthetic ODNs was performed on an Agilent 1100 HPLC system with an Aeris XB-C18 column [4.6 mm × 150 mm, 3.6 μm in particle size and 200 Å in pore size (Phenomenex Inc., Torrance, CA)]. 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 to 25% B over 5 min, 25 to 50% B over 40 min, 50 to 95% B over 10 min, and 95% B over 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 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. A Zorbax SB-C18 column [0.5 mm × 250 mm, 5 μm particle size (Agilent Technologies, Santa Clara, CA)] was used for the online separation; the flow rate was 8 μL/min, and the gradient included 5 to 20% methanol over 5 min followed by 20 to 50% methanol over 35 min in 400 mM HFIP (pH adjusted to 7.0 with triethylamine).

Preparation of the Lesion-Carrying 22-mer ODNs. The 22-mer substrates, d(ATGGCGXGCTATGAGCTCGATC) (*X* represents dT, O^2-EtdT, *N*3-EtdT, or O^4-EtdT), were obtained by ligating the lesion-containing 12-mer described above 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 22-mer 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 22-mer template, d(ATGGCG-XGCTATGAGCTCGATC) (*X* represents dT, O^2-EtdT, *N*3-EtdT, or O^4-EtdT, at 20 nM), was annealed with a 5′-^{32}P-labeled 15-mer primer, d(GATCGAGCTCATAGC) (10 nM). For Kf$^-$, the reactions were conducted in a buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 2 mM EDTA, 1.6 mM β-mercaptoethanol, and 5 μg/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 $MgCl_2$, 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 version 5.2 (GE Healthcare Life Sciences, Piscataway, NJ).

TLS cooperativity experiments were performed following previously described methods.[34] Briefly, the primer−template complexes described above were incubated with hPol ι and all four dNTPs (100 μM) 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 $MgCl_2$, 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 of the Supporting Information).

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 h. 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 were redissolved in a buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, and 1 mM DTT. SacI (40 units) and shrimp alkaline phosphatase (20 units) were subsequently added to the mixture and incubated at 37 °C for 4 h. The proteins in the mixture were again removed by chloroform extraction, and the aqueous layer was dried. The dried residues were reconstituted in 30 μL of H_2O, 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 via MS/MS. The fragment ions observed via MS/MS were manually assigned and the sequences of the ODNs determined (representative MS/MS data for the identification of products from hPol η-mediated reaction of the O^2-EtdT-containing substrate are shown in Figure S8 of the Supporting Information). Following identification of the ODN products, the mass spectrometer was set up for specifically monitoring 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) [9-mer 5′ P, which was the 5′ portion of the primer liberated from SacI cleavage (Figure 1B and Figure S9 of the Supporting Information)]. Areas were determined for the peaks found in the selected ion chromatograms (SICs) by monitoring the formation of one to three unique and abundant fragment ions for each ODN. The peak areas of individual ODNs were then normalized to that of the 9-mer 5′ P and plotted against the molar ratios of these ODNs over 9-mer 5′ P to give the calibration curves (Figure S9 of the Supporting Information). The corresponding normalized ratios for the replication samples were also determined, from which we measured the molar ratios for each extended product over 9-mer 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.

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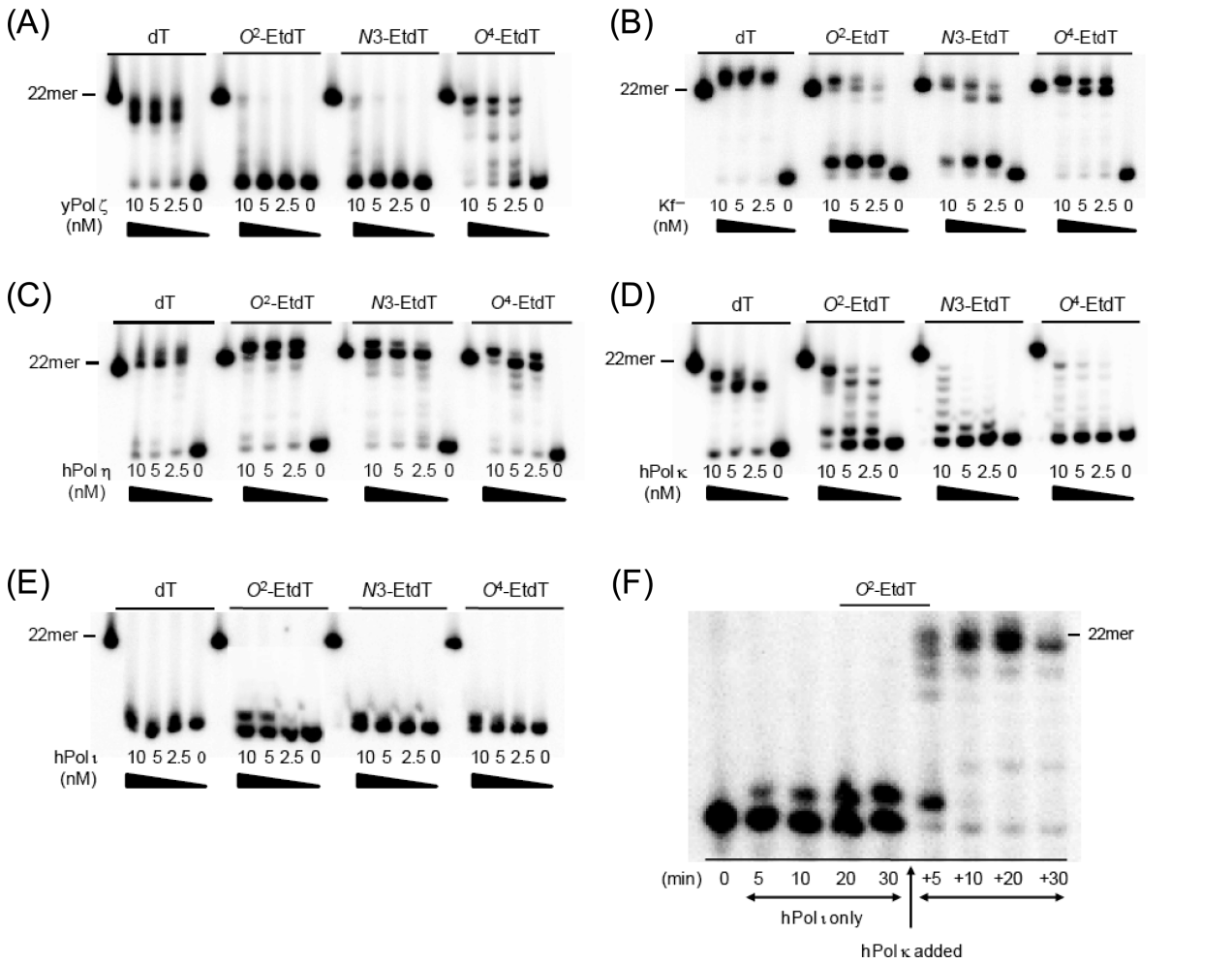

Figure 2. Primer extension assays for O^2-EtdT-, *N*3-EtdT-, and O^4-EtdT-bearing substrates and the control undamaged substrate with yPol ζ (A), Kf^- (B), hPol η (C), hPol κ (D), and hPol ι (E). (F) Cooperativity of human DNA polymerases ι and κ in the bypass of the O^2-EtdT lesion. The primer−template 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 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′-^{32}P-labeled d(GATCGAGCTCATAGC) was used as the primer (see Figure 1B). 22-mer refers to a standard ODN for the fully extended primer strand.

RESULTS

The major objective of this study was to assess systematically how the three site-specifically incorporated, regioisomeric O^2-, *N*3-, and O^4-EtdT lesions 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 the *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 (Figure 2A). yPol ζ, on the other hand, was unable to insert any nucleotide opposite the two lesions (Figure 2B). 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] As 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 conducted 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 (22-mer) products for the templates containing O^2-EtdT, *N*3-EtdT, and O^4-EtdT (Figure 2F and Figure S7 of the Supporting Information), 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

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Table 1. Summary of the Percentages of Products Formed from hPol η-Mediated Replication of dT-, O^2-EtdT-, N3-EtdT-, and O^4-EtdT-Containing Substrates As Determined by LC−MS/MS[a]

name	sequence	dT	O^2-EtdT	N3-EtdT	O^4-EtdT
6-mer unextended primer	5′-CATAGC-3′	28 ± 3	23 ± 3	26 ± 2	26 ± 1
13A + 7	5′-CATAGC**ACGCCAT**-3′	46 ± 3	12 ± 3		2 ± 0
13G + 7	5′-CATAGC**GCGCCAT**-3′		5 ± 1		4 ± 1
14AA (blunt end elongation) + 8	5′-CATAGC**ACGCCATA**-3′	26 ± 1	41 ± 0	42 ± 1	17 ± 2
14GA (blunt end elongation) + 8	5′-CATAGC**GCGCCATA**-3′		19 ± 1	32 ± 2	51 ± 2

[a]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.

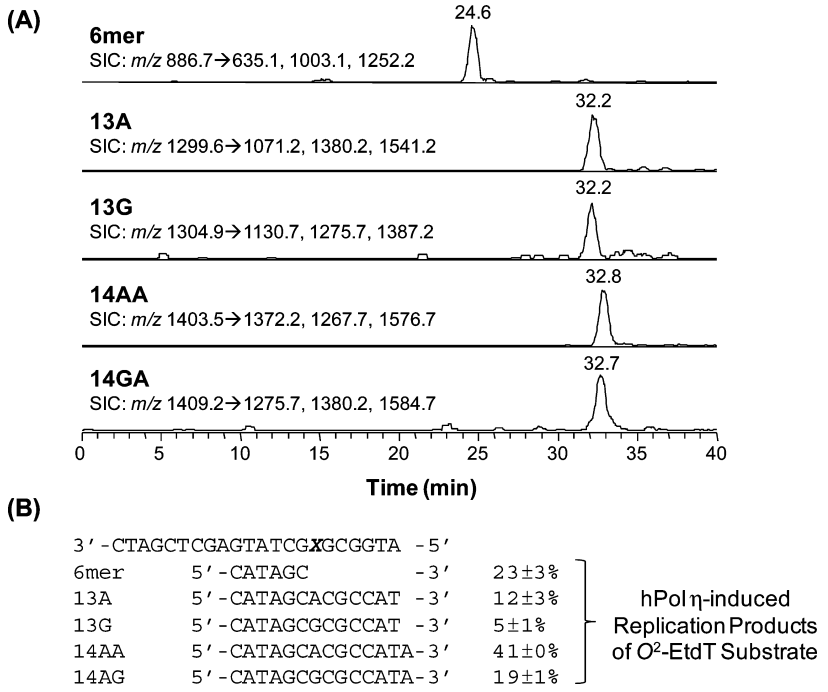

Figure 3. Selected ion chromatograms obtained from the LC−MS and MS/MS analysis of the hPol η-induced replication products for the O^2-EtdT-bearing substrate. The replication products were treated with restriction enzyme SacI and shrimp alkaline phosphatase, and 10 pmol of the replication mixture was injected for the analysis.

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 that, 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 (Figure 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 Materials and Methods).

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 (6-mer) represent 28, 23, 26, and 26% of the total replication products for substrates containing dT, O^2-EtdT, N3-EtdT, and O^4-EtdT, respectively (Table 1). This finding parallels the aforementioned results of PAGE analysis showing that the three EtdT lesions are not strong blocks to DNA replication catalyzed by hPol η (Figure 2C). In addition, we observed a significant amount of blunt-end elongation products where a dAMP was added to the 3′ terminus of the fully extended primer (Table 1, Figure 3, and Figures S10 and S11 of the Supporting Information).

As expected, no mutagenic product could be detected 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 N3-EtdT-containing substrate, and 19 and 55% for the O^4-EtdT-carrying substrate, respectively (Table 1, Figure 3, and Figures S10 and S11 of the Supporting Information). 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

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Table 2. Summary of the Percentages of Products Formed from hPol κ-Mediated Replication of dT-, O^2-EtdT-, N3-EtdT-, and O^4-EtdT-Containing Substrates As Determined by LC−MS/MS[a]

name	sequence	dT	O^2-EtdT	N3-EtdT	O^4-EtdT
6-mer unextended primer	5′-CATAGC-3′	4	59	76	96
7A + 1	5′-CATAGC**A**-3′				2
7G + 1	5′-CATAGC**G**-3′				1
9C + 3	5′-CATAGC**CCG**-3′			11	
9A + 3	5′-CATAGC**ACG**-3′			1	
9G + 3	5′-CATAGC**GCG**-3′			6	
10Del (at lesion site) + 4	5′-CATAGC**CGCC**-3′			3	
10ADel (+1 from lesion site) + 4	5′-CATAGC**AGCC**-3′			3	
11 Del (at lesion site) + 5	5′-CATAGC**CGCCA**-3′		3		
12A + 6	5′-CATAGC**ACGCCA**-3′	96	21		1
12G + 6	5′-CATAGC**GCGCCA**-3′		17		1

[a]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. Summary of the Percentages of Products Formed from Kf⁻-Mediated Replication of dT-, O^2-EtdT-, N3-EtdT-, and O^4-EtdT-Containing Substrates As Determined by LC−MS/MS[a]

name	sequence	dT	O^2-EtdT	N3-EtdT	O^4-EtdT
6-mer unextended primer	5′-CATAGC-3′	16	9	2	22
7T + 1	5′-CATAGC**T**-3′			2	
7A + 1	5′-CATAGC**A**-3′	5	36	12	
7G + 1	5′-CATAGC**G**-3′			8	
8A + 2	5′-CATAGC**AC**-3′	5			
13A + 7	5′-CATAGC**ACGCCAT**-3′	10			
14TA (blunt end elongation) + 8	5′-CATAGC**TCGCCATA**-3′		4	15	
14AA (blunt end elongation) + 8	5′-CATAGC**ACGCCATA**-3′	11	21	17	17
14GA (blunt end elongation) + 8	5′-CATAGC**GCGCCATA**-3′		4	21	35
15TAT (blunt end elongation) + 9	5′-CATAGC**TCGCCATAT**-3′		3	7	
15AAT (blunt end elongation) + 9	5′-CATAGC**ACGCCATAT**-3′	53	20	5	8
15GAT (blunt end elongation) + 9	5′-CATAGC**GCGCCATAT**-3′		2	11	17

[a]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.

substrates housing the thymidine derivatives (Table 1, Figure 3, and Figures S10−S12 of the Supporting Information). 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 EtdT 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 or N3-EtdT. In addition, we found a relatively low frequency [3% (Table 2)] of the −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 to 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 of the Supporting Information). While the insertion of dGMP (64%) was substantially favored over that 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 of the Supporting Information).

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 of the Supporting Information).

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). As seen for 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).

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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 the O^2-EtdT-bearing substrate, while these percentages were 22, 32, and 22%, respectively, for the corresponding *N*3-EtdT substrate (Table 3). By contrast, we did not observe the insertion of dTMP 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 the 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-, *N*3-EtdT-, and O^4-EtdT-bearing templates were degraded to the corresponding unmodified dT-containing substrate (data not shown).

DISCUSSION

Cigarettes and their 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 the levels of nonsmokers.[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 nonsmokers.[15] Despite a substantial amount of work conducted to assess the formation of these DNA lesions, no systematic study has been conducted 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 the O^2-EtdT substrate for hPol κ and the 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 Table S1 of the Supporting Information). In addition, these two lesions in template DNA directed hPol ι or Kf$^-$ to misincorporate dTMP opposite the lesions at appreciable frequencies. The exposure of rats to ENU, a known ethylating agent, was shown to give rise to a specific AT → TA point mutation that 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* conducted 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 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 conduct efficient and error-free nucleotide incorporation opposite the minor groove N^2-dG lesions.[38−43] The poor fidelity and, relative to that of 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.

ASSOCIATED CONTENT

Supporting Information

NMR spectra of synthetic compounds, HPLC trace, and LC−MS and MS/MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

O^2-EtdT, O^2-ethylthymidine; *N*3-EtdT, *N*3-ethylthymidine; O^4-EtdT, O^4-ethylthymidine; *N*7-EtG, *N*7-ethylguanine; ODNs, oligodeoxyribonucleotides; EMS, ethyl methanesulfonate; ENU, *N*-ethyl-*N*-nitrosourea; Kf$^-$, Klenow fragment; hPol, human DNA polymerase; yPolζ, yeast DNA polymerase ζ; DIEA, *N*,*N*-diisopropylethylamine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; DMAP, 4-(dimethylamino)pyridine; DMTr-Cl, dimethoxytrityl chloride; HMBC, heteronuclear multibond correlation.

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