Translesion Synthesis Past Equine Estrogen-Derived 2′-Deoxyadenosine DNA Adducts by Human DNA Polymerases η and κ[†]

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ABSTRACT: Hormone replacement therapy (HRT) increases the risk of developing breast, ovarian, and endometrial cancers. Equilin and equilenin are the major components of the widely prescribed drug used for HRT. 4-Hydroxyequilenin (4-OHEN), a major metabolite of equilin and equilenin, promotes 4-OHEN-modified dC, dA, and dG DNA adducts. These DNA adducts were detected in breast tumor and adjacent normal tissues of several patients receiving HRT. We have recently found that the 4-OHEN-dC DNA adduct is a highly miscoding lesion generating C → T transitions and C → G transversions. To explore the mutagenic potential of another major 4-OHEN-dA adduct, site-specifically modified oligodeoxynucleotides containing a single diastereoisomer of 4-OHEN-dA (Pk-1, Pk-2, and Pk-3) were prepared by a postsynthetic method and used as DNA templates for primer extension reactions catalyzed by human DNA polymerase (pol) η and κ that are highly expressed in the reproductive organs. Primer extension catalyzed by pol η or pol κ occurred rapidly on the unmodified template to form fully extended products. With the major 4-OHEN-dA-modified templates (Pk-2 and Pk-3), primer extension was retarded prior to the lesion and opposite the lesion; a fraction of the primers was extended past the lesion. Steady-state kinetic studies with pol η and pol κ indicated that dTMP, the correct base, was preferentially incorporated opposite the 4-OHEN-dA lesion. In addition, pol η and pol κ bypassed the lesion by incorporating dAMP and dCMP, respectively, opposite the lesion and extended past the lesion. The relative bypass frequency past the 4-OHEN-dA lesion with pol η was at least 2 orders of magnitude higher than that observed with pol κ. The bypass frequency past Pk-2 was more efficient than that past Pk-3. Thus, 4-OHEN-dA is a miscoding lesion generating A → T transversions and A → G transitions. The miscoding frequency and specificity of 4-OHEN-dA varied depending on the stereoisomer of the 4-OHEN-dA adduct and DNA polymerase used.

Hormone replacement therapy (HRT)[1] is widely used among postmenopausal women to alleviate menopausal symptoms and osteoporosis (*1*). Premarin (Wyeth-Ayerst), composed of several estrogens including approximately 50% equine estrogens (equilin and equilenin), is used for this purpose (reviewed in ref *2*). However, HRT increases significantly the risk of developing breast (*3*), ovarian (*4*), and endometrial cancers (*5*); the longer duration of HRT showed the higher cancer risks (*4*, *6*). The occurrence of endometrial hyperplasia, a precancerous stage, was frequently observed in postmenopausal women receiving HRT (*7*). Treatment of hamsters for 9 months with equine estrogens resulted in 100% tumor incidence and many tumor foci in kidneys (*8*). Equine estrogen-derived DNA adducts were detected in breast tumor and adjacent normal tissues of several patients receiving HRT and in paraffin-embedded breast tumor tissues (*9*), indicating that genotoxicity of exogenous estrogens is involved in the initiation of breast, ovarian, and endometrial cancers in humans.

Equilin and equilenin are metabolized to 4-hydroxyl and 2-hydroxyl forms (*2*). 4-Hydroxyequilenin (4-OHEN) is rapidly autoxidized to an *o*-quinone which in turn readily reacts with DNA, resulting in the formation of unique dC, dA, and dG adducts with four possible stereoisomers for each base adduct (*2*, *10*) (Figure 1). 4-Hydroxyequilin (4-OHEQ) is also autoxidized to an *o*-quinone which isomerizes to 4-OHEN *o*-quinone; therefore, 4-OHEQ produces DNA adducts identical to those observed with 4-OHEN (*11*). The absolute configuration of each stereoisomer has not been determined successfully even after using several NMR techniques (*2*, *10*). Using ^{32}P-postlabeling/polyacrylamide gel electrophoresis (PAGE) analysis (*12*, *13*), we have observed that both 4-OHEQ and 4-OHEN are highly reactive with DNA; 4-OHEN-dC, 4-OHEN-dA, and 4-OHEN-dG adducts were detected. Oxidative DNA damage is also generated by reactive oxygen species through the redox cycling between 4-OHEN *o*-quinone and their semiquinone radicals (*14*). When 4-OHEN was incubated with DNA in vitro or exposed

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[1] Abbreviations: HRT, hormone replacement therapy; 4-OHEQ, 4-hydroxyequilin; 4-OHEN, 4-hydroxyequilenin; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; pol η, human DNA polymerase η; pol κ, human DNA polymerase κ; pol $\kappa\Delta$C, a truncated form of pol κ; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; dNTP, deoxynucleoside triphosphate; F_{ins}, frequency of dNTP insertion; F_{ext}, frequency of dNTP extension.

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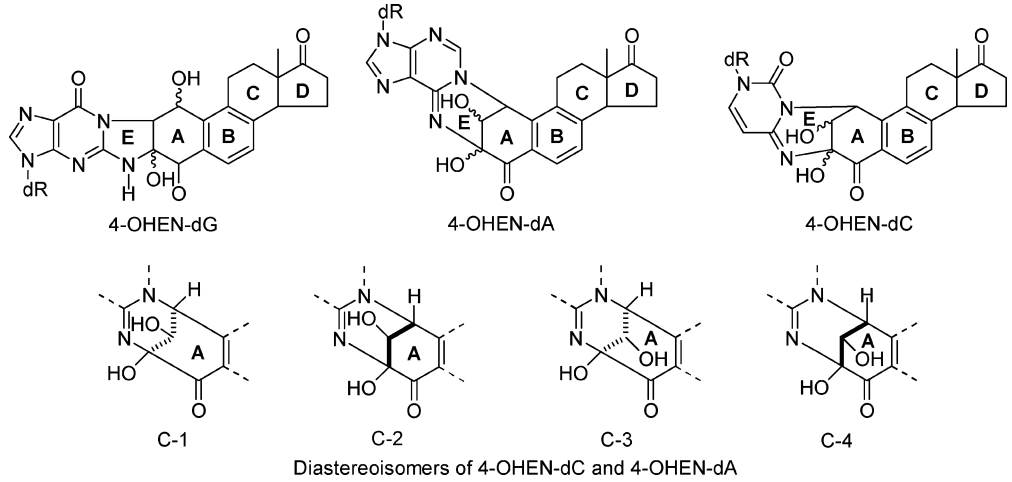


FIGURE 1: Structures of diastereoisomers of 4-OHEN-derived DNA adducts.

to cultured breast cancer cells, increased formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) was observed in the DNA (*14*−*17*). Particularly, when 4-OHEN was injected directly into the mammary fat pads of rats, increased amounts of 8-oxodG as well as 4-OHEN-dA and 4-OHEN-dG adducts were detected in the mammary tissue using liquid chromatography/tandem mass spectroscopy (*18*). These equine estrogen-induced DNA adducts may contribute to initiation of reproductive cancers.

Newly found human DNA polymerases including pol η and pol κ are associated with translesion synthesis past DNA adducts (reviewed in refs *19* and *20*). Pol η and pol κ are highly expressed in human ovary and uterus where steroid hormones, including estrogen, were produced (*21*−*24*). Using these pols, we have found that 4-OHEN-dC adducts are a highly miscoding lesion generating C → T transitions and C → G transversions (*25*). Therefore, the DNA polymerases may miscode the lesion during DNA replication, leading to an increased risk of developing breast, ovary, and endometrial cancers. Structural and thermodynamic studies indicated that 4-OHEN-dC adducts cause distortions in duplex DNA but can reside in the B-DNA major groove with *syn*-damaged cytosine or minor groove with *anti*-damaged cytosine (*26*). The unique structural properties of 4-OHEN-dC adducts may affect the mutagenic events.

To explore the miscoding specificities of other major 4-OHEN-dA adducts, site-specifically modified oligodeoxynucleotides containing a single diastereoisomer of 4-OHEN-dA were prepared by a postsynthetic method and used as DNA templates for primer extension reactions catalyzed by human pol η or pol κ. Steady-state kinetic studies were also performed to measure the relative bypass frequency past the 4-OHEN-dA lesions.

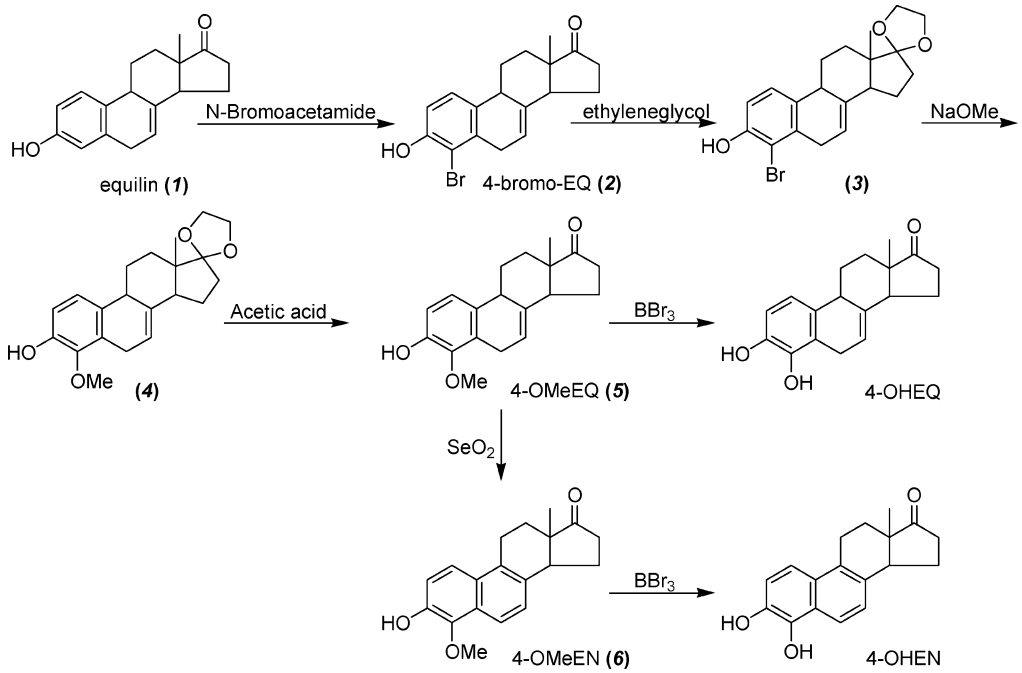
MATERIALS AND METHODS

General. [γ-^{32}P]ATP (specific activity >6000 Ci/mmol) was obtained from Amersham Corp. dNTPs were from Pharmacia; T4 polynucleotide kinase, *Eco*RI restriction endonuclease (100 units/μL), and T4 DNA ligase (400 units/μL) were purchased from New England BioLabs. HPLC-grade acetonitrile and triethylamine were purchased from Fisher Chemical Co. Micrococcal nuclease and spleen phoshodiesterase were obtained from Worthington Biochemical Corp. Alkaline phosphatase was purchased from Sigma Chemical Corp.

Synthesis of 4-OHEN. 4-OHEN (and 4-OHEQ) was prepared following the established methods (*10*, *27*) with some modification (*28*). Briefly, equilin was brominated to 4-bromoequilin (**2**) using *N*-bromoacetamide. The 17-keto group was protected using ethylene glycol to give **3**. The 4-methoxy derivative (**4**) is obtained by nucleophilic displacement of the bromine with methoxide ion in the presence of copper(II) chloride and 15-crown-5-ether. Hydrolysis of the protected 17-keto group (**4**) gave 4-OMeEQ (**5**). Demethylation of **5** with boron tribromide gave 4-OHEQ (Scheme 1). To prepare 4-OMeEN (**6**), compound **5** (290 mg, 1 mmol) was dissolved in *tert*-butyl alcohol (18 mL), and then pyridine (0.09 mL) and selenium dioxide (128 mg, 1.2 mmol) were added. The mixture was refluxed for 1.5 h and then cooled to room temperature. The solvent was evaporated under vacuum and the residue purified by silica gel column chromatography using hexane−ether (2:3) as eluent to give **6** (170 mg, 58%). The ^{1}H NMR was identical to that described previously (*28*). To prepare 4-OHEN, compound **6** (150 mg, 0.5 mmol) was dissolved in anhydrous dichloroethane (27 mL), and the solution was cooled to −15 °C. BBr_3 (5.5 mL of 1 M solution in dichloromethane) was added dropwise and stirred at the same temperature for 8 h. The reaction was quenched with water, and the solution was extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with 10% sodium bicarbonate solution and water and dried over sodium sulfate. After filtration, the solvent was evaporated to give 4-OHEN in quantitative yield from **6** (140 mg). ^{1}H NMR for 4-OHEN (acetone-d_6): 0.76 (s, 3H), 1.84 (m, 1H), 2.01 (m, 1H), 2.18 (m, 1H), 2.40 (m, 1H), 2.70 (m, 2H), 3.15 (m, 1H), 3.28 (m, 2H), 7.17 (d, J = 9 Hz, 1H), 7.22 (d, J = 8.7 Hz, 1H), 7.37 (d, J = 9 Hz, 1H), 8.01 (d, J = 8.7 Hz, 1H); mass FB positive ion m/z = 283 (MH^+). ^{1}H NMR for 4-OHEQ (acetone-d_6): 0.76 (s, 3H), 1.50 (m, 1H), 1.64 (m, 1H), 1.83 (m, 1H), 2.05 (m, 2H), 2.20 (m, 1H), 2.27 (m, 1H), 2.49 (m, 2H), 3.15 (m, 1H), 3.38 (m, 2H), 5.65 (m, 1H), 6.68 (d, J = 9.0 Hz, 1H), 6.76 (d, J = 9.0 Hz, 1H); mass FB positive ion m/z = 285 (MH^+).

Synthesis and Purification of 4-OHEN-dA-Modified Oligodeoxynucleotides. Unmodified DNA templates (5′TTTGTATTTT and 5′TTTGTATTTTCTTCTTTCCTCTCCC),

Scheme 1: Synthetic Protocol of 4-OHEN and 4-OHEQ



primers ($^{5'}$GAAAGAAGAA, $^{5'}$GAAAGAAGAAAA, and $^{5'}$GAAAGAAGAAAAN, where N is C, A, G, or T), and standard markers were prepared by solid-state synthesis on an automated DNA synthesizer. 4-OHEN-dA-modified 25-mers were prepared by a similar procedure described previously (*25*). Briefly, 200 μg of 10-mer oligomer $^{5'}$TTTGTATTTT was incubated with 1 mg of 4-OHEN at 37 °C for 16 h in 1 mL of 25 mM potassium phosphate buffer, pH 7.4. The reaction mixture was centrifuged, and the supernatant was evaporated to dryness and subjected to HPLC by dissolving in 250 μL of distilled water. The 10-mer oligomers containing a single 4-OHEN-dA adduct ($^{5'}$TTTGTA^{4-OHEN}TTTT) were isolated and purified by a reverse-phase μBondapak C_{18} column (0.39 × 30 cm; Waters), using a linear gradient composed of 0.05 M triethylammonium acetate (pH 7.0) containing 10−30% acetonitrile with an elution time of 60 min at a flow rate of 1.0 mL/min. Five micrograms of 15-mer ($^{5'}$CTTCTTTCCTCTCCC) was phosphorylated at the 5′-terminus for 40 min at 37 °C using 7.5 μL of T4 polynucleotide kinase (10 units/μL) and 3 μL of 10 mM ATP, mixed with a 4-OHEN-dA-modified 10-mer (3 μg, $^{5'}$TTTGTA^{4-OHEN}TTTT) and a complementary 22-mer oligomer (10 μg, $^{5'}$AGAGGAAAGAAGAAAATACAAA), and subsequently incubated at 4 °C overnight in 100 μL of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 2.5 μg of BSA, and 3 μL of T4 DNA ligase (400 units/μL). The 25-mer product was isolated using the same HPLC system as described above. The unmodified and modified 25-mers were purified by HPLC and gel electrophoresis. HPLC was performed using a Waters 515 HPLC pump, 996 photodiode array detector, and pump control module.

MALDI-TOF Analysis. Unmodified and modified oligodeoxynucleotides were run on a Voyager-DE STR MALDI-TOF (matrix-assisted laser desorption ionization−time of flight) mass spectrometer system (Applied Biosystems, Framingham, MA) operated in the linear mode. Samples were dissolved in acetonitrile/water (1:1) containing hydroxypicolinic acid (5 mg/mL) and ammonium citrate in an 8:1 ratio and dried on the sample plate. A nitrogen laser operating at 337 nm and a 3 ns pulse rate was employed. The accelerating voltage was set to 20 kV, and a delay of 450 ns was used to accelerate ions into the flight tube of the mass spectrometer. The mass scale (*m*/*z* 1000−10000) was calibrated in the positive ion mode with a mixture of standard oligodeoxynucleotides, and approximately 100 laser shots were used to produce each spectrum.

Enzymatic Hydrolysis of Oligodeoxynucleotides. Oligodeoxynucleotides containing a single diastereoisomer of 4-OHEN-dA (1.5 μg) were digested to deoxynucleoside 3′-monophosphates at 37 °C overnight in 100 μL of 17 mM sodium succinate (pH 7.0) containing 8 mM $CaCl_2$, using micrococcal nuclease (30 units) and spleen phoshodiesterase (0.15 units), and were further incubated at 37 °C for 2 h with alkaline phosphatase (3 units) in 250 μL of 8 mM Tris-HCl (pH 8.5) containing 0.8 mM zinc sulfate. Ethanol (1 mL) was added to the reaction mixture, and the resultant mixture was centrifuged to precipitate digestion enzymes at 12000*g* for 20 min. The supernatant was evaporated to dryness and analyzed by HPLC with a μBondapak C_{18} column (0.78 × 30 cm; Waters), using a linear gradient composed of distilled water containing 10% methanol over 2 min, 10−37% methanol over 5 min, and 37−90% methanol over 19 min at a flow rate of 2.0 mL/min. Diastereoisomers (fr-1, fr-2, fr-3, and fr-4) of monomeric 4-OHEN-dA were prepared as described previously (*10*) and used as standards.

Stability of 4-OHEN-dA-Modified Oligodeoxynucleotide. Each isoform of the 4-OHEN-dA-modified 10-mer (10 pmol) was labeled with ^{32}P and incubated in 100 μL of 50 mM Tris-HCl (pH 7.4) at 37 °C for 24 h. During the incubation, a fraction (1 pmol) was collected at 1, 3, 5, 8, and 24 h from the reaction mixture and stored at −70 °C until analysis. The samples were subjected to 20% PAGE (35 × 42 × 0.04 cm). The radioactivity of extended products was measured by a STORM PhosphorImager (Molecular Dynamics).

Primer Extension Reactions Catalyzed by DNA Polymerases. Human pol η and a truncated form of pol κ (pol $\kappa\Delta$C) were prepared as described previously (*29*, *30*). Pol $\kappa\Delta$C, a catalytically active fragment composed of 560 amino acids, used in this study lacks motifs VIIa and VIIb that denote zinc clusters from intact DINB1 protein (870 amino acids) (*30*). Although pol $\kappa\Delta$C has a lower processivity than full-length pol κ, the miscoding rates on undamaged DNA by pol $\kappa\Delta$C and pol κ were similar (*31*). The ^{32}P-labeled 10-mer primer (100 fmol, 5′GAAAGAAGAA) was annealed with an excess molar amount of the 4-OHEN-dA modified or unmodified 25-mer template (150 fmol) to form a primer-template complex by heating at 95 °C for 5 min and slowly cooling. The replication mixture contained the primer-template complex (10 nM), all four dNTPs (100 μM each), and various concentrations of pol η or pol $\kappa\Delta$C and was incubated at 25 °C for 30 min in a buffer (10 μL). The reaction buffer for pol η contains 40 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM DTT, 250 g/mL BSA, 60 mM KCl, and 2.5% glycerol. A similar reaction buffer was used for pol $\kappa\Delta$C, using 5 mM $MgCl_2$ instead of 1 mM $MgCl_2$. The reaction was stopped by addition of formamide dye containing 25 mM EDTA. The samples were subjected to 20% denaturing PAGE. Quantification of extended products was measured using a STORM PhosphorImager and ImageQuaNT software (Molecular Dynamics).

Steady-State Kinetic Analysis. Kinetic parameters associated with nucleotide insertion opposite the 4-OHEN-dA lesion and chain extension from the 3′ primer terminus were determined at 25 °C for 2 min, using either pol η (1−500 fmol) or pol $\kappa\Delta$C (1−500 fmol). For the insertion kinetics, the reaction mixture containing a single dNTP (0−500 μM) as substrate was incubated in a TE reaction buffer (10 μL), using the 25-mer template (150 fmol, 5′TTTGTXTTTTCTTCTTTCCTCTCCC, where X is dA or 4-OHEN-dA) primed with the ^{32}P-labeled 12-mer (100 fmol, 5′GAAAGAAGAAAA). The analysis of chain extension kinetics was carried out using dATP (0−500 μM) as substrate and the ^{32}P-labeled 13-mer (100 fmol, 5′GAAAGAAGAAAAN, where N is C, A, G, or T) instead of the dNTP and the ^{32}P-labeled 12-mer primer, respectively. Reactions were stopped by addition of 5 μL of formamide dye and the samples subjected to 20% denatureing PAGE. The extent of incorporation was quantitated with the PhosphorImager and ImageQuaNT software. The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were calculated with Hanes−Woolf plots. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dT·dA base pair according to the equation $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair=dT\cdot dA)}$ (*32*, *33*).

RESULTS

Synthesis of 4-OHEN. The earlier method (*34*), in which 4-OHEN was prepared in a single step from equilin (EQ) using Fremy's salt, gave low yield of the product. Therefore, 4-OMeEN was obtained by oxidation of 4-OMeEQ with selenium dioxide (Scheme 1). 4-OHEN was prepared by the demethylation of 4-OMeEN using boron tribromide (*28*). The modified procedure gave a good yield of 4-OHEN. The proton NMR and mass data of 4-OHEN and 4-OHEQ were identical to those reported earlier by Bolton et al. (*11*). These procedures provide enough 4-OHEN to be used for preparation of site-specifically modified oligomers.

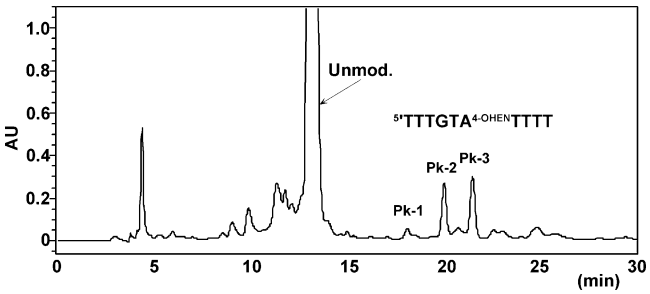


FIGURE 2: HPLC isolation of oligodeoxynucleotides containing a single 4-OHEN-dA adduct. Two hundred micrograms of 5′TTTGTATTTT was reacted at 37 °C for 16 h with 4-OHEN (1 mg/50 μL of acetone) in 1 mL of 25 mM potassium phosphate buffer, pH 7.4. After centrifugation, the supernatant was evaporated in dryness and subjected to HPLC. The 10-mer oligomers containing an isoform of the 4-OHEN-dA adduct (5′TTTGTA^{4-OHEN}TTTT) were isolated on a reverse-phase μBondapak C_{18} column (0.39 × 30 cm; Waters) using a linear gradient composed of 0.05 M triethylammonium acetate (pH 7.0) containing 10−30% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min.

Preparation of Diastereoisomer 4-OHEN-dA-Modified Oligodeoxynucleotides. A 10-mer oligodeoxynucleotide (5′TTTGTATTTT) containing a single dA was reacted with 4-OHEN under neutral pH conditions. One minor (t_R = 18.2 min for Pk-1) and two major (t_R = 20.2 min for Pk-2 and t_R = 21.7 min for Pk-3) products representing 10-mers containing a single 4-OHEN-dA were isolated by HPLC (Figure 2). The molecular weight of three products (Pk-1, Pk-2, and Pk-3) isolated by HPLC was measured using a Voyager-DE STR MALDI-TOF mass spectrometer system. For example, the parent ions of the 4-OHEN-dA-modified 10-mers (Pk-2 and Pk-3) exhibited at *m*/*z* 3309, identifying the molecular mass as 3308 Da (Figure 3). Since potassium forms an adduct with the deprotonated phosphate, peaks representing $(M + K)^+$ = *m*/*z* 3347 were also detected. Thus, the three oligodeoxynucleotides are identified as 10-mers containing a single diastereoisomer of 4-OHEN-dA. To determine which diastereoisomer is incorporated into the modified oligomers, the Pk-2 and Pk-3 of 4-OHEN-dA-modified oligodeoxynucleotides (5′TTTGTA^{4-OHEN}TTTT, 1.5 μg) were enzymatically digested. The resulting deoxynucleosides were analyzed by HPLC and compared with standards (Figure 4). The Pk-2 and Pk-3 contain fr-4 and fr-3 diastereoisomers of 4-OHEN-dA, respectively. The ratio of dG:dT was 1:8 that is consistent with constituents of the oligomer.

Stability of 4-OHEN-dA-Modified Oligodeoxynucleotides. Shen et al. reported that the 4-OHEN-dA adducts are unstable and readily lose the sugar leaving apurinic sites on the DNA (*10*). Using 4-OHEN-dA-modified 25-mers, the stability was confirmed under the condition used in this study. The migration of 4-OHEN-dA-modified oligomers on the gel was slower than that of the corresponding unmodified oligomers (for example, Pk-3 in Figure 5). If the 4-OHEN-dA adduct is depurinated and/or degraded, the shorter fragment could be observed on the gel. However, no significant degradation was detected during 24 h incubation. This indicates that 4-OHEN-dA-modified oligomers are stable under the experimental conditions used in this work.

Primer Extension Reactions Catalyzed by Pol η or Pol $\kappa\Delta$C. Since Pk-2 and Pk-3 of 4-OHEN-dA-modified oligo-

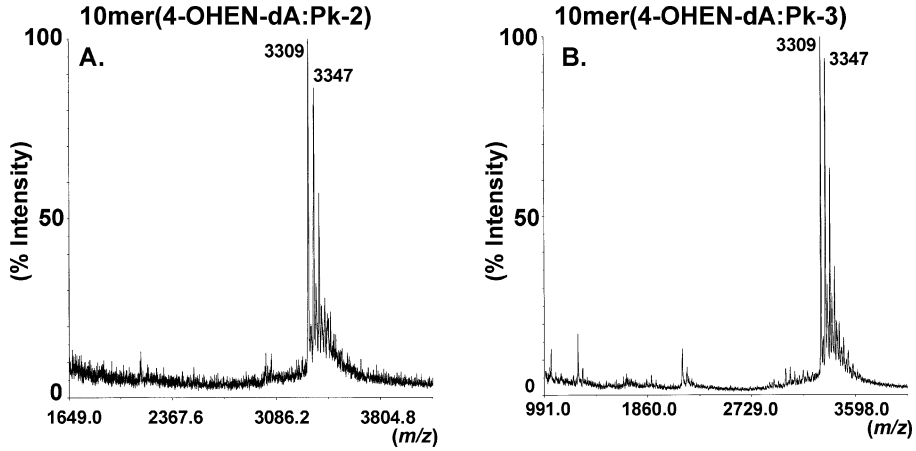


FIGURE 3: MALDI-TOF mass spectra of 4-OHEN-dA-modified oligodeoxynucleotides. The molecular weight of Pk-2 (A) and Pk-3 (B) of 4-OHEN-dA-modified 10-mer oligomers ($^{5'}$TTTGTA^{4-OHEN}TTTT) was measured using MALDI-TOF mass spectroscopy.

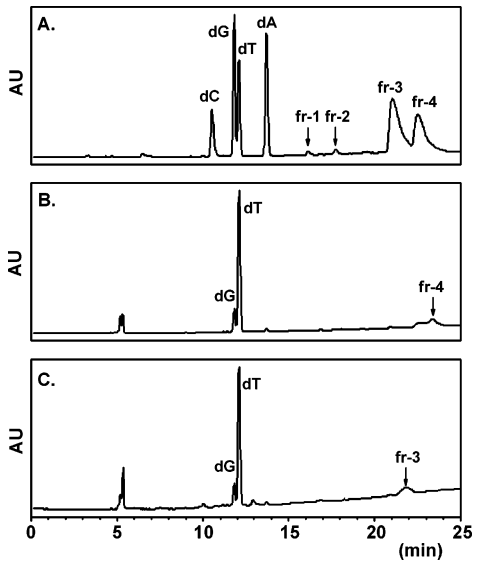


FIGURE 4: Determination of the 4-OHEN-dA isoform incorporated into 4-OHEN-modified oligomers. 4-OHEN-dA-modified oligodeoxynucleotides [$^{5'}$TTTGTA^{4-OHEN}TTTT, 1.5 μg for Pk-2 (B) and Pk-3 (C)] were digested at 37 °C overnight with micrococcal nuclease (30 units) and spleen phoshodiesterase (0.15 unit) and were further incubated at 37 °C for 2 h with alkaline phosphatase (3 units) in a buffer, as described in Materials and Methods. The resulting deoxynucleosides were analyzed using HPLC, using a μBondapak C_{18} column (0.78 × 30 cm; Waters). Elution was carried out using a linear gradient composed of distilled water containing 10% methanol over 2 min, 10−37% methanol over 5 min, and 37−90% methanol over 19 min at a flow rate of 2.0 mL/min. (A) A mixture of dNs and four diastereoisomers of 4-OHEN-dA. Diastereoisomers (fr-1, fr-2, fr-3, and fr-4) of monomeric 4-OHEN-dA were prepared as described previously (*10*).

mers were major adducts (Figure 2), the miscoding potential of these two adducts was determined. Primer extension reactions catalyzed by pol η or pol $\kappa\Delta$C were conducted in the presence of four dNTPs on the 4-OHEN-dA-modified 25-mer templates. Primer extension occurred rapidly on unmodified templates to form fully extended products (Figure 6). Using the 4-OHEN-dA-modified template, primer extension was retarded one base before the lesion and opposite the lesion. The majority of the blockage for pol η was observed opposite the lesion while the blockage for pol $\kappa\Delta$C occurred one base prior to the lesion. With pol η and pol $\kappa\Delta$C, a fraction of the primers was extended past the lesion,

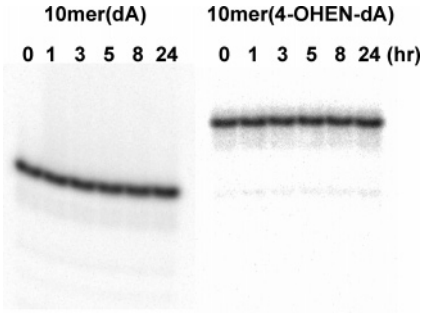


FIGURE 5: Stability of 4-OHEN-dA-modified oligodeoxynucleotides. Unmodified and 4-OHEN-dA-modified (Pk-3, 10 pmol) 10-mers were labeled with ^{32}P and incubated at 37 °C for 24 h in 100 μL of 50 mM Tris-HCl, pH 7.4. A fraction (1 pmol) was collected at 1, 3, 5, 8, and 24 h from the reaction mixture and stored at −70 °C. The samples were subjected to 20% PAGE. The radioactivity of the oligomers was measured by a Molecular Dynamics STORM PhosphorImager.

and the primer extension reactions on the Pk-2 template were slightly more effective than that on the Pk-3 template. With pol η, a broad band was observed opposite the lesion (13X), indicating that more than one dNMP were inserted. When the amount of pol η was increased, blunt-end addition to the fully extended product was observed (*35*, *36*).

Kinetic Studies on 4-OHEN-dA-Modified Templates. Using steady-state kinetic methods, the frequency of dNTP insertion (F_{ins}) and the chain extension (F_{ext}) was measured with pol η or pol $\kappa\Delta$C at the 4-OHEN-dA (Pk-2 or Pk-3) lesion within the linear range of the reaction (Table 1). Typical Hanes−Woolf plots obtained with pol η for dTTP insertion opposite dA or 4-OHEN-dA (Pk-2 and Pk-3) and dATP insertion opposite the 4-OHEN-dA are shown in Figure 7. When pol η was used on both Pk-2 and Pk-3 of the 4-OHEN-dA-modified template, the F_{ins} value for dTMP, the correct base, was similar to that for dAMP and the F_{ext} value for the dTMP·4-OHEN-dA pair was only 5 times higher than that for the dAMP·4-OHEN-dA pair. As a result, the relative bypass frequency ($F_{ins} \times F_{ext}$) for the dT·4-OHEN-dA pair was only 5−6 times higher than that for the dA·4-OHEN-dA. With the Pk-2 template, the $F_{ins} \times F_{ext}$ for dG·4-OHEN-dA was 90 times lower than that for dT·4-OHEN-dA but was only 8 times lower than that for dT·4-OHEN-dA when the Pk-3 template was used. The $F_{ins} \times F_{ext}$ for the dC·4-OHEN-dA was 1 or 2 orders of magnitude lower than that

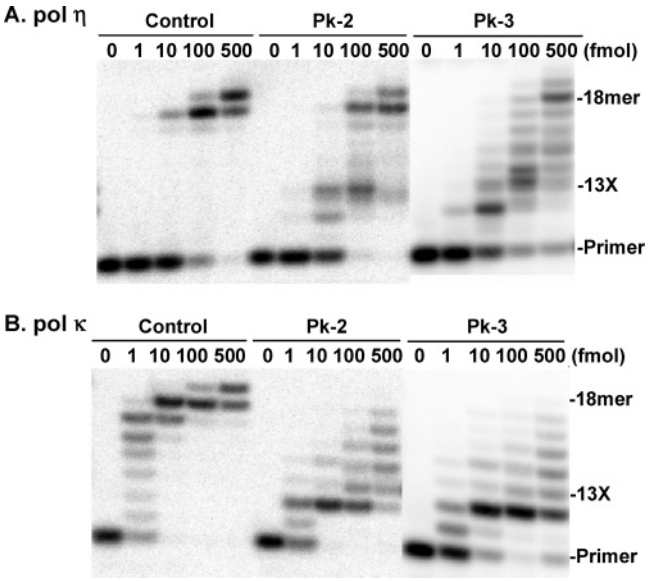


FIGURE 6: Primer extension reactions catalyzed by DNA polymerases on 4-OHEN-dA-modified DNA templates. Unmodified and 4-OHEN-dA-modified 25-mer templates were annealed to a ^{32}P-labeled 10-mer primer (^{32P}GAAAGAAGAA). Primer extension reactions catalyzed by pol η (A) or pol $\kappa\Delta$C (B) were conducted at 25 °C for 30 min in the presence of four dNTPs using variable amounts of enzymes. One-third of the reaction mixture was subjected to PAGE. The radioactivity of extended products was measured by a Molecular Dynamics STORM PhosphorImager and ImageQuaNT software. 13X shows the location opposite the 4-OHEN-dA.

for the dT·4-OHEN-dA. Therefore, pol η may insert dAMP opposite the 4-OHEN-dA lesion, generating A → T transversions during the translesion synthesis.

When pol $\kappa\Delta$C was used for Pk-2 and Pk-3 templates, the F_{ins} value for dTMP, the correct base, was at least 2 orders of magnitudes higher than that of the other dNMP (Table 1). However, the F_{ext} value for the dTMP·4-OHEN-dA pair was 60 and 70 times lower than that for the dCMP·4-OHEN-dA pair on the Pk-2 and Pk-3 templates, respectively. With the Pk-2 template, F_{ext} values for the dAMP·4-OHEN-dA and dGMP·4-OHEN-dA pair were only 4 times lower than that for the dTMP·4-OHEN-dA, but with the Pk-3 template, F_{ext} values for the dAMP·4-OHEN-dA and dGMP:4-OHEN-dA pairs could not be determined due to the inefficient chain extension. Therefore, the $F_{ins} \times F_{ext}$ for the dC·4-OHEN-dA pair was only 2−3 times lower than that for the dT·4-OHEN-dA pair. Therefore, pol $\kappa\Delta$C may insert dCMP opposite the lesion, generating A → G transitions.

DISCUSSION

When a 10-mer oligodeoxynucleotide containing a single dA was reacted with 4-OHEN, one minor (Pk-1) and two major (Pk-2 and Pk-3) oligodeoxynucleotides containing a single 4-OHEN-dA were isolated by HPLC. According to the reports by Geacinotov and colleagues (*37*, *38*), four diastereoisomers of 4-OHEN-dA should be produced. Using enzymatic digestion analysis, the major Pk-2 and Pk-3 oligomers contain fr-4 and fr-3, respectively, of monomeric 4-OHEN-dA. Although we confirmed, using MALDI-TOF mass spectrometry, that the Pk-1 contains a single 4-OHEN-dA, this product was not subjected to the enzymatic digestion analysis because of the poor yield. Therefore, Pk-1 may contain either fr-1 or fr-2 of monomeric 4-OHEN-dA or both. The yield of each diastereoisomer may be influenced by the sequence context of the oligomer, as reported recently (*39*). To explore the miscoding potential, the major 4-OHEN-dA-modified oligodeoxynucleotides were used for preparation of the DNA template that can be used for primer extension reactions.

Table 1: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Pol η and Pol κ[a]

	Insertion dNTP ↓AAAAGAAGAAAG^{32P} 5'TTTGTXTTTTCTTCTTTCCTCTCCC			**Extension dATP** ↓N AAAAGAAGAAAG^{32P} 5'TTTGTXTTTTCTTCTTTCCTCTCCC			
N·X	K_m (μM)[b]	V_{max} (% min^{-1})[b]	F_{ins}[c]	K_m (μM)[b]	V_{max} (% min^{-1})[b]	F_{ext}[c]	$F_{ins} \times F_{ext}$[c]
pol η							
T·A	0.21 ± 0.06	32.5 ± 3.0	1.0	0.46 ± 0.06	37.2 ± 5.6	1.0	1.0
X = 4-OHEN-dA (Pk-2)							
C·X	22.8 ± 1.6	1.38 ± 0.29	3.56×10^{-4}	7.30 ± 1.54	1.11 ± 0.51	1.85×10^{-3}	6.59×10^{-7}
A·X	6.50 ± 0.43	5.77 ± 0.91	5.24×10^{-3}	4.17 ± 0.71	1.53 ± 0.29	4.69×10^{-3}	2.46×10^{-5}
G·X	11.9 ± 2.37	1.55 ± 0.36	7.68×10^{-4}	7.52 ± 2.31	1.34 ± 0.65	2.16×10^{-3}	1.66×10^{-6}
T·X	5.38 ± 0.32	6.08 ± 0.44	6.70×10^{-3}	0.91 ± 0.07	1.60 ± 0.43	2.17×10^{-2}	1.46×10^{-4}
X = 4-OHEN-dA (Pk-3)							
C·X	8.59 ± 3.33	0.71 ± 0.13	5.12×10^{-4}	6.35 ± 1.11	0.93 ± 0.09	1.86×10^{-3}	9.53×10^{-7}
A·X	9.44 ± 1.67	5.42 ± 1.20	3.39×10^{-3}	7.84 ± 0.02	1.71 ± 0.20	2.71×10^{-3}	9.18×10^{-6}
G·X	16.1 ± 4.39	8.33 ± 2.46	3.05×10^{-3}	8.77 ± 0.40	1.47 ± 0.18	2.08×10^{-3}	6.35×10^{-6}
T·X	4.01 ± 0.43	2.18 ± 0.18	3.24×10^{-3}	1.72 ± 0.41	2.05 ± 0.53	1.48×10^{-2}	4.80×10^{-5}
pol κ							
T·A	0.52 ± 0.19	410 ± 35.5	1.0	0.54 ± 0.20	189 ± 62.0	1.0	1.0
X = 4-OHEN-dA (Pk-2)							
C·X	6.15 ± 1.69	0.12 ± 0.03	2.32×10^{-5}	2.62 ± 0.06	6.42 ± 0.31	6.12×10^{-3}	1.42×10^{-7}
A·X	5.87 ± 0.86	0.11 ± 0.05	2.17×10^{-5}	12.8 ± 0.44	0.13 ± 0.04	2.64×10^{-5}	5.72×10^{-10}
G·X	18.3 ± 3.07	0.07 ± 0.02	5.05×10^{-6}	17.5 ± 1.57	0.17 ± 0.04	2.41×10^{-5}	1.22×10^{-10}
T·X	0.16 ± 0.01	0.56 ± 0.11	4.11×10^{-3}	3.66 ± 0.35	0.16 ± 0.05	1.06×10^{-4}	4.36×10^{-7}
X = 4-OHEN-dA (Pk-3)							
C·X	26.2 ± 3.38	0.42 ± 0.04	1.94×10^{-5}	10.6 ± 0.36	14.8 ± 4.83	3.49×10^{-3}	6.77×10^{-8}
A·X	13.7 ± 2.77	0.19 ± 0.03	1.72×10^{-5}	ND[d]	ND	ND	ND
G·X	23.3 ± 1.39	0.11 ± 0.02	5.45×10^{-6}	ND	ND	ND	ND
T·X	0.63 ± 0.02	1.27 ± 0.07	2.40×10^{-3}	13.1 ± 4.57	0.24 ± 0.01	4.76×10^{-5}	1.14×10^{-7}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and Methods. [b] Data are expressed as mean ± SD obtained from three independent experiments. [c] Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair=dT\cdot dA)}$. [d] Not determined.

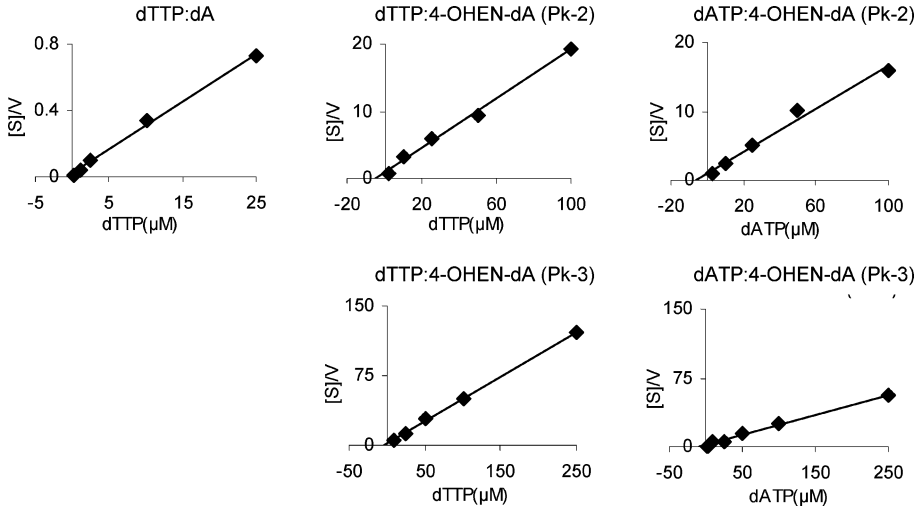


FIGURE 7: Hanes−Woolf plots of deoxynucleotide insertion opposite the 4-OHEN-dA lesion. Using 25-mer templates (150 fmol; 5′CCTTCXCTTCTTTCCTCTCCCTTT, where X is dA or 4-OHEN-dA) primed with a ^{32}P-labeled 12-mer (100 fmol; 5′GAAAGAAGAAAA), kinetic parameters associated with dTTP or dATP insertion opposite 4-OHEN-dA lesion were determined using pol η, as described in Materials and Methods. The K_m and V_{max} were obtained from Hanes−Woolf plots.

The primer extension reactions catalyzed by pol α or pol δ were blocked prior to the 4-OHEN-dA lesion (data not shown). However, during translesion synthesis catalyzed by human pol η and pol κ, both of which are highly expressed in reproductive organs (*21*−*24*), the major 4-OHEN-dA in the oligomers (Pk-2 and Pk-3) showed miscoding potential. On the basis of steady-state kinetic studies, both pols preferentially incorporated dTMP, the correct base, opposite the 4-OHEN-dA lesion. In addition, pol η incorporated dAMP, followed by dGMP, opposite the lesion, suggesting that the enzyme promotes A → T transversions, along with A → C transversions. On the other hand, pol κ incorporated dCMP opposite the 4-OHEN-dA, indicating that this enzyme is associated with generating A → G transitions. The relative bypass frequency past 4-OHEN-dA with pol η was more efficient than that with pol $\kappa\Delta$C, as observed for primer extension reactions with similar molar concentrations. Similar phenomena were observed previously for 4-OHEN-dC lesions (*25*). Therefore, 4-OHEN-dA is a miscoding lesion; the frequency and specificity variy depending on the DNA polymerase used.

When pMY189 plasmid carrying the *supF* gene was exposed to 4-OHEQ and transfected into human fibroblast cells, the mutations were primarily detected at C·G pairs (84%), along with a lesser number of mutations at A·T pairs (16%) (*13*). Supporting the previous results, the miscoding potential of 4-OHEN-dA may be lower than that of the 4-OHEN-dC adduct (*25*). dTMP, the correct base, was preferentially inserted opposite the 4-OHEN-dA lesion and extended past the lesion by both human pol η and pol $\kappa\Delta$C, whereas poor insertion of dCMP, the correct base, and poor chain extension were observed at the 4-OHEN-dC lesion (*25*). Among the base substitutions occurring at A·T pairs, A → T transversions and A → G transitions were primarily observed in the *supF* gene treated with 4-OHEQ (*13*). These mutation spectra were consistent with those observed in our study using pol η and pol $\kappa\Delta$C. Therefore, A → T transversions and A → G transitions may result from miscoding events occurring at the 4-OHEN-dA adduct during DNA synthesis with pol η and pol κ.

Each diastereoisomer of 4-OHEN-derived DNA adducts could exist as mirror image conformations that orient toward either 5′ → 3′ or 3′ → 5′ direction in double-stranded DNA; the opposite orientations could respond differently to DNA replication (*38*). In fact, relative bypass efficiency past 4-OHEN-dC varied depending on the diastereoisomers when pol η was used (*25*). In most cases of base pairs, the bypass frequency past 4-OHEN-dA for the Pk-2 oligomer was approximately 3 times higher than that for the Pk-3 oligomer (Table 1). The adduct stereochemistry may affect the bypass efficiency during translesion synthesis.

In conclusion, pol η and pol $\kappa\Delta$C can bypass 4-OHEN-dA lesion by incorporating dAMP and dCMP opposite the lesion, respectively. If such adducts are not rapidly repaired, pol η and pol κ expressed highly in the reproductive organs (*21*−*24*) are more likely to be associated with mutagenic events generated by equine estrogen−DNA damage.

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