Mutagenic Properties of Estrogen Quinone-Derived DNA Adducts in Simian Kidney Cells†

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ABSTRACT: DNA damage caused by catechol estrogens has been shown to play an etiologic role in tumor formation. Catechol estrogens are reactive to DNA and form several DNA adducts via their quinone forms. To explore the mutagenic properties of 2-hydroxyestrogen-derived DNA adducts in mammalian cells, N^2-(2-hydroxyestrogen-6-yl)-2′-deoxyguanosine and N^6-(2-hydroxyestrogen-6-yl)-2′-deoxyadenosine adducts induced by quinones of 2-hydroxyestrone, 2-hydroxyestradiol, or 2-hydroxyestriol were incorporated site-specifically into the oligodeoxynucleotides (5′TCCTCCTCXCCTCTC, where X is dG, dA, 2-OHE-N^2-dG, or 2-OHE-N^6-dA). The modified oligodeoxynucleotides were inserted into single-stranded phagemid vectors followed by transfection into simian kidney (COS-7) cells. Preferential incorporation of dCMP, the correct base, was observed opposite all 2-OHE-N^2-dG adducts. Only targeted G → T transversions were detected; the highest mutation frequency (18.2%) was observed opposite the 2-OHE_2-N^2-dG adduct, followed by 2-OHE_1-N^2-dG (4.4%) and 2-OHE_3-N^2-dG (1.3%). When 2-OHE-N^6-dA adducts were used, preferential incorporation of dTMP, the correct base, was observed. Targeted mutations representing A → T transversions were detected, accompanied by small numbers of A → G transitions. The highest mutation frequencies were observed with 2-OHE_1-N^6-dA and 2-OHE_3-N^6-dA (14.5 and 14.1%, respectively), while 2-OHE_2-N^6-dA exhibited a mutation frequency of only 6.0%. No mutations were detected with vectors containing unmodified oligodeoxynucleotides. Thus, 2-OHE quinone-derived DNA adducts are mutagenic, generating primarily G → T and A → T mutations in mammalian cells. The mutational frequency varied depending on the nature of the 2-OHE moiety.

Epidemiological studies have shown a link between female reproductive variables and an increased risk of developing cancer in several tissues such as breast and endometrium (*1*). Estrogens are associated with several cancers in humans and are known to induce carcinomas in kidney, liver, uterine, and mammary cells of rodents (*2*−*6*). Although the mitogenic effects of estrogen appear to be responsible for tumor formation during prolonged estrogen exposure, certain observations strongly suggest that nonhormonal events are also involved (*7*−*10*). In particular, catechol metabolites of estrogens are implicated in the incidence of tumor induction (*10*).

Cytochrome P450 enzymes CYP3A4, 1B1, and 1A2 are the major enzymes oxidizing estrogens to 2-hydroxyestrogen (2-OHE)[1] and 4-hydroxyestrogen (4-OHE) (*11*−*13*). 4-OHE was shown to be carcinogenic in the kidneys of male Syrian hamsters, while 2-OHE was not (*8*, *14*). However, 2-OHE and 4-OHE and their quinone forms, except for estrone-3,4-quinone, did not show any significant tumorigenic potential in livers of male B6C3F_1 mice (*15*). Although the carcinogenicity of estrogen may vary depending on the organs that are being examined, the contribution of 2-OHE and 4-OHE to the development of cancer is still unclear.

2-OHE and 4-OHE are formed in breast tissue (*16*−*18*) and can be converted by catechol-*O*-methyltransferase to 2-methoxyestrogen and 4-methoxyestrogen (*19*, *20*). Since the low activity of catechol-*O*-methyltransferase has been known to increase the risk of breast cancer (*21*), nonmethylated 2-OHE and 4-OHE may be involved in the development of breast cancer.

2-OHE and 4-OHE produce DNA damage in animal tissue (*22*, *23*) and induce cell transformation in cultured Syrian hamster embryo cells (*23*). The catechol estrogens produce reactive oxygen species that cause oxidative DNA damage such as strand breaks (*7*, *24*) and 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxodG) (*25*, *26*). The 8-oxodG lesion is mutagenic, generating G → T transversions in mammalian cells (*27*−*29*). The quinone forms of 4-OHE or 2-OHE also react with DNA to form DNA adducts. When estrogen 2,3-quinones react with dG and dA, N^2-(2-hydroxyestrogen-6-yl)-2′-deoxyguanosine (2-OHE-N^2-dG) and N^6-(2-hydrox-

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[1] Abbreviations: dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; E_1, estrone; E_2, estradiol; E_3, estriol; 2-OHE, 2-hydroxyestrogen; 2-OHE_1, 2-hydroxyestrone; 2-OHE_2, 2-hydroxyestradiol; 2-OHE_3, 2-hydroxyestriol; 4-OHE, 4-hydroxyestrogen; 2-OHE-N^2-dG, N^2-(2-hydroxyestrogen-6-yl)-2′-deoxyguanosine; 2-OHE-N^6-dA, N^6-(2-hydroxyestrogen-6-yl)-2′-deoxyadenosine; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; abasic site, apurinic/apyrimidinic site; dG-C8-AAF, *N*-(deoxyguanosin-8-yl)-2-(acetylamino)fluorene; dG-C8-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; pol, DNA polymerase; ss, single-stranded; HPLC, high-performance liquid chromatography.

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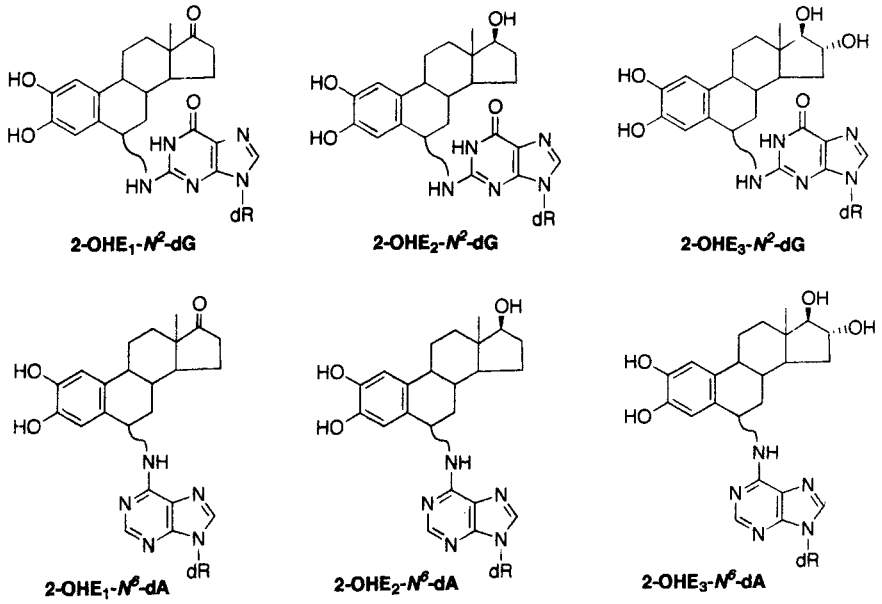

FIGURE 1: Structures of estrogen−DNA adducts.

yestrogen-6-yl)-2′-deoxyadenosine (2-OHE-N^6-dA) (structures in Figure 1), respectively, are formed (*30*). Reaction of estrogen 3,4-quinones with the dG residue produces 7-(4-hydroxyestrogen-1-yl)guanine, resulting in the formation of apurinic/apyrimidinic sites (abasic sites) in DNA (*30*). Although mutagenic properties of 8-oxodG (*27*−*29*) and abasic sites (*31*−*34*) have been reported using mammalian cells, no mutagenesis study has been performed for 2-OHE-induced DNA adducts.

In the study presented here, site-specifically modified oligodeoxynucleotides containing a single dG or dA adduct induced by 2-hydroxyestrone (2-OHE_1), 2-hydroxyestradiol (2-OHE_2), or 2-hydroxyestriol (2-OHE_3) were inserted into a single-stranded (ss) shuttle vector. The modified ss vectors were used to explore the mutagenic specificity and frequency of estrogen 2,3-quinone-derived DNA adducts using simian kidney cells. We found that 2-OHE-N^2-dG and 2-OHE-N^6-dA adducts are mutagenic, generating primarily G → T and A → T transversions, respectively, in mammalian cells.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). *Escherichia coli* DH10B was purchased from Gibco/BRL. The simian kidney (COS-7) cell line was obtained from the tissue culture facility of the State University of New York at Stony Brook. *Eco*RI restriction endonuclease (100 units/μL) and T4 DNA ligase (400 units/μL) were obtained from New England BioLabs Inc. (Beverly, MA). 2-OHEs were purchased from Sigma Chemical Co. (St. Louis, MO) and Steraloids Inc. (Wilton, NH).

Synthesis of Oligodeoxynucleotides. Unmodified 15-mer oligodeoxynucleotides (5′TCCTCCTCGCCTCTC and 5′TCCTCCTCACCTCTC) were prepared by solid-state synthesis, using an automated DNA synthesizer (*35*). As described previously (*36*), 15-mer oligodeoxynucleotides containing a single 2-OHE-N^2-dG or 2-OHE-N^6-dA were prepared by reacting unmodified 15-mer containing a single dG or dA with a 2-OHE quinone solution. After the reaction, the samples were evaporated to dryness and the products were dissolved in water and subjected to HPLC. The 2-OHE-N^2-dG and 2-OHE-N^6-dA-modified oligodeoxynucleotides were isolated on a Waters reverse-phase μBondapak C_{18} column (0.39 cm × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 10 to 35% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min (*36*, *37*). A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for separation and purification of oligodeoxynucleotides. These oligonucleotides were further purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea (35 cm × 42 cm × 0.04 cm) (*38*). The oligonucleotides recovered from the polyacrylamide gel were again subjected to HPLC to remove urea. Oligodeoxynucleotides were labeled at 15 °C for 10 min with ^{32}P at the 5′ terminus by treating with T4 polynucleotide kinase (*39*) and subjected to electrophoresis to establish homogeneity. The position of the oligonucleotides was established by autoradiography, using Kodak Xomat XAR film.

To confirm the molecular mass of 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer oligodeoxynucleotide and to determine the position of the modification by 2-OHE_1 quinone, 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer oligodeoxynucleotides were infused into the source of a Quattro LC/MS/MS spectrometer (Micromass). The instrument was operated in negative ion mode using electrospray ionization. The sample concentration was approximately 1 pmol/μL and produced multiply charged molecular ions ranging from −5 to −11. Collision-induced dissociation of the oligodeoxynucleotide produced sequence-specific fragment ions indicating the mass and position of the modification.

Site-Specific Mutagenesis in COS-7 Cells. The SV40-transformed simian kidney cell line, COS-7, and a single-stranded vector, pMS2, which confers neomycin and ampicillin resistance (*28*), were used to establish mutagenic specificity. Construction of a circular ss DNA containing a single DNA adduct was achieved by following procedures established previously (*28*). The pMS2 ss vector was annealed to a 61-mer and then digested with *Eco*RV to create

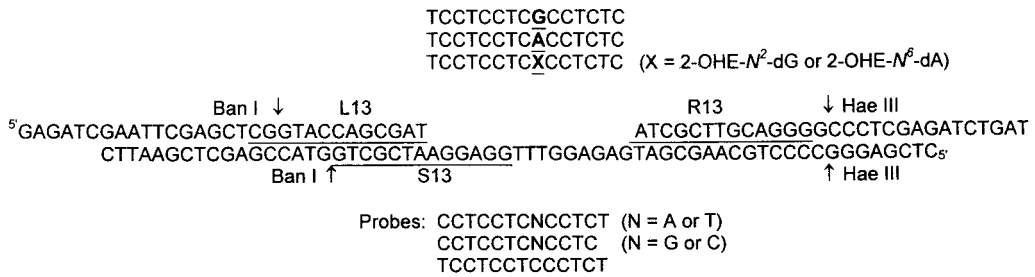

FIGURE 2: Construction of a single-strand vector containing a single estrogen−DNA adduct. The upper strand is a part of the ss pMS2 sequence, where X represents the DNA adduct. The underlined L13 and R13 probes were used to detect the correct insertion. The underlined 13-mer (S13) of 61-mer scaffold (bottom strand) was used to determine the concentration of the ss DNA construct. The probes that are listed were used for oligodeoxynucleotide hybridization to determine the mutation specificity of the DNA adduct.

a 15-mer gap (Figure 2). An unmodified, 2-OHE-N^2-dG or 2-OHE-N^6-dA-modified 15-mer was ligated at 4 °C for 18 h to the gapped vector. The ligation mixture was incubated for 2 h with T4 DNA polymerase (1 unit/pmol of DNA) to digest the hybridized 61-mer, and then treated with *Eco*RV and *Sal*I to cleave residual ss pMS2. The reaction mixture was extracted twice with a 1:1 (v/v) phenol/chloroform mixture and twice with chloroform. Following ethanol precipitation, the DNA was dissolved in distilled water. A portion of the ligation mixture was subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ss DNA. DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labeled S13 probe complementary to DNA containing the 15-mer insert. The absolute amount of closed circular ss DNA was established by comparing the radioactivity in the sample with that in known amounts of ss DNA.

COS-7 cells were transfected with ss DNA (100 fmol) over 18 h using lipofection (*40*), after which the cells were grown for 2 days in a Dulbecco's modified Eagle's medium/10% fetal calf serum mixture. Progeny plasmids were recovered by the method described by Hirt (*41*), treated with S1 nuclease to digest input ss DNA, and used to transform *E. coli* DH10B cells. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization (*42*, *43*). The oligodeoxynucleotide probes used to identify progeny phagemids are shown in Figure 2. Probes L13 and R13 were used to select phagemids containing the correct insert. Transformants that failed to anneal with L13 and R13 were omitted from the analysis. When L13/R13-positive transformants failed to hybridize to the probes designed to detect events targeted to the lesion site, double-stranded DNA was prepared and subjected to dideoxynucleotide sequencing analysis (*44*).

RESULTS

Characterization of 2-OHE Quinone-Modified Oligodeoxynucleotides. 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer oligodeoxynucleotides were prepared by reacting the unmodified oligodeoxynucleotide containing a single dG or dA with the quinone forms of 2-OHE_1, 2-OHE_2, or 2-OHE_3, as described previously (*36*). For example, when a 15-mer oligodeoxynucleotide (5′TCCTCCTCGCCTCTC or 5′TCCTCCTCACCTCTC) was reacted with 2-OHE_1 quinone, 2-OHE_1-N^2-dG- and 2-OHE_1-N^6-dA-modified 15-mers could be isolated at 20.3 and 21.9 min, respectively, by HPLC (Figure 3). The modified oligodeoxynucleotides were purified twice by HPLC and by gel electrophoresis (*36*). Their

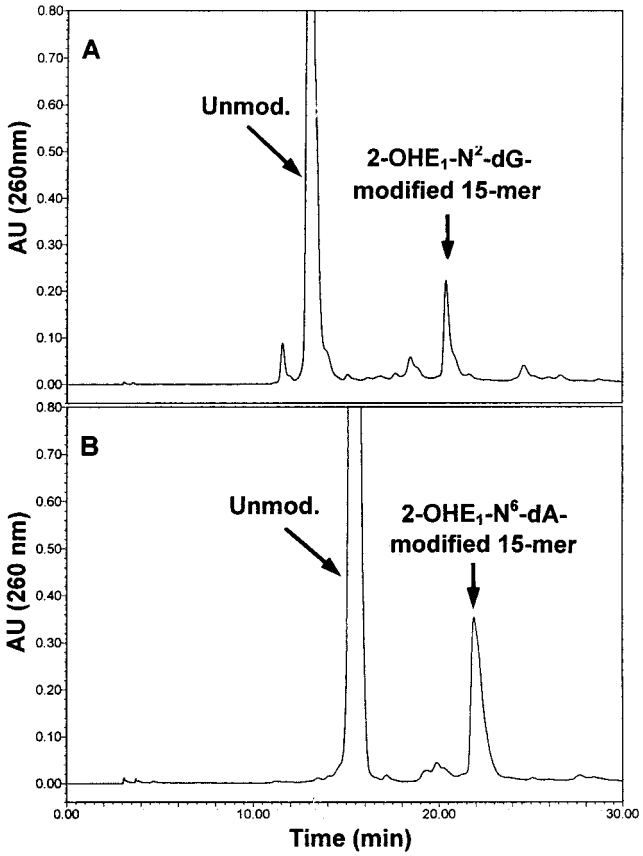

FIGURE 3: HPLC separation of oligodeoxynucleotide containing a single 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA. A 15-mer oligodeoxynucleotide containing a single dG (A) or dA (B) (200 μg) was reacted for 4 h at room temperature with a solution containing 2-OHE_1 quinone. The reaction mixture was evaporated to dryness, dissolved with distilled water, and subjected to HPLC, as described in Materials and Methods.

homogeneities were confirmed after labeling with ^{32}P. The migration of ^{32}P-labeled 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer was slower than that of the corresponding unmodified 15-mers (Figure 4).

To confirm the molecular mass and the position of 2-OHE-N^2-dG or 2-OHE-N^6-dA in the oligodeoxynucleotide, the modified oligodeoxynucleotides were subjected to LC/MS/MS that was carried out in negative ion mode using electrospray ionization. The 2-OHE_1-N^2-dG-modified oligomer sample produced six deprotonated molecular ions whose charges ranged from −7 to −11 (Figure 5A). In this case, the observed molecular mass of 4673.6 Da is consistent with that of a single 2-OHE_1-N^2-dG-adducted 15-mer. The peak at *m*/*z* 666.7 (−7 charge state) was selected for sequence

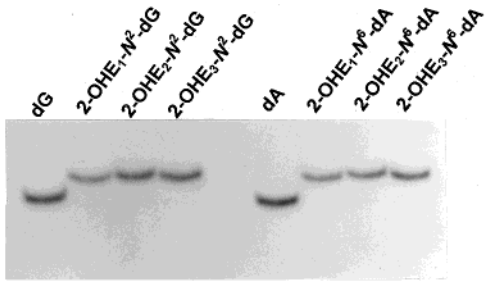

FIGURE 4: Polyacrylamide gel electrophoresis of 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer oligodeoxynucleotides. Unmodified or 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified oligodeoxynucleotides were labeled at 15 °C for 10 min with ^{32}P, as described in Materials and Methods, and then subjected to electrophoresis on 20% polyacrylamide (35 cm × 42 cm × 0.04 cm).

determination by MS/MS. Collision-induced dissociation of the oligodeoxynucleotide produced sequence-specific fragment ions indicating the mass and location of the modification. Using these data, it was found that a nonstandard nucleotide addition of 612.6 Da was positioned at the ninth base from the 5′ terminus. The molecular mass was consistent with that of the 2-OHE_1-N^2-dG adduct (613 Da). Similarly, when the 2-OHE_1-N^6-dA-modified 15-mer sample was subjected to LC/MS/MS, six deprotonated molecular ions whose charges ranged from −6 to −10 were produced (Figure 5B). The observed mass (4659.0 Da) was consistent with that of a single 2-OHE_1-N^6-dA-adducted 15-mer. Collision-induced dissociation of the oligodeoxynucleotide indicated that a nonstandard nucleotide addition of 596.8 Da was positioned at the ninth base from the 5′ terminus. The molecular mass was consistent with that of the 2-OHE_1-N^6-dA adduct (597 Da).

Construction of Vectors Containing 2-OHE Quinone-Derived DNA Adducts. Unmodified and 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mer oligodeoxynucleotides were ligated into a gapped ss vector at 4 °C (Figure 2). When a part of the ligation mixture was cleaved with restriction enzymes, *Ban*I and *Hae*III, and labeled with ^{32}P, a 40-mer product was detected on 12% denaturing polyacrylamide gel electrophoresis (Figure 6). The migration of the digestion product containing 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA was slower than that of unmodified dG or dA, as similarly observed for the unmodified and modified 15-mers (Figure 4). This indicates that 2-OHE_1-N^2-dG and 2-OHE_1-N^6-dA adducts were inserted into the ss vector. Small amounts (14−15%) of the digestion products appeared at the same position of an unmodified dG or dA. A portion of the 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA adduct may have been converted to the normal dG or dA, respectively, during construction.

Mutational Specificity of 2-OHE-N^2-dG and 2-OHE-N^6-dA Adducts. The vectors modified with 2-OHE-N^2-dG and 2-OHE-N^6-dA were used to transfect COS-7 cells. The resulting progeny plasmids were used to transform *E. coli* DH10B for the analysis of mutation. 2-OHE-N^2-dG and 2-OHE-N^6-dA adducts reduced the transformation efficiency to 50−72 and 68−72%, respectively (Table 1). When the 2-OHE_1-N^2-dG-modified vector was used, dCMP (95.6%), the correct base, was preferentially incorporated opposite the lesion (Table 2). Small numbers of targeted G → T transversions (4.4%) were detected. The 2-OHE_2-N^2-dG adduct promoted 18.2% G → T transversions; the mutational frequency was 4.1 times higher than for the 2-OHE_1-N^2-dG adduct. In contrast, the mutational frequency (1.3%) for 2-OHE_3-N^2-dG was 3.4 and 14 times lower than for 2-OHE_1-N^2-dG and 2-OHE_2-N^2-dG, respectively. No mutations were detected with the unmodified vector.

Using the 2-OHE-N^6-dA-modified vectors, dTMP, the correct base, was preferentially incorporated opposite the lesions (Table 2). With the 2-OHE_1-N^6-dA adduct, targeted A → T transversions (10.9%) were detected, accompanied by a small number of A → G transitions (3.6%). The 2-OHE_2-N^6-dA adduct also produced A → T transversions (3.3%) and A → G transitions (2.7%); however, the mutational frequency was 2.4 times lower than that of 2-OHE_1-N^6-dA. 2-OHE_3-N^6-dA promoted only A → T transversions. The mutational frequency (14.1%) was similar to that of 2-OHE_1-N^6-dA. Mutations were also not observed with the unmodified vectors.

DISCUSSION

Mutagenic specificities of DNA adducts induced by quinones of 2-OHE_1, 2-OHE_2, or 2-OHE_3 were compared using a ss plasmid vector and simian kidney cells. The 2-OHE-N^2-dG adducts produced only G → T mutations. The mutational frequency observed with 2-OHE_2-N^2-dG was 4.1 and 14 times higher than that for 2-OHE_1-N^2-dG and 2-OHE_3-N^2-dG, respectively. On the other hand, the 2-OHE-N^6-dA adducts produced primarily A → T mutations, together with fewer A → C mutations. 2-OHE_2-N^6-dA exhibited a 2.4-fold lower mutational frequency than 2-OHE_1-N^6-dA and 2-OHE_3-N^6-dA. Thus, DNA adducts induced by 2-OHEs are mutagenic in mammalian cells. The mutational frequency varies depending on the nature of the 2-OHE moiety.

We recently found that monomeric 2-OHE-N^2-dG and 2-OHE-N^6-dA were degraded, resulting in the formation of normal dG and dA, respectively.[2] Therefore, when 2-OHE-N^2-dG- or 2-OHE-N^6-dA-modified 15-mers were digested using nuclease P1, dG or dA was observed by HPLC; the resulting 2-OHE-N^2-dG or 2-OHE-N^6-dA could not be detected.[2] In place of the enzyme digestion, the presence and position of 2-OHE-N^2-dG or 2-OHE-N^6-dA in the 15-mer oligodeoxynucleotide were confirmed by LC/MS/MS.

2-OHE-N^2-dG- and 2-OHE-N^6-dA-modified 15-mers are much more stable than the monomeric nucleosides. Approximately 1.6% of the 2-OHE-N^2-dG-modified 15-mer and 0.5% of the 2-OHE-N^6-dA-modified 15-mer were converted to the products containing dG and dA, respectively, when incubated at 37 °C for 1 h at neutral pH.[2] During the construction of ss vectors, a portion of the 2-OHE-N^2-dG or 2-OHE-N^6-dA adduct may lose the 2-OHE moiety, resulting in a normal dG or dA (Figure 6). If such degradation does occur, the mutagenic potential of 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA adducts may actually be higher than that observed in this experiment.

The frequency of G → T mutations detected in the *p53* gene of breast cancers was much higher than that of the germline mutation used as background (*45*). This mutagenic spectrum was consistent with that observed for DNA adducts induced by 2-OHE in the study presented here. 8-OxodG lesions produced during redox cycling of catecholestrogens

[2] I. Terashima, N. Suzuki, and S. Shibutani, unpublished data.

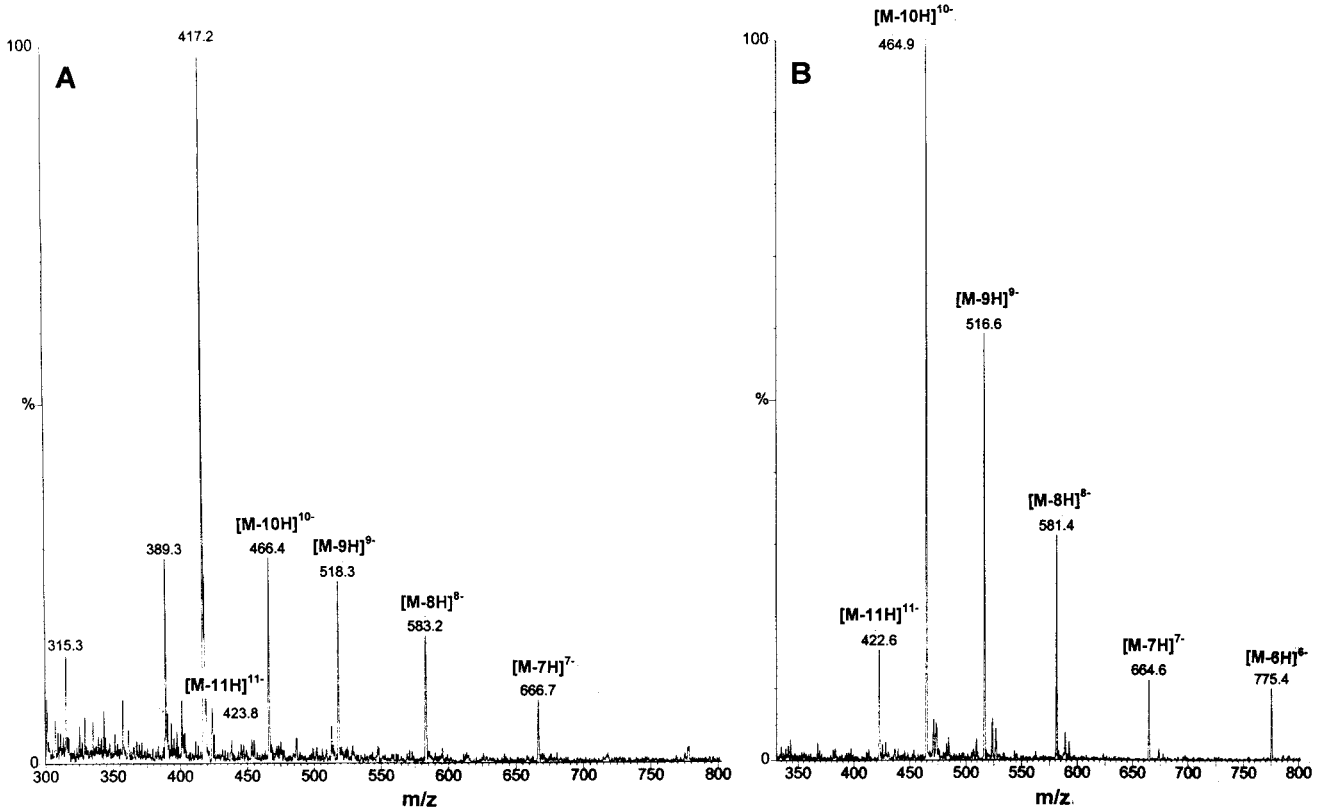

FIGURE 5: LC/MS/MS analysis of 2-OHE$_1$-N^2-dG- or 2-OHE$_1$-N^6-dA-modified 15-mer oligodeoxynucleotide. (A) M: 2-OHE$_1$-N^2-dG-modified d(TCCTCCTCG*CCTCTC) (4674 Da). (B) M: 2-OHE$_1$-N^6-dA-modified d(TCCTCCTCA*CCTCTC) (4659 Da). The instrument was operated in negative ion mode using electrospray ionization, as described in Materials and Methods.

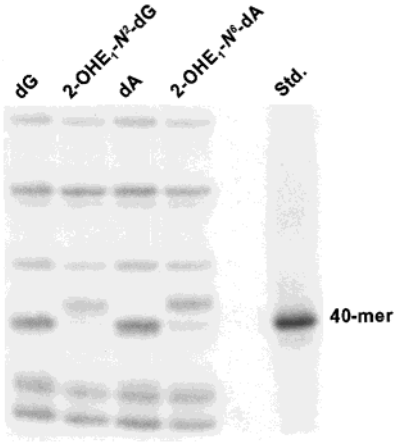

FIGURE 6: Analysis of products inserted into pMS2. A portion of the vector annealing with the 61-mer scaffold was digested with *Ban*I and *Hae*III as described in the legend of Figure 2, and subjected to a 12% denaturing polyacrylamide gel.

Table 1: Transformation of COS-7 Cells with ss DNA Constructs

ss DNA[a]	no. of transformants
dG	26 049 (100%)
2-OHE$_1$-N^2-dG	12 985 (50%)
2-OHE$_2$-N^2-dG	16 185 (62%)
2-OHE$_3$-N^2-dG	18 771 (72%)
dA	23 193 (100%)
2-OHE$_1$-N^6-dA	16 049 (69%)
2-OHE$_2$-N^6-dA	15 851 (68%)
2-OHE$_3$-N^6-dA	16 686 (72%)

[a] ss DNA (100 fmol) was transfected into COS-7 cells. Progeny phagemid was used to transform *E. coli* DH10B for mutation analysis.

also promote G → T mutations (*27*−*29*). Therefore, endogenous estrogen metabolites, including 2-OHE, may also contribute to the development of breast cancer.

Our in vitro mutagenesis studies using 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA adducts inserted site-specifically into oligodeoxynucleotides showed misincorporation of dTMP opposite the 2-OHE$_1$-N^2-dG adduct by DNA pol δ and misincorporation of dCMP opposite the 2-OHE$_1$-N^6-dA adduct by mammalian replication enzymes, pol α and pol δ (*36*). The miscoding spectra detected with the in vitro experimental system were different from that observed in the in vivo studies described here. The mutational properties observed in vivo may be modified by accessory proteins operating during translesional synthesis in mammalian cells (*46*). Moreover, recently discovered DNA polymerases such as pol ζ and pol η (*47*) might be involved in the translesional synthesis past 2-OHE-induced adducts.

Using the same in vivo experimental system and COS-7 cells, mutagenic properties of DNA adducts induced by tamoxifen α-sulfate, an activated form of tamoxifen (*48*), or by 2-acetylaminofluorene (2-AAF), a model chemical carcinogen (*49*), have been determined (Table 3). Since these adducts were positioned in the same sequence context as studied for the 2-OHE-N^2-dG adducts, the mutagenic potential of 2-OHE-N^2-dG adducts can be compared with that of α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-tamoxifen), *N*-(deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dG-C8-AAF), and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) adducts. Both dG-C8-AAF and dG-C8-AF promoted G → T mutations, accompanied by fewer G → A mutations (*49*). The mutational frequency of 2-OHE$_2$-N^2-dG, representing only G → T mutations, was similar to that of dG-C8-

Table 2: Mutational Specificity of 2-Hydroxyestrogen Quinone-Derived DNA Adducts in COS Cells[a]

lesion	experiment	no. of targeted mutations G	T	A	C	Δ[1]	nontargeted[c]
dG	1[b]	69 (100%)	0	0	0	0	0
	2	70 (100%)	0	0	0	0	0
	total	139 (100%)	0	0	0	0	0
2-OHE$_1$-N^2-dG	1	120 (94.5%)	7 (5.5%)	0	0	0	2
	2	118 (96.7%)	4 (3.3%)	0	0	0	1
	total	238 (95.6%)	11 (4.4%)	0	0	0	3[c]
2-OHE$_2$-N^2-dG	1	81 (81%)	19 (19%)	0	0	0	0
	2	117 (82.4%)	25 (17.6%)	0	0	0	0
	total	198 (81.8%)	44 (18.2%)	0	0	0	0
2-OHE$_3$-N^2-dG	1	85 (98.8%)	1 (1.2%)	0	0	0	0
	2	67 (98.5%)	1 (1.5%)	0	0	0	0
	total	152 (98.7%)	2 (1.3%)	0	0	0	0
dA	1	0	0	81 (100%)	0	0	0
	2	0	0	76 (100%)	0	0	0
	total	0	0	157 (100%)	0	0	0
2-OHE$_1$-N^6-dA	1	3 (4.3%)	12 (17.1%)	55 (78.6%)	0	0	0
	2	4 (3.3%)	9 (7.3%)	110 (89.4%)	0	0	5
	total	7 (3.6%)	21 (10.9%)	165 (85.5%)	0	0	5[d]
2-OHE$_2$-N^6-dA	1	1 (1.3%)	2 (2.6%)	75 (96.2%)	0	0	1
	2	3 (4.2%)	3 (4.2%)	66 (91.6%)	0	0	1
	total	4 (2.7%)	5 (3.3%)	141 (94.0%)	0	0	2[e]
2-OHE$_3$-N^6-dA	1	0	17 (14.2%)	103 (85.8%)	0	0	0
	2	0	11 (14.1%)	67 (85.9%)	0	0	0
	total	0	28 (14.1%)	170 (85.9%)	0	0	0

[a] Adducted ss DNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was recovered and used to transform *E. coli* DH10B for mutation analysis. [b] Data from experiments 1 and 2 were obtained using independently prepared progeny phagemid. [c] Nontargeted mutation: 5′TCCTCCTC(→t)GCCTC(→t,a)TC(→g). [d] Nontargeted mutation: 5′TCC(→Δ)TCCTC(→Δ)ACCTCTC, 3× ΔΔΔTCCTCACCTCTC. [e] Nontargeted mutation: 5′TCC(→2t)TCCTCACCTCTC.

Table 3: Comparison of the Mutation Potential of 2-OHE-Derived Adducts and Other Adducts

DNA adduct	targeted mutations (%) T	A	C	Δ[1]
2-OHE$_1$-N^2-dG[a]	4.4	0	0	0
2-OHE$_2$-N^2-dG[a]	18.2	0	0	0
2-OHE$_3$-N^2-dG[a]	1.3	0	0	0
dG-N^2-tamoxifen[b]				
trans-1	1.1	1.5	0.7	0
trans-2	9.6	2.8	0	0
cis-1	10.9	1.7	0.8	0
cis-2	12.3	1.7	0	0
dG-C8-AAF[c]	5.2	3.9	1.3	0
dG-C8-AF[c]	2.0	1.0	0	0

[a] Data were taken from Table 2. [b] Data were taken from ref *48*. [c] Data were taken from ref *49*.

AAF, and 6-fold higher than that of dG-C8-AF. In addition, the mutational frequency of 2-OHE$_2$-N^2-dG was slightly higher than those observed for dG-N^2-tamoxifen adducts except for the *trans*-1 epimer (*48*). Although 2-OHE$_1$-N^2-dG and 2-OHE$_3$-N^2-dG adducts exhibited lower mutational frequencies than dG-C8-AAF and dG-N^2-tamoxifen adducts, the dG-N^2 adduct induced, particularly, by 2-OHE$_2$ has mutagenic potential equivalent to those of AAF- and tamoxifen-derived DNA adducts.

The 2-OHE-N^2-dG and 2-OHE-N^6-dA adducts allow incorporation of dAMP opposite the lesions. Structural studies reveal that the dG-C8-AF adduct adopts the *syn* conformation to pair with *anti*-dA (*50*). Although structural studies have not yet been performed for 2-OHE-induced adducts or dG-N^2-tamoxifen adducts, such studies could explore how these adducts can be paired with dAMP.

Premarin is composed of approximately 50% estrogen (*51*) and is widely used for postmenopausal hormone replacement therapy to decrease menopausal symptoms and to protect from osteoporosis (*52*). This treatment also reduces the risk of cardiovascular disease (*52*), stroke (*53*), and Alzheimer's disease (*54*). However, hormone replacement therapy significantly increases the risk of breast cancer (*55*) and endometrial cancer (*56*). The higher cancer risk was observed particularly for 60−64-year-old women (*55*) or women who took Premarin for more than 5 years (*57*). This indicates that the exogenous estrogens may also be involved in the initiation and/or promotion of breast and endometrial cancers. In this study, we have shown that 2-OHE-induced DNA adducts cause mutations and, therefore, may participate in the initiation of breast and endometrial cancers. Treatment with estrogens may pose a potential risk to women undergoing hormone replacement therapy.

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