# Mutagenic Specificity of Model Estrogen−DNA Adducts in Mammalian Cells†

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ABSTRACT: Site-specifically modified oligodeoxynucleotides were used to explore the mutagenic properties of the model estrogen−DNA adducts $N^2$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyguanosine (dG-$N^2$-3MeE) and $N^6$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyadenosine (dA-$N^6$-3MeE) in simian kidney (COS-7) cells. Oligodeoxynucleotides (5′TCCTCCTCXCCTCTC; X = dG, dA, dG-$N^2$-3MeE, or dA-$N^6$-3MeE) containing an unmodified or model estrogen lesion were inserted into single-stranded (ss) phagemid vectors. These ss vectors were transfected into COS-7 cells. The progeny plasmid obtained were used to transform *Escherichia coli* DH10B. The transformants were analyzed by oligodeoxynucleotide hybridization and sequencing to determine the mutation frequency and spectrum. Preferential incorporation of dCMP, the correct base, was observed opposite the dG-$N^2$-3MeE lesion. Targeted mutations showing G → T transversions were detected, along with a small number of G → C transversions. When a dA-$N^6$-3MeE-modified oligodeoxynucleotide was used, preferential incorporation of dTMP, the correct base, was also observed. Targeted mutations representing A → T transversions were detected, accompanied by a small amount of A → G transitions. The frequency of mutation observed opposite dA-$N^6$-3MeE (17.5%) was 2.3 times higher than that observed opposite dG-$N^2$-3MeE (7.5%). These results indicate that estrogen DNA adducts have mutagenic potential in mammalian cells.

Endogenous and synthetic estrogens have been suspected to be involved in the development of breast and endometrial cancers (*1*−*3*). These hormonal substances induce several different tumors including mammary and uterine tumors in rodents (*3*−*5*). The mechanism of the carcinogenic effect of estrogens is unknown and may relate to promotion and/or initiation of cancer (*1*,*6*). Estrogens increase the frequency of mitotic activity in target organs. As a rare consequence of this estrogen-induced proliferation, malignant phenotypes may occur due to replication and chromosomal translocation errors during cell division (*1*, *7*) and may develop cancers. Alternatively, treatment of estrogens has been known to form DNA adducts in tissues of animals (*5*, *8*, *9*). DNA damage may lead to mutations that initiate human cancer (*10*).

Estrogens are metabolized by estrogen 2- and 4-hydroxylases to catecholestrogens (*11*, *12*), which, in turn, are oxidized to form semiquinones and quinones by cytochrome P450 (*13*). Both 2- and 4-hydroxyestradiols induce DNA adducts in cultured Syrian hamster embryo cells (*14*). The 2,3- and 3,4-quinones of estrogens are thought to be reactive intermediates that directly bind to DNA (*15*). Estrone 2,3-quinone reacts with dG or dA, forming $N^2$-[2-hydroxyestron-6(α,β)-yl]-2′-deoxyguanosine (2-$OHE_1$-6-$N^2$-dG)[1] or $N^6$-[2-hydroxyestron-6(α,β)-yl]-2′-deoxyadenosine (2-$OHE_1$-6-$N^6$-dA) (the structures in Figure 1), respectively (*16*). Reaction of estrone 3,4-quinone with dG produced 7-[4-hydroxyestron-1(α,β)-yl]guanine, with loss of deoxyribose (*16*).

We recently found that pyridinium 3-methoxyestra-1,3,5-(10)-trien-6-yl sulfate (3MeE-6-S) is highly reactive to deoxynucleosides and DNA, forming $N^2$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyguanosine (dG-$N^2$-3MeE) and $N^6$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyadenosine (dA-$N^6$-3MeE) adducts (Figure 1) (*17*, *18*). We proposed (*19*) that the 6-hydroxyl group of 6-hydroxyestrogens is sulfonated and that the highly reactive benzyl esters may form covalent adducts with DNA. Surprisingly, the reacting positions of 3MeE-6-S to dG and dA were the same as those induced by estrone-2,3-quinone (Figure 1). The chemical structures of dG-$N^2$-3MeE and dA-$N^6$-3MeE are quite similar to those of 2-$OHE_1$-6-$N^2$-dG and 2-$OHE_1$-6-$N^6$-dA. Thus, dG-$N^2$-3MeE and dA-$N^6$-3MeE may also be used as model DNA adducts.

We also found that dG-$N^2$-3MeE and dA-$N^6$-3MeE adducts have miscoding potential: the miscoding specificities and frequencies varied depending on the DNA polymerase used (*18*). In these studies, site-specifically-modified oligodeoxynucleotides containing a single dG-$N^2$-3MeE or dA-$N^6$-3MeE were inserted into a single-stranded shuttle vector (*20*). Due to the ss structure, repair of DNA adducts is

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[1] Abbreviations: dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; 3MeE-6-S, pyridinium 3-methoxyestra-1,3,5(10)-trien-6-yl sulfate; dG-$N^2$-3MeE, $N^2$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyguanosine; dA-$N^6$-3MeE, $N^6$-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyadenosine; 2-$OHE_1$-6-$N^2$-dG, $N^2$-[2-hydroxyestron-6(α,β)-yl]-2′-deoxyguanosine; 2-$OHE_1$-6-$N^6$-dA, $N^6$-[2-hydroxyestron-6(α,β)-yl)-2′-deoxyadenosine; abasic site, apurinic/apyridiminic site; HPLC, high-performance liquid chromatography; ss, single strand; ds, double strand; COS-7 cell, simian kidney cell.

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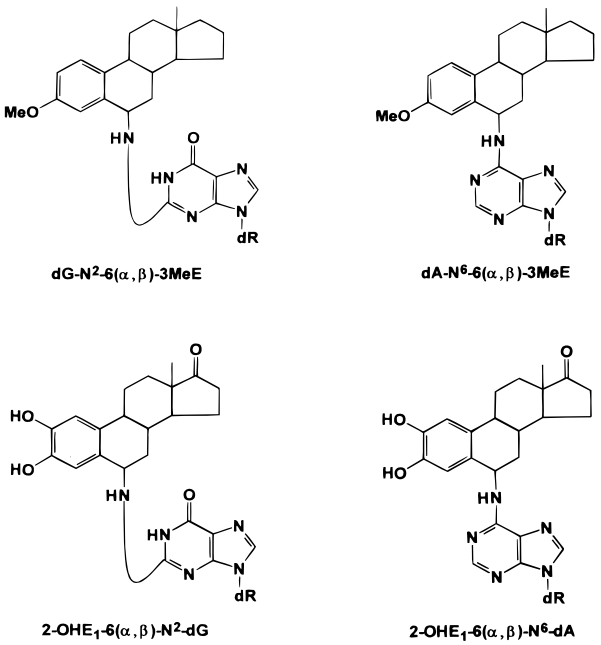


FIGURE 1: Structures of estrogen−DNA adducts.

minimal. These constructed vectors were used to establish the mutagenic specificities and frequencies of dG-$N^2$-3MeE and dA-$N^6$-3MeE. This is the first evidence that the estrogen−DNA adducts are highly mutagenic in mammalian cells.

## MATERIALS AND METHODS

*Materials.* [$\gamma$-$^{32}$P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. *Escherichia coli* DH10B was purchased from Gibco/BRL. The simian kidney (COS-7) cell line was obtained from Cold Spring Harbor Laboratory. *Eco*RI restriction endonuclease (100 units/mL) and T4 DNA ligase (400 units/mL) were obtained from New England Biolabs. A 990 HPLC instrument (Waters), equipped with a photodiode array detector, was used for separation and purification of oligodeoxynucleotides.

*Synthesis of Oligodeoxynucleotides.* Unmodified 15-mer oligodeoxynucleotides (5′TCCTCCTC*G*CCTCTC and 5′TCCTCCTC*A*CCTCTC) were prepared by solid-state synthesis, using an automated DNA synthesizer (*21*). As described previously (*18*), a 15-mer oligodeoxynucleotide containing a single dG-$N^2$-3MeE or dA-$N^6$-3MeE was prepared by reacting 200 $\mu$g of unmodified 15-mer containing a single dG or dA with 2.0 mg of 3MeE-6α-S for 30 min at 37 °C in 500 $\mu$L of 50 mM $NaH_2PO_4$−$Na_2HPO_4$ buffer, pH 7.0. After the reaction, the samples were centrifuged and the supernatants were subjected to HPLC. The dG-$N^2$-3MeE- or dA-$N^6$-3MeE-modified and unmodified oligomers were isolated on a reverse-phase $\mu$Bondapak $C_{18}$ (0.39 × 30 cm, Waters), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10% → 50% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min as described elsewhere (*22*). These oligomers were further purified by electrophoresis on 20% polyacrylamide gel in the presence of 7 M urea (35 × 42 × 0.04 cm) (*23*). The oligomers recovered from PAGE were again subjected to HPLC to remove urea. Oligomers were labeled at the 5′ terminus by treating with T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]ATP (*24*) and subjected to electrophoresis to establish homogeneity. The position of the oligomers was established by autoradiography, using Kodak Xomat XAR film.

*Site-Specific Mutagenesis in COS-7 Cells.* SV40-transformed simian kidney cell lines COS-7 and a single-strand (ss) vector, pMS2, which confers neomycin ($Neo^R$) and ampicillin ($Amp^R$) resistance (*20*), were used to establish mutagenic specificity. Construction of a circular ss DNA containing a single DNA adduct followed procedures established previously in this laboratory (*20*). pMS2 ss DNA was repurified on a Nucleogen 4000-7 DEAE column (0.6 × 12.5 cm), using a linear gradient of 0.02 M potassium phosphate and 5 M urea, pH 6.9 (eluent A), containing 40% → 100% (eluent A) and 1.5 M KCl (eluent B), with an elution time of 90 min and a flow rate of 1.0 mL/min. The fraction containing pMS2 ($t_R$ = 33.0) was concentrated on Centricon 100 filters, washed three times with distilled water, and subjected to ethanol precipitation. pMS2 DNA was annealed to a 61-mer and then digested with *Eco*RV to create a 15-mer gap (Figure 2). An unmodified or dG-$N^2$-3MeE- or dA-$N^6$-3MeE-modified 15-mer was ligated to the gapped vector. A portion of the ligation mixture was used to establish ligation efficiency. 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 phenol/chloroform, 1:1 (vol/vol), and twice with chloroform. Following ethanol precipitation, the DNA was dissolved in distilled water. A portion of the ligation mixture and known amounts of ss pMS2 were subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ssDNA. 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 ssDNA was established by comparing the radioactivity in the sample with that in known amounts of ssDNA.

COS-7 cells were transfected with ssDNA (100 fmol) over 18 h using lipofection (*25*), after which the cells were grown for 2 days in Dulbecco's modified Eagle's medium/10% fetal calf serum. Progeny plasmids were recovered by the method described by Hirt (*26*), treated with S1 nuclease to digest input ssDNA, and used to transform *E. coli* DH10B. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization (*27, 28*). 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-strand (ds) DNA was prepared and subjected to dideoxynucleotide sequencing analysis (*29*).

## RESULTS

*Preparation of Vectors Containing Estrogen−DNA Adducts.* Oligodeoxynucleotides (15-mers) containing a dG-$N^2$-3MeE, dA-$N^6$-3MeE, or unmodified dG or dA were prepared as described previously (*18*) and purified twice by HPLC and by gel electrophoresis. Their homogeneities were confirmed after labeling with $^{32}$P (Figure 3). The migration

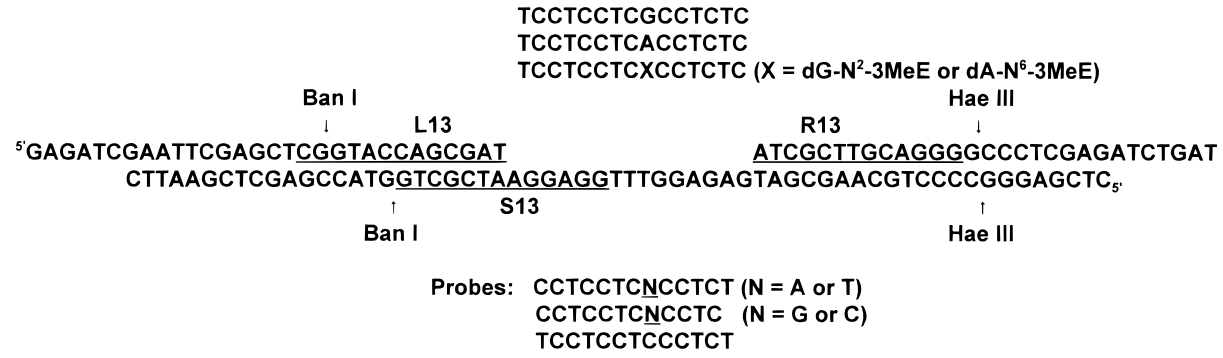


FIGURE 2: Construction of a single-strand vector containing a single estrogen−DNA adduct. The upper stand is a part of ss pMS2 sequence: X represents dG-$N^2$-3MeE or dA-$N^6$-3MeE. The underlined L13 and R13 probes were used to detect the correct insertion. The underlined 13-mer (S13) of 61-mer scafford (bottom strand) was used to determine the concentration of ss DNA construct. The probes listed were used for oligodeoxynucleotide hybridization to determine mutation specificity of dG-$N^2$-3MeE or dA-$N^6$-3MeE.


FIGURE 3: Polyacrylamide gel electrophoresis of 15-mer containing a single estrogen−DNA adduct. Oligodeoxynucleotides (5′TCCTC-CTCXCCTCTC; X = dG, dG-$N^2$-3MeE, dA, or dA-$N^6$-3MeE) were labeled with $^{32}$P, as described in Materials and Methods, and then subjected to electrophoresis on a 20% polyacrylamide gel (15 × 45 × 0.04 cm).

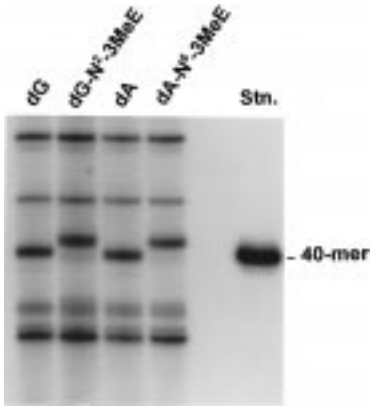


FIGURE 4: Analysis of products inserted into pMS2. A portion of the vector annealing with the 61-mer scafford was digested with *Ban*I and *Hae*III as described in Figure 2 and subjected to denaturing 12% polyacrylamide gel electrophoresis.

of dG-$N^2$-3MeE- and dA-$N^6$-3MeE-modified 15-mers was slower than that of the corresponding unmodified 15-mers, respectively. These oligodeoxynucleotides were ligated into a gapped single-strand vector as described in Figure 2. When a part of the ligation mixture was cleaved with *Ban*I and *Hae*III restriction enzymes and labeled with $^{32}$P, a 40-mer product was detected on denaturing 12% polyacrylamide gel electrophoresis (Figure 4). The migration of the digestion product containing dG-$N^2$-3MeE or dA-$N^6$-3MeE was slower than that of unmodified dG or dA, respectively, as shown similarly for the unmodified and modified 15-mers (Figure 3). This indicates that dG-$N^2$-3MeE and dA-$N^6$-3MeE

Table 1: Transformation of COS-7 Cells with ssDNA Constructs

| ssDNA[a] | no. of transformants |
|---|---|
| pMS2 (dG) | 24 700 (100%) |
| pMS2 (dG-$N^2$-3MeE) | 9490 (38.4%) |
| pMS2 (dA) | 20 500 (100%) |
| pMS2 (dA-$N^6$-3MeE) | 12 500 (61.0%) |

[a] ssDNA (100 fmol) was transfected into COS-7 cells. Progeny phagemid was used to transform *E. coli* DH10B for ampicillin resistance.

lesions were inserted into the ss vector. No significant difference in the ligation efficiency was observed between the unmodified and modified oligodeoxynucleotide. Minor bands were observed below the 40-mers containing a dG-$N^2$-3MeE or dA-$N^6$-3MeE. Since the migration of the minor bands was faster than that of unmodified 40-mers, the minor band does not result from unmodified oligomers. The modified 40-mers may be shortened by digestion with some contamination in restriction enzymes.

The final concentration of ss DNA vector was quantified by Southern blot hybridization. The S13 probe was hybridized to the ligation site of the ss vector (Figure 2). By use of a $\beta$-phosphorimager, the net product of the closed circular (cc) DNA of each construct was estimated by comparison with various amounts of pMS2 DNA standards (data not shown). The concentrations of the cc vector sample were 1.51 and 5.40 ng/$\mu$L for the unmodified oligomer containing a dG and dA and 6.04 and 4.83 ng/$\mu$L for the dG-$N^2$-3MeE- and dA-$N^6$-3MeE-modified oligomers, respectively.

*Mutational Specificity.* The vectors modified with dG-$N^2$-3MeE and dA-$N^6$-3MeE were used to transfect COS-7 cells; the number of transformants recovered was compared to the unmodified control (Table 1). The presence of a single dG-$N^2$-3MeE and dA-$N^6$-3MeE residue reduced transformation efficiency to 38% and 61%, respectively. When a dG-$N^2$-3MeE-modified vector was used, dCMP (93%), the correct base, was preferentially incorporated opposite the lesion (Table 2). Fifteen targeted mutants showing G → T transversions were detected, along with two mutants showing G → C. The frequency of the targeted mutation was 7.5%. No mutations were observed with the unmodified vector. When a dA-$N^6$-3MeE-modified vector was used, preferential incorporation of dTMP (83%), the correct base, was also observed opposite the lesion (Table 2). Thirty-three targeted mutants representing A → T transversions were detected,

Table 2: Mutational Specificity of Model Estrogen−DNA Adducts in COS-7 Cells[a]

| plasmid | | no. of targeted mutations (dG, dG-N$^2$-3MeE, dA, or dA-N$^6$-3MeE → X) | | | | | |
|---|---|---|---|---|---|---|---|
| | | G | T | A | C | Δ | others[b] |
| pMS2 (dG) | expt 1[c] | 64 | 0 | 0 | 0 | 0 | 0 |
| | expt 2 | 134 | 0 | 0 | 0 | 0 | 0 |
| | total | 198 (100%) | 0 (<0.5%) | 0 (<0.5%) | 0 (<0.5%) | 0 (<0.5%) | 0 |
| pMS2 (dG-N$^2$-3MeE) | expt 1 | 112 | 6 | 0 | 2 | 0 | 0 |
| | expt 2 | 100 | 9 | 0 | 0 | 0 | 0 |
| | total | 212 (93%) | 15 (6.6%) | 0 | 2 (0.9%) | 0 | 0 |
| pMS2 (dA) | expt 1[c] | 0 | 0 | 65 | 0 | 0 | 0 |
| | expt 2 | 0 | 0 | 135 | 0 | 0 | 1 |
| | total | 0 (<0.5%) | 0 (<0.5%) | 200 (100%) | 0 (<0.5%) | 0 (<0.5%) | 1 |
| pMS2 (dA-N$^6$-3MeE) | expt 1 | 1 | 16 | 81 | 0 | 0 | 2 |
| | expt 2 | 2 | 17 | 90 | 0 | 0 | 3 |
| | total | 3 (1.5%) | 33 (16%) | 171 (83%) | 0 | 0 | 5 |

[a] Adducted ssDNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was recovered and used to transform *E. coli* DH10B for mutation analysis. [b] Nontargeted mutations are 5′TCC(→t)TCCTCACCTCTC and 5′TCCTCCTCA*CC(→g2 or →t3)TCTC. [c] Data of experiments 1 and 2 were obtained from independently prepared progeny phagemid.

accompanied by a small number of mutants showing A → G transitions. The frequency of the targeted mutation was 17.5%. Some nontargeted mutations were detected opposite dC two bases 3′ to the dA-N$^6$-3MeE lesion. The frequency of targeted mutation observed opposite dA-N$^6$-3MeE was 2.3 times higher than that observed opposite dG-N$^2$-3MeE. Thus, the dA-N$^6$-3MeE lesion was much more mutagenic than the dG-N$^2$-3MeE lesion.

## DISCUSSION

A single-stranded plasmid vector was used to establish mutagenic specificity for dG-N$^2$-3MeE and dA-N$^6$-3MeE replicated in COS-7 cells. Targeted mutations representing G → T transversions were detected at the dG-N$^2$-3MeE lesion, along with a small number of G → C transitions. On the other hand, A → T transversions and a lesser number of A → G transitions were observed opposite dA-N$^6$-3MeE. The mutational frequency of dA-N$^6$-3MeE was much higher than that of dG-N$^2$-3MeE, as similarly observed in the primer extension reactions catalyzed by all DNA polymerases on the dG-N$^2$-3MeE- and dA-N$^6$-3MeE-modified templates (*18*). Thus, both estrogen−DNA adducts have mutagenic potential.

Miscoding specificities of dG-N$^2$-3MeE and dA-N$^6$-3MeE have been analyzed during DNA synthesis in reactions catalyzed by DNA polymerases (*18*). Exo$^-$ Klenow fragment of *E. coli* DNA pol I and mammalian pol $\beta$, enzymes that are involved in repair (*30*), catalyzed misincorporation of dAMP and/or dGMP opposite dG-N$^2$-3MeE and misincorporation of dAMP and dCMP, respectively, opposite dA-N$^6$-3MeE. Thus, the miscoding spectra observed in vitro were similar to the mutational spectra observed in COS-7 cells. In contrast, primer extension reactions catalyzed by pol α and pol $\delta$, mammalian replicative enzymes, were blocked at the dG-N$^2$-3MeE and dA-N$^6$-3MeE lesions. Pol α showed only small amounts of misincorporation of dCMP and deletions at the dA-N$^6$-3MeE lesions. The miscoding properties varied depending on the DNA polymerases used. Thus, the miscoding properties are quite different from the mutagenic properties observed in COS-7 cells. Although it is not clear which mammalian polymerases are involved in DNA replication on the single-strand vector, the mutational properties observed in vitro may be modified by accessory proteins operating during the translesional synthesis in mammalian cells (*30*). Alternatively, repair system may also modify mutagenic properties after translesional synthesis past the modified lesion on the single-strand vector.

When estrogen quinones are reduced to the semiquinones during redox cycling, free radicals produce oxidative damage, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in DNA (*31*). 8-Oxo-dG is a mutagenic lesion, generating primarily G→T transversions (*20*, *32*−*34*). This mutagenic lesion has been detected in mammary DNA from breast cancer patients (*35*). Thus, if 8-oxo-dG is not repaired, oxidative damage may also contribute to the initiation of cancer.

Estrogen 3,4-quinone directly reacts with dG residue, resulting in *N*7-(4-hydroxyestron-1-yl)guanine and an apurinic site in DNA (*16*, *36*). Apurinic/apyridiminic (abasic) sites have been shown to generate mutations in mammalian cells. In our in vitro studies using pol α and pol $\delta$ (*37*, *38*), preferential misincorporation of dAMP was detected opposite a natural or synthetic abasic site during DNA synthesis. In COS-7 cells, dAMP, dCMP, and dTMP were incorporated at similar frequencies opposite a natural abasic site (*39*). In another study in COS-7 cells, preferential incorporation of dAMP was observed opposite a synthetic abasic site, accompanied by a small number of deletions (*40*). In human lymphoblastoid cells, dGMP was incorporated primarily opposite natural abasic sites (*41*). Although the mutation spectra observed in the three studies were different, unstable depurinating sites induced by estrogen 3,4-quinone may promote mutations. Alternatively, the unstable adduct, *N*7-(4-hydroxyestron-1-yl)-2′-deoxyguanosine, itself may also have a mutagenic potential. On the other hand, since premalin metabolite, a 4-hydroxyequilenin semiquinone radical, is reactive with dG, forming unusual cyclic adducts (*42*), estrogen 3,4-quinone may also produce such a cyclic adduct.

The increased formation of 16α-hydroxyestrogen metabolites may be associated with an increased risk for developing mammary cancer in rodents (*6*). Since 16α-hydroxyestrone reacts covalently with amino groups in proteins through the Schiff base (*43*), 16α-hydroxyestrogen may also react with DNA, forming DNA adducts.

Both 2- and 4-hydroxyestradiols have been shown to form DNA adducts in cultured hamster embryo cells (*14*). In our studies, stable model estrogen−DNA adducts similar to that generated by 2-hydroxyestrogen have highly mutagenic potential. Thus, estrogen-derived DNA adducts, depurinic sites, as well as oxidative damage may be involved in the development of human cancers including breast and endometrial cancers. 4-Hydroxyestrogens have been shown to be carcinogenic in the kidneys of male Syrian hamsters, while 2-hydroxyestrogens were not (*44*, *45*). However, 2- and 4-hydroxyestrogens and their major metabolites, estradiol 2,3-quinone and estradiol 3,4-quinone, did not show any tumorigenic potency in liver of B6C3F$_1$ male mice (*36*). Carcinogenicity of estrogen may vary depending on the organs examined, the species, and the sex of the animal. So far no carcinogenic studies of estrogen metabolites such as hydroxyestrogens and estrogen quinones have been investigated in mammary and uterine tissues of animals. Further studies will be required to explore the carcinogenicity of estrogen and its metabolites.

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