Published in final edited form as:
Int J Cancer. 2009 March 15; 124(6): 1276–1284. doi:10.1002/ijc.24113.

Mechanism of metabolic activation and DNA adduct formation by the human carcinogen diethylstilbestrol: The defining link to natural estrogens

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Abstract

Diethylstilbestrol (DES) is a human carcinogen, based on sufficient epidemiological evidence. DES is mainly metabolized to its catechol, 3′-hydroxyDES (3′-OH-DES), which can further oxidize to DES-3′,4′-quinone (DES-3′,4′-Q). Similarly to estradiol-3,4-quinone, the reaction of DES-3′,4′-Q with DNA would form the depurinating 3′-OH-DES-6′-N3Ade and 3′-OH-DES-6′-N7Gua adducts. To prove this hypothesis, synthesis of DES-3′,4′-Q by oxidation of 3′-OH-DES with Ag_2O was tried; this failed due to instantaneous formation of a *spiro*-quinone. Oxidation of 3′-OH-DES by lactoperoxidase or tyrosinase in the presence of DNA led to the formation of 3′-OH-DES-6′-N3Ade and 3′-OH-DES-6′-N7Gua adducts. These adducts were tentatively identified by LC-MS/MS as 3′-OH-DES-6′-N3Ade, *m/z* = 418 $[M+H]^+$, and 3′-OH-DES-6′-N7Gua, *m/z* = 434 $[M+H]^+$. Demonstration of their structures derived from their oxidation by MnO_2 to the DES quinone adducts and subsequent tautomerization to the dienestrol (DIES) catechol adducts, which are identical to the standard 3′-OH-DIES-6′-N3Ade, *m/z* = 416 $[M+H]^+$, and 3′-OH-DIES-6′-N7Gua, *m/z* = 432 $[M+H]^+$, adducts. The reaction of DIES-3′,4′-Q or lactoperoxidase-activated 3′-OH-DIES with DNA did not produce any depurinating adducts, due to the dienic chain being perpendicular to the phenyl planes, which impedes the intercalation of DIES into the DNA. Enzymic oxidation of 3′-OH-DES suggests that the catechol of DES intercalates into DNA and is then oxidized to its quinone to yield N3Ade and N7Gua adducts. These results suggest that the common denominator of tumor initiation by the synthetic estrogen DES and the natural estrogen estradiol is formation of their catechol quinones, which react with DNA to afford the depurinating N3Ade and N7Gua adducts.

Introduction

Diethylstilbestrol (DES), a potent synthetic estrogen, was obtained in 1938 and thereafter used to prevent spontaneous abortions and premature deliveries [1]. In 1971 an association was found between prenatal exposure to DES and clear cell adenocarcinoma of the vagina and cervix in young women [2]. Evidence for a causal relationship between transplacental exposure to DES and clear cell adenocarcinoma is conclusive [3–5]. Furthermore, it was later reported that women who took DES during pregnancy had a higher incidence of breast cancer [6,7]. More recently, it was found that women exposed to DES prenatally also have a higher incidence of breast cancer after age 40 [8,9].

The natural estrogens estradiol (E_2) and estrone (E_1) are metabolically oxidized to their respective catechols, and the catechols are subsequently oxidized to their quinones, in particular the E_1(E_2)-3,4-quinones (Q). We have proposed and demonstrated that reaction of

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the $E_1(E_2)$-3,4-Q with DNA is the first critical step in the initiation of cancer [10–13]. The depurinating adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, as well as the resulting apurinic sites in the DNA, are formed in this reaction. These apurinic sites generate mutations that we think can lead to cancer initiation [13–15].

The same mechanism of metabolic activation and reaction with DNA has been demonstrated for the synthetic estrogen hexestrol (HES) [16,17]. The catechol quinone of HES has chemical and biochemical properties similar to those of $E_1(E_2)$-3,4-Q; namely, it forms analogous depurinating N3Ade and N7Gua adducts after reaction with DNA. In addition, depurination of the N7Gua adduct occurs rather slowly, analogously to the respective adducts of the natural $E_1(E_2)$-3,4-Q [16,17]. HES is the derivative of DES hydrogenated at the ethylenic bond.

The major metabolites of HES and DES are their catechols [18–21]. The natural estrogens E_1 and E_2, as well as the synthetic HES and DES, are carcinogenic in the kidney of male Syrian golden hamsters [22]. In this article, we report that the human carcinogen DES is activated similarly to the natural estrogens and the synthetic estrogen HES. The catechol quinone of DES reacts with DNA to form N3Ade and N7Gua adducts analogously to the quinones of E_1, E_2 and HES.

Materials and methods

Chemicals, reagents and enzymes

DES, lactoperoxidase (LP, from bovine milk, 78 units/mg solid), H_2O_2, methemoglobin, and tyrosinase (Tyr, 2, 130 units/mg solid) were purchased from Sigma-Aldrich (St. Louis, Mo). Prostaglandin H synthase (PHS, ovine, 5K) and arachidonic acid were purchased from Cayman Chemical Co. (Ann Arbor, MI). *E,E*-Dienestrol (*E,E*-DIES) was purchased from Steraloids (Newport, RI). *Z,Z*-DIES was synthesized from DES as reported [23]. 3′-OH-DES, 3′-OH-*E,E*-DIES, 3′-OH-*Z,Z*-DIES and 3′-OH-4″-OCH_3-DES were synthesized as reported previously [24]. Ag_2O, Ade, 2-iodoxybenzoic acid (IBX), trifluoroacetic acid (TFA), DMSO-d_6 and acetone-d_6 were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Instrumentation

UV—The UV spectra were obtained during HPLC by using the photodiode array detector (Waters 996, Milford, MA) for all compounds synthesized.

NMR—^{1}H NMR spectra of all new compounds were recorded in deuterated solvents on a Varian Inova 500 instrument at 499.562 MHz at 25 °C. Labile protons on oxygen and nitrogen atoms were detected by recording spectra after shaking the sample with one drop of D_2O. Chemical shifts are reported relative to DMSO (2.50 ppm) or acetone (2.04 ppm) and coupling constants are given in hertz (Hz).

HPLC—Preparative HPLC was conducted on a Waters 600E solvent delivery system equipped with a 996 photodiode array detector and a Luna C-18(2) column (10 μm 100 Å, 250 × 21.2 mm) (Phenomenex, Torrance, CA). A gradient elution system began with 10% CH_3CN/90% H_2O (containing 0.4% acetic acid) and then linearly increased the proportion of CH_3CN up to 50% in 30 min and 100% in the next 5 min at a flow rate of 9 mL/min. Eluting compounds were monitored at 270 nm. Analysis of reaction mixtures was conducted on a Waters 2690 (Alliance) separation module equipped with Waters 996 photodiode array detector interfaced to a digital Venturis Fx 5100 computer. A 50-μL aliquot of a reaction mixture was injected and compounds were separated on a Luna C-18 (2) column (5 μm, 100

Å, 250 × 4.6 mm, Phenomenex) by using a gradient elution system starting from 10% CH_3CN/90% H_2O (containing 0.25% TFA) and changing linearly to 100% CH_3CN in 30 min at a flow rate of 1 mL/min. Analytical HPLC using electrochemical detection was conducted on a system equipped with dual ESA Model 580 solvent delivery modules, an ESA Model 540 autosampler and a 12-channel CoulArray detector (ESA, Chelmsford, MA). Depurinating adducts from the reaction of chemically formed quinones or enzyme-activated catechols with DNA were separated on a reverse phase Luna C-18 (2) column (5 μm, 100 Å, 250 × 4.6 mm) with two mobile phases, A [$CH_3CN/CH_3OH/H_2O$/buffer, pH 3.7 (15:5:70:10)] and B [$CH_3CN/CH_3OH/H_2O$/buffer, pH 3.7(50:20:20:10)]. The buffer was a 1:1 mixture of 1 M NH_4OAc and 1 M citric acid and pH (3.7) was adjusted with conc NH_4OH. Elution was started with 90%A/10%B and the proportion of B was increased linearly up to 35% in 25 min, eluting isocratically for 5 min and, finally, raising the proportion of B linearly up to 90% in 20 min with a flow rate of 0.5 mL/min at 35 °C. The serial array of 12 coulometric electrodes was set at potentials between −10 and 440 mV (−10, 80, 150, 200, 230, 260, 290, 320, 350, 380, 410, and 440). The system was controlled and data were acquired and processed using the CoulArray software package. Peaks were identified by both retention time and peak height ratios between the dominant peak and the peaks in the two adjacent channels. The depurinating adducts were quantified by comparison of peak response ratios with known amounts of standards. Triplicate samples were analyzed for each data point.

MS—Fast atom bombardment tandem mass spectrometry (FAB-MS) was conducted at the Nebraska Center for Mass Spectrometry (University of Nebraska-Lincoln) using a MicroMass (Manchester, England) AutoSpec high resolution magnetic sector mass spectrometer. Xenon was admitted to the collision cell at a level to attenuate the precursor ion signal by 75%. Data acquisition and processing were accomplished using OPUS software that was provided by the manufacturer (Microcasm). Samples were dissolved in 5–10 μL of methanol; 1-μL aliquots were placed on the sample probe tip along with 1 μL of a 1:1 mixture of glycerol/thioglycerol.

To verify the presence and identity of adducts, the reaction mixtures were analyzed by direct infusion tandem mass spectrometry (MS/MS) using a MicroMass QuattroMicro triple quadrupole mass spectrometer (Waters). A 100-μL aliquot of the reaction mixture was diluted 1:1 in triple distilled H_2O then, passed through a 0.22 μm syringe filter prior to infusion into the mass spectrometer. Flow from the syringe was 5 μL/min directed to an interface at 120 °C using nitrogen as both the desolvation and auxiliary gas. Reaction components were ionized in positive mode with the capillary voltage at 3.5 kV and cone voltage at 25 V. Full scan spectra were obtained between 20 and 700 amu. MS/MS conditions were optimized for each individual peak identified in the full-scan spectra. Argon was used as the collision gas.

For analysis of depurinating adducts, samples were run on a Waters Acquity UPLC equipped with a MicroMass QuattroMicro triple stage quadrupole system (Waters). The 10-μL injections were carried out on a Waters Acquity UPLC™ $BEHC_{18}$ column (1.7 μm, 1 × 100 mm). The instrument was operated in positive electrospray ionization mode. All aspects of system operation, data acquisition and processing were controlled using QuanLynx v4.0 software (Waters). The column was eluted starting with 20% CH_3CN in H_2O (0.1% formic acid) for 1 min at a flow rate of 75 μL/min; raised to 35% CH_3CN in H_2O (0.1% formic acid) in 5 min and then to 80% in 1 min. Ionization was achieved by using the following settings: capillary voltage 3 kV; cone voltage 30–66 V; source temperature 100 °C; desolvation temperature 200 °C with a nitrogen flow of 400 L/hr. MS/MS conditions were optimized for each compound using pure standard adducts and argon as the collision gas. Triplicate samples were analyzed for each data point.

Syntheses

Synthesis of 3′-OH-Z,Z-DIES—Synthesis of 3′-OH-*Z,Z*-DIES was achieved by reacting *Z,Z*-DIES with IBX, as described previously [24]. Yield 75%; ^{1}H NMR (DMSO-d_6): δ 9.35 (s, 1H, Ar-OH, exchangeable with D_2O), 8.81 (s, 1H, Ar-OH, exchangeable with D_2O), 8.75 (s, 1H, Ar-OH, exchangeable with D_2O), 7.12 (d, *J* = 8.79 Hz, 2 H, H-2″,6″), 6.71 (d, *J* = 1.9, 1H, H-2′), 6.63-6.57 (m, 4 H, H-5′,6′,3″,5″), 6.13 (q, *J* = 6.34 Hz, 1 H, CH), 6.08 (q, *J* = 6.82 Hz, 1 H, CH), 1.60 (bt, *J* = 7.32 Hz, 6 H, 2 × CH_3).

Synthesis of DIES-3′,4′-Q—To a stirred solution of 3′-OH-*Z,Z* DIES or 3′-OH-*E,E* DIES (15.4 mg, 0.05 mmol) in acetone (2 mL) was added Ag_2O (76.6 mg, 0.3 mmol) at room temperature. After 15–20 min, the orange-colored solution of quinone was filtered and used as such for further reactions.

Z,Z-DIES-3′,4′-Q—Yield 95%, ^{1}H NMR (acetone-d_6): δ 8.40 (s, 1H, Ar-OH, exchangeable with D_2O), 7.75 (dd, *J* = 2.4, 10.7 Hz, 1 H, H-6′), 7.24 (d, *J* = 8.3 Hz, 2 H, H-2″,6″), 7.02 (q, *J* = 7.32 Hz, 1 H, CH), 6.78 (d, *J* = 8.30 Hz, H-3″,5″), 6.42 (d, *J* = 10.7 Hz, 1 H, H-5′), 6.33 (q, *J* = 6.83 Hz, 1 H, CH), 6.11 (d, J = 2.0 Hz, 1 H, H-2′), 1.84 (d, *J* = 6.83 Hz, 3 H, CH_3), 1.67 (d, *J* = 6.83 Hz, 3 H, CH_3).

E,E-DIES-3′,4′-Q—Yield 99%, ^{1}H NMR (acetone-d_6): δ 8.46 (s, 1H, Ar-OH, exchangeable with D_2O), 7.04 (dd, *J* = 2.4, 10.2 Hz, 1 H, H-6′), 7.00 (d, *J* = 8.3 Hz, 2 H, H-2″,6″), 6.86 (d, *J* = 8.30 Hz, H-3″,5″), 6.36 (d, *J* = 10.2 Hz, 1 H, H-5′), 6.17 (d, J = 1.5 Hz, 1 H, H-2′), 5.75 (q, *J* = 6.84 Hz, 1 H, CH), 5.50 (q, *J* = 6.84 Hz, 1 H, CH), 1.76 (d, *J* = 7.32 Hz, 3 H, CH_3), 1.58 (d, *J* = 6.83 Hz, 3 H, CH_3).

Synthesis of standard depurinating adducts

3′-OH-Z,Z-/E,E-DIES-6′-N3Ade—A freshly prepared solution of *Z,Z*- or *E,E*-DIES-3′,4′-Q (0.05 mmol) in acetone (1 mL) was reacted with a solution of Ade (67.5 mg, 0.5 mmol) in DMF/H_2O/acetic acid (1:1:1, 5 mL) at room temperature for 24 h. The reaction mixture was filtered and solvent was removed at low pressure. The residue was re-dissolved in 2 mL of DMF/CH_3OH (1:1) and analyzed by HPLC as described above. Separation of the reaction mixture was carried out by preparative HPLC, as described above.

3′-OH-Z,Z-DIES-6′-N3Ade—Yield 25%. ^{1}H NMR (DMSO-d_6): δ 9.50 (bs, 1H, Ar-OH, exchangeable with D_2O), 9.40 (bs, 2 H, Ar-OH, exchangeable with D_2O), 8.00 (bs, 2 H, NH_2-Ade), 7.67 (s, 1 H, H-2/8-Ade), 7.62 (s, 1 H, H-2/8-Ade), 6.85 (d, *J* = 8.30 Hz, 2 H, H-2″,6″), 6.82 (s, 1 H, H-5′/2′), 6.60 (d, *J* = 8.30 Hz, H-3″,5″), 6.58 (s, 1 H, H-5′/2′), 5.55 (bs, 2 H, 2 × CH), 1.87 (d, *J* = 7.32 Hz, 3 H, CH_3), 1.39 (d, *J* = 6.83 Hz, 3 H, CH_3). FAB-MS: *m/z* 416.1699 ($[M+H]^+$ $C_{23}H_{21}N_5O_3$, calc. 416.1644).

3′-OH-E,E-DIES-6′-N3Ade—Yield 20%. ^{1}H NMR (DMSO-d_6): δ 9.58 (bs, 1H, Ar-OH, exchangeable with D_2O), 9.35 (bs, 2 H, Ar-OH, exchangeable with D_2O), 7.96 (bs, 2 H, NH_2-Ade), 7.84 (s, 1 H, H-2/8-Ade), 7.66 (s, 1 H, H-2/8-Ade), 7.03 (s, 1 H, H-5′/2′), 6.63 (d, *J* = 8.30 Hz, 2 H, H-2″,6″), 6.63 (s, 1 H, H-5′/2′), 6.39 (d, *J* = 8.30 Hz, H-3″,5″), 5.10 (q, *J* = 6.83 Hz, 1 H, CH), 5.04 (q, *J* = 6.83 Hz, 1 H, CH), 1.39 (d, *J* = 7.32 Hz, 3 H, CH_3), 1.04 (d, *J* = 6.83 Hz, 3 H, CH_3). FAB-MS: *m/z* 416.1735 ($[M+H]^+$ $C_{23}H_{21}N_5O_3$, calc. 416.1644).

3′-OH-Z,Z-/E,E-DIES-6′-N7Gua—A freshly prepared solution of *Z,Z*- or *E,E*-DIES-3′,4′-Q (0.05 mmol) in acetone (1 mL) was reacted with a solution of dG (142.5 mg, 0.5 mmol) in DMF/H_2O/acetic acid (1:1:1, 5 mL) at room temperature for 24 h. The reaction mixture was filtered and solvent was removed at low pressure. The residue was redissolved in 2 mL of

DMF/MeOH (1:1) and analyzed by HPLC as described above. Separation of the reaction mixture was carried out by preparative HPLC as described above.

3′-OH-Z,Z-DIES-6′-N7Gua—Yield 30%. ^{1}H NMR (DMSO-d_6): δ 10.68 (s, 1 H, NH-Gua, exchangeable with D_2O), 9.36 (s, 1 H, Ar-OH, exchangeable with D_2O), 9.32 (s, 1 H, Ar-OH, exchangeable with D_2O), 9.24 (s, 1 H, Ar-OH, exchangeable with D_2O), 7.31 (s, 1 H, H-8-Gua), 6.88 (d, *J* = 8.8 Hz, 2 H, H-2″,6″), 6.69 (s, 1 H, H-5′/2′), 6.60 (d, *J* = 8.30 Hz, 2 H, H-3″,5″), 6.46 (s, 1 H, H-5′/2′), 6.09 (bs, 2 H, NH_2-Gua), 5.64 (bs, 1 H, CH), 5.55 (bs, 1 H, CH), 1.45 (d, *J* = 7.32 Hz, 6 H, 2 × CH_3). FAB-MS: *m/z* 432.1678 ([M+H]$^+$ $C_{23}H_{21}N_5O_4$, calc. 432.1594).

3′-OH-E,E-DIES-6′-N7Gua—Yield 28%. ^{1}H NMR (DMSO-d_6): δ 10.68 (s, 1 H, NH-Gua, exchangeable with D_2O), 9.36 (bs, 3 H, Ar-OH, exchangeable with D_2O), 7.46 (s, 1 H, H-8-Gua), 6.82 (s, 1 H, H-5′/2′), 6.67 (d, *J* = 8.8 Hz, 2 H, H-2″,6″), 6.54 (s, 1 H, H-5′/2′), 6.50 (d, *J* = 8.30 Hz, 2 H, H-3″,5″), 6.03 (bs, 2 H, NH_2-Gua), 5.16 (q, *J* = 6.83 Hz, 1 H, CH), 5.02 (q, *J* = 6.83 Hz, 1 H, CH), 1.41 (d, *J* = 7.32 Hz, 3 H, CH_3), 1.17 (d, *J* = 7.32 Hz, 3 H, CH_3). FAB-MS: *m/z* 432.1647 ([M+H]$^+$ $C_{23}H_{21}N_5O_4$, calc. 432.1594).

3′-OH-4″-OCH$_3$-DES-6′-N3Ade—A freshly prepared solution of 4″-OCH_3-DES-3′,4′-Q[2] (0.05 mmol) in CH_3CN (1 mL) was reacted with a solution of Ade (67.5 mg, 0.5 mmol) in DMF/H_2O/acetic acid (1:1:1, 5 mL) at room temperature for 24 h. The reaction mixture was filtered and solvent was removed at low pressure. The residue was redissolved in 2 mL of DMF/CH_3OH (1:1) and analyzed by HPLC as described before. Separation of the reaction mixture was carried out by preparative HPLC as described before. Yield 35%, ^{1}H NMR (DMSO-d_6): δ 9.74 (bs, 1 H, Ar-OH, exchangeable with D_2O), 9.62 (bs, 1 H, Ar-OH, exchangeable with D_2O), 9.54 (bs, 1 H, Ar-OH, exchangeable with D_2O), 8.91 (s, 1 H, H-2/8-Ade), 8.54 (bs, 2 H, NH_2-Ade), 7.05 (s, 1 H, H-2/8-Ade), 6.88-6.70 (m, 6 H, H-5′,2′, 2″,3″,5″,6″), 3.71 (s, 3 H, OCH_3), 2.23 (m, 2 H, CH_2), 1.74 (m, 2 H, CH_2), 0.61 (m, 6 H, 2 × CH_3). FAB-MS: *m/z* 432.1965 ([M+H]$^+$ $C_{24}H_{25}N_5O_3$, calc. 432.1957).

3′-OH-4″-OCH$_3$-DES-6′-N7Gua—A freshly prepared solution of 4″-OCH_3-DES-3′,4′-Q [24] (0.05 mmol) in CH_3CN (1 mL)was reacted with a solution of dG (142.5 mg, 0.5 mmol) in DMF/H_2O/acetic acid (1:1:1, 5 mL) at room temperature for 24 h. The reaction mixture was filtered and solvent was removed at low pressure. The residue was re-dissolved in 2 mL of DMF/CH_3OH (1:1) and analyzed by HPLC as described before. Separation of the reaction mixture was carried out by preparative HPLC as described before. Yield 32%, 9.43 (bs, 3 H, Ar-OH, NH-Gua), 7.87 (s, 1 H, H-8-Gua), 6.87 (s, 4 H, H-2″,3″,5″,6″), 6.80 (s, 1 H, H-2′/5′), 6.63 (s, 1 H, H-2′/5′), 6.47 (bs, 2 H, NH_2-Gua), 3.72 (s, 3 H, OCH_3), 2.20 (m, 2 H, CH_2), 1.79 (m, 2 H, CH_2), 0.61 (t, J = 7.32 Hz, 6 H, 2 × CH_3). FAB-MS: *m/z* 448.1978 ([M+H]$^+$ $C_{24}H_{25}N_5O_4$, calc. 448.1907).

Covalent binding of quinones to DNA

Z,Z- or *E,E*-DIES-3′,4′-Q (2.5 mg/500 μL of acetone), or 4″-OCH_3-DES-3′,4′-Q (2.5 mg/500 μL of CH_3CN) was mixed with a 10-mL solution of calf thymus DNA (3 mM) in 67 mM sodium-potassium phosphate buffer (pH 7.0) and incubated at 37 °C. After 10 h, DNA was precipitated with two volumes of ethanol, and processed for measurement of stable adducts by the ^{32}P-postlabeling technique [25]. The supernatant, containing depurinating adducts, was dried under low pressure, the residue redissolved in CH_3OH/DMF (500 μL), filtered and semipurified by preparative HPLC. Samples of standard depurinating adducts were first injected in the preparative HPLC to determine the exact retention times of the adducts. The DNA samples were then injected under the same conditions and the fractions that had the retention times of the standards were collected and dried under vacuum for a second HPLC

analysis, which was conducted by using an analytical HPLC equipped with a series of electrochemical detectors and the UPLC-MS/MS technique, as described above.

Covalent binding of enzyme-activated catechols to DNA

3′-OH-DES, 3′-OH-*Z,Z*-DIES or 3′-OH-*E,E*-DIES was incubated with DNA in the presence of Tyr, LP or PHS. The reaction volume in each experiment was 10 ml. In the Tyr-catalyzed reaction, the mixture containing 3mM calf thymus DNA in 67 mM sodium-potassium phosphate (pH 7.0), 3′-OH-DES (2.5 mg/500μL DMSO) and 1 mg of enzyme (2000 units) was incubated at 37 °C for 10 h. For the LP-catalyzed reaction, the mixture containing 3mM calf thymus DNA, 3′-OH-DES (2.5 mg/500μL DMSO), H_2O_2 (0.5 mM) and 1 mg of enzyme (78 units) was incubated at 37 °C for 10 h. For the PHS-catalyzed reaction, the mixture containing 3 mM calf thymus DNA, 3′-OH-DES (2.5 mg/500μL DMSO), 1 ml of methemoglobin (2.95 mg/ml in 75 mM KH_2PO_4, pH 7.5), 1 ml of arachidonic acid (50 mM in ethanol) and 800 μL of PHS (400 units) was incubated at 37 °C for 10 h. After incubation, DNA was precipitated with 2 volumes of ethanol and processed for ^{32}P-postlabeling analysis of stable adducts [25]. The supernatant, containing depurinating adducts, was evaporated, resuspended in CH_3CN, filtered and treated with 10 mg of solid MnO_2 to convert the DES adducts to DIES adducts. After stirring at room temperature for 10 min, the reaction mixture was filtered to remove solid material, CH_3CN was evaporated and the residue was redissolved in 200 μL of CH_3OH/H_2O. The solution was passed through a 5000 MW cut-off filter and analyzed on UPLC-MS/MS by comparing with synthesized depurinating DIES adducts. Control reactions were carried out under identical conditions either with no enzyme or no cofactor. 3′-OH-4″-OCH_3-DES, 3′-OH-*Z,Z*-DIES, and 3′-OH-*E,E*-DIES were reacted with DNA under the same conditions, but without treating the supernatant with MnO_2.

Results

Synthesis of quinones

Due to the presence of the double bond between C3 and C4 of 3′-OH-DES (Fig. 1), its chemical oxidation afforded a highly unstable DES-3′,4′-Q, which cyclized instantaneously to *spiro*-catechol and then to *spiro*-quinone (Fig. 2) [26]. Use of different solvents and reaction conditions did not help to stabilize DES-3′,4′-Q.

On the other hand, the two dehydrogenated derivatives of 3′-OH-DES, 3′-OH-*Z,Z*-DIES and 3′-OH-*E,E*-DIES, were easily converted into their respective DIES-3′,4′-Q, when reacted with silver oxide (Ag_2O) in acetone (Figs. 3 and 4). The structure of the quinones was confirmed by ^{1}H NMR, which along with HPLC analysis, indicated quantitative conversion of the catechols to the corresponding *ortho*-quinones.

Synthesis and structure determination of standard adducts

Reaction of *Z,Z*-DIES-3′,4′-Q (Fig. 3) or *E,E*-DIES-3′,4′-Q (Fig. 4) with Ade or dG led to formation of the N3Ade or N7Gua adduct.

3′-OH-DIES-6′-N3Ade—Reaction of *Z,Z*-DIES-3′,4′-Q or *E,E*-DIES-3′,4′-Q with Ade afforded one major product with some unknown minor impurities, as observed by analytical HPLC. The reaction mixture was purified by using preparative HPLC as described above and structure elucidation was carried out with the help of spectroscopic techniques.

FAB-MS of the adducts showed a protonated molecular ion $[M+H]^+$ at *m/z* 416, which was consistent with addition of the DIES moiety to an Ade base. Furthermore, the presence of the DIES moiety in the ^{1}H NMR spectrum was easily recognized due to their characteristic

correlation patterns of two aliphatic methyl groups as two doublets resonating between δ 1.00 to 1.90 ppm and two olefinic methine protons resonating as a quartet between δ 5.00 to 5.65 ppm, in the ^{1}H NMR spectra. In general, the chemical shifts of a particular proton attached to the *Z,Z*-isomer were found to be upfield with respect to those attached to the *E,E*-isomer. Characteristic signals of H-2′ (d), H-5′ (d), and H-6′ (dd) in the starting catechol [24] were replaced by two sharp singlets around δ 7.03 and 6.63 ppm, which were assigned to H-5′ and H-2′, respectively, indicating that substitution had taken place at C-6′ of the DIES moiety. On the other hand, the presence of two additional sharp singlets around δ 7.84 and 7.66 ppm, assigned to H-8 (Ade) and H-2 (Ade), indicated binding of Ade to DIES. The presence of a broad signal, with integration of two protons around δ 7.95 ppm ruled out the possibility of an exocyclic NH_2 adduct. The bond of Ade *via* the N3 position was established by using detailed HMQC and HMBC analyses (data not shown).

3′-OH-DIES-6′-N7Gua—The reaction of *Z,Z*-DIES-3′,4′-Q or *E,E*-DIES-3′,4′-Q with dG yielded initially two peaks in HPLC analysis (data not shown). Interestingly, the parent ion scan by direct infusion in mass spectrometry of the reaction mixture after 2 h showed the presence of a molecular ion $[M+H]^+$ at *m/z* 548, indicating formation of the 3′-OH-DIES-6′-N7dG adduct. The concentration of this adduct initially increased and then it started to decrease after 5 h and became a trace after 24 h. At the same time, the concentration of the depurinating adduct, 3′-OH-DIES-6′-N7Gua, formed after the loss of deoxyribose from the initial adduct, increased continuously and became constant after 24 h. Formation of dG adducts was already reported when E_2-3,4-Q or HES-3′,4′-Q was reacted with dG [16]. HPLC analysis after 24 h indicated the presence of one major compound with some unknown less polar impurities. The reaction mixture was then separated by using preparative HPLC, as described above.

^{1}H NMR and MS techniques were used for structure elucidation of the N7Gua adduct. FAB-MS analysis of the compounds showed an $[M+H]^+$ ion at *m/z* 432, corresponding to the proposed elemental composition. Again, assignments of chemical shifts corresponding to the DIES moiety were easily accomplished by their characteristic coupling patterns. The most upfield chemical shift around δ 10.68 ppm, assigned to NH-1, as well as two proton resonances around δ 6.00 ppm, assigned to the exocyclic NH_2, were used as diagnostic signals for the formation of the N7Gua adducts [11,17,27]. Moreover, the presence of a one-proton sharp singlet at δ 7.31 (*Z,Z*-isomer) or 7.46 (*E,E*-isomer), assigned to C-8 of Gua, further ruled out the possibility of the formation of adducts with the DIES moiety bound to the C-8 or NH_2 of Gua. Hence, binding of the DIES moiety to Gua occurred *via* the N7 position. Furthermore, the binding site of the DIES moiety at C-6′ was confirmed by the absence of the characteristic d, d, and dd pattern of 3′-OH-DIES [24] (see above), and the appearance of two sharp singlets around δ 6.82 and 6.54 ppm in the ^{1}H NMR spectra of the adducts.

3′-OH-4″-OCH_3-DES-6′-N3Ade and 3′-OH-4″-OCH_3-DES-6′-N7Gua—The rapid conversion of DES-3′,4′-Q to a *spiro*-quinone was due to the presence of the ethylenic double bond between the two rings (Fig. 2), which rendered the DES-3′,4′-Q extremely unstable. To impede rearrangement of the quinone to the *spiro*-quinone, the 4″-OH was methylated (Fig. 5). In this case, oxidation of the catechol to the quinone provided a stable compound that could react with Ade or dG in a 1,4-Michael addition.

Formation of the standard depurinating adducts 3′-OH-4″-OCH_3-DES-6′-N3Ade and 3′-OH-4″-OCH_3-DES-6′-N7Gua (Fig. 5) was accomplished by reacting 4″-OCH_3-DES-3′,4′-Q with Ade or dG, respectively. Structure elucidation was carried out by using NMR and MS techniques as described above. Assignment of the chemical shifts and confirmation of

proposed structures of the adducts were accomplished by following the same spectroscopic analyses as described above.

Covalent binding of quinones to DNA

DES-3′,4′-Q instantaneously formed a *spiro*-quinone. When the *spiro*-quinone was incubated with DNA, it was recovered unreacted with no adduct present in the supernatant (data not shown).

Reaction of *Z,Z*- DIES-3′,4′-Q or *E,E*-DIES-3′,4′-Q with DNA yielded no depurinating adducts (Table 1). The precipitated DNA was analyzed for stable adduct formation by ^{32}P-postlabeling [25] and stable adducts were observed as 1.10 μmol/mol DNA-P with *Z,Z*-DIES-3′,4′-Q, and 0.89 μmol/mol DNA-P with *E,E*-DIES-3′,4′-Q (Table 2).

When 4″-OCH_3-DES-3′,4′-Q was reacted with DNA, two depurinating adducts, 3′-OH-4″-OCH_3-DES-6′-N3Ade and 3′-OH-4″-OCH_3-DES-6′-N7Gua were formed (Fig. 5 and Table 1). The amount of 3′-OH-4″-OCH_3-DES-6′-N7Gua was 32.3 ± 10.6 μmol/mol DNA-P, which was almost double the amount of 3′-OH-4″-OCH_3-DES-6′-N3Ade, 17.7 ± 10.2 μmol/mol DNA-P. Precipitated DNA was then analyzed for stable adducts and the level of unknown adduct formation was 0.59 μmol/mol DNA-P. When the level of stable adducts was compared with the level of depurinating adducts, about 99% of the adducts were depurinating adducts.

Covalent binding of enzyme-activated catechols to DNA

3′-OH-DES was enzymatically activated by PHS, LP or Tyr in the presence of DNA at 37 °C for 10 h. After processing the supernatant as described in the Methods section, the level of depurinating adducts was measured by UPLC-MS/MS. Activation with PHS did not yield any detectable depurinating adducts, and the only product identified was the *spiro*-catechol, observed as *spiro*-quinone (Fig. 2) after oxidation with MnO_2.

Activation with Tyr yielded a very low level of 3′-OH-DIES adducts (5.4 ± 2.2μmol/mol DNA-P for the N3Ade adducts and 8.6 ± 5.5 μmol/mol DNA-P for the N7Gua) compared to activation carried out with LP (28.4 ± 5.2 μmol/mol DNA-P for the N3Ade adducts and 39.6 ± 9.5 μmol/mol DNA-P for the N7Gua), as shown in Table 1. Precipitated DNA was then analyzed for stable adducts, and the level of unknown adducts was 0.71 μmol/mol DNA-P with Tyr-activation, 1.00 μmol/mol DNA-P with LP-activation, and 0.80 μmol/mol DNA-P with PHS-activation. Again, the level of depurinating adduct formation was 95–99% higher with Tyr and LP compared to the level of stable adducts (Table 2).

Incubation of both 3′-OH-*Z,Z*-DIES and 3′-OH-*E,E*-DIES with DNA in the presence of Tyr, LP or PHS produced none of the depurinating adducts 3′-OH-DIES-6′-N7Gua and 3′-OH-DIES-6′-N3Ade (Table 1). However, analysis of the DNA yielded various low amounts of unknown stable adducts, as shown in Table 2.

Discussion

In vitro and *in vivo* data generated from our laboratory over several years [10–15], as well as our recent studies on human urine [28–31], have clearly demonstrated the relevance of the formation of depurinating natural estrogen-DNA adducts to the initiation of breast and prostate cancer. Because the synthetic estrogen DES shares many similarities with the natural estrogen E_2, i.e., it is a potent estrogen and initiates cancer, it is plausible to hypothesize that the initiation of cancer by DES occurs by formation of depurinating adducts. Toward this goal, the first hindrance was the synthesis of the standard depurinating adducts of DES.

Previously, we synthesized depurinating adducts of the natural estrogen E_2 and synthetic estrogen HES, by chemical oxidation of the catechol estrogens to their respective electrophilic quinones, followed by reaction with Ade or dG [11,17,27]. Under similar conditions, chemical oxidation of 3′-OH-DES with Ag_2O in acetone afforded a highly unstable DES-3′,4′-Q, which underwent novel cyclization to *spiro*-quinone (Fig. 2) [26]. Efforts to synthesize the standard depurinating adducts of DES suffered because of the unavailability of DES-3′,4′-Q to react with Ade or dG.

Cyclization of DES-3′,4′-Q to *spiro*-compound takes place in a state where the molecule is free to undergo various rotations with a high degree of freedom. We hypothesized that if free rotations of these chemical bonds were blocked, for instance by a physical complex with DNA, then there would be a chance that the DES-3′,4′-Q might be sufficiently stabilized to react with the DNA. To test this hypothesis, 3′-OH-DES was enzymatically activated in the presence of DNA. Under these conditions the quinone DES-3′4′-Q was stable enough to react specifically at the N3 position of Ade and N7 position of Gua, thus forming the depurinating adducts. This result can only be explained by intercalation of the 3′-OH-DES in the DNA, followed by enzymatic oxidation to its quinone. The N3Ade and N7Gua adducts could be identified by rapidly determining their molecular weight by MS (Fig. 6) before the adducts autoxidized to their corresponding DIES adducts. For further demonstration that these adducts were formed, the freshly obtained DES adducts were oxidized with MnO_2 and converted to their DIES adducts (Fig. 6). The structures of these DIES adducts were elucidated by comparison with the synthesized standard DIES adducts.

To confirm that the DIES-DNA adducts were formed from DES and not from DIES, we treated DNA with DIES-3′,4′-Q or 3′-OH-DIES after enzymatic activation. No depurinating adducts were observed in these experiments (Table 1), which indicated that the DIES-DNA adducts observed following reaction of enzyme-activated 3′-OH-DES with DNA were solely formed from DES and not from DIES.

The lack of formation of depurinating adducts in the reaction of DIES-3′,4′-Q with DNA derives from the chemical structures of these compounds. Data from X-ray crystallography of DES and DIES derivatives indicate that the orientation of the dienic chain of the DIES derivatives is perpendicular to the phenyl planes [32], whereas it is almost linear with the phenyl planes in DES [33]. Thus, the perpendicular dienic chain of DIES derivatives impedes the appropriate intercalation of DIES, but not DES, into DNA. Hence, the quinones of DES give rise to the formation of depurinating adducts, whereas the quinones of DIES do not.

It was surprising to observe the formation of stable adducts even with DIES metabolites. Although DIES metabolites are not suitable candidates for intercalation in DNA, they can generate free radicals. Kalyanaraman and Sealy [34] have studied the formation of free radicals from stilbene catechol estrogens. The DIES radicals generated under the quinone-semiquinone-catechol system may damage DNA, thus giving rise to lesions observed as stable adducts.

Unified mechanism of depurinating estrogen-DNA adduct formation

The totality of experiments on estrogen metabolism [35–41], formation of DNA adducts [10–13,28,30,42,43], mutagenicity [13–15,44,45], cell transformation [46–49] and carcinogenicity [50–53] have led to the hypothesis that certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, react with DNA to cause mutations that lead to the initiation of cancer [13]. Catechol estrogen-3,4-quinones can react with DNA to form the depurinating adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, generating

apurinic sites in the DNA (Fig. 7). Various types of evidence indicate that error-prone repair of these apurinic sites to mutations that can initiate breast cancer [13–15,44,45].

The more abundant formation of depurinating N3Ade and N7Gua adducts obtained from $E_1(E_2)$-3,4-Q versus $E_1(E_2)$-2,3-Q [12] is in line with other studies that indicate the important role of $E_1(E_2)$-3,4-Q in the initiation of cancer [13]. In fact, in animal models 4-$OHE_1(E_2)$ is more carcinogenic than the extremely weak carcinogen 2-$OHE_1(E_2)$ [50–52]. The catechol of DES, when oxidized to its corresponding catechol quinone and reacted with DNA, yields N3Ade and N7Gua adducts analogous to those formed by $E_1(E_2)$-3,4-Q and HES-3′,4′-Q [16,17].

This common mechanism of metabolic activation for natural estrogens and DES (Fig. 7) is extremely important because DES is a proved human carcinogen [2–9]. Furthermore, through the studies reported here, we have demonstrated that intercalation of the catechol estrogen in DNA is the necessary preliminary step to generate specificity in the formation of the depurinating adducts.

Acknowledgments

This research was supported by U.S. Public Health Service grant P01 CA49210 from the National Cancer Institute and the U.S. Army Breast Cancer Research Program grant DAMD 17-03-1-0229. Core support at the Eppley Institute was provided by grant P30 CA36727 from the National Cancer Institute.

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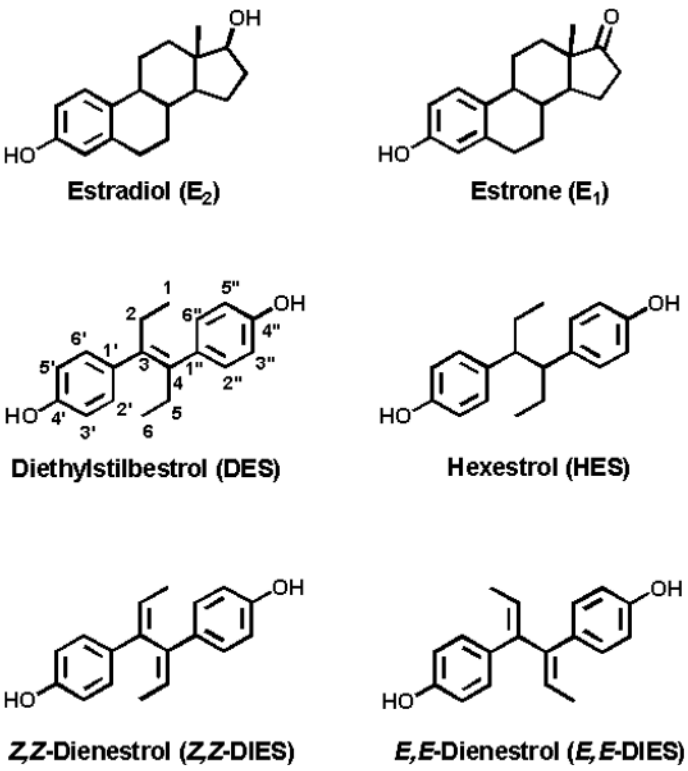

Figure 1.
Structures of natural and synthetic estrogens.

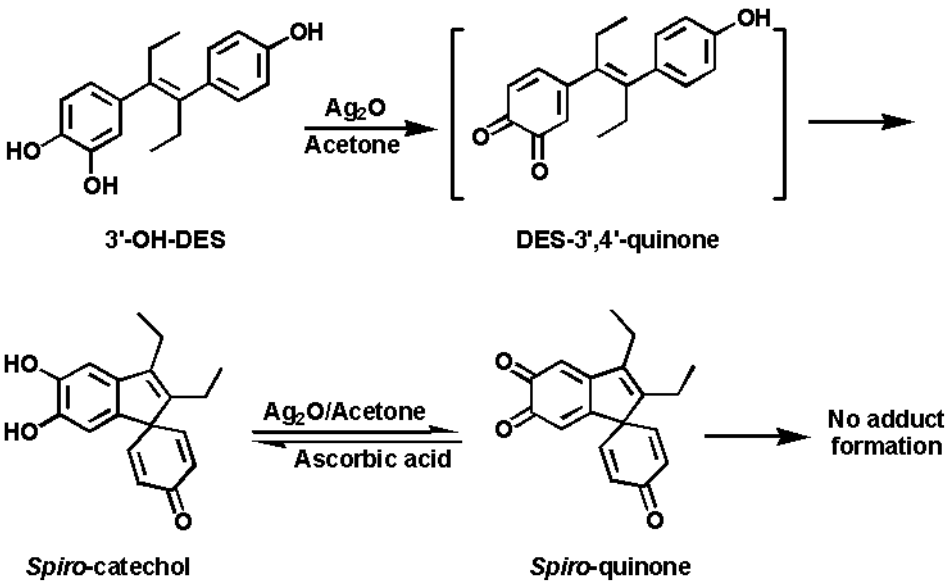

Figure 2.
Formation of *spiro*-quinone by oxidation of 3′-OH-DES.

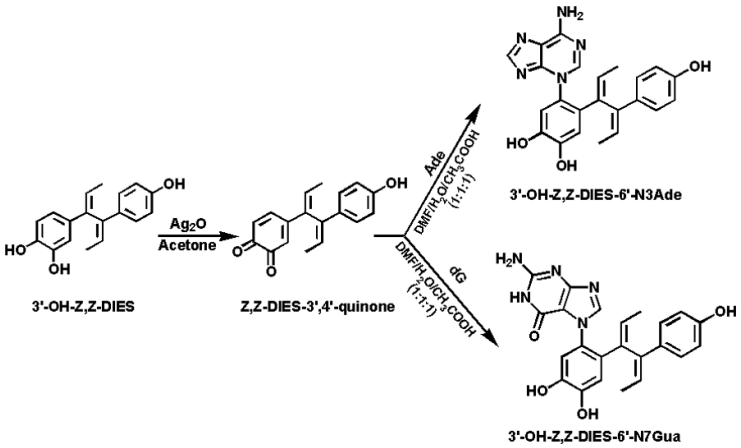

Figure 3.

Oxidation of 3′-OH-*Z*,*Z*-DIES to *Z*,*Z*-DIES-3′,4′-Q and reaction of the quinone with Ade or dG to form N3Ade or N7Gua adducts.

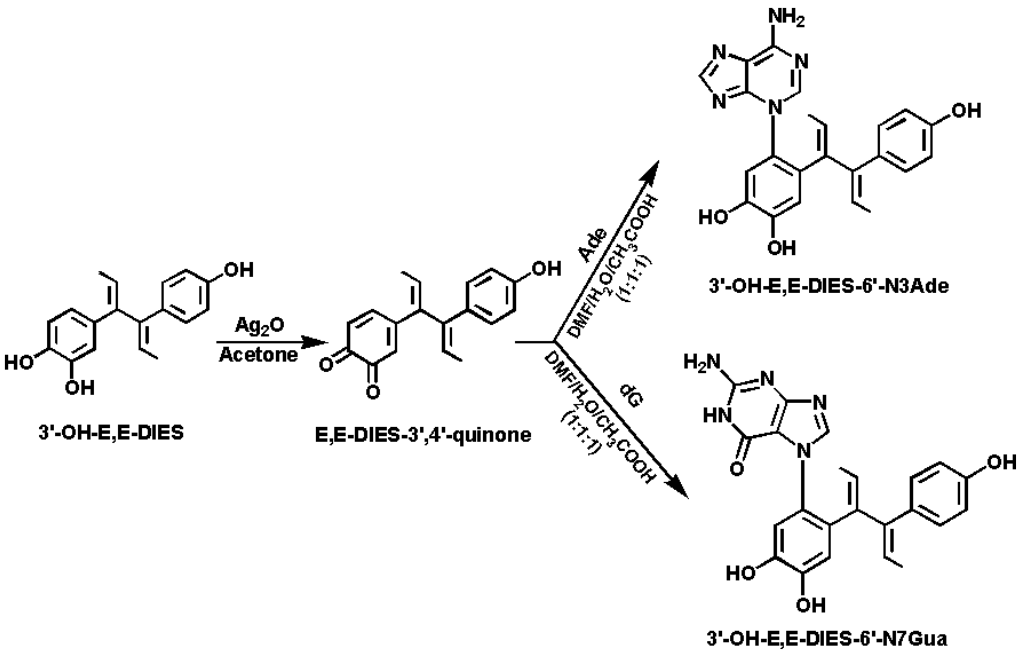

Figure 4.
Oxidation of 3′-OH-*E,E*-DIES to *E,E*-DIES-3′,4′-Q and reaction of the quinone with Ade or dG to form N3Ade or N7Gua adducts.

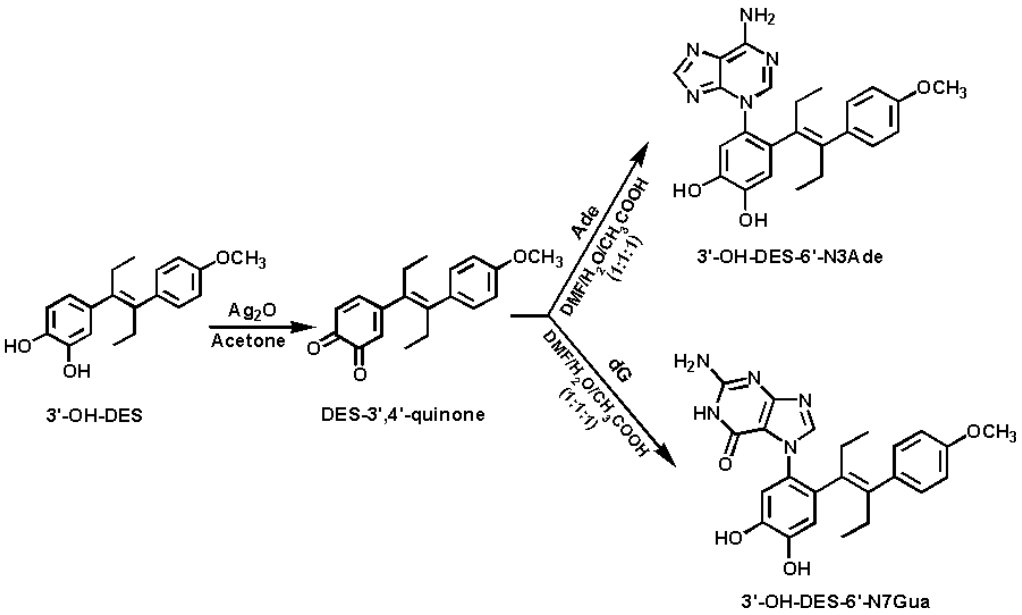

Figure 5.
Oxidation of 3′-OH-4″-OCH_3-DES to 4″-OCH_3-DES-3,4-Q and reaction of the quinone with Ade or dG to form N3Ade or N7Gua adducts.

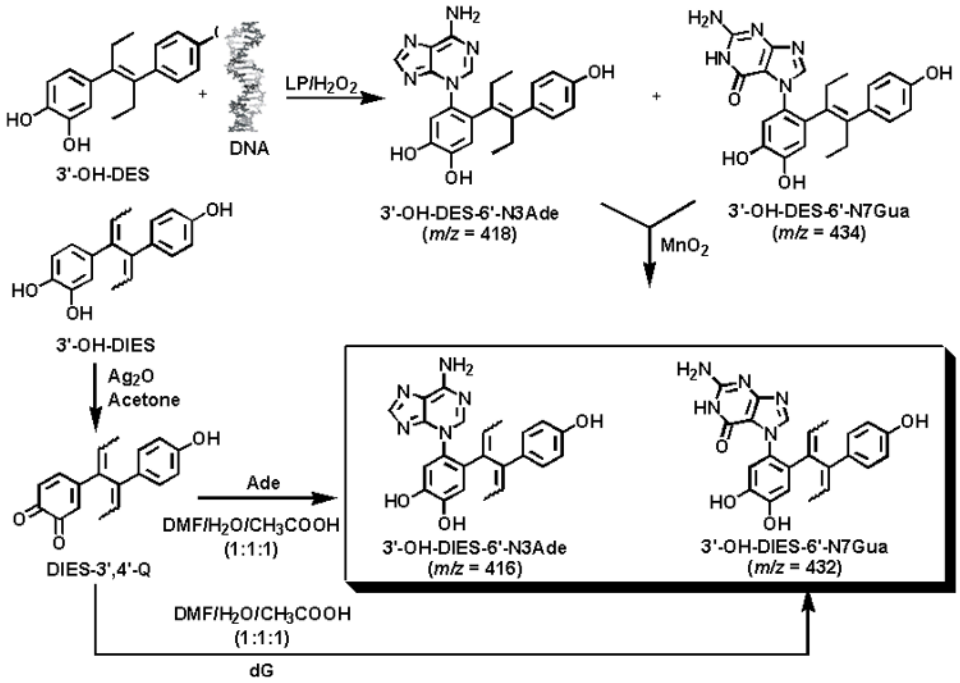

Figure 6.

Formation of depurinating N3Ade and N7Gua adducts of DES following enzymic activation of 3′-OH-DES in the presence of DNA. The DES adducts were oxidized to the corresponding DIES adducts.

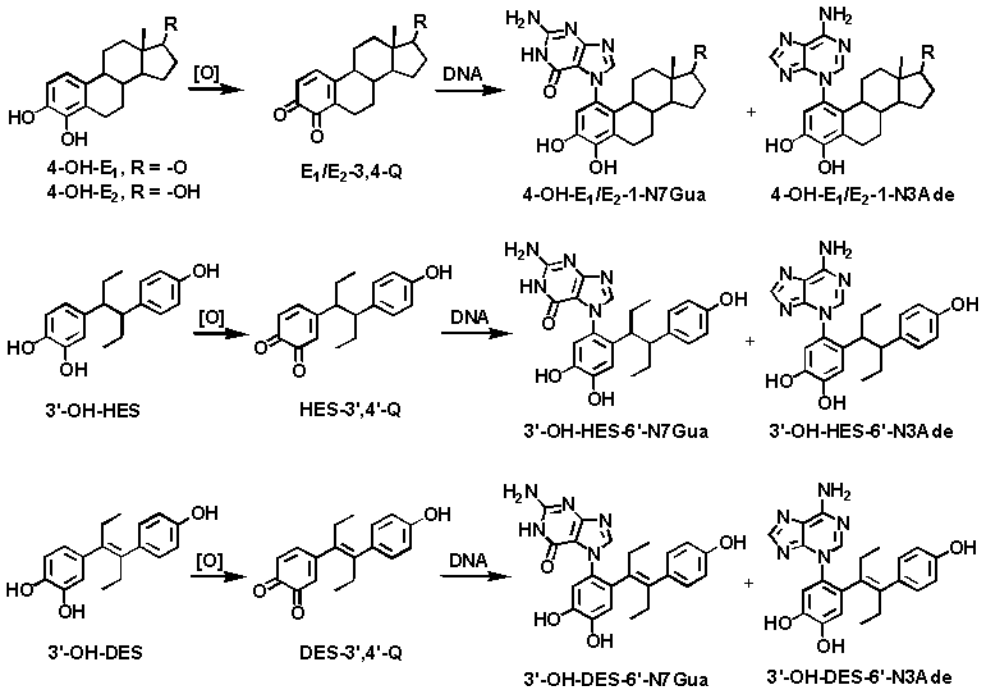

Figure 7.
Unified mechanism of metabolic activation and reaction with DNA of the natural estrogens and the synthetic estrogens HES and DES.

Table 1

Formation of depurinating adducts after incubation of DNA with quinones or enzyme-activated catechols

	Depurinating adducts, μmol/mol DNA-P					
Reactant	**3′-OH-*Z*,*Z*-DIES-6′-N3Ade**	**3′-OH-*Z*,*Z*-DIES-6′-N7Gua**	**3′-OH-*E*,*E*-DIES-6′-N3Ade**	**3′-OH-*E*,*E*-DIES-6′-N7Gua**	**4″-OCH_3-3′-OH-DES-6′-N3Ade**	**4″-OCH_3-3′-OH-DES-6′-N7Gua**
Quinones						
Z,*Z*-DIES-3′,4′-Q	<0.5[a]	<0.5				
E,*E*-DIES-3′,4′-Q			<0.5	<0.5		
4″-OCH_3-DES-3′,4′-Q					17.7 ± 10.2	32.3 ± 10.6
Catechol/enzymes						
3′-OH- DES/Tyr	3.7 ± 2.0[b]	5.8 ± 1.7	1.7 ± 0.2	2.8 ± 1.5		
3′-OH- DES/LP	16.7 ± 2.0	23.8 ± 5.7	11.7 ± 3.2	15.8 ± 3.8		
3′-OH- DES/PHS	<0.5[c]	<0.5	<0.5	<0.5		
3′-OH-*Z*,*Z*- DIES/Tyr	<0.5	<0.5				
3′-OH-*Z*,*Z*-DIES/LP	<0.5	<0.5				
3′-OH-*Z*,*Z*-DIES/PHS	<0.5	<0.5				
3′-OH-*E*,*E*- DIES/Tyr			<0.5	<0.5		
3′-OH-*E*,*E*-DIES/LP			<0.5	<0.5		
3′-OH-*E*,*E*-DIES/PHS			<0.5	<0.5		

[a] Less than the limit of detection of the instrument.

[b] Based on LC-Ms/MS analysis of the reaction mixture, N3Ade and N7Gua adducts of DES were tentatively identified. Their structures were definitely demonstrated after treatment of supernatant with MnO_2 to convert DES adducts to DIES adducts that were compared with the synthesized depurinating adducts.

[c] The major compound found in this experiment was *spiro*-catechol which after oxidation with MnO_2 was observed as *spiro*-quinone.

Table 2

Formation of stable adducts in DNA after incubation with quinones or enzyme-activated catechols

Reactant	Stable adducts, μmol/mol DNA-P
Quinones	
Z,*Z*-DIES-3′,4′-Q	1.10
E,*E*-DIES-3′,4′-Q	0.89
4″-OCH_3-DES-3′,4′-Q	0.59
Catechol/enzymes	
3′-OH- DES/Tyr	0.71
3′-OH- DES/LP	1.00
3′-OH- DES/PHS	0.80
3′-OH-*Z*,*Z*- DIES/Tyr	0.32
3′-OH-*Z*,*Z*-DIES/LP	0.45
3′-OH-*Z*,*Z*-DIES/PHS	0.44
3′-OH-*E*,*E*- DIES/Tyr	0.35
3′-OH-*E*,*E*-DIES/LP	0.47
3′-OH-*E*,*E*-DIES/PHS	0.46

Int J Cancer. Author manuscript; available in PMC 2010 March 15.