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Formation of Two Novel Estrogen Guanine Adducts and HPLC/MS Detection of 4-Hydroxyestradiol-*N*⁷-Guanine in Human Urine

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Abstract

Estrogen-DNA adducts are potential biomarkers for assessing the risk of developing of a number of hormonally-modified cancers, including breast cancer. Formation of the 4-hydroxyestradiol-*N*⁷-guanine (4-OHE₂-*N*⁷-guanine) adduct from reaction of estradiol-3,4-quinone with DNA and its detection *in vivo* has been established. With the ultimate goal of exploring estrogen-DNA adducts as biomarkers in experimental and human investigations, the 4-OHE₂-*N*⁷-guanine was synthesized and preliminary studies demonstrated that this adduct was detectable in all ten female human urine samples examined. Therefore, more extensive investigations were conducted to study this compound's chemical-physical properties and to examine the stability of 4-OHE₂-*N*⁷-guanine under a range of pH conditions that might influence biomarker measurement. Under neutral to alkaline conditions 4-OHE₂-*N*⁷-guanine could completely oxidize to an 8-oxo-guanine derivative. This derivative was isolated by HPLC and mass spectrometry confirmed the oxidized compound by demonstrating the formation of an *m/z* 168 fragment, generated by oxygen addition to guanine. Further, investigation of the 4-OHE₂-*N*⁷-2'-deoxyguanosine nucleoside adduct showed that under alkaline conditions a formamidopyrimidine analogue was produced. The formamidopyrimidine derivative forms from ring opening of the guanine imidazole ring following C-8 oxidation in the *N*⁷,*N*⁹ disubstituted guanine. Formation of both of these oxidized estrogen-guanine DNA adducts has precedent with other chemical agents that covalently bind to the *N*⁷ position in guanine. Therefore, the development and application of methods to measure estrogen-guanine adducts will need to also consider these new adducts and the biological implications of these compounds will need to be explored to determine their contribution to estrogen toxicology.

Introduction

Estrogens are endogenous hormones that are involved in the development and maintenance of reproductive organs, tissue differentiation and gene expression(1). Estrogens are also known contributors to the development of breast cancer through long term exposure. There are two main hypotheses about how estrogen initiates cancer; one through estrogen receptor-mediated processes and the other by DNA damage caused from the interaction of specific estrogen metabolites with DNA(2). The metabolism of estrogen is extremely complex involving many enzymes which produce a multitude of metabolites. One metabolite of toxicological interest is estradiol-3,4-quinone (E₂-3,4-Q) which is formed from the oxidation of the catechol estrogen 4-hydroxyestradiol (4-OHE₂)(3,4). To mitigate the formation of a potentially deleterious quinone, 4-OHE₂ can be methylated by catechol-o-methyltransferase (COMT) creating an

inactive metabolite(5,6). The estradiol-3,4-quinone also reacts directly with DNA to form covalent estrogen-DNA adducts(7). One of these DNA lesions is the 4-hydroxyestradiol- N^7 -guanine (4-OHE₂- N^7 -guanine) adduct (Structure IV, Scheme 1), which undergoes spontaneous depurination at the glycosidic bond creating potentially mutagenic abasic sites(8).

Previous studies have demonstrated that 2,3- and 3,4-catechol estrogens can be oxidized to their respective quinones both chemically and enzymatically(4,7) and it is these reactive electrophiles that directly react with DNA and its constituents forming a variety of estrogen adducts(8,9). As a cellular protective mechanism, the estrogen quinones can also react with other nucleophiles such as glutathione which reduces their reactive burden with DNA(10,11). Although the estrogen-2,3-quinone reacts with DNA to form adducts, the extent of adduct formation was much less when compared to the E₂-3,4-Q(12); however, the estrogen-2,3-quinone forms a stable adduct with guanine which does not depurinate(9). Experimental animal studies found that 4-hydroxyestradiol, but not 2-hydroxyestradiol induces tumor formation (13), supporting the hypothesis that E₂-3,4-Q pathway significantly contributes to tumorigenesis.

The ability to measure the amount of DNA adducts in urine of both humans and experimental models would provide information on DNA damage caused through estrogen metabolism. Furthermore, if there is an association between the levels of these adducts in urine and the risk of developing disease then these compounds would be important biomarkers for risk assessment. To achieve these biomarker goals, it is important to characterize the physical properties of these adducts as a foundation to developing a quantitative method for measuring estrogen-DNA adducts in biological samples. Thus, in the studies reported here, the 4-OHE₂- N^7 -guanine adduct and the analogous isotopically labeled internal standard were synthesized. This adduct was detected in all ten human urine samples examined providing a rationale to examine its chemical properties. These studies revealed that 4-OHE₂- N^7 -guanine can be transformed into at least two oxidative derivatives and these findings will need to be incorporated into developing quantitative molecular dosimetry methods for estrogen-DNA adduct biomarker studies.

Materials and Methods

Chemicals and Reagents

4-Hydroxyestradiol was purchased from Steraloids (Newport, RI). ¹³C-2'-Deoxyguanosine labeled compounds were obtained from Spectra Stable Isotopes (Columbia, MD). ¹³C₅, ¹⁵N₅-2'-Deoxyguanosine-5'-monophosphate, sodium salt, dansyl chloride, nuclease P1, and all HPLC grade reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Mass spectrometer grade solvents and alkaline phosphatase were purchased from VWR (West Chester, PA). All chemicals were used without further purification except for the solvents used for HPLC and mass spectrometric analysis which were filtered through a 2 μm filter and degassed before use. Bond Elut C18 columns were purchased from Varian (Palo Alto, CA). MCX columns were purchased from Waters (Milford, MA)

Synthesis of 4-Hydroxyestradiol- N^7 -Guanine (Structure IV, Scheme 1)

Synthesis of the 4-OHE₂- N^7 -guanine adduct was carried out based on a modification of a previously published method(9). The catechol estrogen, 4-OHE₂ (5 mg, 17.3 μmol), was suspended in acetonitrile (0.5 mL), cooled to 0°C, and activated manganese dioxide (15 mg, 172.5 μmol) was added. The solution was then stirred for 10 min, filtered using a 2 μm filter directly into another solution of 50 % acetic acid in water (1.0 mL) containing 2'-deoxyguanosine (25 mg, 87.6 μmol). This solution was then stirred at ambient temperature for 5 h. Purification of the 4-OHE₂- N^7 -guanine adduct was carried out by HPLC with water and

methanol as solvents as described below. Synthesis of 4-OHE₂-¹³C₅-N⁷-guanine and 4-OHE₂-¹³C₅,¹⁵N₅-N⁷-guanine adduct were carried out in the same way employing ¹³C₅-2'-deoxyguanosine and ¹³C₅,¹⁵N₅-2'-deoxyguanosine-5'-monophosphate sodium salts, respectively. Semi-preparative purification of estrogen-guanine adducts was done using a Waters HPLC with Waters 510 pumps and a Waters 996 PDA UV detector, monitoring at 291 nm, with a Zorbax C18 column (9.4 mm × 250 mm). Purification was achieved with a 3 mL/min flow rate with the following mobile phases and gradient: solvent A, water; solvent B, methanol; initial conditions, 25% B; from 0 to 30 min, linear change to 69% B; from 30 to 33 min, linear change to 100% B; holding at 100% B for 2 min; from 35 to 38 min, linear change to 25% B; from 38 to 50 min, 25% B.

Structural Analysis by Ion Trap Mass Spectrometry/Mass Spectrometry

Samples were introduced into a ThermoFinnigan LCQ Deca ion trap mass spectrometer with a Surveyor HPLC auto sampler and pump system. Data was analyzed with Xcalibur software. Samples were run with a gradient at 50 µL/min using 0.1% acetic acid for buffer A and 0.1% acetic acid in methanol for buffer B. Gradient conditions were the same as above. The mass spectrometer was set to the positive ion mode to acquire both a fullscan mass spectrum with mass range 100–600 amu and for MS/MS daughter ion full scan mass spectra of the protonated molecular ions at m/z 438.5 and 448.5 of the unlabeled and isotopically-labeled adducts, respectively. Mass range was 120–600 amu and a 50% collision energy. The capillary temperature was set at 180°C.

Determination of the Extinction Coefficient for 4-Hydroxyestradiol-N⁷-Guanine

4-OHE₂-N⁷-guanine (0.204 mg, 46.5 µmol) was weighed out in a temperature and humidity monitored room on a micro scale balance. Prior to weighing, the adduct was placed in a vacuum desiccator with Drierite for 72 h to ensure that all moisture was removed from the solid. The 4-OHE₂-N⁷-guanine adduct was initially dissolved in 50 % MeOH (0.5 mL). UV-Vis spectra were taken at four concentrations (0.93 mM, 93.0 µM, 37.2 µM, and 18.6 µM) in 50 % MeOH. Spectra were obtained in triplicate for 93.0 and 37.2 µM. UV-Vis spectra were obtained using a Beckman Coulter DU 800 spectrophotometer. The extinction coefficient at 291 nm in 50% MeOH was calculated to be 1,400 ± 104 M⁻¹cm⁻¹.

Formation of 4-Hydroxyestradiol-N⁷-2'-Deoxyguanosine (Structure III, Scheme 1)

4-OHE₂ (1 mg, 3.48 µmol) was suspended in acetonitrile (100 µL) and cooled to 0°C. Activated MnO₂ (3 mg, 34.5 µmol) was added and the solution was stirred for 10 min. The solution was filtered directly into another solution containing 2'-deoxyguanosine hydrate (5 mg, 18.7 µmol) in 50 % acetic acid in water (200 µL) and stirred at room temperature for 24 h. Aliquots (20 µL) were removed at 0, 1, 3, 5, 24 h and analyzed by HPLC/UV to monitor progress of the reaction. Analysis was carried out on a Thermo Finnigan Surveyor LC pump with Surveyor PDA detector and a Phenomenex Luna C18 column (250 mm × 4.60 mm, 5 µ). Buffer A was water and buffer B was methanol. Gradient conditions were as follows: initial conditions, 100 % A, holding for 2.0 min; from 2.0 to 12.0 min, linear change to 24 % B, holding for 2.0 min; from 14.0 to 37.0 min, linear change to 69 % B; from 37.0 to 39.0 min, linear change to 100 % B, holding for 1.0 min; from 40 to 48 min, linear change to 100 % A. The peak corresponding to 4-OHE₂-N⁷-2'-deoxyguanosine (4-OHE₂-N⁷-dG) was collected and verified by mass spectrometry.

Formation of 4-Hydroxyestradiol-N⁷-2'-Deoxyguanosine Formamidopyrimidine (Structure VI, Scheme 1)

4-OHE₂ (1 mg, 3.48 µmol) was suspended in acetonitrile (100 µL) and cooled to 0°C. Activated MnO₂ (3 mg, 34.5 µmol) was added and the solution stirred for 10 min. The mixture was

filtered directly into another solution containing 2'-deoxyguanosine hydrate (5 mg, 18.7 μ mol) in a solution of 50 % acetic acid in water (200 μ L) and then stirred at room temperature for 1 h. An aliquot (40 μ L) was analyzed by HPLC and the peak corresponding to 4-OHE₂-N⁷-dG was collected. An aliquot (400 μ L) of the purified 4-OHE₂-N⁷-dG was treated with concentrated ammonium hydroxide (10 μ L) at 37°C for 20 min. The solution was neutralized with acetic acid (10 μ L), separated by HPLC and the peak corresponding to 4-hydroxyestradiol-N⁷-2'-deoxyguanosine formamidopyrimidine (4-OHE₂-N⁷-dG fapy) was collected and analyzed by mass spectrometry.

Formation of 4-Hydroxyestradiol-N⁷-Guanine in Calf Thymus DNA

4-OHE₂ (3.0 mg, 10.4 μ mol) was suspended in 200 μ L acetonitrile and cooled to 0°C. Activated MnO₂ (6.0 mg, 69.0 μ mol) was added to the suspension and stirred at 0°C for 10 min. The solution was filtered into a solution of calf thymus DNA (1 mg/mL), sodium/potassium phosphate, pH 7.0 (0.67 mM, total volume 5.0 mL). The solution was covered with aluminum foil and incubated at 37°C with mild shaking for 9 h. Two volumes of cold ethanol (10 mL) was added to the solution and the sample was placed in a -80°C freezer overnight to precipitate the DNA. The solution was centrifuged at 4°C for 15 min (650 rpm) to pellet the DNA. The supernatant, containing the depurinated guanine adduct, was decanted and concentrated by rotary evaporation. The residue was dissolved in a solution of DMF:MeOH (1:1, 1 mL). The peak corresponding to the adduct was collected and analyzed by mass spectrometry.

For the time course of reaction with DNA, 4-OHE₂ (1 mg, 3.47 μ mol) was suspended in acetonitrile (150 μ L) and cooled to 0°C. Activated MnO₂ (3 mg, 34.5 μ mol) was added and the solution stirred for 10 min. The solution was filtered directly into another solution containing calf thymus DNA (2 mg/mL) and sodium/potassium phosphate, pH 7.0 (67 mM). The solution was stirred at 37°C for 24 h. Aliquots (50 μ L) were removed at 0, 1, 2, 5, 10, and 24 h. To precipitate the DNA, three volumes of cold ethanol (150 μ L) were added and the solution was placed on ice for 30 min. The solution was spun at 283 rcf for 1 minute to pellet the DNA. The supernatant was removed and acetic acid (1 μ L) was added to acidify the solution. An aliquot (25 μ L) of supernatant from each sample was removed, spiked with 4-OHE₂-¹³C₅, ¹⁵N₅-N⁷-guanine (1 ng, 2.23 pmol) and the resulting mixture was concentrated under a flow of nitrogen. The volume was brought to 40 μ L with 50% MeOH, 0.5% acetic acid and 20 μ L was injected for mass spectrometry.

Isolation and Analysis of 4-Hydroxyestradiol-N⁷-guanine from Human Urine

Overnight urines from ten women (9 pre- and 1 post-menopausal) were collected one day prior to their scheduled elective reduction mammoplasty as described previously(14). After collection, urines were stored in their home refrigerators. Within 6 h of receiving the urine on the morning of surgery, it was centrifuged and frozen -80°C. Before processing, the urine, was thawed, centrifuged and 1 mg/mL of ascorbic acid added to 2 mL aliquots. Urine was diluted with 4% acetic acid (2 mL), spiked with 4-OHE₂-¹³C₅, ¹⁵N₅-N⁷-guanine (1 ng, 2.23 pmol), and passed through a MCX column by the following conditions: The column was washed with MeOH (4 mL) and 1% acetic acid (4 mL). The sample was loaded on the column and the sample vial was rinsed with 1% acetic acid (1 mL) and the rinse solution was also loaded on the column. The column was washed sequentially with 1% acetic acid (10 mL), 95% MeOH (10 mL), and 2.5% NH₄OH in MeOH (1 mL). 4-OHE₂-N⁷-guanine was eluted with a mixture of pyridine/H₂O/MeOH (5/15/80, 5 mL). The elution fraction was concentrated under a flow of nitrogen to $\geq$ 200 μ L. The sample was diluted with MeOH/acetic acid/H₂O (50/0.5/49.5, 200 μ L) followed by 1% acetic acid (5 mL) and passed through a Bond Elut column using the following conditions: The column was washed with MeOH (4 mL), and 1% acetic acid (4 mL). The diluted sample was loaded on the column and the sample vial rinsed with 1% acetic acid (1 mL) which was also loaded on the column. The column was washed with 1% acetic acid (4

mL), MeOH/acetic acid/H₂O (5/1/96, 4 mL), MeOH/acetic acid/H₂O (15/1/84, 4 mL) and finally eluted with MeOH/acetic acid/H₂O (50/0.5/49.5, 5 mL). The elution fraction was concentrated under a flow of nitrogen to ~100 μ L. DMF (10 μ L) was added and the solution further concentrated to >10 μ L being careful to not take the sample to complete dryness. The concentrated sample was derivatized by diluting with a solution of 0.2 M Na₂CO₃, pH 10.7 containing 1 mg/mL ascorbic acid (150 μ L) followed by a solution of 1 mg/mL dansyl chloride in acetone (100 μ L) following a method described previously(15). Handling of dansyl chloride was done under reduced lighting. The sample was heated at 55°C for 20 min. The solution was diluted with 1% acetic acid (4 mL) and passed through another Bond Elut column with the following conditions: The column was washed with acetonitrile (4 mL) and 1% acetic acid (4 mL). The sample was loaded on the column and the vial washed with 1% acetic acid (1 mL) which was also loaded on the column. The column was sequentially washed with 15% acetonitrile, 1% acetic acid (3 mL) and the sample eluted with 90% acetonitrile (3 mL) followed by acetonitrile (1 mL). The two elution fractions were combined and concentrated under a flow of nitrogen to >20 μ L. The volume was adjusted to 40 μ L with MeOH/acetic acid/H₂O (50/0.5/49.5). Liquid chromatography/tandem mass spectrometry was carried out using a ThermoFinnigan Surveyor liquid chromatography pump with a Surveyor autosampler coupled to a ThermoFinnigan TSQ Quantum Ultra triple quadrupole mass spectrometer. For chromatographic separation, a Varian Pursuit 5 C18 column (150 mm $\times$ 1.0 mm) was utilized. The flow rate was set to 75 μ L/min and buffer A was 5% acetonitrile, 0.1% acetic acid and buffer B was acetonitrile containing 0.1% acetic acid. Gradient conditions were as follows: initial conditions, 3% B; from 0 to 10.0 min, linear change to 80% B; from 10.0 to 20.0 min, linear change to 90% B; from 20.0 to 21.0 min, linear change to 3% B, holding for 9 min to re-equilibrate the column to initial conditions. The mass spectrometer was operated in the positive ion electrospray mode and was set to monitor simultaneously two MS/MS transitions: dansylated 4-OHE₂-N⁷-guanine [M + H]⁺ = 671 $\rightarrow$ 437 and dansylated 4-OHE₂-¹³C₅, ¹⁵N₅-N⁷-guanine [M + H]⁺ = 681 $\rightarrow$ 447.

Results

Mass Spectrometry of 4-Hydroxyestradiol-N⁷-Guanine

Initial experiments were conducted to prepare estradiol-3,4-quinone (E₂-3,4-Q), an electrophilic species that was subsequently reacted with 2'-deoxyguanosine leading to the formation of 4-OHE₂-N⁷-guanine. The 4-OHE₂-N⁷-guanine isolated by HPLC was used to determine a molar extinction coefficient of 1,400 M⁻¹cm⁻¹ in 50% MeOH at 291 nm. To produce isotopically labeled DNA adducts, E₂-3,4-Q was reacted with ¹³C- and ¹⁵N labeled 2'-deoxyguanosine. Positive ion electrospray full scan daughter ion mass spectra of the 4-OHE₂-N⁷-guanine, 4-OHE₂-¹³C₅-N⁷-guanine, and 4-OHE₂-¹³C₅, ¹⁵N₅-N⁷-guanine protonated molecular ions were obtained, and the fragmentation pattern of the non-isotopically labeled adduct, [M + H]⁺ = 438.5, was consistent with the literature(9,16,17) (Figure 1A). A collision energy of 46% was sufficient to fragment the 4-OHE₂-N⁷-guanine into daughter fragments at 152.2, 272.3, 286.3, 298.3, 312.4, 420.3, and 436.4 mass units with 4, 100, 7, 20, 27, 41, and 69% relative abundance, respectively, with 18% relative abundance of the parent ion remaining (Figure 1A). 4-OHE₂-¹³C₅-N⁷-guanine [M + H]⁺ = 443.5 produced daughter fragments of 157.1, 277.2, 291.2, 303.2, 313.4, 425.2, 441.4 mass units with 3, 100, 10, 17, 25, 27, and 18% relative abundance, respectively, with 0.5% relative abundance of the parent ion remaining (Figure 1B). 4-OHE₂-¹³C₅, ¹⁵N₅-N⁷-guanine [M + H]⁺ = 448.5 produced the daughter fragments 162.2, 282.2, 296.2, 308.3, 314.4, 430.3, 446.4 mass units with 5, 100, 9, 16, 26, 25, and 15% relative abundance, respectively with 0.5% relative abundance of the parent ion remaining (Figure 1C).

Detection of 4-Hydroxyestradiol- N^7 -guanine in Female Human Urine

To verify our ability to detect 4-OHE₂- N^7 -guanine in human urine, samples from ten females were spiked with isotopically labeled internal standard and analyzed by mass spectrometry. The adducts isolated from these urine samples were derivitized prior to mass spectrometry to form the dansylated product. This process resulted in a higher molecular weight, reduced background and increased ionization during analysis. Two forms/MS transitions were monitored: 4-OHE₂- N^7 -guanine $[M + H]^+ = 671 \rightarrow 437$ and the isotopically labeled internal standard $[M + H]^+ = 681 \rightarrow 447$. Both the estrogen-guanine adduct and the isotopically labeled internal standard eluted at 14.3 min (Figure 2A, 2B) confirming the presence of the 4-OHE₂- N^7 -guanine adduct in the urine sample. This adduct was detected in all 10 samples analyzed. Estimation using a semi-quantitative analysis of the mean level of 4-OHE₂- N^7 -guanine in these samples was 190 ± 100 pg/mL urine with a range of 70 – 300 pg/mL urine. Encouraged by these results, we undertook a detailed examination of the chemical stability of these adducts, required for the development of a quantitative method.

Stability of 4-Hydroxyestradiol- N^7 -Guanine under Aqueous Conditions

Since the ultimate goal of this work is to measure the 4-OHE₂- N^7 -guanine and other estrogen DNA adducts in biological samples, the stability of this adduct was examined by incubation, at pH 5.3, 6.5, and 9.4, over time at 37° C. While there was no discernable degradation detected by HPLC/UV after 48 h at pH 5.3, raising the pH to 6.5 resulted in a slow degradation of the adduct with less than half remaining at 96 h. This decomposition was accelerated at pH 9.4 with almost no detectable adduct present after 24 h. At the two higher pHs, there was concomitant formation of a new, more polar, product detected by HPLC/UV. This new compound was isolated and analyzed by mass spectrometry and was determined to be a heretofore undescribed DNA adduct with a parent molecular ion ($[M + H]^+ = 454.5$) 16 mass units higher than 4-OHE₂- N^7 -guanine, indicative of oxygen addition. MS/MS analysis (Figure 3) revealed a daughter ion fragment of 168 mass units with no fragment ion corresponding to guanine (m/z 152), consistent with formation of an estrogen-8-oxo-guanine adduct. Further experiments using radiolabeled 4-OHE₂- N^7 -guanine revealed a subsequent degradation of the estrogen-8-oxo-guanine adduct into an even more polar compound that eluted in the void volume on HPLC (not shown). The structures of these later compounds have yet to be explored.

Formation of 4-Hydroxyestradiol- N^7 -2'-Deoxyguanosine Formamidopyrimidine from 4-Hydroxyestradiol- N^7 -2'-Deoxyguanosine

Formation of 4-OHE₂- N^7 -dG, the nucleoside precursor to 4-OHE₂- N^7 -guanine was examined by HPLC and mass spectrometry. Aliquots of the reaction between E₂-3,4-Q and 2'-deoxyguanosine were studied at various time points by HPLC/UV. At the initial time point, the only chromatographic peak in the HPLC profile was E₂-3,4-Q. At 1 h, there was a decrease in this peak, which continued to decline to undetectable level at 24 h (not shown). Additionally, at 1 hour there was formation of another peak. This new compound was collected and analyzed by mass spectrometry using both MS and MS/MS. Mass spectrometric analysis confirmed this compound as 4-OHE₂- N^7 -dG (Figure 4A and 4B). The formation of this nucleoside adduct reached its maximum at 3 h, followed by a slight decline at 5 h with a further decrease to undetectable levels at 24 h. HPLC and mass spectrometry revealed a parallel increase in the 4-OHE₂- N^7 -guanine adduct starting at 1 h and increasing over time consistent with depurination of the nucleoside adduct.

Previous studies with aflatoxin B₁- N^7 -guanine adducts showed that the imidazole ring also was susceptible to ring opening to form a formamidopyrimidine (fapy) analogue that restabilized the glycosidic bond and inhibited depurination(18). Similar experiments were carried out to determine whether this phenomenon occurred with the 4-OHE₂- N^7 -dG adduct. The purified estrogen-nucleoside adduct was incubated with ammonium hydroxide and

analyzed by HPLC/UV. A previously unidentified polar peak from this reaction was collected and analyzed by mass spectrometry. From this unresolved HPLC peak, two compounds were identified by MS/MS and MS/MS/MS. The putative estrogen fapy adduct with $[M+H]^+ = 572.4$ was analyzed by MS/MS and produced a daughter ion fragment at m/z 455.3, corresponding to loss of the deoxyribose moiety (Figure 4C). In addition, MS/MS/MS analysis was carried out on the m/z 455.3 daughter ion fragment (Figure 4D) and two sets of peaks were identified; 437.3, 409.3 and 167.9, 140.1. The mass difference between the two peaks in both sets was 28 mass units, indicating loss of a CO molecule. Loss of CO likely comes from the amide functional group that was formed from opening of the imidazole ring in the guanine moiety. These data support the formation of the fapy adduct derivative from 4-OHE₂-N⁷-dG.

Further examination of the reaction products by mass spectrometry revealed a protonated molecular ion at m/z 570.6, which upon MS/MS analysis fragmented to a daughter ion peak at m/z 454.2 (Figure 5A). The daughter ion fragment at m/z 454.2 encompasses two separate structures (I and II in Figure 5A), both having identical mass but differing in the location of an aldehyde group. In structure II, the aldehyde group has migrated from guanine to the estrogen moiety. Again, MS/MS/MS was carried out on the daughter fragment m/z 454.2 giving the fragmentation pattern shown in Figure 5B. Comparing this to the fragmentation seen when MS/MS/MS analysis was conducted on the m/z 572→455 transition (Figure 4C and 4D), there were similarities. Both spectra contain daughter ion peaks at m/z 168 and m/z 140 corresponding to the fapy-guanine moiety and loss of CO from fapy-guanine as seen in structure (I) in Figure 5A. In contrast, analysis of the m/z 572 peak resulted in daughter ion fragments at m/z 409.3 and m/z 437.3 whereas analysis of the m/z 570 peak resulted in daughter ion fragments at m/z 408.3 and m/z 436.3. The disparity of two mass units in the parent molecular ion peak was attributed to oxidation of the catechol to its respective quinone resulting in $[M+H]^+ = 570.1$. The difference of one mass unit in the daughter fragments resulted from initial migration of the CO moiety from guanine to the estrogen forming structure (II) shown in Figure 5A. From structure (II) there was loss of water followed by loss of CO producing fragments m/z 436.3 and m/z 408.3 respectively (Figure 5B). Collectively these data are consistent with the formation of a heretofore undetected fapy adduct.

Reaction of E₂-3,4-Q with Calf Thymus DNA

Reaction of E₂-3,4-Q with calf thymus DNA was carried out to examine whether the estrogen guanine adducts described above for dG also occur in DNA. HPLC/UV analysis of the reaction mixture showed a peak corresponding to the retention time of 4-OHE₂-N⁷-guanine. This compound was collected, and mass spectrometry revealed a parent molecular ion peak identical to the authentic standard $[M+H]^+ = 438.5$. MS/MS of this material induced the same fragmentation pattern as authentic standard (not shown). This result confirmed the identity of the peak as the 4-OHE₂-N⁷-guanine adduct and also demonstrated that the adduct does form in the reaction between E₂-3,4-Q and calf thymus DNA *in vitro* and that depurination occurs.

Time course experiments were conducted to determine the rate of 4-OHE₂-N⁷-guanine formed in DNA (Figure 6). Aliquots of the E₂-3,4-Q calf thymus DNA reaction mixture were removed at various time points and measured by isotope dilution mass spectrometry. There was a substantial amount of adduct detected at the initial time point (225 pmol/mg calf thymus DNA) which was sampled immediately after the reaction was started but then underwent a 30 min precipitation to isolate the DNA from solution; however, there was no adduct detected in control samples containing just calf thymus DNA (not shown). 4-OHE₂-N⁷-guanine adduct formation continued to increase over the course of the experiment resulting in over 400 pmol adduct per mg calf thymus DNA by 24 h.

Formation of oxidized 4-OHE₂-N⁷-guanine was also investigated in *in vitro* DNA binding experiments. Supernatant from the reaction between E₂-3,4-Q and calf thymus DNA was

analyzed by MS/MS in the single reaction monitoring mode monitoring for 454.5 → 436.8. A small peak with an area close to the limit of detection corresponding to the oxidized 4-OHE₂-N⁷-guanine adduct was found but the relative amount of this oxidized adduct compared to 4-OHE₂-N⁷-guanine detected in the supernatant was less than 1% (not shown).

The formation of the fapy derivative in calf thymus DNA was also investigated. The estrogen quinone E₂-3,4-Q was allowed to react with DNA for various times before raising the pH of the reaction mixture to induce imidazole ring opening. Samples were analysed by either HPLC/UV or mass spectrometry. Unidentified peaks in the HPLC chromatograms were collected and further analyzed by mass spectrometry, but no fapy derivative was detected. To confirm that fapy adducts could form under the conditions used, aflatoxin B₁-N⁷-guanine modified DNA, synthesized as previously published(19), was subjected to the same treatment as in the experiments aimed at forming the estrogen-fapy adducts. These samples were analyzed by mass spectrometry and both the aflatoxin B₁ adduct and its fapy derivative were detected confirming that the conditions employed for fapy formation were valid. Therefore, there appeared to be a distinct difference in the capacity of aflatoxin N⁷-guanine compared to estrogen N⁷-guanine adducts in DNA to form stable fapy derivatives.

Discussion

Metabolism of catechol estrogens to their respective quinone forms, such as the estrogen-3,4-quinone, is known to result in formation of adenine and guanine adducts in DNA. Several of these adducts rapidly depurinate from DNA resulting in abasic sites(7–9). Based on studies in animals, the estrogen-3,4-quinones are thought to be contributors to the carcinogenicity of estradiol, making it important to the study the ultimate fate of these metabolites(20). In addition, since the depurinating adducts give an indication of the amount of induced DNA damage from estrogen-related exposure, they are good candidates for biomarkers of exposure to genotoxic estrogen metabolites. Other N⁷-guanine adducts, such as the aflatoxin B₁-N⁷-guanine adduct have been used as biomarkers of exposure to aflatoxin B₁, a known carcinogen, in both animal and human studies(19,21). An obligatory step in the development of the 4-OHE₂-N⁷-guanine adduct as a reliable biomarker of estrogen exposure, and ultimately a determinant of risk for the development of breast cancer and other hormonally driven cancers, is to understand its chemical and physical properties.

The studies presented here show that the base adduct 4-OHE₂-N⁷- guanine is unstable under neutral and basic conditions but relatively stable under acidic conditions. The instability of this adduct under neutral or alkaline conditions made it susceptible to oxidation at the guanine moiety forming an 8-oxo-derivative. The novel 8-oxo-adduct was also unstable under the conditions used to detect it and promoted its further degradation into a more polar compound. This outcome was not surprising since it has been documented that the oxidation potential of an oxidized guanine is much lower than guanine itself(22–24). Under acidic conditions where 4-OHE₂-N⁷-guanine was stable, the oxidized adduct was not detected. These experiments suggest that the 4-OHE₂-N⁷-guanine adduct may not be a terminal metabolite of estrogen exposure. Therefore, if this adduct is to be used as a quantitative biomarker, samples potentially containing the adduct need to be acidified at the time of sample collection to help prevent degradation during storage prior to future analysis.

An alternative pathway to depurination of the estrogen-guanine adduct is opening of the imidazole ring in guanine. Studies to explore this possibility led to detection of another novel adduct, a formamidopyrimidine analogue. In contrast to the estrogen-8-oxo-guanine adduct, which was a base derivative, the estrogen-guanine fapy adduct retained its sugar moiety forming an estrogen-nucleoside analogue. Formamidopyrimidine derivatives have also been detected with aflatoxin-guanine adducts(25–27), which are stable in DNA and may require

cellular repair for removal. If the estrogen-formamidopyrimidine adduct is formed *in vivo*, it could lead to mutations or incomplete replication resulting in an altered genome which may enhance the carcinogenic properties of E₂-3,4-Q. The experiments presented provide evidence that the metabolic profile of estrogen may not be fully elucidated and that there are other possible candidates contributing to the carcinogenicity of estrogen exposure. Experiments designed to measure and quantify only the non-oxidized estrogen-guanine adducts in a biological sample may be underestimating their level due to the formation of these identified oxidative analogues, especially if preparation and storage of urine is not carefully controlled.

Also reported herein, is the synthesis of stable isotopically labeled 4-OHE₂-N⁷-guanine that will be used as an internal standard in the development and implementation of a mass spectrometric method designed to quantitatively measure 4-OHE₂-N⁷-guanine in human urine. Previous studies have used HPLC with electrochemical detection(17,28) or liquid chromatography/mass spectrometry(29–31) to measure estrogen-DNA adducts in various biological samples, but without the use of a stable isotopically labeled internal standard. Using 4-OHE₂-¹³C₅,¹⁵N₅-N⁷-guanine as internal standard, we estimated that the mean level of 4-OHE₂-N⁷-guanine in ten female human urine samples was 190 ± 100 pg/mL urine with a range of 70–300 pg/mL urine. Such levels of urinary 4-OHE₂-N⁷-guanine are within the range of reported literature values(31).

In conclusion, the development and application of a quantitative method to accurately measure estrogen-guanine adducts will require investigation of not only the parent estrogen-guanine adducts but also their respective novel oxidative derivatives presented here. The biological implications of these compounds will need to be explored further to determine their contribution to estrogen toxicology.

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References

1. Wotiz HH, Davis JW, Lemon HM, Gut M. Studies in Steroid Metabolism V. The Conversion of Testosterone-4-¹⁴C to Estrogens by Human Ovarian Tissue. *J. Biol. Chem* 1956;222:487–495. [PubMed: 13367020]
2. Yager JD, Davidson NE. Estrogen Carcinogenesis in Breast Cancer. *The New England Journal of Medicine* 2006;354:270–282. [PubMed: 16421368]
3. Nelson SD, Mitchell JR, Dybing E, Sasame HA. Cytochrome P-450-Mediated Oxidation of 2-Hydroxyestrogens to Reactive Intermediates. *Biophys. Res. Commun* 1976;70:1157–1165.
4. Abul-Hajj YJ. Synthesis of 3,4-Estrogen-*O*-Quinone. *J. Steroid Biochem* 1984;21:621–622. [PubMed: 6513561]
5. Letsinger RL, Caruthers MH, Miller PS, Ogilvie KK. Studies on the Directive *O*-Methylation of Catechol Estrogens. *J. Am. Chem. Soc* 89:7147–7148. [PubMed: 6064359]
6. Suzuki E, Saegusa K, Matsuki Y, Nambara T. Assay of Enzymatic *O*-Methylation of Catechol Estrogens by High-Performance Liquid Chromatography with Coulometric Detection. *Journal of Chromatography, Biomedical Applications* 1993;617:221–225. [PubMed: 8408387]
7. Dwivedy I, Devanesan P, Cremonesi P, Rogan E, Cavalieri E. Synthesis and Characterization of Estrogen 2,3- and 3,4-Quinones. Comparison of DNA Adducts Formed by the Quinones Versus Horseradish Peroxidase-Activated Catechol Estrogens. *Chem. Res. Toxicol* 1992;5:828–833. [PubMed: 1336990]
8. Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Goodne J, Ramanathan R, Cerny RL, Rogan EG. Molecular Origin of Cancer:

- Catechol Estrogen-3,4-Quinones as Endogenous Tumor Initiators. *Proc. Nat. Acad. Sci. U.S.A* 1997;94:10937–10942.
9. Stack DE, Byun J, Gross ML, Rogan EG, Cavalieri EL. Molecular Characteristics of Catechol Estrogen Quinones in Reactions with Deoxyribonucleosides. *Chem. Res. Toxicol* 1996;9:851–859. [PubMed: 8828920]
 10. Zahid M, Gaikwad NW, Rogan EG, Cavalieri EL. Inhibition of Depurinating Estrogen-DNA Adduct Formation by Natural Compounds. *Chem. Res. Toxicol* 2007;20:1947–1953. [PubMed: 18039013]
 11. Cao K, Stack DE, Ramanathan R, Gross ML, Rogan EG, Cavalieri EL. Synthesis and Structure Elucidation of Estrogen Quinones Conjugated with Cysteine, N-Acetylcysteine and Glutathione. *Chem. Res. Toxicol* 1998;11:909–916. [PubMed: 9705753]
 12. Zahid M, Kohli E, Saeed M, Rogan E, Cavalieri E. The Greater Reactivity of Estradiol-3,4-quinone vs Estradiol-2,3-quinone with DNA in the Formation of Depurinating Adducts: Implications for Tumor-Initiating Activity. *Chem. Res. Toxicol* 2006;19:164–172. [PubMed: 16411670]
 13. Liehr JG, Fang W-F, Sirbasku DA, Ari-Ulubelen A. Carcinogenicity of Catechol Estrogens in Syrian Hamsters. *J. Steroid Biochem* 1986;24:353–356. [PubMed: 3009986]
 14. Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, Chen M-SA, Stierer T, Garrett-Mayer E, Argani P, Davidson NE, Talalay P, Kensler TW, Visvanathan K. Preclinical and Clinical Evaluation of Sulforaphane for Chemoprevention in the Breast. *Carcinogenesis* 2007;28:1485–1490. [PubMed: 17347138]
 15. Xu X, Keefer L, Ziegler R, Veenstra T. A Liquid Chromatography-Mass Spectrometry Method for the Quantitative Analysis of Urinary Endogenous Estrogen Metabolites. *Nat. Protoc* 2007;2:1350–1355. [PubMed: 17545972]
 16. Devanesan P, Todorovic R, Zhao J, Gross ML, Rogan EG, Cavalieri EL. Catechol Estrogen Conjugates and DNA Adducts in the Kidney of Male Syrian Golden Hamsters Treated with 4-Hydroxyestradiol: Potential Biomarkers for Estrogen-Initiated Cancer. *Carcinogenesis* 2001;22:489–497. [PubMed: 11238191]
 17. Todorovic R, Devanesan P, Higginbotham S, Zhao J, Gross ML, Rogan EG, Cavalieri EL. Analysis of Potential Biomarkers of Estrogen-Initiated Cancer in the Urine of Syrian Golden Hamsters Treated with 4-Hydroxyestradiol. *Carcinogenesis* 2001;22:905–911. [PubMed: 11375897]
 18. Croy RG, Wogan GN. Temporal Patterns of Covalent DNA Adducts in Rat Liver After Single and Multiple Doses of Aflatoxin B₁. *Cancer Res* 1981;41:197–203. [PubMed: 7448760]
 19. Egner PA, Groopman JD, Wang J-S, Kensler TW, Freiesen MD. Quantification of Aflatoxin-B₁-N⁷-Guanine in Human Urine by High-Performance Liquid Chromatography and Isotope Dilution Tandem Mass Spectrometry. *Chem. Res. Toxicol* 2006;19:1191–1195. [PubMed: 16978023]
 20. Cavalieri E, Chakravarti D, Guttentplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T. Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers: Implications for Biomarkers of Susceptibility and Cancer Prevention. *Biochim. Biophys. Acta* 2006;1766:63–78. [PubMed: 16675129]
 21. Walton M, Egner P, Scholl PF, Walker J, Kensler TW, Groopman JD. Liquid Chromatography Electrospray-Mass Spectrometry of Urinary Aflatoxin Biomarkers: Characterization and Application to Dosimetry and Chemoprevention in Rats. *Chem. Res. Toxicol* 2001;14:919–926. [PubMed: 11453740]
 22. Sheu C, Foote CS. Reactivity Toward Singlet Oxygen on a 7,8-Dihydro-8-oxoguanosine ("8-Hydroxyguanosine") Formed by Photooxidation of a Guanosine Derivative. *J. Am. Chem. Soc* 1995;117:6439–6442.
 23. Burrows CJ, Muller JG. Oxidative Nucleobase Modifications Leading to Strand Scission. *Chem. Rev* 1998;98:1109–1151. [PubMed: 11848927]
 24. Steenken S, Jovanovic SV, Bietti M, Bernhard K. The Trap Depth (in DNA) of 8-Oxo-7,8-dihydro-2'-deoxyguanosine as Derived from Electron-Transfer Equilibria in Aqueous Solution. *J. Am. Chem. Soc* 2000;122:2373–2374.
 25. Swenson DH, Miller EC, Miller JA. Aflatoxin B₁-2,3-Oxide: Evidence for its Formation in Rat Liver *In Vivo* and by Human Liver Microsomes *In Vitro*. *Biophys. Res. Commun* 1974;60:1036–1043.

26. Stark AA, Essigmann JM, Demain AL, Skopek TR, Wogan GN. Aflatoxin B₁ Mutagenesis, DNA Binding, and Adduct Formation in *Salmonella Typhimurium*. Proc. Nat. Acad. Sci. U.S.A 1979;76:1343–1347.
27. Groopman JD, Kensler TW. Molecular Biomarkers for Human Chemical Carcinogen Exposures. Chem. Res. Toxicol 1993;6:764–770. [PubMed: 8117914]
28. Lu F, Zahid M, Saeed M, Cavalieri EL, Rogan EG. Estrogen Metabolism and Formation of Estrogen-DNA Adducts in Estradiol-Treated MCF-10F Cells The Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Induction and Catechol-O-Methyltransferase Inhibition. J. Steroid Biochem. Mol. Biol 2007;105:150–158. [PubMed: 17582757]
29. Markushin Y, Gaikwad N, Zhang H, Kapke P, Rogan EG, Cavalieri EL, Trock BJ, Pavlovich C, Jankowiak R. Potential Biomarker for Early Risk Assessment of Prostate Cancer. The Prostate 2006;66:1565–1571. [PubMed: 16894534]
30. Saeed M, Rogan E, Fernandez SV, Sheriff F, Russo J, Cavalieri E. Formation of Depurinating N3Adenine and N7Guanine Adducts by MCF-10F Cells Cultured in the Presence of 4-Hydroxyestradiol. International Journal of Cancer 2007;120:1821–1824.
31. Gaikwad NW, Yang L, Muti P, Meza JL, Pruthi S, Ingle JN, Rogan EG, Cavalieri EL. The Molecular Etiology of Breast Cancer: Evidence From Biomarkers of Risk. International Journal of Cancer 2008;122:1949–1957.

Figure 1.

Full scan MS/MS daughter ion mass spectra of 4-OHE₂-N⁷-guanine and 2 stable isotope-labeled analogues. (A) 4-OHE₂-N⁷-guanine [M + H]⁺ = 438.4. (B) 4-OHE₂-¹³C₅-N⁷-guanine [M + H]⁺ = 443.2. (C) 4-OHE₂-¹³C₅,¹⁵N₅-N⁷-guanine [M + H]⁺ = 448.4.

Figure 2. Selected Reaction Monitoring chromatograms for HPLC-MS/MS analysis of one female human urine sample. (A) $[M + H]^+ = 671 \rightarrow 437$ The peak at retention time = 14.3 min corresponds to the dansyl derivative of 4-OHE₂-N⁷-guanine. (B) $[M + H]^+ = 681 \rightarrow 447$ The internal standard peak at retention time = 14.3 min corresponds to the dansyl derivative of 4-OHE₂-¹³C₅,¹⁵N₅-N⁷-guanine.

Figure 3.

Full scan MS/MS daughter ion mass spectrum of the oxidized 4-OHE₂-N⁷-guanine adduct [M + H]⁺ = 454.5.

Figure 4.

Full scan MS/MS and MS/MS/MS daughter ion mass spectra of 4-OHE₂-N⁷-dG and its fapy derivative. (A) MS/MS of 4-OHE₂-N⁷-dG [M + H]⁺ = 554.1. (B) MS/MS/MS of 4-OHE₂-N⁷-dG [M + H]⁺ = 554.1 → 438.3. (C) MS/MS of the fapy analogue of 4-OHE₂-N⁷-dG [M + H]⁺ = 572.1. (D) MS/MS/MS of the fapy analogue [M + H]⁺ = 572.1 → 455.3.

Figure 5.

Full scan MS/MS and MS/MS/MS daughter ion mass spectra of estradiol-3,4-quinone-*N*⁷-2'-deoxyguanosine. (A) MS/MS of the quinone form of the fapy analogue $[M + H]^+ = 570.1$. The parent compound fragmented into two different daughter structures (I) and (II), each with $[M + H]^+ = 454.2$ (B) MS/MS/MS of the quinone form of the fapy analogue.

Figure 6. 4-OHE₂-N⁷-guanine adduct formation from E₂-3,4-Q reacting with calf thymus DNA. Aliquots from the reaction solution were removed and analyzed by isotope dilution mass spectrometry. Experiments were done in triplicate and error bars represent $\pm$ standard deviation.

Scheme 1.

Depurination, oxidation, and fapy formation from 4-hydroxyestradiol-*N*⁷-2'-deoxyguanosine. A positive charge on the imidazole ring in guanine results in destabilization of the glycosidic bond of 4-hydroxyestradiol-*N*⁷-2'-deoxyguanosine (III) leading to depurination. The depurinated adduct, 4-hydroxyestradiol-*N*⁷-guanine (IV) undergoes subsequent oxidation of the guanine forming the novel oxidized analogue (V). Alternatively, under basic conditions, the positive charge on (III) is quenched by attack from a hydroxide ion leading to ring opening of the imidazole ring resulting in formation of the also novel fapy analogue (VI). The ring opening leading to formation of the fapy analogue results in re-stabilization of the glycosyl bond evidenced by retention of the sugar moiety.