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Inhibition of Depurinating Estrogen−DNA Adduct Formation by Natural Compounds

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Specific metabolites of estrogens, catechol estrogen-3,4-quinones, if produced in relatively large amounts, can become chemical carcinogens by reacting with DNA to form predominantly depurinating DNA adducts. Estradiol (E_2)-3,4-quinone (Q) reacts with DNA to form predominantly the depurinating DNA adducts, 4-hydroxyestradiol (OHE_2)-1-N3Ade and 4-OHE_2-1-N7Gua. The depurinating adducts induce mutations by error-prone repair. We have conducted a study in which selected natural chemopreventing agents, *N*-acetylcysteine (NAcCys), melatonin, reduced lipoic acid, and resveratrol, have been tested for their ability to prevent the reaction of E_2-3,4-Q with DNA. When DNA was incubated with E_2-3,4-Q or lactoperoxidase-activated 4-OHE_2 in the presence of an antioxidant, the formation of the N3Ade and N7Gua adducts was reduced. E_2-3,4-Q or lactoperoxidase-oxidized 4-OHE_2 (87 μM final concentration) was incubated with calf-thymus DNA and one of the antioxidants at different ratios (1:0, 1:0.3, 1:1, and 1:3 with respect to E_2-3,4-Q or 4-OHE_2) at 37 °C. After 10 h, the DNA was precipitated, and the supernatant was analyzed by using ultraperformance liquid chromatography/tandem mass spectrometry (LC/MS/MS). As anticipated, resveratrol and melatonin did not affect the formation of the depurinating adducts when E_2-3,4-Q was reacted with DNA in their presence. On the other hand, NAcCys and lipoic acid (reduced form) showed a significant inhibition of the formation of the depurinating adducts by E_2-3,4-Q. With reaction of lactoperoxidase-activated 4-OHE_2 with DNA, resveratrol achieved the highest level of inhibition, NAcCys and reduced lipoic acid produced moderate inhibition, and melatonin had the least inhibition. These results demonstrate that all four selected compounds can inhibit the formation of depurinating estrogen−DNA adducts and set the stage for studies of their ability to inhibit adduct formation and malignant transformation in mammary epithelial cells. This approach is highly useful for identifying agents to prevent the initiation of human cancers, especially breast and prostate cancer.

Introduction

Experiments on the metabolism of the estrogens estrone (E_1)[1] and estradiol (E_2) (*1–5*), the formation of depurinating estrogen−DNA adducts (*6–12*), mutagenicity (*12–16*), cell transformation (*17–19*), and carcinogenicity (*20–23*) have suggested that specific estrogen metabolites can become endogenous chemical carcinogens by reaction with DNA to initiate breast, prostate, and other cancers. These metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q) and, to a much lesser extent, catechol estrogen-2,3-quinones, form depurinating DNA adducts, 4-hydroxyestrone(estradiol)[4-OHE_1(E_2)]-1-N3Ade, 4-OHE_1(E_2)-1-N7Gua (Figure 1), and 2-OHE_1(E_2)-6-N3Ade (not shown in Figure 1) (*6–12*). Apurinic sites resulting from the formation of these adducts can undergo error-prone repair to generate the critical mutations that initiate breast, prostate, and other cancers (*12–14*). This initiating mechanism can occur in both hormone-dependent and -independent tissues. Elimination of the initiating step, namely, the reaction of catechol estrogen quinone (CE-Q) with DNA, should enable us to prevent a variety of human cancers, including breast and prostate.

This paradigm of cancer initiation by estrogens hinges on estrogen metabolism that involves a disrupted homeostatic balance between activating and deactivating pathways. Several factors can unbalance estrogen homeostasis, namely, the equilibrium between estrogen-activating and -deactivating pathways with the scope of averting the reaction of endogenous CE-Q with DNA (*12*). This is presented in Figure 1 for the major pathway leading to the initiation of cancer by estrogens.

In view of our hypothesized mechanism in the initiation of breast, prostate, and other cancers, there is the possibility of preventing the critical steps, namely, the formation of CE-Q and their reactions with DNA. Such prevention could be obtained with natural compounds, such as *N*-acetylcysteine (NAcCys), lipoic acid, melatonin, and resveratrol (Figure 2). These compounds can prevent oxidative and/or electrophilic damage to DNA by inhibiting the formation of the electrophilic CE-Q and/or reacting with them. Some of these natural chemopreventing agents could also act by modulating estrogen-activating enzymes such as cytochrome P450 (CYP) 1B1 or inducing protective enzymes such as quinone reductase.

The antimutagenic and anticarcinogenic properties of NAcCys (Figure 2) are attributed to multiple protective mechanisms, including its nucleophilicity, antioxidant activity, and inhibition of DNA adduct formation (*24, 25*). Hydrolysis of NAcCys by acylase in the liver and gut yields cysteine (Cys), the precursor to the formation of intracellular glutathione (GSH), guaranteeing replenishment of the critical tripeptide. Changes in GSH

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[1] Abbreviations: CE-Q, catechol estrogen quinone; Cys, cysteine; DNA-P, DNA-phosphate; E_1, estrone; E_2, estradiol; E_2-3,4-Q, estradiol-3,4-quinone; IBX, 2′-iodoxybenzoic acid; LP, lactoperoxidase; NAcCys, *N*-acetylcysteine; OHE_2, hydroxyestradiol; PDA, photodiode array; TFA, trifluoroacetic acid.

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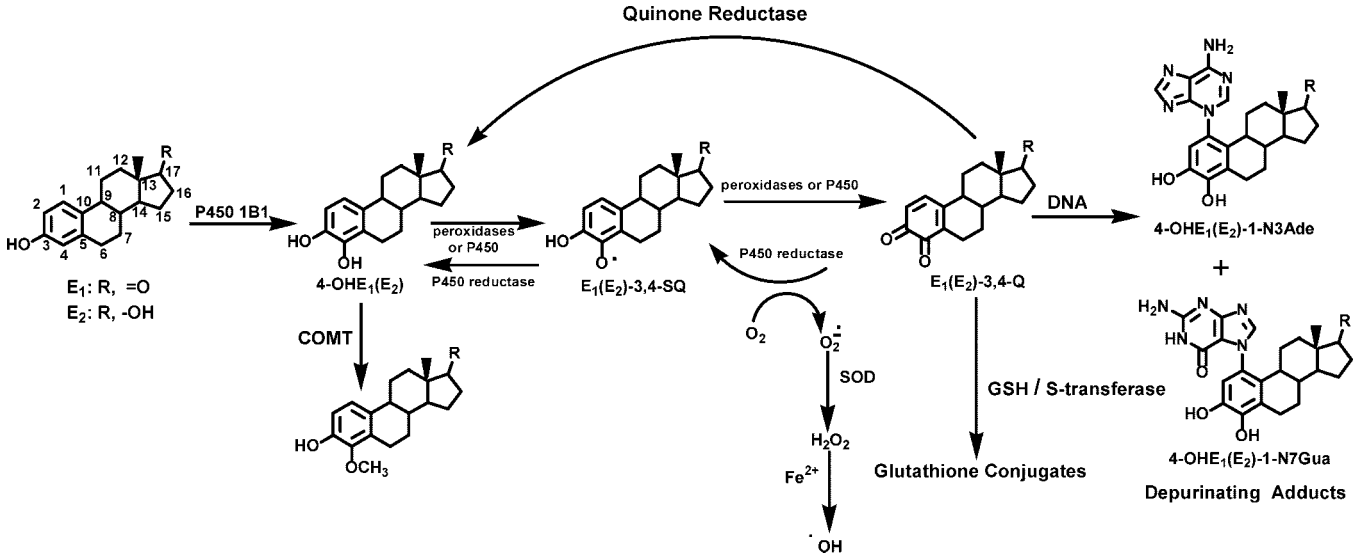

Figure 1. Metabolism of E_2 to 4-OHE_2 (4-hydroxyestradiol) and formation of depurinating adducts in the major pathway leading to cancer initiation.

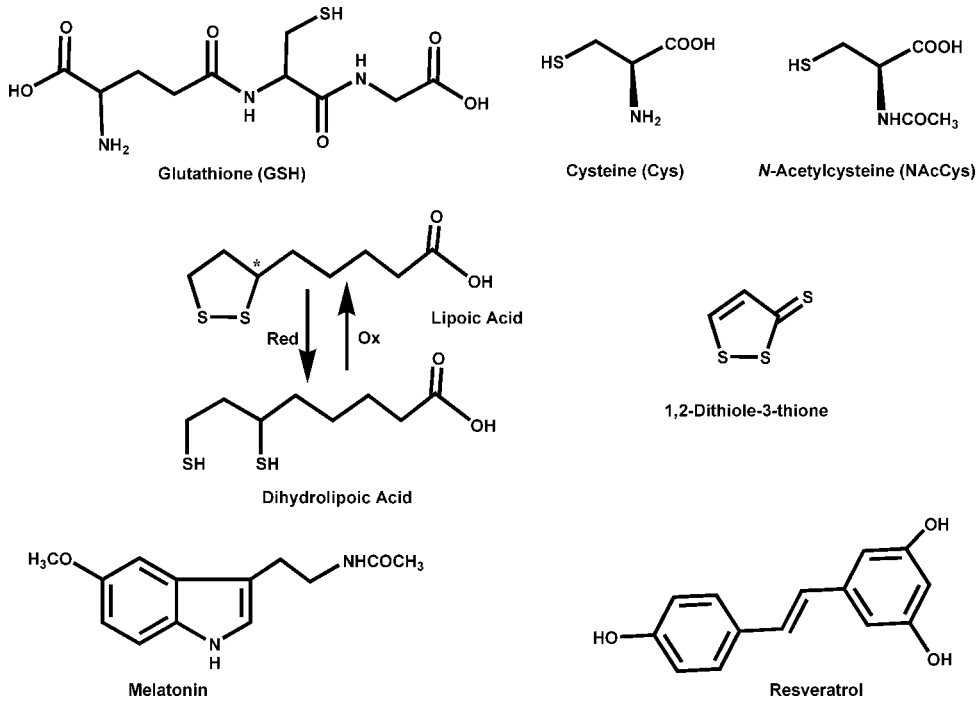

Figure 2. Structures of preventing agents.

homeostasis have been implicated in the etiology and progression of a variety of human diseases, including cancer (*26*). GSH cannot be used as a preventive agent because it does not cross cell membranes. The use of Cys as a preventive agent is limited by its toxicity in humans. In contrast, NAcCys has very low toxicity and can cross the blood−brain barrier (*24, 25*). NAcCys reacts with the electrophilic $E_1(E_2)$-3,4-Q (*27, 28*) to prevent their reaction with DNA. Like Cys, NAcCys operates as an antioxidant in reducing catechol estrogen semiquinones to catechol estrogens (*29*).

Lipoic acid is a natural compound present in most prokaryotic and eukaryotic cells. It can be found in our diet, in meat, liver, heart, spinach, broccoli, and tomatoes. The antioxidant properties of lipoic acid are related to the dithiolane structure, a strained five-membered ring (Figure 2). The relatively high energy content of the disulfide group in lipoic acid is responsible for its reactivity with oxidizing species (*30*). The antioxidant activity of reduced lipoic acid, dihydrolipoic acid (Figure 2), which is always present biologically, is greater than that of lipoic acid. Lipoic acid/dihydrolipoic acid exhibits free radical (superoxide and hydroxyl radicals) scavenger properties where oxidative stress is involved. Lipoic acid readily crosses the blood−brain barrier (*31*). The structure and chemical properties of lipoic acid resemble those of 1,2-dithiole-3-thione (Figure 2), which induces the estrogen-protective enzyme quinone reductase (*32*). Therefore, lipoic acid could exert its preventive effect by not only reducing oxidative stress but also reducing the levels of CE-Q, both by reacting with them and by inducing quinone reductase (*33*).

Melatonin is a direct free radical scavenger and antioxidant (*34*). Melatonin is a tryptophan derivative (Figure 2) and is produced in the pineal gland. There is evidence for its formation in other organs, such as the ovary, lens, gastrointestinal tract, etc. (*35*). Melatonin crosses the blood−brain barrier and the

placenta (*36, 37*). As a direct free radical scavenger, melatonin interacts with highly toxic hydroxyl radicals, with a rate constant equivalent to those of other efficient radical scavengers (*38*). Antioxidative enzymes are stimulated by melatonin; these include superoxide dismutastase, GSH peroxidase, and GSH reductase (*39*).

Resveratrol, 3,5,4′-trihydroxy-*trans*-stilbene (Figure 2), is found in foods, including grapes, peanuts, and wine. This compound exerts chemopreventive effects in diverse in vitro and in vivo systems (*40, 41*). Resveratrol was first shown to function as an antioxidant and antimutagenic agent, thus acting as an anti-initiation agent in cancer (*42*). These properties are attributed to the easy hydrogen abstraction from the 4′-OH bond, with the formation of a hydroxyl radical. This easy abstraction is a consequence of the greater resonance stabilization energy of the intermediate (*42*). Resveratrol is also a modulator of CYP1B1 (*43*) and an inducer of quinone reductase (*44*). Resveratrol was found to inhibit dioxin-induced expression of CYP 1A1 and 1B1 (*45*). Therefore, the preventive effects of resveratrol, its modulating effects on estrogen-activating enzymes, and its protective effects by inducing quinone reductase suggest that this compound should be an excellent candidate for protection against estrogen-induced cancer.

A series of studies are necessary to understand the preventive effects of these compounds. To this end, their preventive properties are first being investigated in vitro, as reported in this article, and, second, in cell culture, in which estrogen-induced transformation of mammary epithelial cells is inhibited (*46*). In addition, analysis of estrogen compounds in human urine (*47*) can be used to determine the ability of these compounds to balance estrogen metabolism.

Experimental Procedures

Chemicals and Reagents. MnO_2, 2-iodobenzoic acid, oxone, ascorbic acid, ammonium acetate, formic acid, sodium phosphate, DMSO-d_6, CH_3CN (HPLC grade), H_2O_2, NAcCys, GSH, Cys, lipoic acid, melatonin, resveratrol, and lactoperoxidase (LP, from bovine milk) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Calf thymus DNA was purchased from USB (Cleveland, OH), and CH_3OH was from Merck KGaA (Darmstadt, Germany). Bond Elute Certify II SPE cartridges were purchased from Varian (Palo Alto, CA). Ultrafree-MC centrifugal filters (5000 MW cutoff) were purchased from Millipore (Bedford, MA). Activated MnO_2 was prepared as previously described (*48*). Lipoic acid was reduced to dihydrolipoic acid by using $NaBH_4$ (*49*).

4-OHE_2 was synthesized by reacting E_2 with 2′-iodoxybenzoic acid (IBX) and then separating the mixture of 2-OHE_2 and 4-OHE_2 by HPLC, as described (*50*). To synthesize E_2-3,4-Q, 4-OHE_2 (0.25 mg, 0.87 μmol) was dissolved in 200 μL of CH_3CN and stirred at 0 °C. Then, activated MnO_2 (0.75 mg, 8.62 μmol) was slowly added. After 15 min, the yellowish green solution was filtered through a Gelman acrodisc and saved on dry ice until further use. Under these conditions, the yield of E_2-3,4-Q was nearly 100%, which was verified by analytical HPLC as described below. Depurinating adducts (4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua) were synthesized by published procedures (*8, 51*).

The formation and yield of E_2-3,4-Q was confirmed by analytical HPLC, which was conducted on a Waters (Milford, MA) 2690 (Alliance) Separations Module equipped with a Waters 996 photodiode detector array (PDA) by using a Luna-2 C-18 column (5 μm, 120 Å, 250 mm × 4.6 mm, Phenomenex, Torrence, CA). A linear gradient of 30% CH_3CN/70% H_2O [0.4% trifluoroacetic acid (TFA)] to 100% CH_3CN in 15 min at a flow rate of 1 mL/min was used with UV detection at 280 and 432 nm. The disappearance of the 4-OHE_2 peak at 5.85 min and the appearance of a new peak at 5.75 min suggested the quantitative conversion of catechol to its quinone.

Formation of Depurinating Adducts by Reaction of E_2-3,4-Q or LP-Activated 4-OHE_2 with DNA. Various concentrations of E_2-3,4-Q (870, 87, or 8.7 μM final concentration, 0.2 mL of CH_3CN) were mixed with DNA (3 mM in 0.067 M sodium potassium phosphate buffer, pH 7.0) with or without NAcCys (1:1 with E_2-3,4-Q) in a total volume of 10 mL and incubated at 37 °C for 10 h. In addition, various concentrations of LP-activated 4-OHE_2 (870, 87, or 8.7 μM final concentration, 0.2 mL of CH_3CN) were mixed with DNA (3 mM in 0.067 M sodium potassium phosphate buffer, pH 7.0) with or without NAcCys (1:1 with 4-OHE_2) in a total volume of 10 mL and incubated at 37 °C for 10 h. After 10 h, the DNA was precipitated with two volumes of ethanol, and the supernatant was centrifuged and condensed to 5 mL by evaporation. After the pH of all samples was adjusted to 8 with 1 M potassium phosphate, the volume was adjusted to 10 mL. The samples were passed through Sep Pac Certify II cartridges using minimal vacuum, which were pre-equilibrated by sequentially passing 1 mL of methanol, distilled water, and potassium phosphate buffer (100 mM), pH 8. Adducts, which were retained by the C8 matrix, were then eluted with 1 mL of CH_3OH: CH_3CN:H_2O:CF_3COOH (8:1:1:0.1). The resulting eluates were further concentrated by evaporation in a speed-vac apparatus, then resuspended in 100 μL of CH_3OH:H_2O (1:1), and finally passed through 5000 MW cutoff filters. Each reaction was carried out at least three times, and the data are the average of replicate determinations.

Preventive Effect of Natural Compounds on Covalent Binding of E_2-3,4-Q or LP-Activated 4-OHE_2 with DNA. Freshly prepared E_2-3,4-Q (87 μM final concentration, 0.2 mL of CH_3CN) was added to a solution of calf thymus DNA (3 mM in 0.067 M sodium potassium phosphate buffer, pH 7.0) mixed with or without different ratios (1:0, 1:0.3, 1:1, and 1:3) of preventive compound [NAcCys, GSH, Cys, lipoic acid (oxidized and reduced form), melatonin, or resveratrol]. After thorough mixing, the reaction was incubated at 37 °C. After 10 h, the samples were processed as described above.

For the LP-catalyzed reactions, the 10 mL reaction mixture containing 3 mM calf thymus DNA, 87 μM 4-OHE_2 (0.25 mg in 0.2 mL of CH_3CN), 0.5 mM H_2O_2, and 1 mg of enzyme (97 units) was incubated at 37 °C for 10 h individually, as well as in the presence of different ratios of antioxidant [NAcCys, GSH, Cys, lipoic acid (oxidized and reduced form), melatonin, or resveratrol]. After precipitation of the DNA, the supernatant was used for analysis of depurinating adducts, as described above. Control reactions were carried out under identical conditions with either no enzyme or no cofactor.

Analysis of Depurinating Adducts by Ultraperformance LC/MS/MS. The resulting samples were analyzed by using a Waters Acquity ultraperformance liquid chromatography apparatus, monitored with a MicroMass QuattroMicro triple stage quadrupole mass spectrometer (Waters, Milford, MA) in the electrospray positive ionization mode, using the supplied QuanLynx v4.0 software. Samples (10 μL aliquots) were injected into a BEHC18 (1.7 μm, 1 mm × 100 mm) column using a linear gradient of 30% CH_3CN/70% H_2O (0.1% TFA) to 100% CH_3CN in 10 min at a flow rate of 150 μL/min. Ionization of the separated adducts was achieved in the mass spectrometer under these settings: 3 kV capillary voltage, 55–60 V cone voltage, 100 °C source block temperature, 200 °C desolvation temperature, using nitrogen as both desolvation and auxiliary gas, flowing at 400 L/h. The adducts were characterized by tandem mass spectrometry, by comparing their daughter ion spectra (using argon as the collision gas) with those obtained with synthesized pure standard adducts (4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua). Quantification of the eluted adducts was based on three-point calibration curves prepared for each standard adduct. Recovery of the internal standard was ~70–80% at the end of the analytical steps. This value was used to normalize the adduct levels in the samples. DNA adduct levels were determined by comparing the areas of mass spectrometric peaks of the standard adducts with the corresponding ones present in the samples. The mean value from the integrated peak area from each concentration was used to calculate the percent inhibition with respect to the

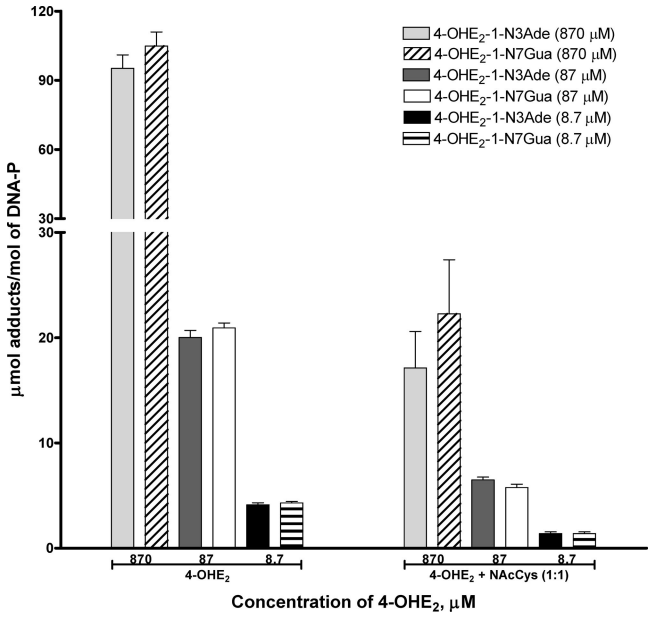

Figure 3. Formation of depurinating adducts at various concentrations of LP-activated 4-OHE_2 with or without NAcCys.

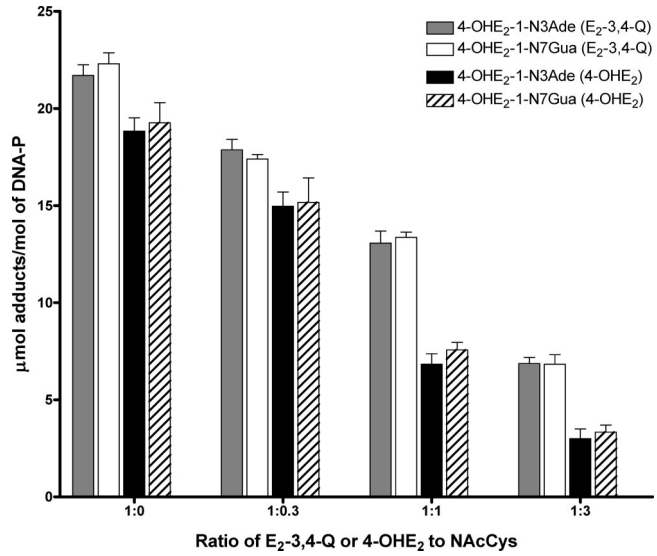

Figure 4. Effect of NAcCys on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of NAcCys, the levels of adducts were lower, with $p < 0.002$–0.04.

control experiments performed in the absence of preventing agents. This analysis provided the absolute amounts of DNA adducts (pg/μL) in the samples, which were then related to the μmol adducts/mol of deoxyribonucleic acid-phosphate (DNA-P).

Results and Discussion

To discover whether the four preventing agents can inhibit the oxidation of 4-OHE_2 to E_2-3,4-Q and/or its reaction with DNA, various concentrations of LP-activated 4-OHE_2 were reacted with DNA for 10 h at 37 °C in the presence or absence of NAcCys, and the amounts of the two depurinating adducts, 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua, were determined. The long incubation was necessary to allow complete depurination of the 4-OHE_2-1-N7Gua adduct, which has a half-life of 3 h (*9*). Roughly equal amounts of the two depurinating adducts were obtained at all three concentrations (870, 87, and 8.7 μM) of 4-OHE_2, and NAcCys inhibited the formation of the adducts at all three concentrations (Figure 3). The medium concentration of 87 μM was selected as optimal for determining the inhibitory effects of the preventing agents on the formation of adducts by reaction of E_2-3,4-Q or LP-activated 4-OHE_2 with DNA.

NAcCys. One of the predicted inhibitory effects of NAcCys in these in vitro studies is the reaction of E_2-3,4-Q with the nucleophilic sulfur group of NAcCys when DNA is incubated with the quinone in the presence of NAcCys. When LP-activated 4-OHE_2 is reacted with DNA, we expect that the total inhibitory effect is the sum of two inhibitory processes: When 4-OHE_2 is oxidized by LP to E_2-3,4-semiquinone, this intermediate can be reduced by NAcCys to 4-OHE_2 (*29*). In addition, NAcCys reacts with the end product of oxidation, E_2-3,4-Q (*27, 28*).

In fact, when E_2-3,4-Q or 4-OHE_2 and NAcCys were present in a ratio of 1:0.3, only a slight inhibitory effect (~20%) on the formation of the adducts was observed for the quinone or the catechol (Table 1 and Figure 4). When the concentrations of estrogen and NAcCys were the same, the inhibitory effect with LP-activated 4-OHE_2 was much larger than with E_2-3,4-Q (~62 vs 40%, Table 1 and Figure 4). The inhibitory effect on adduct formation when the ratio of estrogen to NAcCys was 1:3 was 69% with the quinone, whereas 84% inhibition was observed with LP-activated 4-OHE_2 (Table 1 and Figure 4). These results are consistent with one mechanism of inhibition with E_2-3,4-Q [reaction with NAcCys (*28*)], whereas the preventive effect on the LP-activated catechol is the sum of two processes [reaction of the E_2-3,4-Q product with NAcCys plus the reduction of E_2-3,4-semiquinone back to 4-OHE_2 (*29*)].

GSH and Cys. Since NAcCys is hydrolyzed to Cys and Cys is the rate-limiting precursor of GSH, it was important to investigate the relative inhibitory action of these two compounds in comparison to that of NAcCys. When E_2-3,4-Q was reacted with DNA in the presence of GSH, the level of inhibition of formation of N3Ade and N7Gua adducts was slightly less than that observed with Cys or NAcCys (Table 1, Figures 5 and 6). With ratios of GSH to LP-activated 4-OHE_2 of 1:1 and 3:1,

Table 1. Inhibition of Depurinating Adduct Formation by Preventing Agents

	percent inhibition											
	4-OHE_2-1-N3Ade						4-OHE_2-1-N7Gua					
ratio	GSH	Cys	NAcCys	dihydrolipoic acid	melatonin	resveratrol	GSH	Cys	NAcCys	dihydrolipoic acid	melatonin	resveratrol
	E_2-3,4-Q: preventing agent											
1:0	0	0	0	0	0	0	0	0	0	0	0	0
1:0.3	12 ± 2	15 ± 3	17 ± 5	13 ± 3	0	0	10 ± 3	15 ± 3	18 ± 4	14 ± 3	0	0
1:1	32 ± 4	35 ± 5	40 ± 6	32 ± 1	0	0	31 ± 2	37 ± 4	40 ± 3	34 ± 2	0	0
1:3	61 ± 3	66 ± 2	68 ± 3	60 ± 2	0	0	58 ± 2	67 ± 3	70 ± 5	60 ± 3	0	0
	4-OHE_2: preventing agent											
1:0	0	0	0	0	0	0	0	0	0	0	0	0
1:0.3	13 ± 3	17 ± 3	20 ± 5	19 ± 2	13 ± 2	25 ± 3	15 ± 4	16 ± 4	21 ± 8	18 ± 1	12 ± 3	25 ± 5
1:1	45 ± 6	57 ± 4	64 ± 3	55 ± 2	37 ± 2	61 ± 2	44 ± 5	56 ± 3	61 ± 6	57 ± 6	35 ± 2	60 ± 6
1:3	62 ± 4	76 ± 3	84 ± 5	71 ± 1	55 ± 1	97 ± 1	64 ± 3	78 ± 4	83 ± 4	72 ± 4	54 ± 3	97 ± 1

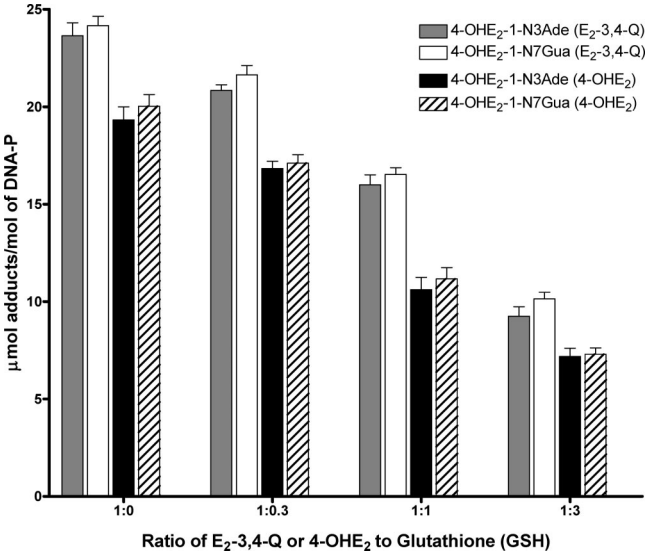

Figure 5. Effect of GSH on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of GSH, the levels of N3Ade were lower, with $p < 0.0001$–0.02; the levels of N7Gua were lower ($p < 0.0001$–0.008), except with E_2-3,4-Q (1:0.3).

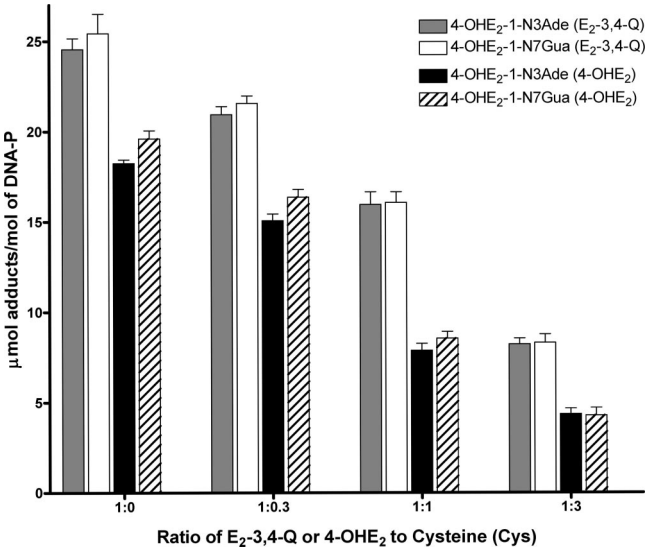

Figure 6. Effect of Cys on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of Cys, the levels of both adducts were lower with $p < 0.0001$–0.02.

inhibition of adduct formation is similar to that obtained with E_2-3,4-Q, with the exception of the 1:1 ratio, which is larger (Table 1). These results suggest that the inhibition occurs only in the reaction of GSH with E_2-3,4-Q and not by reduction of E_2-3,4-semiquinone to 4-OHE_2. With Cys and either E_2-3,4-Q or LP-activated 4-OHE_2, formation of the adducts is inhibited almost at the same extent as with NAcCys (Figure 6, Table 1).

Reduced Lipoic Acid (Dihydrolipoic Acid). The biological action of lipoic acid as an antioxidant is mainly due to the reduced form (Figure 2), which is obtained after ingestion of lipoic acid. Therefore, the inhibitory effects of lipoic acid were investigated with dihydrolipoic acid. This compound has two sulfhydryl groups. Therefore, its properties in vitro should be analogous to those of NAcCys. This compound can exert its action by reacting with E_2-3,4-Q, thereby inhibiting the formation of DNA adducts. This effect was observed with all ratios of dihydrolipoic acid to E_2-3,4-Q or LP-activated 4-OHE_2

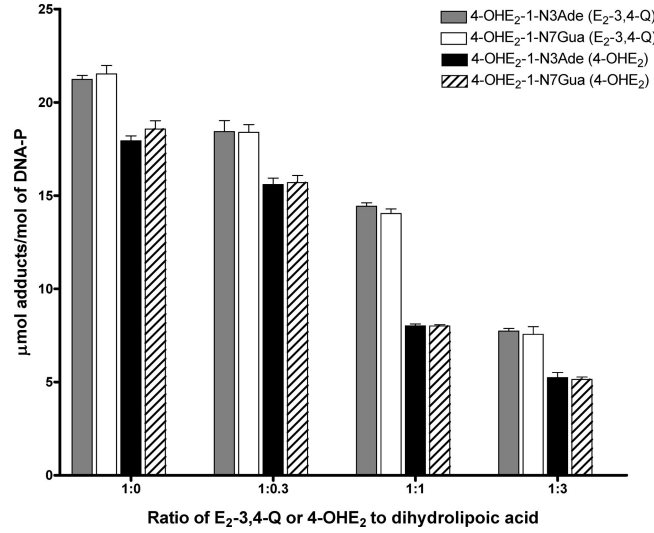

Figure 7. Effect of dihydrolipoic acid on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of dihydrolipoic acid, the levels of adducts formed were lower, with $p < 0.00002$–0.03.

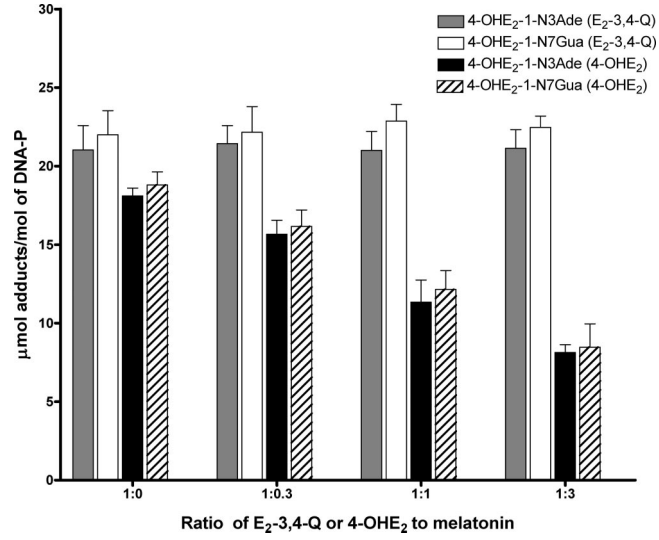

Figure 8. Effect of melatonin on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of melatonin, the levels of adducts formed from 4-OHE_2 were lower, with $p < 0.004$–0.04, except for N7Gua at the ratio 1:0.3.

(Figure 7). With LP-activated 4-OHE_2, an additional inhibitory effect is also possible because dihydrolipoic acid may be able to reduce the E_2-3,4-semiquinone back to the catechol. The results obtained offer support for this second inhibitory effect when dihydrolipoic acid is present at a concentration equal to that of the estrogen or three times greater. Specifically, at a ratio of 1:1, 32% inhibition was observed with E_2-3,4-Q and 55% inhibition was observed with 4-OHE_2 (Table 1); at a ratio of 1:3, 60% inhibition was seen with E_2-3,4-Q vs 71% inhibition with LP-activated 4-OHE_2 (Table 1).

Melatonin. The antioxidant effect of melatonin is thought to be due to the reduction of the E_2-3,4-semiquinone to 4-OHE_2 because melatonin does not react with E_2-3,4-Q. In fact, with the quinone, no inhibition of adduct formation was observed (Figure 8). The antioxidant effect was observed at all ratios when LP-activated 4-OHE_2 was reacted with DNA: 13% inhibition at 1:0.3, 37% at 1:1, and 55% at 1:3 (Table 1 and Figure 8).

Resveratrol. Analogously to melatonin, resveratrol cannot react with E_2-3,4-Q to prevent adduct formation, as seen in

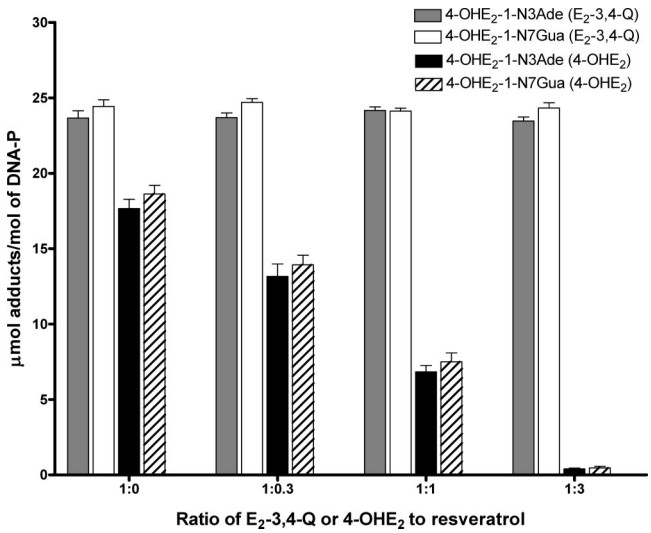

Figure 9. Effect of resveratrol on the formation of depurinating adducts obtained by reaction of 87 μM E_2-3,4-Q or LP-activated 4-OHE_2 with DNA. In the presence of resveratrol, the levels of both adducts formed from 4-OHE_2 were lower, with $p < 0.0003$–0.04.

Figure 9. Because of the high energy of resonance of its one-electron oxidized form (*42*), corresponding to the 4′-oxyradical, resveratrol presumably reduces the E_2-3,4-semiquinone to 4-OHE_2 very efficiently, leading to a reduction in adduct formation when LP-activated 4-OHE_2 reacts with DNA (Figure 9). This mechanism is very effective, resulting in 25% inhibition of adduct formation at a ratio of 1:0.3, 60% at a ratio of 1:1, and 97% inhibition at a ratio of 1:3 (Table 1 and Figure 9).

Comparison of Inhibitory Effects for the Four Preventing Agents. The relative inhibitory effects of the four preventing agents can be seen from the percent inhibition of adduct formation at the three ratios of estrogen and agent (Table 1). The chemical inhibitory effects of NAcCys and dihydrolipoic acid are based on two mechanisms, the reaction of E_2-3,4-Q with the thiol group in the preventive agent and the ability of both molecules to reduce the semiquinone of 4-OHE_2 back to the catechol. Both mechanisms express the total inhibitory effect of the two compounds. NAcCys exerts these effects more strongly than dihydrolipoic acid in the formation of 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua, with 70 vs 60% at a ratio of 1:3 and 40 vs 34% at a ratio of 1:1 with E_2-3,4-Q and 84 vs 72% at a ratio of 1:3 with 4-OHE_2 (Table 1). The other two preventing agents, melatonin and resveratrol, exert their inhibitory effect through the reduction of E_2-3,4-semiquinone to 4-OHE_2. With these two compounds, the inhibition of formation of the two depurinating adducts is by far more efficient with resveratrol, a 61% inhibition at a ratio of 1:1, as compared to 37% with melatonin and 97% with a resveratrol at a ratio of 1:3 vs 61% with melatonin (Table 1).

Conclusion

To gain a comprehensive picture of the protective effects of potential agents to prevent initiation of estrogen-induced cancer, an essential starting point has been measuring the chemical effects that lead to inhibiting the formation of estrogen−DNA adducts in vitro. Some of these agents can also affect the expression of estrogen-activating and -deactivating enzymes. For example, resveratrol induces the expression of quinone reductase (*44*), which reduces the estrogen quinone to its catechol estrogen (*52*), thereby preventing adduct formation. In addition, resveratrol modulates the induction of cytochrome P450 1B1 (*53*). By analogy with dithiole-3-thione, lipoic acid also induces quinone reductase (*32, 33*). To gain knowledge of the additional biochemical effects that can inhibit adduct formation, the next step is the study of the inhibitory effects of these compounds in relevant cell culture systems. To this end, Venugopal et al. have discovered that NAcCys can prevent the formation of depurinating estrogen−DNA adducts and the malignant transformation of mouse mammary epithelial cells treated with 4-OHE_2 or E_2-3,4-Q (*46*). Similar studies have been conducted with resveratrol in the MCF-10F human breast epithelial cell line (*53*). Once the studies with cultured cells are complete, the ability of these preventive agents to reduce the formation of depurinating estrogen−DNA adducts will be investigated in humans. Inhibiting the formation of these adducts in humans is expected to reduce the incidence of cancer by preventing cancer initiation.

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