Depurinating estrogen–DNA adducts in the etiology and prevention of breast and other human cancers

Ercole L Cavalieri[†] and

Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA

Eleanor G Rogan

Eppley Institute for Research in Cancer and Allied Diseases and Department of Environmental, Agricultural and Occupational Health, College of Public Health, University of Nebraska Medical Center, NE, USA

Abstract

Experiments on estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity have led to and supported the hypothesis that the reaction of specific estrogen metabolites, mostly the electrophilic catechol estrogen-3,4-quinones, with DNA can generate the critical mutations to initiate breast and other human cancers. Analysis of depurinating estrogen–DNA adducts in urine demonstrates that women at high risk of, or with breast cancer, have high levels of the adducts, indicating a critical role for adduct formation in breast cancer initiation. Men with prostate cancer or non-Hodgkin lymphoma also have high levels of estrogen–DNA adducts. This knowledge of the first step in cancer initiation suggests the use of specific antioxidants that can block formation of the adducts by chemical and biochemical mechanisms. Two antioxidants, *N*-acetylcysteine and resveratrol, are prime candidates to prevent breast and other human cancers because in various *in vitro* and *in vivo* experiments, they reduce the formation of estrogen–DNA adducts.

Keywords

adducts; cancer; cancer initiation; catechol estrogen quinines; cell transformation by estrogens; depurinating estrogen–DNA adducts; estrogen genotoxicity; estrogen metabolism balance; *N*-acetylcysteine; prevention; resveratrol

Estrogens in the etiology of cancer

A variety of chemicals have been demonstrated to be carcinogenic to humans. Epidemiological evidence has suggested that 30% of human cancers are due to tobacco

smoking [1]. The causative factors related to diet, pollution and lifestyle remain unknown [1]. Our environment, in general, is not sufficiently polluted to account for the majority of human cancer incidence. Therefore, it has been fruitful to investigate the potential of endogenous chemicals to initiate cancer.

A few investigators had the intuition that endogenous estrogens can be cancer-causing agents, since the estrogens contain a benzene ring in their structure, and benzene and poly-cyclic aromatic hydrocarbons (PAH) are carcinogenic. Benzene has long been known to induce leukemia [2] and, more recently, lymphoma [3]. The predominant endogenous bio-molecules containing a benzene ring are the estrogens and dopamine. Benzene, estrogens and dopamine are all metabolized to electro-philic *ortho*-quinones, which can react with DNA to form specific depurinating DNA adducts at the N-3 of adenine (Ade) and N-7 of guanine (Gua) [4].

Testing of the endogenous estrogens, estrone (E_1) and estradiol (E_2), and their catechols demonstrated that they induce tumors in hormone-dependent and -independent organs [5–8]. Despite these results, the scientific community did not accept estrogens as chemical carcinogens, largely because the compounds were not found to induce mutations in bacterial and mammalian test systems [6,9–13]. These findings have led scientists to classify E_1 and E_2 as epigenetic carcinogens that function mainly by stimulating abnormal cell proliferation in estrogen receptor (ER)-mediated processes [10,14–18]. The stimulated cell proliferation would generate more opportunities for mutations leading to carcinogenesis [14,18,19]. The ER-mediated processes can be involved in accelerating or delaying the process of carcinogenesis, but they do not play a central role in initiation because the hypothetical mutations obtained during cell proliferation are random, whereas the intercalation of catechol estrogens and catechol estrogen quinones in DNA leads to specific mutations that can initiate cancer.

The endogenous estrogens are true carcinogens that induce mutations. Since estrogens are chemicals and are mutagenic, they must act as other carcinogens do. This line of reasoning builds on the basic principles of chemical carcinogenesis established by James and Elizabeth Miller in the late 1960s [20,21]. Most chemical carcinogens are metabolically activated to electrophilic forms that bind covalently to DNA and other cellular macromolecules. The discovery that specific oxidative metabolites of estrogens can react with DNA led to and supports the hypothesis that estrogen metabolites can become endogenous chemical carcinogens by generating the mutations leading to the initiation of cancer [22,23]. This paradigm suggests that specific, critical mutations generate abnormal cell proliferation leading to cancer, rather than ER-mediated ab normal cell proliferation giving rise to random mutations [10,14–18]. The specificity of the critical mutations is generated by an intercalating physical complex between the estrogen and DNA before the conversion to a covalent bond between them, as demonstrated with the human carcinogen diethylstilbestrol (DES) [24].

Experiments on estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity led to and support the hypothesis that reaction of specific estrogen metabolites, mostly catechol estrogen-3,4-quinones, with DNA can generate the

critical mutations to initiate breast, prostate and other human cancers [4,23]. The major initiating pathway is illustrated in **Figure 1**. E_1 and E_2 can be metabolically transformed to 4-hydroxy$E_1(E_2)$ (4-OHE$_1$[E$_2$]) by cyto-chrome P450 (CYP) 1B1. Oxidation of these catechol estrogens leads to the corresponding 3,4-quinones (E_1[E$_2$]-3,4-Q), which can react with DNA to form small amounts of stable adducts, which remain in the DNA unless removed by repair, and predominant amounts of depurinating adducts, 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua **(Figure 1)**, which detach from DNA, leaving behind apurinic sites. Errors in the repair of these sites can lead to the critical mutations initiating breast, prostate and other human cancers [4,23].

Evidence for this paradigm

Estrogen metabolism

Aromatization of androstenedione and testosterone catalyzed by CYP19 (aromatase) yields E_1 and E_2, respectively. E_1 and E_2 are inter-converted by the enzyme 17β-estradiol dehydrogenase and are metabolized by two major pathways. The first is the formation of catechol estrogens by hydroxy lation at the 2- or 4-position **(Figure 2)**, and the second is the 16a-hydroxylation (not shown in **Figure 2**). CYP1A1 preferentially hydroxylates E_1 and E_2 at the 2-position, whereas CYP1B1 almost exclusively catalyzes the formation of 4-OHE$_1$ and 4-OHE$_2$ [25–27]. The two catechol estrogens are inactivated by conjugating reactions such as glucuronidation and sulfation, especially in the liver. However, the most common pathway of conjugation in extrahepatic tissues is *O*-methylation of the hydroxyl group, catalyzed by the ubiquitous catechol-*O*-methyl transferase (COMT) [28]. This conjugation pathway can become insufficient if the activity of COMT is low. In that case, the competitive oxidation of 2-OHE$_1$(E$_2$) and 4-OHE$_1$(E$_2$) to their respective semiquinones and quinones by CYP or peroxidases can increase **(Figure 2)**.

The oxidation of semiquinones to quinones can also occur by molecular oxygen. The reduction of estrogen quinones to semiquinones, catalyzed by CYP reductase, completes the redox cycle. In this process, the molecular oxygen is reduced to superoxide anion radical, which is converted to H_2O_2. In the presence of Fe^{++}, H_2O_2 yields hydroxyl radicals. The first damage by hydroxyl radicals is the formation of lipid hydroperoxides, which can act as unregulated cofactors of CYP. The lack of regulation of lipid hydroperoxides can generate an ab normal increase in the oxidation of catechol estrogens to their respective quinones. Thus, redox cycling can be one of the major contributors to the formation of catechol estrogen quinones, which are the ultimate carcinogenic metabolites of estrogens.

4-OHE$_1$(E$_2$) have greater carcinogenic potency than 2-OHE$_1$(E$_2$) [6–8]. This effect cannot be attributed to formation of hydroxyl radicals obtained by redox cycling, because 2-OHE$_1$(E$_2$) and 4-OHE$_1$(E$_2$) have the same redox potentials [29,30]. Instead, the greater carcinogenic potency of 4-OHE$_1$(E$_2$) must be related to the much higher levels of de purinating adducts formed by E_1(E$_2$)-3,4-Q with DNA, compared with E_1(E$_2$)-2,3-Q with DNA [31].

Once the catechol estrogen quinones are formed, they can be inactivated by conjugation with glutathione (GSH) **(Figure 2)**. Another in activation pathway for the quinones is

reduction to their respective catechols by quinone reductase [32]. Quinone reductase is a protective enzyme that can be induced by a variety of compounds [33]. If all of the inactivating (protective) processes are insufficient, the catechol estrogen quinones may react with DNA to form pre dominantly depurinating adducts.

Formation of estrogen–DNA adducts

Carcinogens react with DNA to form two types of adducts – stable adducts and depurinating adducts. Investigators in chemical carcinogenesis have always dealt with stable adducts, which remain in the DNA unless removed by repair. These adducts are routinely detected and quantified by ^{32}P-postlabeling techniques, but their identification has rarely been accomplished. Stable adducts are formed when carcinogenic electrophiles react with the exocyclic amino group of Ade or Gua. However, formation of adducts at the N-3 and N-7 of Ade, and the N-7 of Gua destabilizes the glycosyl bond and subsequent depurination of the adduct from DNA occurs [22,34,35].

Evidence that depurinating DNA adducts play a major role in tumor initiation was first derived from a correlation between the levels of depurinating PAH–DNA adducts and oncogenic Harvey (H)-*ras* mutations in mouse skin papillomas [36–38]. The potent carcinogens 7,12-dimethylbenz[*a*]anthracene [39] and dibenzo[*a,l*]pyrene [40,41] form predominantly depurinating Ade adducts and induce A>T transversions in codon 61. By contrast, benzo[*a*]pyrene forms approximately twice as many Gua depurinating adducts as Ade depurinating adducts [42], and twice as many codon 13 G>T transversions as codon 61 A>T transversions [38]. A similar correlation between the sites of formation of depurinating DNA adducts and H-*ras* mutations was observed in mouse skin and rat mammary gland treated with E_2-3,4-Q [43,44].

When $E_1(E_2)$-3,4-Q react with DNA, they predominantly form (>99%) the de purinating adducts 4-OHE$_1$(E$_2$) -1–N3Ade and 4-OHE$_1$(E$_2$)-1–N7Gua by 1,4-Michael addition (**Figure 2**) [22,31,34,35], whereas $E_1(E_2)$-2,3-Q forms much lower levels of 2-OHE$_1$(E$_2$)-6–N3Ade by 1,6-Michael addition after tautomerization of the quinone to the $E_1(E_2)$-2,3-quinone methide [31]. The levels of DNA adducts formed by the catechol estrogen quinones are concordant with the greater carcinogenic activity of 4-OHE$_1$(E$_2$) compared with the borderline carcinogenic potency of 2-OHE$_1$(E$_2$) [6–8]. The critical role of depurinating DNA adducts and the apurinic sites they generate has also been observed in the mutagenic activity of E_2-3,4-Q *in vivo* [43,44].

Imbalances in estrogen homeostasis

Normally, estrogen metabolism occurs as a balanced set of activating and deactivating pathways (homeostasis), which minimize the oxidation of catechol estrogens to quinones and their reaction with DNA. Initiation of cancer by estrogens is based on an estrogen metabolism, in which the homeostatic balance is disrupted (**Figure 2**). Several factors can unbalance homeostasis. The first factor could be excessive synthesis of estrogens by overexpression of *CYP19* (aromatase) in target tissues [45–47]. The observation that breast tissue can synthesize estrogens *in situ* suggests that much more estrogen is present in target tissues than would be predicted from plasma concentrations [47]. In fact, plasma

concentrations of estrogens are 50–100-fold lower in post menopausal women than premenopausal women, but the levels of estrogens in the breast are similar in both groups of women [48,49].

A second factor that can create imbalances in estrogen metabolism is represented by high levels of 4-OHE$_1$(E$_2$), generated by over expression of CYP1B1, which converts estrogen predominantly to 4-OHE$_1$(E$_2$) **(Figure 2)** [26,27]. Expression of CYP1B1 can be induced by exposure to potential environmental pollutants, such as dioxin [50]. In addition, CYP1B1 with the polymorphic variation of codon 432 exhibits higher enzymatic activity, thus producing more 4-OHE$_1$(E$_2$) [51]. Large amounts of 4-catechol estrogens produced could generate more E$_1$(E$_2$)-3,4-Q. This imbalance could be further compromised by a low level of COMT activity due to polymorphic variation [52], resulting in in sufficient methylation of 4-OHE$_1$(E$_2$) and greater oxidation to E$_1$(E$_2$)-3,4-Q. Furthermore, polymorphisms in quinone reductase (NQO1) that decrease the conversion of quinones to catechols could also lead to higher levels of E$_1$(E$_2$)-3,4-Q [53]. For example, a proline to serine polymorphism at codon 609 creates a null phenotype [54]. Low cellular levels of GSH, which reacts with the quinones, could be an additional factor in producing higher levels of E$_1$(E$_2$)-3,4-Q.

However, one factor that can help maintain estrogen homeostasis is the feedback inhibition exerted by methoxy estrogens on the expression of CYP1A1 and CYP1B1 [55]. This would help to keep the level of catechol estrogens regulated.

The effects of some of these factors have been observed in animal models for estrogen carcinogenicity and in the human breast. Imbalanced estrogen metabolism leading to substantial formation of catechol estrogen–GSH conjugates and depurinating catechol estrogen–DNA adducts has been observed in the kidney of male Syrian golden hamsters [56], prostate of Noble rats [57] and mammary gland of ER-α knockout mice [58]. In addition, comparison of breast tissue from women with and without breast cancer provides key evidence for imbalances in estrogen homeostasis [59]. In normal breast tissue from women with breast carcinoma, the levels of 4-OHE$_1$(E$_2$) were nearly four-times higher than the levels in breast tissue from women without breast cancer (p < 0.01). In addition, the levels of catechol estrogen–GSH conjugates in normal breast tissue from women with breast carcinoma were three-times the level in breast tissue from control women (p < 0.003). Thus, the levels of 4-OHE$_1$(E$_2$) and quinone conjugates appear to be significantly associated with breast cancer [59]. Further evidence of imbalances in estrogen homeostasis comes from the higher expression of estrogen deactivating enzymes (COMT and NQO1) in breast tissue of women without breast cancer and greater expression of estrogen activating enzymes (CYP19 and CYP1B1) in breast tissue of women with breast cancer **(Figure 3)** [60].

In addition to the endogenous factors that can disrupt estrogen homeostasis, there are environmental factors that can also unbalance estrogen metabolism. These factors include substances we ingest through the nose, mouth and skin. For example, pesticides and herbicides in the soil and food, pollutants in the air, cigarette smoke and other contaminants in food can affect estrogen metabolism. In fact, it is thought that certain environ mental pollutants generally induce cancer by imbalancing estrogen homeostasis and increasing

formation of catechol estrogen quinones, rather than by acting as direct carcinogens themselves.

Mutagenicity of estrogens

The formation of estrogen–DNA adducts, in particular the depurinating adducts, has indicated that $E_1(E_2)$-3,4-Q are indeed the predominant carcinogenic metabolites of estrogens. The next important step in determining the role of these quinones in cancer initiation was assessing their mutagenic activity. The failure to detect estrogen-induced mutations through *in vitro* assays was the cornerstone of denying the genotoxicity of estrogens [61].

More recently, by determining the major pathway of metabolic activation of estrogens, using sensitive mutagenicity assays and selecting more appropriate assay conditions, we have been able to demonstrate that both 4-OHE_2 and E_2-3,4-Q are mutagenic [43,44,62]. The mutagenicity of 4-OHE_2, under conditions in which it can be metabolized to E_2-3,4-Q, and the mutagenic activity of E_2-3,4-Q itself, provide solid evidence for the genotoxicity of estrogens.

Mutagenicity in SENCAR mice & ACI rats

The mutagenicity of E_2-3,4-Q was first demonstrated by treating the dorsal skin of female 'sensitivity to carcinogenesis' (SENCAR) mice, excising the treated skin and determining both the estrogen–DNA adducts formed and the H-*ras* mutations generated in the skin. Although equal amounts of the de purinating 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts were detected, A.T to G.C mutations were predominantly observed [43]. Similarly, when the mammary glands of female ACI rats were treated with E_2-3,4-Q by intra mammillary injection, roughly equal amounts of the N3Ade and N7Gua adducts were detected and A.T to G.C. mutations in the H-*ras* gene predominated [44]. The high levels of mutations at A residues in the H-*ras* gene and small numbers of mutations at G residues may result from the rapid de purination of N3Ade adducts and much slower de purination of N7Gua adducts [31,63].

Mutagenicity in Big Blue® rats

The Big Blue® (BB) rat is a Fischer 344 rat with about 80 copies of the λ-LIZ vector in every cell. The transgene is not expressed and has no effect on the biochemistry or physiology of the rat. Female BB rats were implanted with E_2, 4-OHE_2 or both compounds, and, after 20 weeks mutations in the *cII* gene in the mammary cells, were analyzed. The mutational spectra in rats treated with E_2 were similar to those in untreated control rats. By contrast, only in the rats treated with 4-OHE_2 (with or without E_2) were A.T to G.C mutations detected [23]. These results demon strate the mutagenicity of 4-OHE_2 under conditions in which the catechol can be metabolized to E_2-3,4-Q *in vivo*.

Mutagenicity in BB rat2 embryonic cells

Cells in the BB rat2 embryonic cell line contain approximately 60 copies of the λ-LIZ vector per cell. Treatment of these cells with 4-OHE_2 or E_2-3,4-Q generates mutations, but six

treatments with either compound are needed to obtain statistically significant results [62]. By contrast, no mutagenic activity could be demonstrated with 2-OHE$_2$. The mutational spectrum obtained with 4-OHE$_2$ contains predominant mutations at A.T base pairs, presumably due to rapid de purination of N3Ade adducts.

In summary, 4-OHE$_2$ and E$_2$-3,4-Q have been demonstrated to be mutagenic both *in vitro* and *in vivo* under appropriate assay conditions, whereas 2-OHE$_2$ was not mutagenic in the BB rat2 cells under the same assay conditions. The mutational spectra, both *in vitro* and *in vivo*, were consistent with those expected from the DNA adducts formed by E$_2$-3,4-Q and enzyme-activated 4-OHE$_2$. These results provide further support for the hypothesis that estrogens are carcinogenic through their genotoxicity.

Transformation of human cells lacking ER-α

Further evidence for the initiation of cancer by estrogen–DNA adducts has been acquired by the use of cultured human breast epithelial cells. MCF-10F cells are an immortalized, non transformed ER-α-negative cell line. When these cells are treated with E$_2$ or 4-OHE$_2$ at doses of 0.007 nM to 1 ng/ml, transformation of the cells is detected by their ability to form colonies in soft agar and their aggressiveness [64,65]. Transformation of the cells is not affected by the inclusion of the antiestrogens tamoxifen or ICI-182,780 [66], supporting the induction of transformation by genotoxic effects of estrogens. 2-OHE$_2$ also induces these changes, but to a much lesser extent. Implantation of estrogen-transformed MCF-10F cells, selected on the basis of their invasiveness, into severely-compromised immune-deficient mice leads to the development of tumors [67]. The depurinat ing 4-OHE$_2$-1-N3Ade and 4-OHE$_2$-1-N7Gua adducts were detected following treatment of the MCF-10F cells with 4-OHE$_2$ [68].

These results demonstrate that ER-negative breast epithelial cells can be transformed by genotoxic effects of estrogen metabolites. Thus, they support the hypothesis that formation of specific estrogen–DNA adducts is the critical event in the initiation of estrogen-induced cancer.

Carcinogenicity in estrogen-receptor-α knockout mice

The carcinogenicity of catechol estrogens was first demonstrated in hamsters [5–7] and mice [8]. 4-OHE$_1$(E$_2$) was carcinogenic in both species, while 2-OHE$_2$ had borderline carcinogenic activity only in the mice.

Studies of transgenic mice with ER-α knocked-out (ERKO)/*wnt-1* mice provide further important evidence for the role of estrogen-induced genotoxic events in the initiation of cancer. Bocchinfuso *et al.* found that the *wnt-1* transgene in female ERKO/*wnt-1* mice induced mammary tumors in 100% of the mice, despite the lack of ER-α and ER-β [69,70]. The mammary tissue of these mice was found to contain 4-OHE$_1$(E$_2$) and GSH conjugates formed by E$_1$(E$_2$)-3,4-Q, but no methoxycatechol estrogens [58], indicating that estrogen metabolism is unbalanced toward excess activation and reduced protection. When the mice were ovariectomized at 15 days of age to remove the major source of estrogens and implanted with E$_2$, the E$_2$-treated mice developed mammary tumors in a dose-dependent

manner [71,72]. Furthermore, mammary tumors developed when the mice were implanted with both E_2 and the anti estrogen ICI-182,780 [73]. These results provide strong evidence for tumor initiation by estrogen-induced genotoxic events, and not by ER-mediated processes.

Unifying mechanism of cancer initiation by natural & synthetic estrogens

The oxidation of estrogens to catechols and then to semiquinones and quinones is the pre dominant metabolic pathway that can initiate cancer by natural estrogens **(Figure 2)**. Reaction of the estrogen quinones with DNA by 1,4-Michael addition yields N3Ade and N7Gua adducts, and depurination of the N7Gua adducts occurs rather slowly [31,63]. Synthetic estrogens, such as the human carcinogen DES [74] and its hydrogenated derivative hexestrol, are similar to the natural estrogens:

■ The major metabolites are their catechols [75–78];

■ They are carcinogenic in the Syrian golden hamster [5,75];

■ The catechol quinones of DES and hexestrol have similar properties to those of $E_1(E_2)$-3,4-Q and form their N3Ade and N7Gua adducts by 1,4-Michael addition after reaction with DNA **(Figure 4)**;

■ Analogously to the natural estrogens, depuri-nation of the N7Gua adducts occurs rather slowly [24,63,79,80].

These strong similarities between natural and synthetic estrogens suggest that the catechol quinones are the ultimate carcinogenic metabolites for the synthetic estrogens and substantiate the hypothesis that $E_1(E_2)$-3,4-Q are the endogenous initiators of breast, prostate and other human cancers.

Analysis of estrogen–DNA adducts in human subjects with & without cancer

If formation of estrogen–DNA adducts is the critical event in the initiation of cancer by estrogens, one would expect to observe increased levels of estrogen–DNA adduct formation in tissues at risk for cancer. When depurinating estrogen–DNA adducts are released from DNA, they are shed from cells and tissues, pass into the bloodstream and are eventually excreted in urine. Therefore, ana lysis of urine or serum samples could provide information on the presence or risk of developing cancer.

High levels of estrogen–DNA adducts have been observed in analyses of urine from women who are at high risk of breast cancer or have the disease **(Figure 5)** [81,82]. These analyses are conducted by using ultraperformance liquid chromatography/tandem mass spectrometry, a methodology developed in our laboratory [81]. In two studies of such women, highly significant differences in relative levels of estrogen–DNA adducts were observed when urine samples from normal-risk women were compared with those from high-risk women or those with breast cancer **(Figure 5)** [81,82]. Subject characteristics did not affect these highly significant differences. These studies demonstrate that elevation of estrogen– DNA adducts is associated with high risk of developing breast cancer. In addition, ana lysis of

urine samples from men with and without prostate cancer demonstrated that men with the disease have relatively high levels of estrogen–DNA adducts in their urine samples [83]. These results have been confirmed in a second study of men with and without prostate cancer **(Figure 6)** [84].

Most recently, we have found that men diagnosed with non-Hodgkin lymphoma have significantly higher levels of depurinating estrogen–DNA adducts in their urine, when compared with healthy control men [85]. Thus, formation of estrogen–DNA adducts may play a critical role in the etiology of non-Hodgkin lymphoma, and these adducts could be potential biomarkers of risk for this disease.

Novel approach to cancer prevention

We hypothesize that prevention of breast and other cancers can arise from blocking the first critical step in initiation, reaction of catechol estrogen quinones with DNA. Such prevention could be obtained with natural compounds, such as *N*-acetylcysteine (NAcCys) and resveratrol (Resv) **(Figure 7)**. These compounds can prevent oxidative and/or electrophilic damage to DNA by inhibiting formation of the electro-philic quinones and/or reacting with them [86]. The antimutagenic and anticarcinogenic properties of NAcCys are attributed to multiple protective mechanisms, including its nucleophilicity, antioxidant activity and inhibition of DNA adduct formation. Hydrolysis of NAcCys by acylase in the liver and gut yields cysteine (Cys), the precursor to GSH biosynthesis, guaranteeing replenishment of this critical tri-peptide. GSH has similar chemical properties to Cys and NAcCys. The use of Cys as a preventive agent is limited by its toxicity in humans. By contrast, NAcCys has very low toxicity [87]. NAcCys reacts with $E_1(E_2)$-3,4-Q [88] to prevent their reaction with DNA **(Figure 2)**. NAcCys, like Cys, also operates as an antioxidant in reducing catechol estrogen semiquinones to catechol estrogens **(Figure 2)** [89].

A normal mouse mammary epithelial cell line, E6, can be transformed by a single treatment with 4-OHE$_2$ or E$_2$-3,4-Q [90]. NAcCys inhibited the oxidation of 4-OHE$_2$ to its quinone and consequent formation of the DNA adducts 4-OHE$_1$(E$_2$)-1–N3Ade and 4-OHE$_1$(E$_2$)-1–N7Gua. It also highly reduced the level of estrogen-induced transformation of the E6 cells. These results implicate formation of the estrogen–DNA adducts in the malignant transformation of these mammary cells. These results also provide a proof-of-principle and warrant exploration of NAcCys in the prevention of breast cancer.

Resv, 3,5,4′-3-OH-*trans*-stilbene **(Figure 7)**, is found in foods, including grapes, peanuts and wine. This compound exerts chemo-preventive effects in diverse *in vivo* and *in vitro* systems [91]. Resv was first demonstrated to function as an antioxidant and antimutagenic agent, thus acting as an anti-initiation agent in cancer [91]. These properties are attributed to the easy hydrogen abstraction from the 4′-hydroxy bond with formation of a hydroxyl radical [92]. This easy abstraction is a consequence of the greater resonance stabilization of the intermediate. Resv is also an inducer of the protective enzyme NQO1 and modulator of the activating enzyme CYP1B1 **(Figure 2)** [93,94]. Furthermore, Resv prevents formation of depurinating estrogen–DNA adducts and neoplastic transformation in human breast epithelial cells treated with E$_2$ [93].

Resv inhibits formation of estrogen–DNA adducts both biochemically and chemically. First, it induces NQO1 [93], which reduces $E_1(E_2)$-3,4-Q to 4-OHE$_1$(E$_2$) [32], thus minimizing reaction of the quinones with DNA. Second, Resv reacts with estrogen semi-quinones, reducing them to their catechol estrogens [93]. These effects were observed in MCF-10F cells treated with 4-OHE$_2$ and Resv **(Figure 8)**. When the cells were incubated with 4-OHE$_2$, 4-OHE$_1$(E$_2$)-1–N3Ade and 4-OHE$_1$(E$_2$)-1–N7Gua were formed in a dose-dependent manner. When the cells were preincubated with Resv for 48 h to induce NQO1 before 4-OHE$_2$ was added, formation of the N3Ade and N7Gua adducts was reduced at all doses of 4-OHE$_2$. When fresh Resv was also added to the cells along with the 4-OHE$_2$, formation of the estrogen–DNA adducts was no longer detected **(Figure 8)**. Therefore, the preventing effects of Resv, its modulating effect on estrogen-activating enzymes, and its protective effect by inducing quinone reductase suggest that this compound should be an excellent candidate for protection against estrogen-initiated cancer.

When MCF-10F cells were cultured in the presence of 4-OHE$_2$, and Resv and/or NAcCys were added to the culture medium at different doses, the formation of estrogen–DNA adducts by the cells was greatly inhibited in a dose-dependent manner [Cavalieri EL, Rogan EG *et al*. Unpublished data]. The same methods were used as in the published studies with MCF-10F cells [93,94]. Inhibition of estrogen–DNA adduct formation was greater in the presence of both compounds at all doses.

We have also conducted a small pilot study of the ability of one natural antioxidant compound, NAcCys, to reduce the level of depurinating estrogen–DNA adducts in the urine of healthy subjects. The same analytical procedures were used as in previous studies with human subjects [81,82,84]. Healthy people at normal risk of developing cancer have low, but detectable, levels of adducts **(Figures 5 & 6)** [81–84]. After 1 month of daily ingestion of NAcCys, the level of estrogen–DNA adducts in urine decreased an average of 55%, and 60% of the subjects experienced an average decrease of 84% [Cavalieri EL, Rogan EG *et al*. Unpublished data]. People at elevated risk of cancer are anticipated to have elevated levels of estrogen–DNA adducts, based on the higher levels observed in women at high risk for breast cancer **(Figure 5)** [81,82]. The results of this pilot study suggest that selected antioxidant compounds can reduce formation of estrogen–DNA adducts, presumably decreasing the risk of initiating and, thus, developing breast and other human cancers.

Conclusion

Evidence has been obtained for a unifying mechanism of initiation of breast and prostate cancer, as well as non-Hodgkin lymphoma. We think that other prevalent types of human cancer are also initiated by this same mechanism. This mechanism entails the metabolic formation of endogenous catechol estrogen-3,4-quinones that can react with DNA to yield predominantly the depurinating N3Ade and N7Gua adducts. The N3Ade adducts de purinate instantaneously, while the N7Gua adducts depurinate with a half-life of a few hours. These adducts leave apurinic sites in the DNA that can generate mutations leading to the initiation of cancer.

The genotoxicity and mutagenicity of 4-OHE$_2$ and E$_2$-3,4-Q have been demonstrated in cultured mammalian cells and in animal models.

Treatment of ER-α-negative human breast epithelial cells, MCF-10F cells, with E$_2$ or 4-OHE$_2$ in culture, generates not only estrogen–DNA adducts, but also malignant trans formation of the cells. Injection of these transformed cells into severely compromised immuno deficient mice results in the growth of tumors. These results are consistent with estrogen induction of malignant transformation of the cells beginning with reaction of the catechol estrogen-3,4-quinones with DNA.

Catechol estrogens have also been demonstrated to be carcinogenic in Syrian golden hamsters and SENCAR mice. The 4-OHE$_1$(E$_2$) are carcinogenic in both animal models, but the 2-OHE$_2$ are noncarcinogenic or borderline carcinogenic in these models. In addition, ERKO/*wnt-1* mice develop mammary tumors, despite the lack of ERs. Once again, these results indicate that estrogen genotoxic effects initiate the series of events leading to the development of cancer.

The synthetic estrogen DES, a human carcinogen, and its dihydrogenated derivative, hexestrol, are metabolized to their catechols and quinones and react with DNA to form N3Ade and N7Gua adducts analogous to those of the natural E$_1$(E$_2$)-3,4-Q.

From all of the similarities described above for the natural and synthetic estrogens, we foresee a common mechanism of initiation for many types of cancer.

Estrogen metabolites, conjugates and de purinating DNA adducts can be analyzed in human urine samples by using ultra performance liquid chromatography/tandem mass spectrometry. Such analyses have demonstrated that women with breast cancer or at high risk for the disease, have significantly higher levels of estrogen–DNA adducts than healthy women at normal risk for breast cancer. In addition, men with prostate cancer or non-Hodgkin lymphoma have significantly higher levels of estrogen–DNA adducts in their urine than do healthy men. The higher levels of depurinating estrogen–DNA adducts observed in subjects with cancer and women at high risk of breast cancer suggest that these adducts could serve as biomarkers of susceptibility that can potentially be used to monitor the risk of breast, prostate and other prevalent types of human cancer.

Having understood how estrogens can initiate a variety of types of cancer and knowing what to target, cancer prevention could be tackled. By preventing the first critical step in cancer initiation, the formation of estrogen–DNA adducts, the series of events leading to the development of cancer can be blocked. Two suitable candidates for cancer prevention in humans are the natural antioxidants NAcCys and Resv. These agents act by inhibiting formation of catechol quinones and/or reacting with the quinones themselves. They may also induce expression of protective enzymes such as quinone reductase **(Figure 2)**, and/or inhibit activating enzymes such as CYP19 (aromatase) and CYP1B1 **(Figure 2)** Both NAcCys and Resv inhibit formation of catechol estrogen quinones and their reaction with DNA in cultured mammary cells, as well as the malignant transformation of these cells. Thus, this approach to cancer prevention can be widely applicable and successful.

Future perspective

We have clear and compelling evidence that specific oxidized metabolites of estrogens react with DNA to form predominantly depurinating estrogen–DNA adducts. These adducts are critical in the initiation of cancer by estrogens. We can capitalize on the fact that the depurinating adducts responsible for cancer initiation by estrogens can be detected in tissues and body fluids, as we have already found in people with breast and prostate cancer. The levels of these adducts in urine are significantly higher than the levels in healthy people without cancer. Therefore, these adducts can become important biomarkers for assessing the risk for cancer before tumors are detected.

The future of this research has a two-pronged approach – screening of people with various types of cancer to discover the levels of estrogen–DNA adducts in their urine, and development of targeted strategies for cancer prevention. To screen various types of cancer, subject populations with the following types of cancer need to be identified and their urine samples analyzed. These cancers include brain, colon, endometrium, kidney, leukemia, lymphoma, lung of nonsmokers, melanoma, myeloma, ovary, pancreas, testis and thyroid. Such studies will provide new information related to the possible role of estrogen–DNA adducts in the initiation of these types of cancer.

The strategy of using estrogen–DNA adduct bio-markers is not limited to the assessment of cancer risk, but it is also a very important tool for studying cancer prevention. If the levels of estrogen–DNA adducts are reduced by a preventive compound, the likelihood of cancer initiation should also decrease. This strategy of prevention does not require knowledge of the genes involved or the complex series of events that occur after initiation of cancer.

The study of cancer prevention begins with short-term trials (a few months) assessing the ability of the selected agents to reduce the levels of estrogen–DNA adducts in urine samples from healthy subjects. In addition, a study of women at high risk for breast cancer, for example, women with 5-year Gail Model scores greater than 1.66% would provide a very useful target population in which to discover the ability of the selected antioxidants to reduce formation of estrogen–DNA adducts. An added benefit for these women is that reduction in the level of estrogen–DNA adducts would presumably lower their risk of actually developing breast cancer. If populations at high risk for other types of cancer can be identified, they also could be targeted in these short-term studies of reduction in the levels of estrogen–DNA adducts.

Following these short-term studies, the next effective step would be to conduct a medium-term and medium-size study of the ability of the selected antioxidants to prevent breast cancer in women at high risk for breast cancer. In this population, sufficient numbers of breast cancer cases could be anti cipated to develop in 4–5 years so that the ability of the antioxidants to decrease the incidence of breast cancer could be determined.

Finally, a long-term, large clinical trial of cancer prevention should be conducted in both men and women to determine the efficacy of the selected antioxidants, presumably NAcCys and Resv, to prevent the initiation and, thus, development of breast, prostate and other prevalent types of human cancer. In such a trial, subjects would ingest one or more of the

selected anti-oxidants in comparison with placebo, and regular, periodic medical monitoring and assessments would be conducted.

Acknowledgements

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Executive summary

Estrogens in the etiology of cancer

■ Experiments on estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity led to and support the hypothesis that reaction of specific estrogen metabolites, mostly catechol estrogen-3,4-quinones with DNA, can generate the critical mutations to initiate breast and other human cancers.

■ Estrogen metabolism is normally a balanced set of activating and deactivating pathways that minimizes formation of the quinones and their reaction with DNA.

■ When this balance is disrupted, more formation of the adducts 4-OHE$_1$(E$_2$)-1– N3Ade and 4-OHE$_1$(E$_2$)-1–N7Gua can occur, generating mutations that can lead to cancer initiation.

■ The estrogens are genotoxic and mutagenic and induce malignant transformation of human breast epithelial cells, as well as tumors in estrogen-receptor knockout mice.

■ Estrogen metabolites, conjugates and DNA adducts can be analyzed in human urine samples. Women with breast cancer or at high risk have significantly higher levels of estrogen–DNA adducts than healthy, normal-risk women. Men diagnosed with prostate cancer or non-Hodgkin lymphoma also have significantly higher levels of urinary adducts than healthy men.

■ It is thought that the most prevalent human cancers are initiated by mutations generated by depurinating estrogen–DNA adducts.

Novel approach to cancer prevention

■ Specific, selected natural antioxidants, through chemical and biochemical mechanisms, can reduce formation of catechol estrogen quinones and their reaction with DNA.

■ Both *N*-acetylcysteine and resveratrol reduce the formation of estrogen–DNA adducts and malignant transformation of cultured breast epithelial cells. In combination, they are even more effective in reducing adduct formation in human breast cells.

■ Pilot data indicate that ingestion of these two natural antioxidants reduces formation of estrogen–DNA adducts in humans.

■ This is a novel approach to prevention of human cancer based on the etiology of the disease.
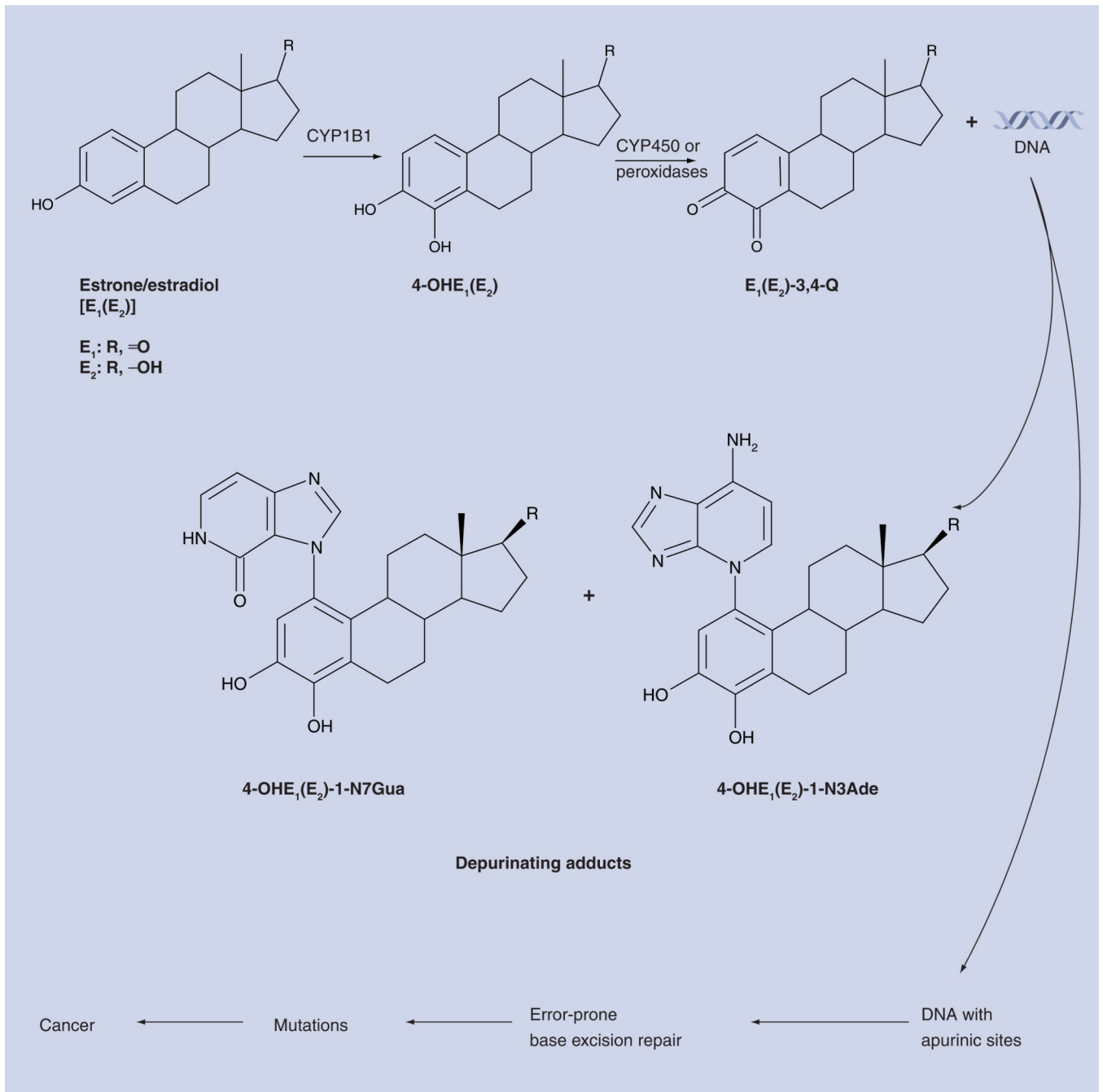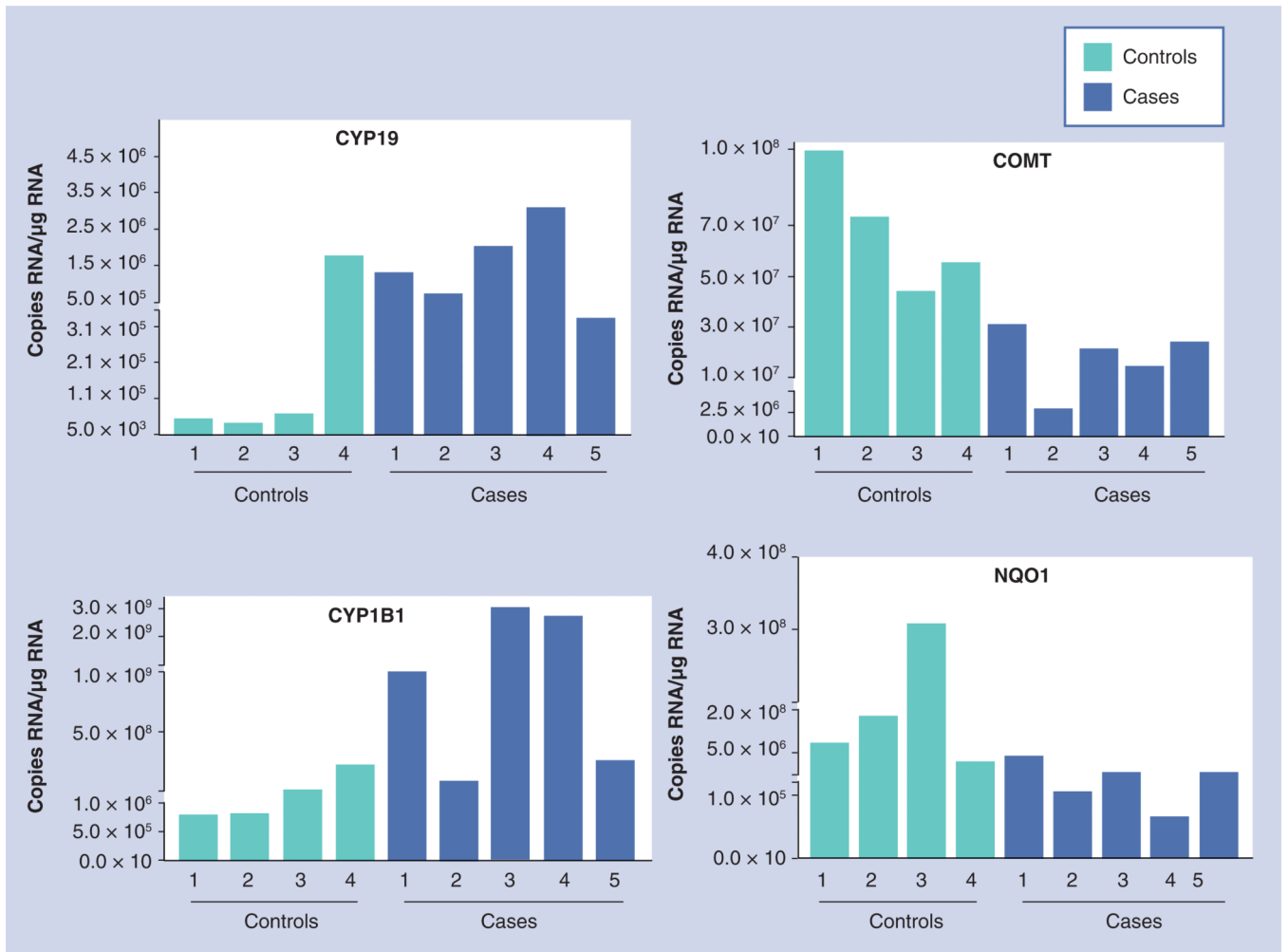
Figure 1.
Major metabolic pathway in cancer initiation by estrogens.
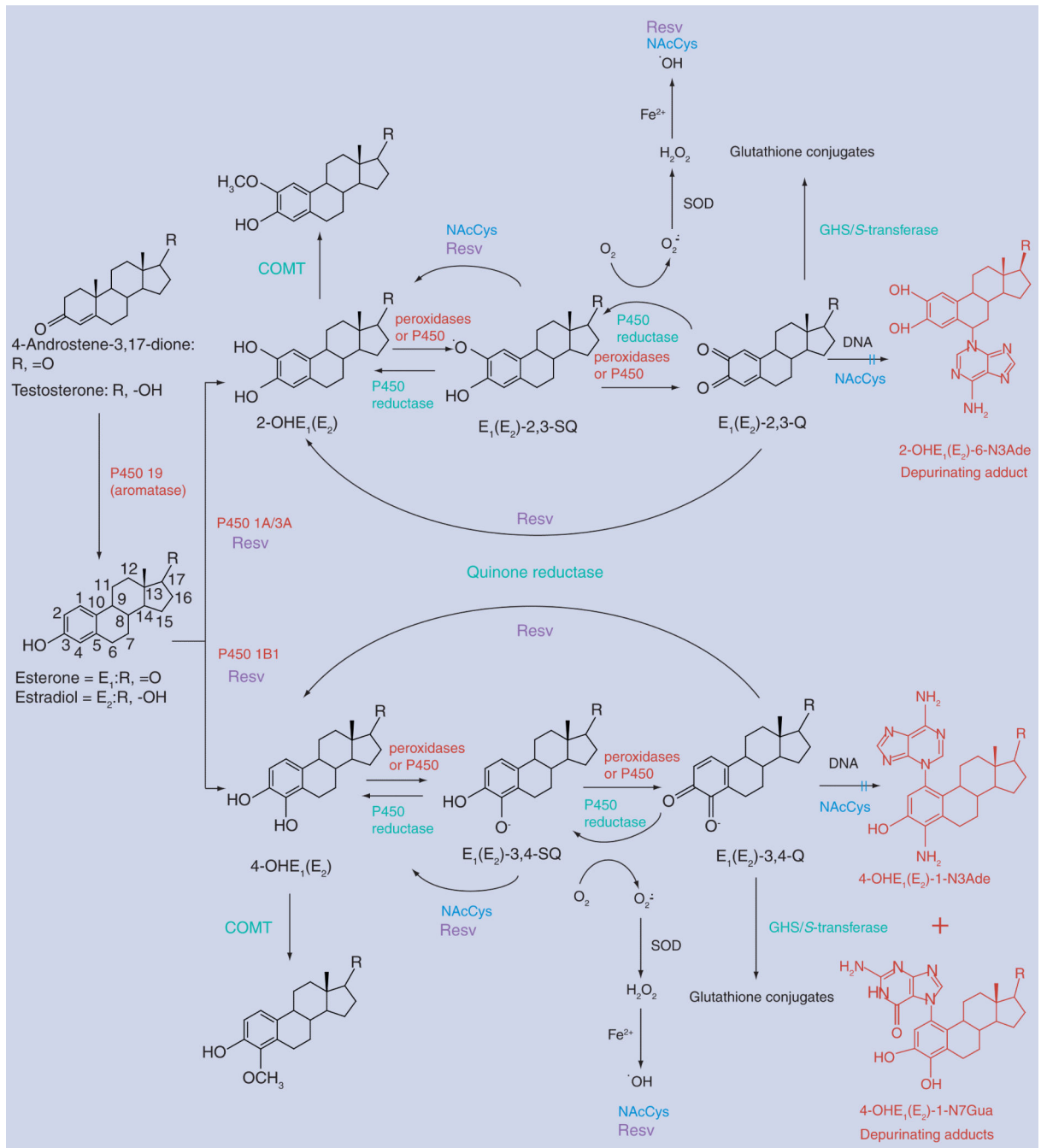
Figure 2. Formation, metabolism and DNA adducts of estrogens
Activating enzymes and depurinating DNA adducts are in red and protective enzymes are in turquoise. *N*-acetylcysteine (NAcCys, shown in blue) and resveratrol (Resv, purple) indicate the various points where NAcCys and Resv could improve the balance of estrogen metabolism and minimize the formation of depurinating DNA adducts.
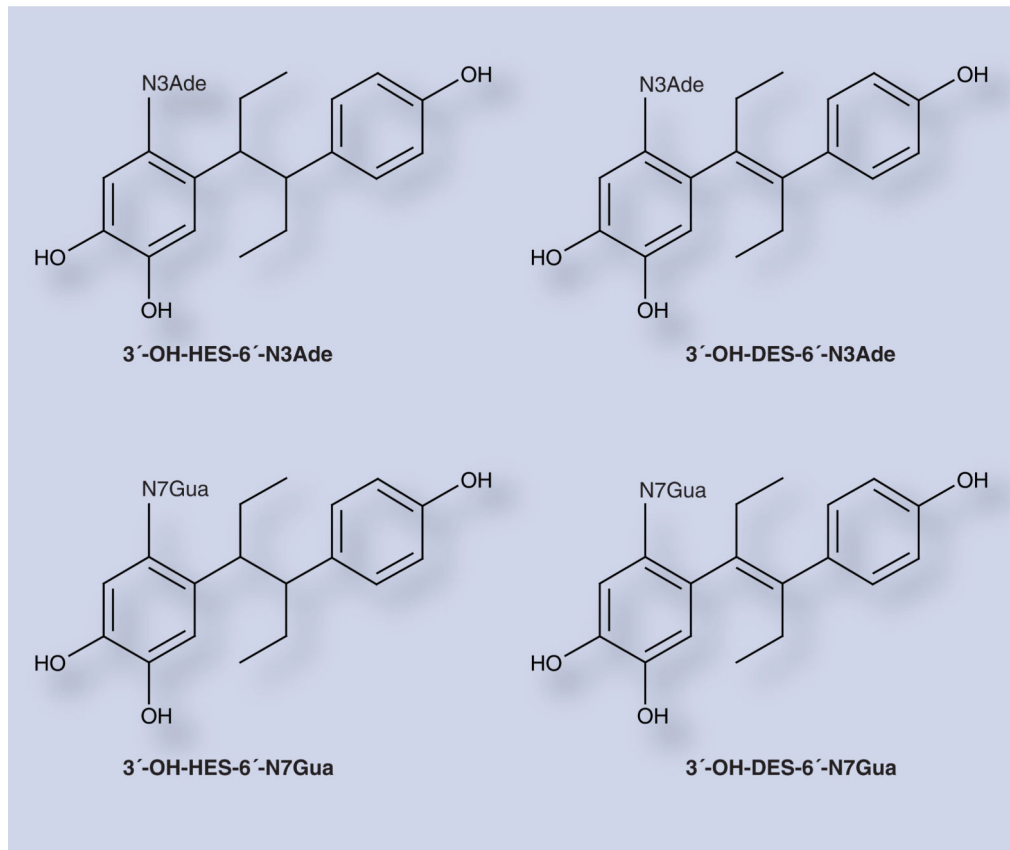
Figure 3. Expression of estrogen-metabolizing enzymes in the human breast
Reproduced with permission from [60].

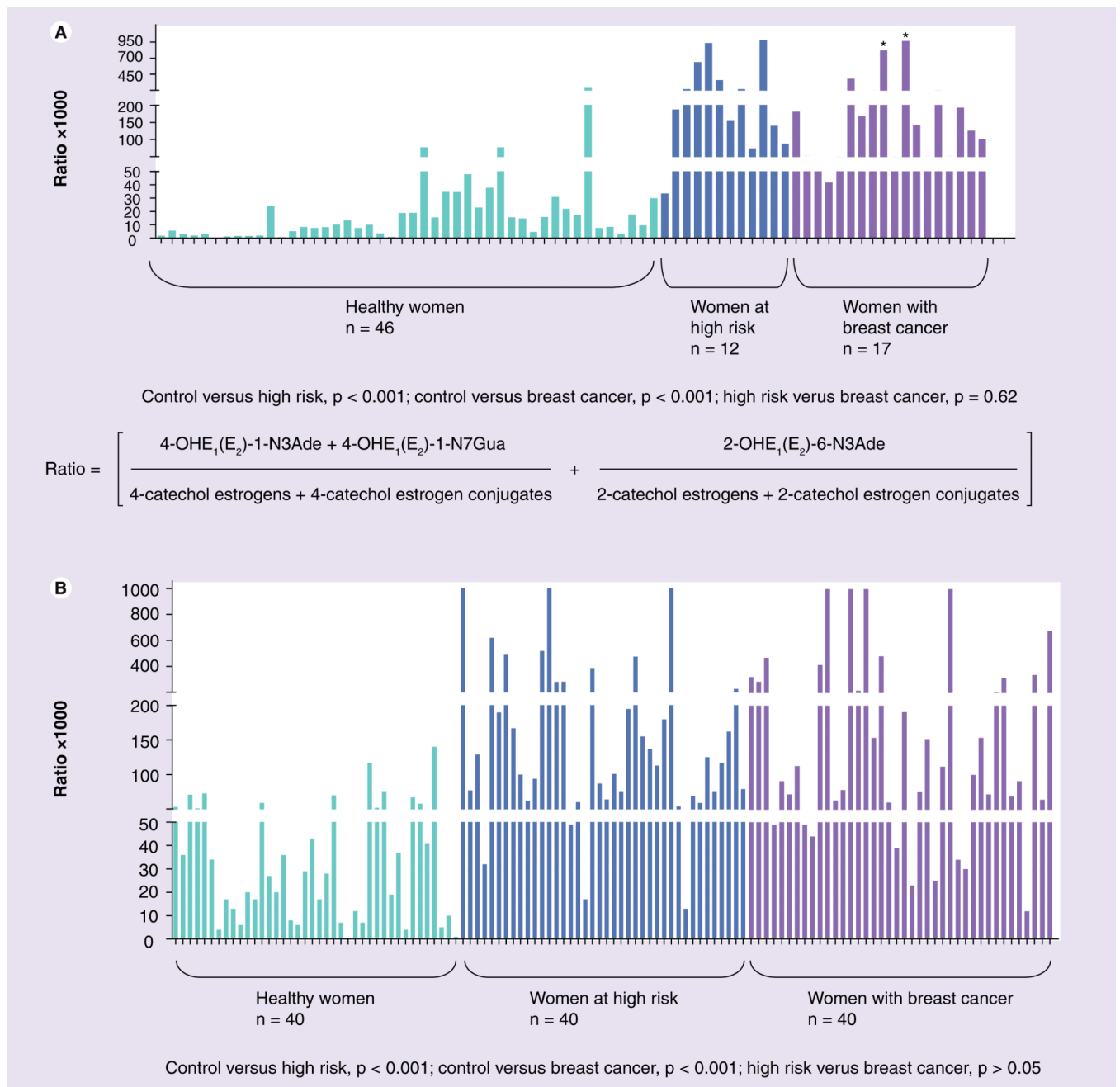**Figure 4. Structures of the depurinating N3Ade and N7Gua adducts formed by reaction of the catechol quinones of hexestrol and DES with DNA**
DES: Diethylstilbestrol; HES: Hexestrol.

$$\text{Ratio} = \left[\frac{\text{4-OHE}_1(\text{E}_2)\text{-1-N3Ade} + \text{4-OHE}_1(\text{E}_2)\text{-1-N7Gua}}{\text{4-catechol estrogens} + \text{4-catechol estrogen conjugates}} + \frac{\text{2-OHE}_1(\text{E}_2)\text{-6-N3Ade}}{\text{2-catechol estrogens} + \text{2-catechol estrogen conjugates}} \right]$$

Figure 5. Depurinating estrogen–DNA adducts in urine of healthy women, high-risk women and women with breast cancer

(A) First study. **(B)** Second study [81,82]. In the ratio of depurinating estrogen–DNA adducts to the sum of their respective estrogen metabolites and conjugates, the 4-catechol estrogen–DNA adducts account for more than 98% of the value of the ratio.
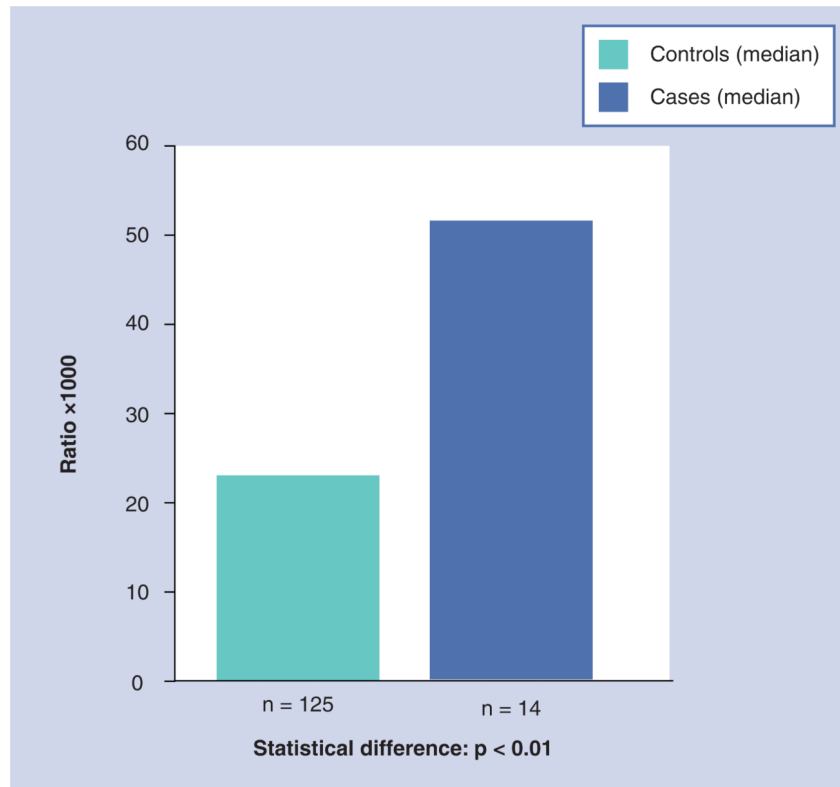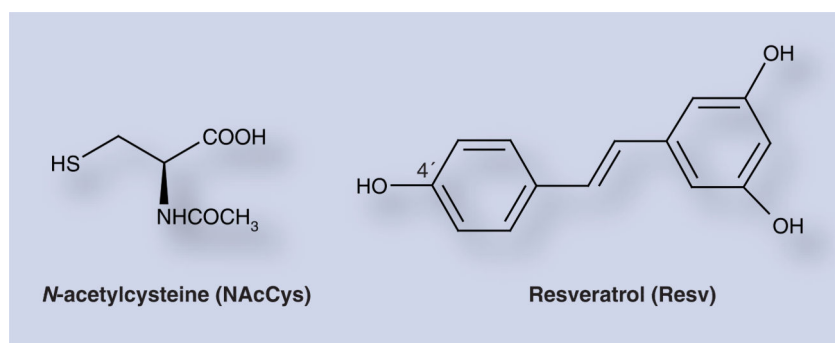
Figure 6. Levels of the estrogen–DNA adducts in urine samples from men with and without prostate cancer
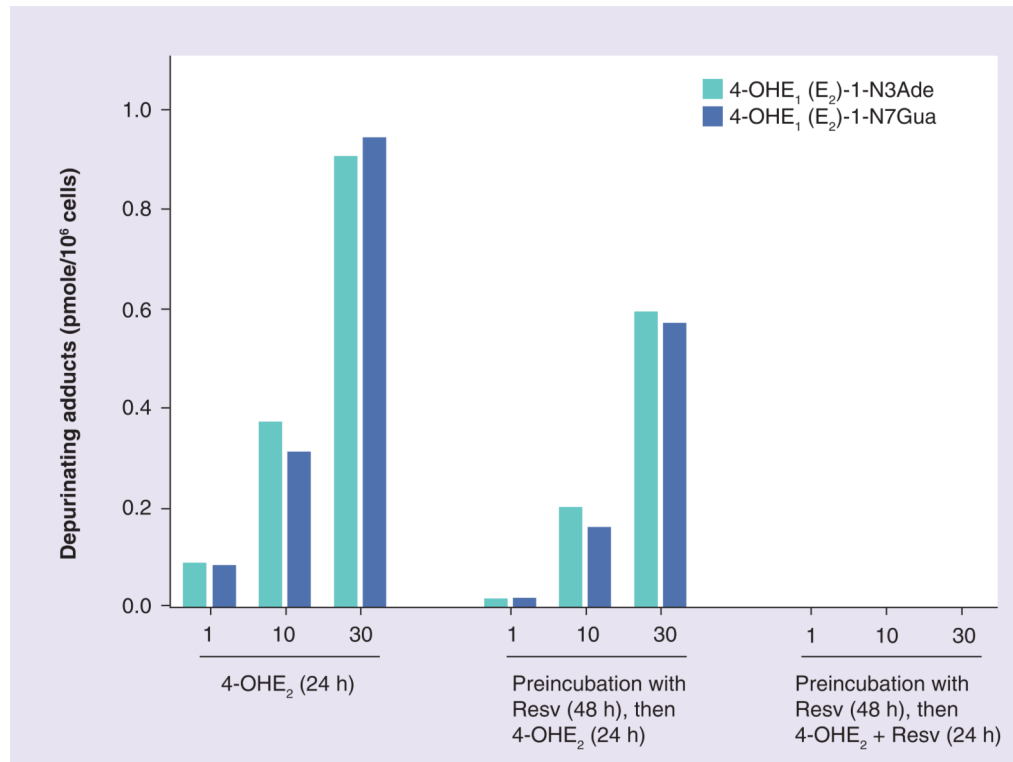Reproduced with permission from [84].

Figure 7.
Structures of antioxidant compounds, *N*-acetylcysteine and resveratrol.

Figure 8. Levels of depurinating estrogen–DNA adducts in culture medium from cells treated with 4-OHE$_2$, with or without Resv

The levels of DNA adducts in Resv-treated cells were significantly lower than those in the cells not treated with Resv ($p < 0.05$) as determined by analysis of variance.

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