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Review

Initiation of cancer and other diseases by catechol ortho-quinones: a unifying mechanism

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Abstract. Exposure to estrogens is a risk factor for breast and other human cancers. Initiation of breast, prostate and other cancers has been hypothesized to result from reaction of specific estrogen metabolites, catechol estrogen-3,4-quinones, with DNA to form depurinating adducts at the N-7 of guanine and N-3 of adenine by 1,4-Michael addition. The catechol of the carcinogenic synthetic estrogen hexestrol, a hydrogenated derivative of diethylstilbestrol, is metabolized to its quinone, which reacts with DNA to form depurinating adducts at the N-7 of guanine and N-3 of adenine. The catecholamine dopamine and the metabolite catechol (1,2-dihydroxybenzene) of the leukemogen benzene can also be oxidized to their quinones, which react with DNA to form predominantly analogous depurinating adducts. Apurinic sites formed by depurinating adducts are converted into tumor-initiating mutations by error-prone repair. These mutations could initiate cancer by estrogens and benzene, and Parkinson's disease by the neurotransmitter dopamine. These data suggest a unifying molecular mechanism of initiation for many cancers and neurodegenerative diseases and lay the groundwork for designing strategies to assess risk and prevent these diseases.

Key words. Catechol estrogens; catecholamines; depurinating DNA adducts; error-prone DNA repair; estrogen homeostasis; 1,4-Michael addition; tumor initiation.

Introduction

One of the major obstacles in cancer research is related to the concept that cancer is a problem of 200 diseases. This viewpoint has impeded researchers from looking at the etiology of cancers because the search would be prohibitively complex. For this reason, the etiology of breast, prostate and other human cancers remains virtually unknown. While the expression of various cancers coincides with the above concept, some scientists consider there to be a common origin for many prevalent types of cancer.

There is widespread agreement in the scientific community that cancer is basically a genetic disease – not in the sense that most cancers are inherited (they are not), but in the sense that cancer is triggered by genetic mutations. Thus, cancer can be considered a disease of mutated critical genes that modulate cell growth and death. These include oncogenes and tumor suppressor genes, which give rise to transformation and abnormal cell proliferation [1]. Understanding the origin of these mutations opens the door to strategies for controlling and preventing cancer.

A second barrier to the progress of cancer research is related to the reluctance of the scientific community to recognize that the natural estrogens, estrone (E_1) and estradiol (E_2), are true carcinogens, which induce tumors in various hormone-dependent and independent organs of several animal species and strains [2–4].

A third obstacle to the progress of research on breast and other hormone-dependent cancers is related to the stan-

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dard paradigm, stated by Feigelson and Henderson, that estrogens, through receptor-mediated processes, 'affect the rate of cell division and, thus, manifest their effect on the risk of breast cancer by causing proliferation of breast epithelial cells. Proliferating cells are susceptible to genetic errors during DNA replication, which, if uncorrected, can ultimately lead to a malignant phenotype' [5]. While there is no doubt that estrogen-mediated control of cell proliferation plays a role in the development of breast and other hormone-dependent cancers, accumulating evidence suggests that specific oxidative metabolites of estrogens, if formed, can be the endogenous ultimate carcinogens [6]. By reacting with DNA, they cause the mutations leading to cancer. This initiating mechanism occurs in hormone- dependent and independent tissues.

Covalent binding of carcinogens to DNA: Stable and depurinating adducts

Chemical carcinogens covalently bind to DNA to form two types of DNA adducts: stable ones that remain in DNA unless removed by repair and depurinating ones that are released from DNA by destabilization of the glycosyl bond (fig. 1) [7, 8]. Stable adducts are formed when carcinogens react with the exocyclic N^6 amino group of adenine (Ade) or N^2 amino group of guanine (Gua), whereas depurinating adducts are obtained when carcinogens covalently bind at the N-3 or N-7 of Ade or the N-7 or sometimes C-8 of Gua. The loss of Ade or Gua by depurination leads to formation of apurinic sites that can generate the mutations leading to tumor initiation.

Identification and quantification of polycyclic aromatic hydrocarbon (PAH)-DNA adducts led us to discover that there is a correlation between depurinating adducts and oncogenic mutations, suggesting that these adducts are the primary culprits in the tumor-initiating pathway [8-10]. This discovery was made by identifying the DNA adducts formed in mouse skin by dibenzo[*a,l*]pyrene (DB[*a,l*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (BP) and, at the same time, determining the mutations in the Harvey (H)-*ras* oncogene in mouse skin papillomas initiated by these three PAHs (table 1). These mutations correlate with the predominant formation of depurinating Ade adducts by DMBA and DP[*a,l*]P and the two-to-one ratio of depurinating Gua to

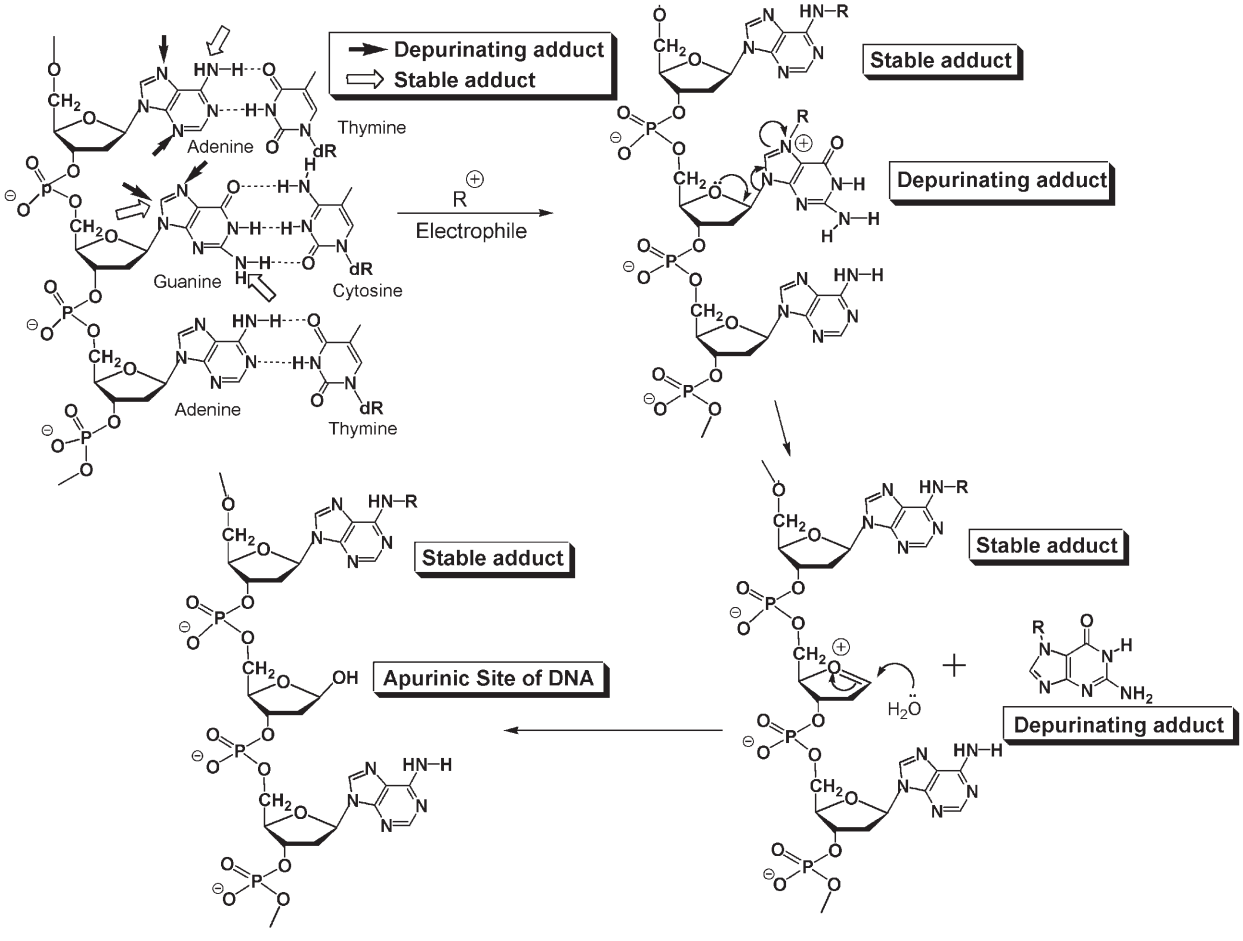


Figure 1. Formation of stable and depurinating DNA adducts, and generation of apurinic sites.

Table 1. Correlation of depurinating adducts with H-*ras* mutations in mouse skin papillomas.

		H-*ras* mutations	
		No. of mutations	
PAH	Major DNA adducts	No. of mice	Codon
DMBA	N7Ade (79%)	4/4 CAA → CTA	61
DB[*a,l*]P	N7Ade (32%) N3Ade (49%)	10/12 CAA˚CTA	61
BP	C8Gua + N7Gua (46%) N7Ade (25%)	10/20 CGC → GTC 5/20 CAA → CTA	13 61

Ade adducts formed by BP. This pattern of *ras* mutations suggests that the oncogenic mutations in mouse skin papillomas induced by these PAHs are generated by misrepair of the apurinic sites derived from loss of the depurinating adducts (see below) [10]. Because thousands of apurinic sites are formed by cells each day [11], repair of apurinic sites induced by PAH might be expected. The level of apurinic sites arising from treatment with PAH is, however, 15–120 times higher than those formed spontaneously, suggesting that this large increase in apurinic sites could lead to misrepair [10, 12]. In summary, apurinic sites can generate the mutations that play the critical role in the initiation of cancer, and formation of depurinating adducts has become the common denominator for recognizing the potential of a chemical to initiate cancer [8–10, 12].

Formation, metabolism and DNA adducts of estrogens

Evidence that depurinating PAH-DNA adducts play a major role in tumor initiation [7–9] provided the impetus for discovering the estrogen metabolites that form depurinating DNA adducts and can be potential endogenous initiators of cancer [13]. Catechol estrogens (CEs) are among the major metabolites of E_1 and E_2. If these metabolites are oxidized to the electrophilic CE quinones (CE-Qs), they may react with DNA. Specifically, the carcinogenic 4-CEs [14–16] are oxidized to CE-3,4-Qs, which react with DNA to form depurinating adducts [13, 17]. These adducts generate apurinic sites that may lead to oncogenic mutations [10, 12, 18], thereby initiating cancer.

Estrogen metabolism

E_1 and E_2 are obtained by aromatization of 4-androsten-3,17-dione and testosterone, respectively, catalyzed by cytochrome P450 (CYP)19, aromatase (fig. 2). The estrogens E_1 and E_2 are biochemically interconvertible by the enzyme 17β-estradiol dehydrogenase. E_1 and E_2 are metabolized via two major pathways: formation of CE and, to a lesser, extent, 16α-hydroxylation (not shown in fig. 2). The CEs formed are the 2- and 4-hydroxylated estrogens. The major 4-hydroxylase in extrahepatic tissues is CYP1B1 [19–21]. In general, the CEs are inactivated by conjugating reactions such as glucuronidation and sulfation, especially in the liver (not shown in fig. 2). The most common pathway of conjugation in extrahepatic tissues, however, occurs by *O*-methylation catalyzed by the ubiquitous catechol-*O*-methyltransferase (COMT) [22]. A reaction that is competitive with the conjugation of CEs is their catalytic oxidation to CE-semiquinones (CE-SQs) and CE-Q (fig. 2). CE-SQs and CE-Qs can be neutralized by conjugation with glutathione (GSH). A second inactivating pathway for CE-Qs is their reduction to CEs by quinone reductase and/or cytochrome P450 reductase [23, 24]. If these two inactivating processes are insufficient, CE-Qs may react with DNA to form stable and depurinating adducts (fig. 2) [25]. The carcinogenic 4-CEs [14–16] are oxidized to form predominantly the depurinating adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua [13, 17, 26]. The weakly carcinogenic 2-CEs [16] are oxidized to form predominantly stable adducts, 2-$OHE_1(E_2)$-6-N^6dA and 2-$OHE_1(E_2)$-6-N^2dG, but also depurinating adducts to a much lesser extent [26–28].

Redox cycling of CE-SQs and CE-Qs

Redox cycling (figs. 2 and 3) generated by reduction of CE-Q to CE-SQ, catalyzed by cytochrome P450 reductase, and subsequent oxidation back to CE-Q by molecular oxygen forms superoxide anion radicals ($O_2^{\cdot}$) [29]. These $O_2^{\cdot}$ dismutate to H_2O_2, either spontaneously or, even faster, when the reaction is catalyzed by superoxide dismutase. H_2O_2 is rather nonreactive, except in the presence of reduced transition metal ions, namely, Fe^{2+} and Cu^{+}, which cause formation of indiscriminate oxidants, the hydroxyl radicals. These reactive species can damage DNA by formation of oxygenated bases [29–32]. Concurrently, hydroxyl radicals can initiate the lipid peroxidation process [33], generating lipid hydroperoxides that can serve as unregulated cofactors for oxidation of CE by cytochrome P450. In contrast, under normal conditions

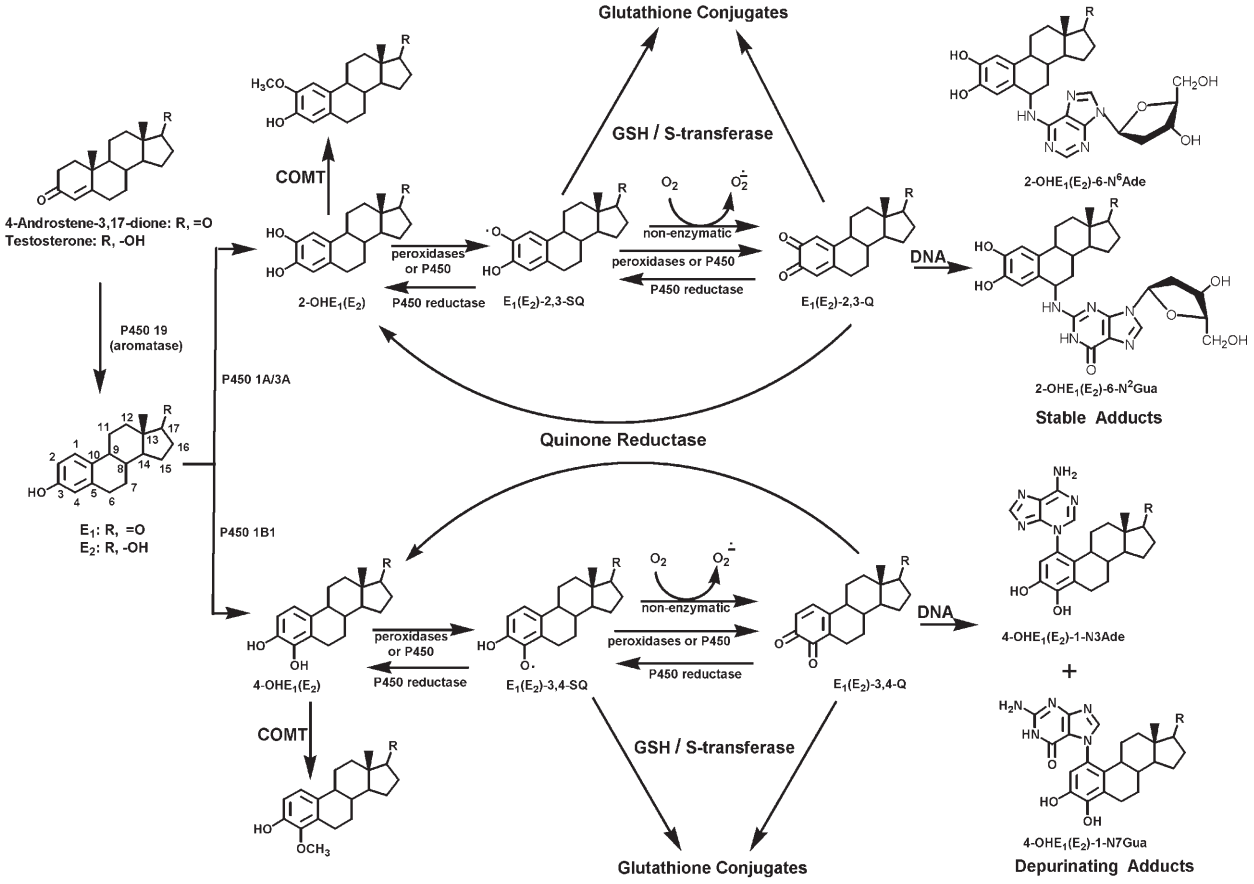


Figure 2. Formation, metabolism, conjugation and DNA adducts of estrogens.

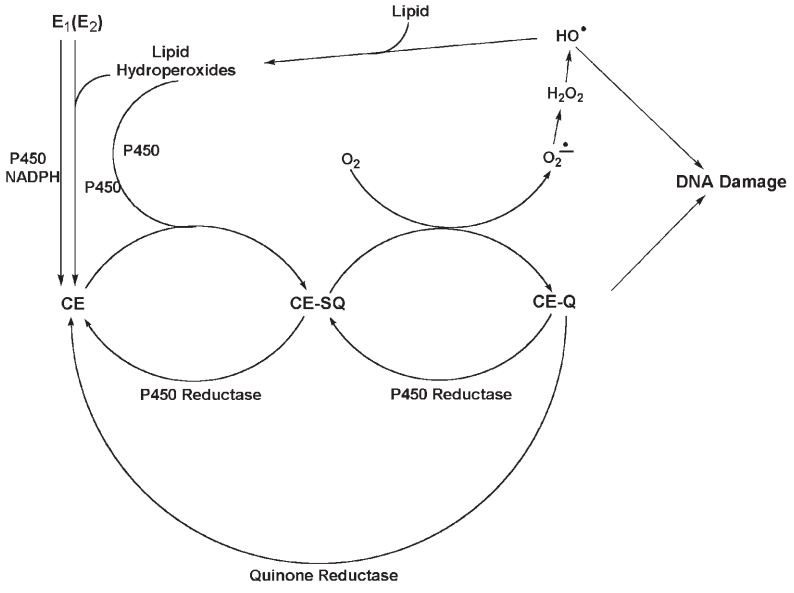


Figure 3. Redox cycling of CE-SQs and CE-Qs: DNA damage and formation of lipid hydroperoxides.

nicotinamide adenine dinucleotide phosphate (NADPH) serves not only as a cofactor, but also regulates cytochrome P450 in the oxidation of CE. Thus, once lipid hydroperoxides are formed, the oxidation of CE to CE-SQ and CE-Q can become a self-generating process that unbalances estrogen homeostasis and leads to formation of CE-Q.

Binding of CE-Qs to DNA

To determine whether DNA adducts are formed in biological systems, E_2-3,4-Q or enzymatically-activated 4-hydroxyestradiol (4-OHE_2) was reacted with DNA for 2 h at 37 EC [17]. The stable adducts were quantified by the ^{32}P-postlabelling method, and the depurinating adducts were analyzed by high-performance liquid chromatography (HPLC) interfaced with an electrochemical detector. When E_2-3,4-Q reacted with DNA, almost the same amount of the depurinating adducts 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua were obtained, and the amount of stable adducts was 0.02% of the depurinating ones. Activation of 4-OHE_2 by horseradish peroxidase gave similar results, whereas the mammalian lactoperoxidase produced a similar amount of N3Ade adduct, but about 50% more N7Gua adduct. The same two depurinating adducts were obtained in equal but smaller amounts when 4-OHE_2 was activated with tyrosinase or phenobarbital-induced rat liver microsomes. In all cases, the level of stable adducts was 0.02% or less compared with the depurinating adducts [E. L. Cavalieri et al., unpublished results].

DNA adducts were analyzed in vivo in rat mammary gland and mouse skin after treatment of the animals with E_2-3,4-Q or 4-OHE_2. Female ACI rats, which are susceptible to E_2-induced mammary tumors [34], were treated by intramammillary injection of E_2-3,4-Q or 4-OHE_2 (200 nmol in 20 µl of DMSO/gland at four teats) for 1 h. The mammary tissue was excised, extracted and analyzed for stable and depurinating adducts. N3Ade and N7Gua adducts from both 4-OHE_2 and 4-OHE_1 were detected in the range of 100–300 µmol/mol DNA-P [E. L. Cavalieri et al., unpublished results]. The level of stable adducts was not above the low level detected in untreated mammary tissue. Similarly, female SENCAR mice were treated topically on a shaved area of dorsal skin with E_2-3,4-Q [200 nmol in 50 µl of acetone/DMSO (9:1)] for 1 h [18]. The treated area of skin was excised, extracted and analyzed for stable and depurinating adducts. Equal amounts of 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua, ~12 µmol/mol DNA-P, were detected, and the amount of stable adducts was 0.02% of the depurinating adducts. These results in rats and mice demonstrate that the depurinating CE-DNA adducts are formed in vivo, generating apurinic sites in the DNA that could lead to oncogenic mutations.

Depurinating adducts and induction of mutations

Mouse skin provides a model system to study the conversion of DNA lesions, such as carcinogen-induced depurinating and stable DNA adducts, into mutations. In mouse skin, tumor initiation occurs when these DNA lesions are converted into oncogenic mutations in the H-*ras* gene. Previous studies indicated that stable adducts are inefficiently removed by excision repair, and cells containing these adducts enter the S-phase [35]. In the S-phase, occasional mutations are induced when replicative DNA polymerases go over adducted templates [36, 37]. Therefore, it was concluded that adduct-induced mutagenesis occurs in proliferating cells. These studies, however, did not address the fate of apurinic sites formed by the depurinating adducts.

Resting cells are greatly susceptible to tumor formation

Mouse skin is most susceptible to tumor formation by carcinogens during the telogen phase of the hair cycle. At telogen, epidermal thickness is low, indicating that at these times epidermal cells are in the resting phase. DMBA, which forms 99% depurinating adducts and 1% stable adducts [38], induces severalfold more tumors when applied to resting phase skin [39]. This suggests that apurinic sites induced by the depurinating DNA adducts may be most efficiently converted into oncogene-activating mutations in G0-G1-phase cells. We examined these questions with DB[*a,l*]P, the strongest among PAH carcinogens, which also forms 99% depurinating adducts and 1% stable adducts in mouse skin DNA [8] and induces the H-*ras* codon 61 (CAA-to-CTA) mutation in tumors [9].

Using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, we found that treatment of mouse skin with 200 nmol of DB[*a,l*]P resulted in the induction of these codon 61 mutations as early as 1 day after treatment [12]. In this technique, a segment of the H-*ras* gene is PCR amplified and the product is restricted with *Xba*I to examine the induction of a RFLP from the codon 61 mutation. We observed that at 1 day, 0.1% of the H-*ras* genes in the treated area of skin contained the codon 61 mutation, and then the population of these mutations increased to a maximum of 5% between 3 and 4 days after DB[*a,l*]P treatment. Subsequently, the level of mutations was reduced to background levels (0.0001%) [12]. The early time of induction of the codon 61 mutations (one day after DB[*a,l*]P treatment) coincides with suppression of DNA synthesis [40] and induction of excision repair [41, 42]. Therefore, we proposed that DB[*a,l*]P-induced DNA damage is converted into mutations by error-prone excision repair in pre-S-phase cells [12].

Error-prone repair of apurinic sites is a mechanism of tumor initiation

Evidence in support of this hypothesis was obtained from a comparative study of mutations in the mouse skin H-*ras* gene induced by 200 nmol of DB[*a,l*]P or 200 nmol of *anti*-DB[*a,l*]P-11,12-dihydrodiol-13,14-epoxide (*anti*-DB[*a,l*]PDE) [10]. Unlike DB[*a,l*]P, *anti*-DB[*a,l*]PDE forms 97% stable adducts and 3% depurinating adducts in DNA [8]. In these experiments, we identified the types of mutations that are induced in the early preneoplastic times (12 h to 9 days after treatment) and then analyzed whether these mutations were induced as a result of error-prone repair (fig. 4). The mutations were identified by PCR amplifying a segment of the H-*ras* gene from DNA extracted from mouse skin treated with one of the carcinogens, cloning the PCR products in a plasmid, isolating individual subclones and sequencing the H-*ras* inserts to identify mutations.

The mutation spectra induced by DB[*a,l*]P contained 90% A/T-to-G/C mutations at day 1. This correlated with the abundant DB[*a,l*]P-Ade depurinating adducts (81% of total adducts) and suggested that these A/T-to-G/C mutations were induced at Ade depurinations. Thus, the adducts could be correlated with these early preneoplastic mutations, as well as with the clonal H-*ras* mutations found in the tumors (table 1). If, according to our hypothesis, Ade depurinations induce these A/T-to-G/C mutations, they are A-to-G mutations generated as G.T heteroduplexes by error-prone excision repair (fig. 5).

Using a novel technique, we determined that these A-to-G mutations in the H-*ras* gene are initially induced as G.T heteroduplexes [10]. In this technique, G.T heteroduplexes in skin DNA are converted to G. apyrimidinic sites by treatment with T.G-DNA glycosylase (TDG) (fig. 5).

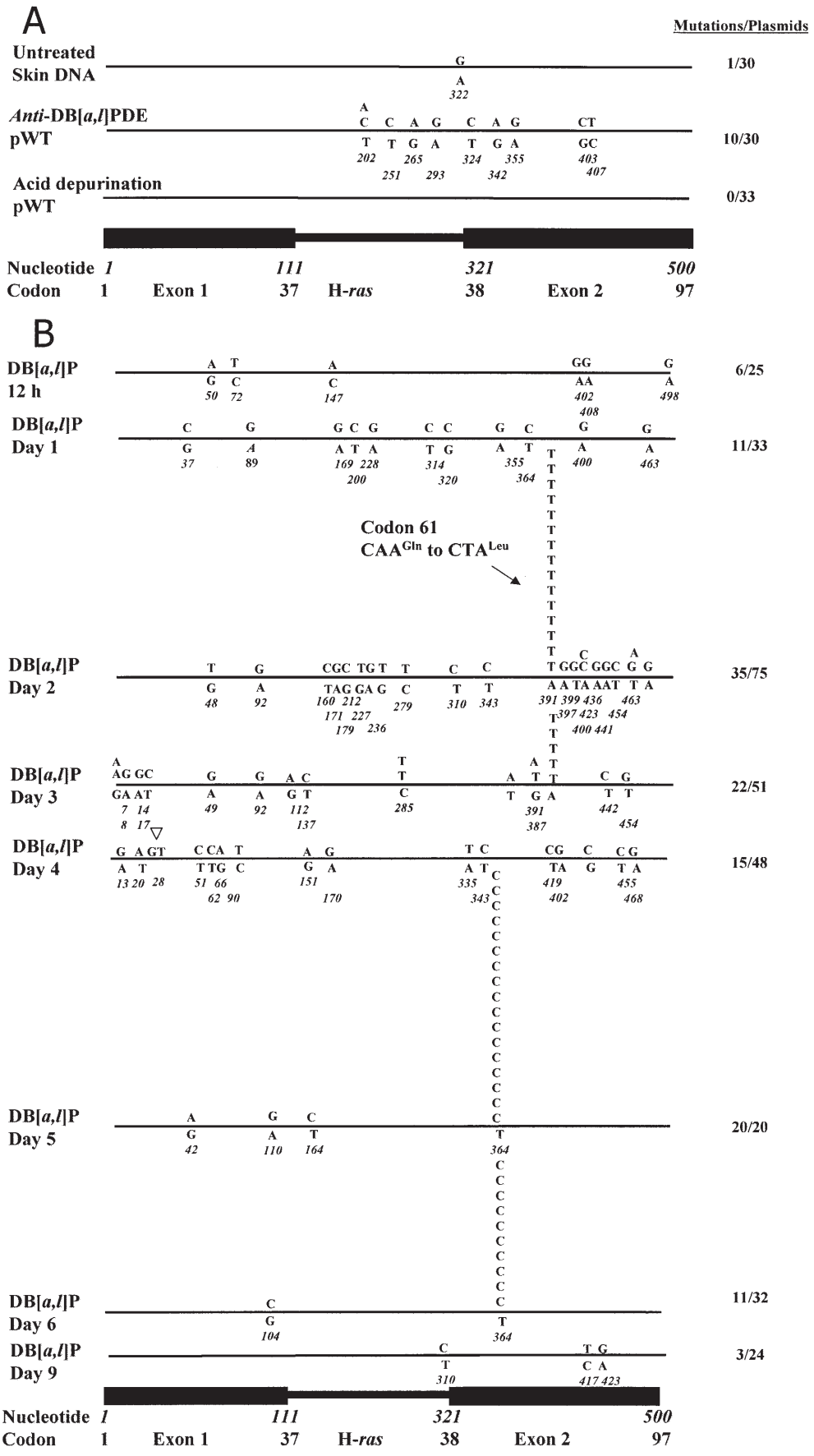
Depurinated DNA templates are refractory to PCR amplification [10, 43]. To demonstrate this point, we PCR amplified a mixture of two plasmids [one contained the wild-type H-*ras* exon 1–2 segment (pWT) and the other contained the same DNA with the codon 61 (CAA to CTA) mutation (pMUTX)]. The yield of pMUTX in the PCR product was determined by XbaI digestion. When pMUTX was incubated in an acidic buffer to induce a relatively small amount of depurination that did not significantly degrade the plasmid (~1 depurination/H-*ras* segment), mixed with untreated pWT and PCR amplified, a drastically reduced amount of the product was XbaI digestible [10]. This confirmed that depurinated templates are refractory to PCR amplification. Failure to score mutations in pWT depurinated either by acid treatment (fig. 4A) or through depurinating adduct formation by E_2-3,4-Q (fig. 7A) may be related to the unavailability of depurinated templates for PCR amplification.

Since abasic-site-containing DNA molecules are refractory to PCR amplification, the conversion of G.T heteroduplexes into G. apyrimidinic sites makes H-*ras* molecules containing these heteroduplexes unamplifiable. Under these circumstances, PCR preferentially amplifies templates that do not contain G.T heteroduplexes. As a result, PCR amplification of the H-*ras* gene from TDG-treated skin DNA, followed by cloning the PCR product and isolating and determining the sequence of individual subclones, causes a specific, drastic reduction of A-to-G mutations in the mutation spectra. In addition, the preferential PCR amplification artificially enriches low-abundance mutations that are observed only in TDG-treated spectra.

Following the entry of skin cells into S-phase, however, the G.T heteroduplexes are converted into G.C and A.T base pairs by one round of replication. At this stage, TDG treatment does not reduce the frequency of A-to-G mutations in the spectra. Thus, the specific reduction of A-to-G mutations in the mutation spectra by the TDG-PCR procedure characterizes these mutations as G.T heteroduplexes. The TDG-PCR procedure resulted in a drastic reduction in the population of A/T-to-G/C mutations on day 1, but did not make a significant change at days 2 and 3 (table 2). Therefore, A-to-G mutations remained as G.T heteroduplexes until 1 day after DB[*a,l*]P treatment of the skin, beyond which they were present as G.C and A.T mutations, presumably by replication [10]. Flow cytometric analysis of epidermal keratinocytes isolated from DB[*a,l*]P-treated mouse skin confirms that cells begin to enter the S-phase 1 day after the treatment [D. Chakravarti et al., unpublished results].

A major difference in mutation spectra induced by DB[*a,l*]P and *anti*-DB[*a,l*]PDE was the presence or absence of multiple codon 61 (CAA to CTA) mutations in early preneoplastic skin. These mutations were detectable 1 day after DB[*a,l*]P treatment by the PCR-RFLP procedure, which indicated that they constitute 0.1% of H-*ras* genes [12], and in the mutation spectrum obtained after TDG treatment of skin DNA [10]. Since these CAA-to-CTA mutations were observed during the active repair period, we hypothesize that they were also induced by error-prone repair as T.T heteroduplexes. Since these mutations were present in days 2–3 in a significantly greater

Figure 4A, B. H-*ras* mutations induced by DB[*a,l*]P or its metabolite, *anti*-DB[*a,l*]PDE. Wild-type sequences and nucleotide numbers (GenBank accession no. U89950) are indicated below, and mutations above the line. (*A*) PCR artifact mutations induced in untreated skin DNA and in a cloned H-*ras* gene (pWT) treated with *anti*-DB[*a,l*]PDE or with acid. Under the treatment conditions, *anti*-DB[*a,l*]PDE induces 1 adduct per 1000 bases, and acid induces 1 depurination per 170 bases [10]. (*B*) H-*ras* mutations in mouse skin DNA after treatment with 200 nmol DB[*a,l*]P in 100 µL of acetone. At 12 h – 1 day, the spectra contained mostly A/T-to-G/C mutations. At days 2 and 3, multiple codon 61 mutations were observed. At 4 days, no clear pattern of mutations could be determined. At days 5 and 6, multiple codon 52 (CTA-to-CCA) mutations were observed. Few mutations were observed at day 9. ▽, insertion.


A
Mutations/Plasmids
Untreated Skin DNA
1/30
Anti-DB[a,l]PDE pWT
10/30
Acid depurination pWT
0/33
Nucleotide 1 111 321 500
Codon 1 Exon 1 37 H-ras 38 Exon 2 97
B
DB[a,l]P 12 h
6/25
DB[a,l]P Day 1
11/33
Codon 61
CAA^Gln to CTA^Leu
DB[a,l]P Day 2
35/75
DB[a,l]P Day 3
22/51
DB[a,l]P Day 4
15/48
DB[a,l]P Day 5
20/20
DB[a,l]P Day 6
11/32
DB[a,l]P Day 9
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Nucleotide 1 111 321 500
Codon 1 Exon 1 37 H-ras 38 Exon 2 97

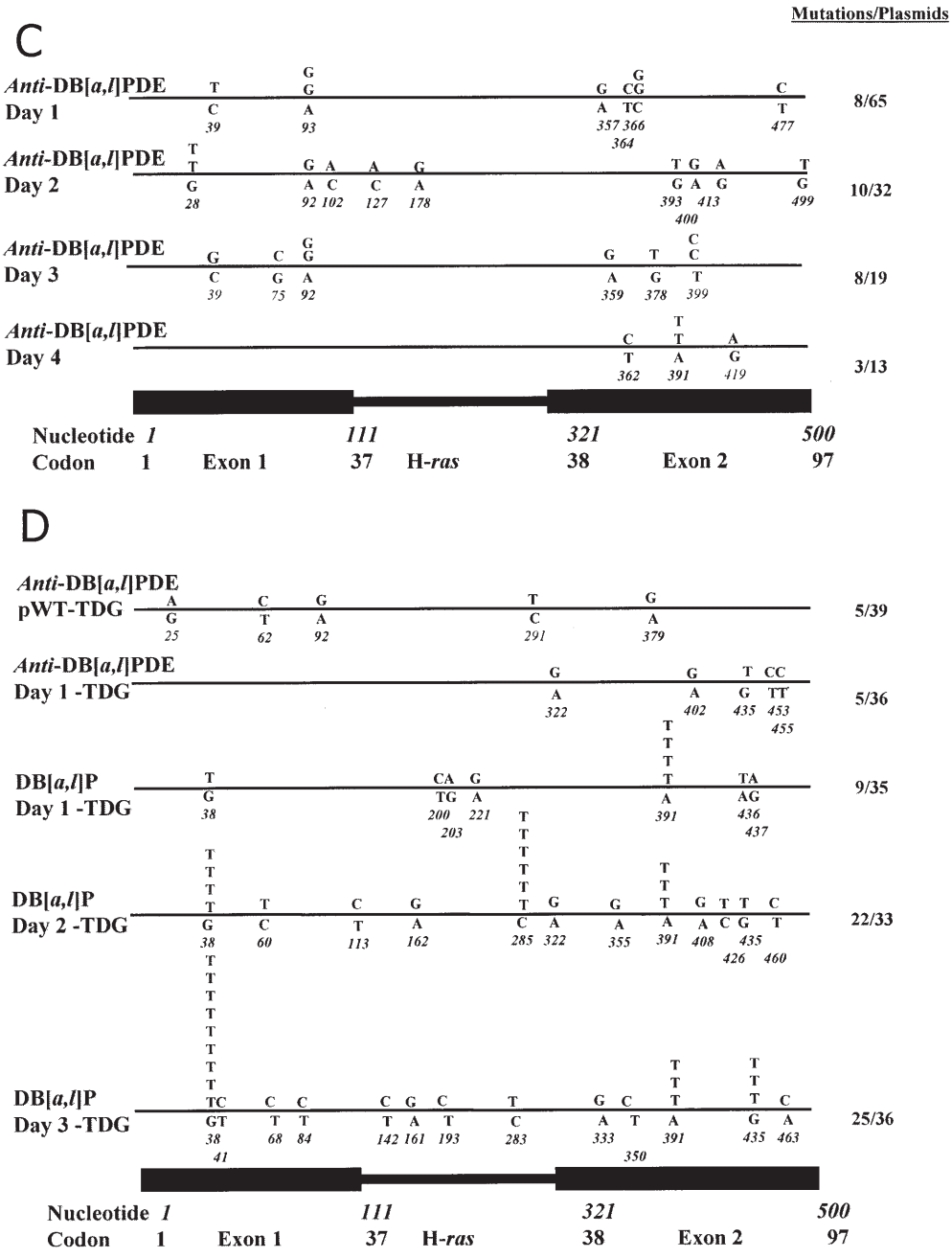

Figure 4C, D. (*C*) H-*ras* mutations in mouse skin DNA after treatment with 200 nmol of *anti*-DB[*a,l*]PDE in 100 µL of acetone. Fifty to 60% of mutations between days 1 and 4 were A/T-to-G/C mutations. (*D*) H-*ras* mutations after TDG treatment of DNA from *anti*-DB[*a,l*]PDE-treated pWT and from DB[*a,l*]P- or *anti*-DB[*a,l*]PDE-treated mouse skin. TDG treatment resulted in drastic reduction of A/T-to-G/C mutations and the observation of multiple codon 61 (CAA-to-CTA) mutations at day 1. These mutations were also observed at days 2 and 3. In addition, at days 2 and 3, multiple codon 13 (GGC-to-GTC) mutations were observed.

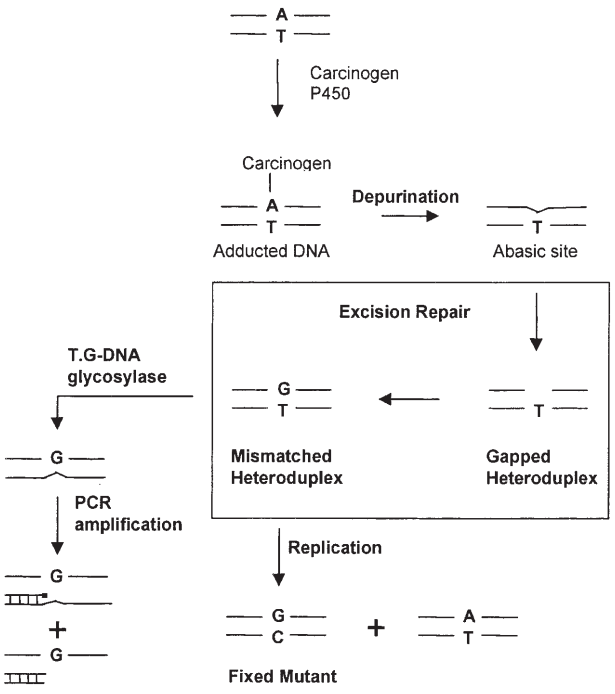

Figure 5. Proposed pathway of formation of A-to-G mutations by error-prone base excision repair of carcinogen-induced apurinic sites and the detection of the resulting G.T heteroduplexes by the TDG-PCR technique. The conversion of G.T heteroduplexes into G.apyrimidinic sites results in a drastic reduction in the formation of A/T-to-G/C mutations. G.T heteroduplexes are converted into fixed mutations (G.C and A.T pairs) by one round of replication.

Sequence	Mutation (nt)
CTGGAGGCGTG	C (37)
TGTGGACGAGT	G (89)
TGACCAAACAG	G (169)
TGGGGTATGAT	C (200)
GTGCAAGGGTG	G (228)
TGCAAAACAAC	*G (314)*
TTGCAGGACTC	C (320)
TGGGGAGACAT	G (355)
ATGTCTACTGG	C (364)
AGAGTATAGTG	G (400)
CATCAACAACA	G (463)

Figure 6. Sequence similarity among sites of DB[*a,l*]P-induced mutations in H-*ras* DNA of mouse skin at day 1. A putative conserved sequence is shaded. The mutated base is underlined. The italicized sequence ($A^{314} \rightarrow G$ mutation) is from the bottom strand.

frequency relative to other mutations in the spectra, we hypothesize that the increase in frequency was due to a clonal proliferation of codon 61-mutated (initiated) cells. Further studies suggest that at days 2–3, these codon 61-mutated cells express activated Ras protein [D. Chakravarti et al., unpublished observations].

On the other hand, *anti*-DB[*a,l*]PDE formed approximately 50% A/T-to-G/C mutations, which correlated with 48.5% formation of *anti*-DB[*a,l*]PDE-Ade stable adducts in mouse skin DNA (table 2). The frequency of these mutations was not significantly reduced by the TDG-PCR procedure, indicating that these mutations were not induced by error-prone repair. Studies conducted in other laboratories also indicate that nucleotide excision repair of bulky stable adducts is error-free [44]. When the pWT plasmid was treated with *anti*-DB[*a,l*]PDE in vitro (97% bulky stable adducts) and subjected to PCR amplification, A/T-to-G/C mutations were also found to constitute 50% of all mutations (5 out of 10) (fig. 4A). These mutations are induced by translesional synthesis over bulky stable adducts by the PCR polymerases. If, as has been proposed by others [35], only a small population of PAH-induced bulky stable adducts is removed by prereplication repair, a large fraction of *anti*-DB[*a,l*]PDE-induced adducts would persist in the mouse skin DNA. It is, therefore, possible that the mutations found in *anti*-DB[*a,l*]PDE-treated mouse skin DNA are adduct-induced PCR artifacts. The similarity of the frequencies of A/T-to-G/C mutations in vitro and in skin is consistent with this idea.

Four days after treating mouse skin with DB[*a,l*]P, we observed no codon 61 mutations in 48 plasmids that contained 15 other mutations (fig. 4B). No definite patterns of mutations were recognized at this time. At days 5 and 6, the mutation spectra were mainly limited to codon 52 (CTA-to-CCA) mutations. This coincided with the early phase of DB[*a,l*]P-induced hyperplasia that starts at day 5 and persists beyond day 10 [45, 46]. The codon 52 mutation may be oncogenic, but further study is required.

Table 2. The frequency of changes in DB[*a,l*]P-induced A/T to G/C mutations by TDG treatment followed by PCR.

PAH	DNA	Day	A/T-to-G/C mutations/total mutations	
			–TDG	+TDG
anti-DB[*a,l*]PDE	pWT	–	5/10 (50%)	3/5 (60%)
	skin	1	5/8 (62.5%)	4/5 (80%)
DB[*a,l*]P	skin	1	10/11 (90%)	2/10 (20%)
		2	11/35 (31%)	6/22 (27%)
		3	7/22 (31%)	8/25 (32%)

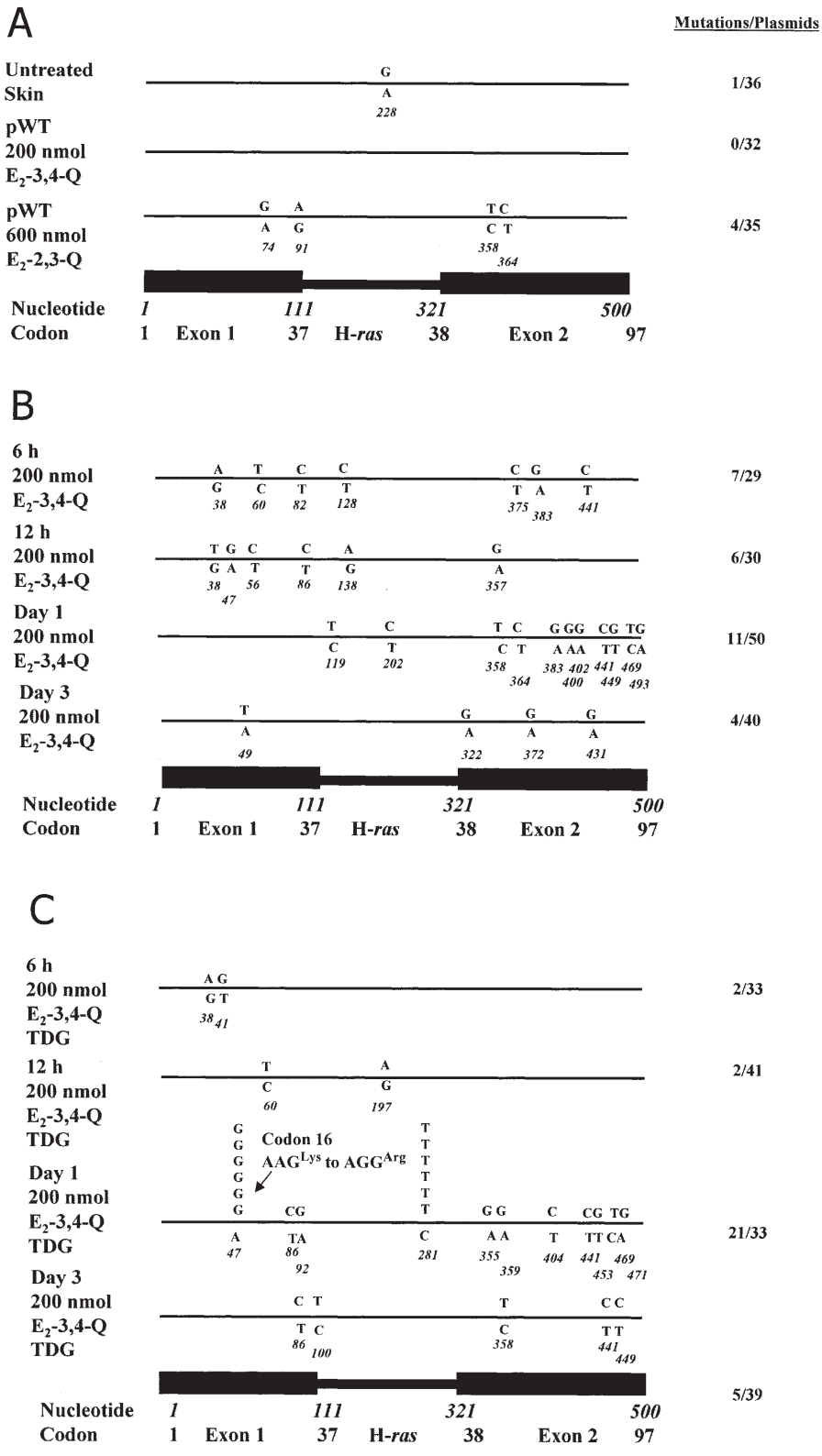

Figure 7. H-*ras* mutations induced by E_2-3,4-Q. (*A*) PCR artifact mutations induced in untreated skin DNA and in a cloned H-*ras* gene (pWT) treated with E_2-2,3-Q or with E_2-3,4-Q. (*B*) H-*ras* mutations in mouse skin DNA after treatment with 200 nmol E_2-3,4-Q in 100 µL of acetone/ethanol (70:30). The spectra contained mostly A/T-to-G/C mutations. (*C*) H-*ras* mutations after TDG treatment of DNA from E_2-3,4-Q-treated mouse skin. TDG treatment resulted in drastic reduction of A/T-to-G/C mutations in 6- and 12-h samples, but not in 1- and 3-days samples. This suggests that these mutations were in the form of G.T heteroduplexes between 6–12 h, but were converted into fixed mutations after that.

The repair error-induced A/T-to-G/C mutations in DB[*a,l*]P-treated mouse skin frequently occurred 3′ to a sequence element, TGN doublet (fig. 6), whereas these mutations in *anti*-DB[*a,l*]PDE-treated skin did not show a sequence context preference (not shown in fig. 6) [10]. This suggests that the sequence context of the depurinated base may determine the erroneous base incoporated by repair. We also noted that DB[*a,l*]P induces approximately 120-fold more depurinations through the depurinating adducts [8,9] than are formed by spontaneous base loss (10,000–20,000 depurinations/cell/day) [11, 47]. This raised the possibility that abundant depurination may be a factor in inducing infidelity in repair.

Effect of a burst of DNA depurination

Treatment of mouse skin with E_2-3,4-Q provided evidence that abundant depurination may induce errors in repair. Like DB[*a,l*]P, E_2-3,4-Q forms predominantly depurinating adducts in mouse skin DNA, consisting of roughly equal amounts of two depurinating adducts (4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua) [18]. The N3Ade adduct depurinates instantaneously after its formation, whereas the N7Gua adduct depurinates slowly, with a half-life of 5 h [48]. The difference in the rate of depurination of the two adducts provided a way to examine the effect of abundant depurination on repair fidelity. Briefly, E_2-3,4-Q would challenge the mouse skin repair machinery with a burst of Ade-specific depurination and slow-release Gua-specific depurination. Should a burst of depurination be a contributing factor in causing repair to be error-prone, a greater frequency of Ade-specific mutations compared with Gua-specific mutations would be expected in the mouse skin DNA.

Treatment of mouse skin with 200 nmol of E_2-3,4-Q induced primarily A/T-to-G/C mutations in the H-*ras* gene (fig. 7) [18]. For example, 6 h after E_2-3,4-Q treatment, 7 mutations were identified among 29 H-*ras* inserts. Five of the seven were A/T to G/C mutations. At 12 h, 4 out of the 6 mutations found in 30 H-*ras* inserts were A/T to G/C mutations. At day 1, 7 out of the 11 mutations found among 50 plasmids were A/T-to-G/C mutations. Cells do not have enough time to replicate by 6 h, but they may undergo repair. The observation that E_2-3,4-Q induces mutations at 6 h is, therefore, a basis to propose that these mutations are induced by error-prone repair. To confirm this, we conducted TDG-PCR analysis of these mutations (fig. 7). Specifically, at 6 h, TDG treatment reduced the frequency of the A/T-to-G/C mutations from 5 in 29 H-*ras* inserts to 0 in 33 H-*ras* inserts. At 12 h, the change was from 4 in 30 H-*ras* inserts to 0 in 41 plasmids. These results suggest that at 6–12 h, A/T-to-G/C mutations were in the form of G.T heteroduplexes. By day 1, a major change in the frequency of A/T-to-G/C mutations was not observed, following TDG treatment, suggesting that G.T heteroduplexes were present as G.C and A.T pairs. The TDG-treated 1-day spectrum was dominated by two clonal mutations of equal frequency (codon 16 AAG-to-AGG and intronic C-to-T mutations). Because it is unlikely that the intronic mutation would affect Ras activity and the two clonal mutations were found in the same frequency, we speculate that these mutations were allelic, belonging to a clonally proliferating population. In contrast, TDG treatment of day 3 DNA did not make any perceptible changes from the TDG-untreated spectrum. This suggests that the mutations found at day 3 were double-stranded and could not be affected by TDG treatment.

E_2-3,4-Q-induced early A/T-to-G/C mutations were frequently found at Ade depurinations 5′ to G residues. This supports the hypothesis that the sequence context of depurination influences the selection of which base is incorporated during error-prone repair.

Although these studies suggest that depurinating adducts play a major role in inducing transforming mutations to begin the process of tumorigenesis, the stable adducts can also contribute to these processes. Studies indicate that erroneous base incorporation during replication over various bulky stable adducts contributes to the induction of transforming mutations. For example, the BP-7,8-dihydrodiol-9,10-epoxide-N^2dG stable adduct induces A incorporation, forming G-to-T mutations [37], and the corresponding N^6dA stable adduct induces C incorporation, forming A-to-G mutations [49]. Similar studies indicate that E_2-2,3-Q, which induces primarily bulky stable adducts [26–28], is also mutagenic. The 2-OHE_2-N^6dA stable adducts cause mostly A-to-T mutations and some A-to-G mutations, whereas 2-OHE_2-N^2dG stable adducts cause mainly G-to-T mutations [50–52].

Estrogen Homeostasis

There are several factors that unbalance estrogen homeostasis, namely, the equilibrium between activating and deactivating metabolic pathways with the scope of averting the reaction of endogenous CE-Q with DNA (fig. 2). The first critical factor could be excessive synthesis of E_2 by overexpression of aromatase, CYP19, in target tissues [53–57] and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1 [58]. The observation that breast tissue can synthesize E_2 in situ suggests that much more E_2 is present in some sites of target tissues than would be predicted from plasma concentrations [57].

A second critical factor in unbalancing estrogen homeostasis might be the presence of high levels of 4-CE due to overexpression of CYP1B1, which converts E_2 predominantly to 4-OHE_2 (fig. 2) [19–21]. A relatively large amount of 4-CEs could lead to more extensive oxidation to CE-3,4-Q, with increased likelihood of damaging DNA.

A third factor could be a lack or low level of COMT activity. If this enzyme is insufficient, either through a low

level of expression or its low-activity allele, 4-CEs will not be effectively methylated, facilitating their oxidation to the ultimate carcinogenic metabolites, CE-3,4-Qs (fig. 2).

A fourth factor might be a low level of GSH and/or low levels of quinone reductase and/or cytochrome P450 reductase [23, 24]. This imbalance can leave available a higher level of CE-Q that may react with DNA. The effects of some of these factors have already been observed in model studies with Syrian golden hamsters [59] and transgenic knockout mice [60], as well as in human breast tissue samples [61].

Studies in Syrian golden hamsters

The hamster provides an excellent model for studying activation and deactivation (protection) of estrogen metabolites in relation to formation of CE-Q. In fact, implantation of E_1 or E_2 in male Syrian golden hamsters induces renal carcinomas in 100% of the animals, but does not induce liver tumors [62]. Therefore, comparison of the profiles of estrogen metabolites, conjugates and DNA adducts in the two organs should provide information concerning the imbalance in estrogen homeostasis generated by treatment with E_2. Hamsters were injected with 8 µmol of E_2 per 100 g of body weight, and liver and kidney extracts were analyzed for 31 estrogen metabolites, conjugates and depurinating DNA adducts by HPLC interfaced with an electrochemical detector [59]. Neither the liver nor the kidney contained 4-methoxyCE, presumably due to the known inhibition of COMT by 2-CE [63]. More *O*-methylation of 2-CE was observed in the liver, whereas more formation of CE-Q was detected in the kidney (table 3) [59]. These results suggest less protective methylation of 2-CE and more pronounced oxidation of CE to CE-Q in the kidney. To further investigate the rationale behind this interpretation, hamsters were first pretreated with L-buthionine (*SR*)-sulfoximine, an inhibitor of GSH synthesis, to deplete GSH levels. The hamsters were then treated with E_2. Very low levels of CE and methoxyCE were observed in the kidney compared with the liver, suggesting little protective reduction of CE-Q to CE in the kidney (table 3). Most significantly, the 4-$OHE_1(E_2)$-1-N7Gua depurinating adduct, arising from reaction of CE-3,4-Q with DNA, was detected in the kidney, but not in the liver (table 3) [59]. From these results, it seems that tumor initiation in the kidney occurs because of poor methylation of CE, which favors the competitive oxidation of CE to CE-Q, and poor reductase activity to remove CE-Q. Thus, these two effects lead to a large amount of CE-Q, which can react with biological nucleophiles, including those in DNA.

Studies in estrogen receptor-α knockout (ERKO)/Wnt-1 mice

A novel model for breast cancer was established by crossing mice carrying the *Wnt*-1 transgene (100% of adult females develop spontaneous mammary tumors) with the ERKO mouse line, in which the mice lack estrogen receptor-α, and estrogen receptor-β is not detected in the mammary tissue [64]. Mammary tumors develop in these mice despite the lack of functional estrogen receptor-α [64]. To begin investigating whether estrogen-metabolite-mediated genotoxicity may play an important role in the initiation of mammary tumors, the pattern of estrogen metabolites and conjugates was analyzed in ERKO/Wnt-1 mice. Extracts of hyperplastic mammary tissue and mammary tumors were analyzed by HPLC interfaced with an electrochemical detector [60]. Picomole amounts of the 4-CEs were detected, but their methoxy conjugates were not. Neither the 2-CEs nor 2-methoxyCEs were detected. 4-CE-GSH conjugates or their hydrolytic products (conjugates of cysteine and *N*-acetylcysteine) were detected in picomole amounts in both tumors and hyperplastic mammary tissue, demonstrating the formation of

Table 3. Selected estrogen metabolites, conjugates and adducts formed in hamsters treated with E_2 or E_2 plus BSO.[a]

Metabolites/conjugates[b]/adducts	nmol/g tissue			
	Kidney		Liver	
	E_2	E_2 + BSO	E_2	E_2 + BSO
2-$OHE_1(E_2)$	2.66	1.02	4.75	10.27
4-$OHE_1(E_2)$	0.29	0.14	0.44	1.04
2-$OCH_3E_1(E_2)$	1.13	0.42	4.16	4.46
$E_1(E_2)$-2,3-Q conjugates[b]	1.36	0.21	0.63	0.13
$E_1(E_2)$-3,4-Q conjugates[b]	0.30	0.09	0.06	0.01
$E_1(E_2)$-3,4-Q N7Gua adducts	< 0.01	0.27	< 0.01	< 0.01

[a] Data are from [59]. BSO, *L*-buthionine (*SR*)-sulfoximine. The notation $E_1(E_2)$ indicates that the metabolites, conjugates or adducts of both E_1 and E_2 are detected.

[b] Conjugates include all compounds produced by reaction of CE-Q with GSH and detected as GSH, cysteine or *N*-acetylcysteine conjugates.

CE-3,4-Qs. These preliminary findings indicate that estrogen homeostasis is unbalanced in the mammary tissue, in that the normally minor 4-CE metabolites were detected in the mammary tissue, but not the normally predominant 2-CEs. In addition, methylation of CE was not detected, whereas formation of 4-CE-GSH conjugates was. These results are consistent with the hypothesis that mammary tumor development is primarily initiated by metabolism of estrogens to CE-3,4-Qs, which may react with DNA to induce oncogenic mutations.

Studies in human breast tissue specimens

Imbalances in estrogen homeostasis were also observed in women with breast carcinoma compared with women without breast cancer (table 4) [61]. Breast tissue specimens obtained from women undergoing breast biopsy or surgery were analyzed for 31 estrogen metabolites, conjugates and depurinating DNA adducts by HPLC with electrochemical detection. In women without breast cancer, a larger amount of 2-CE than 4-CE was observed. In women with breast carcinoma, the 4-CEs were 3.5 times more abundant than the 2-CEs and were four times higher than in the women without breast cancer. Furthermore, a statistically lower level of methylation was observed for the CEs in cancer cases compared with controls. Finally, the level of CE-Q conjugates in women with cancer was three times that in controls, suggesting a larger probability for the CE-Qs to react with DNA in the breast tissue of women with carcinoma. These data suggest that initiation of human breast cancer is due to imbalances in estrogen homeostasis that result in excessive formation of the electrophilic CE-Qs. In particular, the CE-3,4-Qs can react with DNA to generate successively depurinating adducts, apurinic sites and oncogenic mutations leading to breast cancer.

Unifying mechanism of initiation of cancer and other diseases

Oxidation of catechols to semiquinones and quinones is a postulated pathway to initiate cancer not only with endogenous estrogens but also with synthetic estrogens such as the human carcinogen diethylstilbestrol [65] and its hydrogenated derivative hexestrol. In fact, these two compounds are also carcinogenic in the kidney of Syrian golden hamsters [62, 66], and the major metabolites are their catechols [66–69]. These catechols may be metabolically converted to catechol quinones. The catechol quinone of hexestrol has chemical and biochemical properties similar to those of CE-3,4-Q, namely it specifically forms N7Gua and N3Ade adducts by 1,4-Michael addition after reaction with dG or Ade, respectively, as well as DNA (fig. 8A) [70 and unpublished results]. These data suggest that the hexestrol catechol quinone is the electrophile involved in tumor initiation by hexestrol. In turn, these results substantiate the hypothesis that CE-3,4-Q may be the major endogenous tumor initiators.

The oxidation of phenols to catechols and then to semiquinones and quinones is not only a mechanism of tumor initiation for natural and synthetic estrogens, but it could also be the mechanism of tumor initiation for the leukemogen benzene (fig. 8B). Certain metabolites of benzene may be responsible for both its cytotoxic and genotoxic effects [71–73]. Benzene is metabolized to phenol in the liver by cytochrome P450 2E1 [73–75]. Other metabolites include catechol, hydroquinone (1,4-dihydroxybenzene) and muconaldehyde [76–78]. Catechol and hydroquinone accumulate in the bone marrow [79,80], where they can be oxidized by peroxidases, including myeloperoxidase and prostaglandin H synthase [81–87]. The resulting quinones can yield DNA adducts [85–87].

In fact, catechol, one of the metabolites of benzene, when oxidized to catechol quinone, reacts with dG and Ade to form the catechol-4-N7Gua and catechol-4-N3Ade

Table 4. Estrogen metabolites and conjugates in breast tissue from women with and without breast cancer.

Breast Tissue	Compounds,[a] pmol/g tissue						
	4-OHE$_1$(E$_2$)	2-OHE$_1$(E)$_2$	4-OHE$_1$(E$_2$) / 2-OHE$_1$(E$_2$)	4-OMeE$_1$(E$_2$)	2-OMeE$_1$(E$_2$)	4-+2-OMeE$_1$(E$_2$)	CE-Q conjugates
Controls[b]	3.6 ± 2.1 (10)[c]	6.9 ± 6.1 (25)	0.52	4.9 ± 1.8 (24)	3.6 ± 2.3 (16)	8.5	2.6 ± 1.3 (29)
Breast cancer cases[b]	14.7 ± 11.5 (53)	4.2 ± 4.6 (46)	3.5	31. ± 2.3 (39)	1.7 ± 1.0 (29)	4.8	8.2 ± 6.4 (57)
p[d]	0.047	n.s.[e]		0.049	0.050		0.003

[a] The notation E$_1$(E$_2$) indicates that the metabolites, conjugates or adducts of both E$_1$ and E$_2$ were detected.
[b] Controls include 18 women with benign breast tissue and 31 with benign fibrocystic changes for a total of 49 women. Breast cancer cases include 28 women with carcinoma of the breast.
[c] Number in parentheses indicates the percentage of specimens in which the compound was detected.
[d] p was calculated by the Student's t-test.
[e] n.s.: not significant.

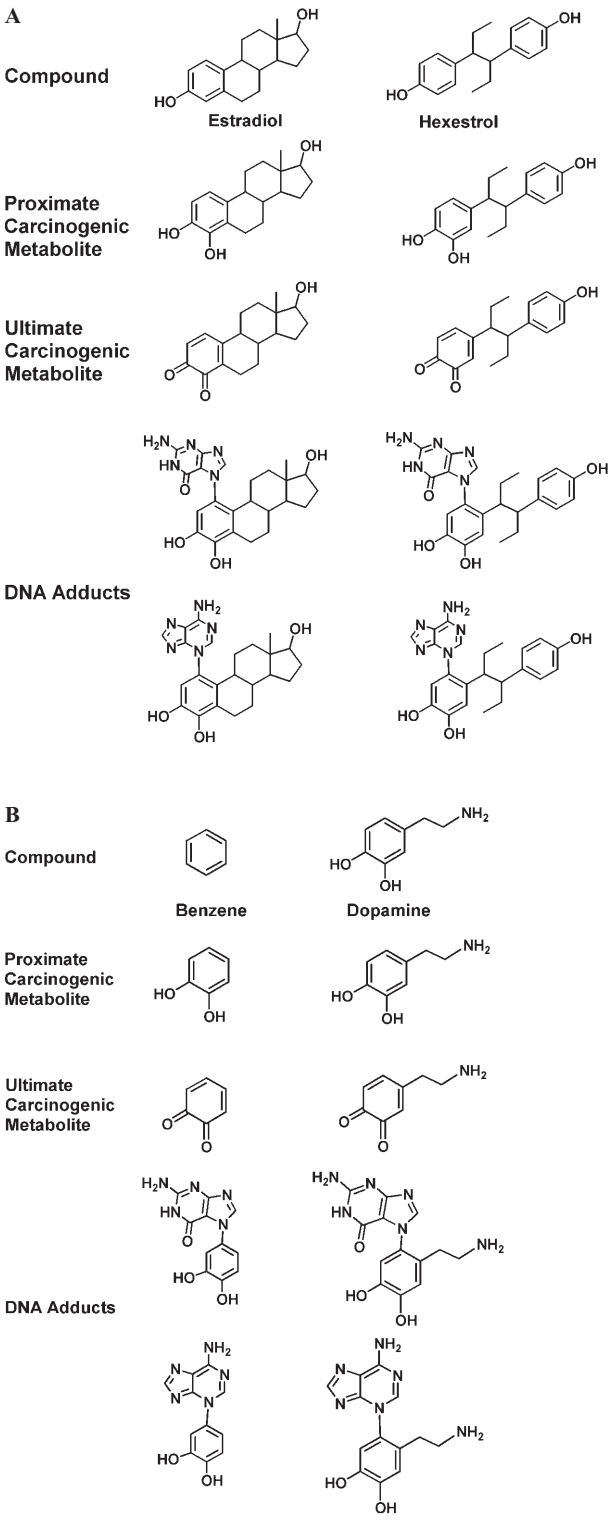

Figure 8. A unifying mechanism of activation and formation of DNA adducts. (*A*) Natural and synthetic estrogens, and (*B*) benzene and dopamine.

adducts in high yields, respectively [88, 89]. Oxidation of catechol catalyzed by horseradish peroxidase, tyrosinase or phenobarbital-induced rat liver microsomes in the presence of DNA yielded the catechol-4-N7Gua adduct, while the catechol-4-N3Ade adduct was obtained only with tyrosinase [89].

The formation of depurinating adducts specifically at the N-7 of Gua and N-3 of Ade by 1,4-Michael addition to catechol quinone suggests that the metabolite catechol may play a major role in tumor initiation by benzene. The leukemogenicity of benzene could result from a synergistic response to catechol quinone, which predominantly produces depurinating DNA adducts, and 1,4-benzoquinone, which produces only stable DNA adducts [85, 86, 90, 91].

Catecholamine neurotransmitters such as dopamine may produce semiquinones and quinones via autoxidation, metal ion oxidation and peroxidative enzyme or cytochrome P450 oxidation [92–94]. This oxidative process is similar to the one described for the benzene metabolite catechol and the 4-CEs, and it may initiate Parkinson's disease and other neurodegenerative disorders. The etiology of Parkinson's disease and the basic mechanism of loss of dopamine neurons are unknown. One of the functions of dopamine is the synthesis of neuronmelanin via oxidation of dopamine to its quinone. We hypothesize that if oxidation of dopamine to its quinone does not occur in a properly controlled environment, dopamine quinone may react with DNA to cause damage by formation of specific depurinating adducts. In fact, N7Gua and N3Ade adducts (fig. 8B) are obtained by reaction of the dopamine quinone with dG or Ade, respectively [88, 89], and the same adducts are formed when dopamine is enzymatically activated in the presence of DNA [89]. The mutations generated by this damage may play a role in the initiation of Parkinson's disease and other neurodegenerative disorders.

Conclusions

The carcinogenicity of estrogens in animal models led us to investigate the plausible estrogen metabolites that could react with DNA and lead to mutations initiating cancer. The electrophilic CE-3,4-Qs can, indeed, react with DNA to form the specific depurinating adducts bonded at the N-7 of Gua and N-3 of Ade [25]. The apurinic sites formed by depurinating adducts are converted into tumor-initiating mutations by error-prone repair [10, 18]. The specificity of the reaction of the electrophiles with DNA is not limited to the natural estrogens, but also includes the carcinogenic synthetic estrogens such as hexestrol. In this case metabolic formation of its catechol and further oxidation to its catechol quinone lead to formation of analogous specific depurinating

adducts at the N-7 of Gua and N-3 of Ade [70]. In addition, the metabolite catechol of the leukomogenic benzene and the catecholamine neurotransmitter dopamine, when oxidized to quinone, bind to DNA to form N7Gua and N3Ade adducts.

Thus, a unifying mechanism, namely formation of catechol quinones and reaction with DNA by 1,4-Michael addition to yield depurinating adducts, could be at the origin of cancers induced by oxidation of endogenous and synthetic estrogens, leukemia by oxidation of benzene and neurodegenerative diseases by oxidation of dopamine. This proposed unifying mechanism in the initiation of these diseases lays the groundwork for developing strategies to assess risk and prevent diseases.

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