Bioreductive activation of catechol estrogen-ortho-quinones: Aromatization of the B ring in 4-hydroxyequilenin markedly alters quinoid formation and reactivity

Li Shen, Emily Pisha, Zhiwen Huang, John M. Pezzuto, E. Krol, Zishan Alam, Richard B. van Breemen and Judy L. Bolton

Department of Medicinal Chemistry and Pharmacognosy (M/C 781), College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St, Chicago, IL 60612–7231, USA

Introduction

A firm link between female reproductive variables and increased risk of developing cancer in several tissues, especially the breast, has been established from epidemiological studies (1, 2). The longer women are exposed to estrogens either through early menarche and late menopause (3) and/or through estrogen replacement therapy (ERT*), the higher is the risk of developing cancer (4, 5). Recent data indicate that as many as 30% of post-menopausal women in the United States are currently receiving ERT (6). Although there are many benefits of ERT, including a substantial reduction in the risk of coronary heart disease or osteoporosis, such statistics emphasize the importance of fully understanding all the deleterious effects of estrogens including their potential to initiate and/or promote the carcinogenic process.

Premarin (Wyeth-Ayerst) is the estrogen replacement treatment of choice and in 1995, it was the most widely dispensed prescription in the USA according to ‘The Top 200 Drugs’ survey (7). In spite of the high level of exposure of post-menopausal women to this formulation, very little is known about the metabolism and potential toxic metabolites that could be produced from the 10 different estrogens which make up Premarin (Table I). It is known that treating hamsters for 9 months with either estrone, equilin + equilenin, or sulfatase-treated Premarin, resulted in 100% tumor incidences and 100% mortality (8). In this study, we synthesized the catechol metabolite of equilenin, 4-hydroxyequilenin, and examined how aromatization of the B ring affects the formation and reactivity of the o-quinone (3, 5-cyclohexadien-1,2-dione). 4-Hydroxyequilenin-o-quinone is much more redox-active and longer-lived than the endogenous catechol estrone-o-quinones, which suggests that the mechanism(s) of toxicity of the former could be quite different. Interestingly, the rate of reduction of the 4-hydroxyequilenin-o-quinone is increased at least 13-fold in the presence of NAD(P)H:quinone oxidoreductase (DT-diaphorase). Once NADH is consumed however, the catechol auto-oxidized rapidly to the o-quinone. NADH consumption was accompanied by dicumarol-sensitive oxygen uptake both with the purified enzyme and with cytosol from human melanoma cells with high levels of DT-diaphorase activity. P450 reductase and rat liver microsomes also catalyzed NADPH consumption and oxygen uptake. 4-Hydroxyestrone-o-quinone was also rapidly reduced by NADPH; however, this o-quinone does not auto-oxidize and once the o-quinone is reduced the reaction terminates. Including oxidative enzymes in the incubation completes the redox couple and 4-hydroxyestrone-o-quinone behaves like 4-hydroxyequilenin-o-quinone. These data suggest that reduction of estrogen-o-quinones may not result in detoxification. Instead this could represent a cytotoxic mechanism involving consumption of reducing equivalents (NADH/NADPH) as well as formation of superoxide and other reactive oxygen species leading to oxidative stress. Finally, we have compared the cytotoxicity of 4-hydroxyequilenin with that of the estrone catechols in human melanoma cells. 4-Hydroxyequilenin is 5-fold more toxic in these cells compared with 4-hydroxyestrone (ED50 = 7.8 versus 38 µM, respectively) suggesting that formation of the longer-lived redox-active 4-hydroxyequilenin-o-quinone was responsible for the cytotoxic differences. These results substantiate the conclusion that the involvement of quinoids in catechol estrogen toxicity depends on a combination of the rate of formation of the o-quinone, the lifetime of the o-quinone, and the electrophilic/redox reactivity of the quinoids.

Abbreviations: 2-OHE, 2-hydroxyestrone, 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHE, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHEN, 4-hydroxyequilenin, 3,4-dihydroxy-1,3,5(10),6,8-estrapentaen-17-one; estrone, 3-hydroxy-1,2,5,10(1)-oestratrien-17-one; equilenin, 1,3,5(10),6,8-estrapentaen-3-ol-17-one; equilin, 1,3,5(10),7-estratetraen-3-ol-17-one; 4-NC, 1,2-dihydroxynaphthalene; 2-THNC, 3,4-dihydroxy-5,6,7,8-tetrahydroquinophthylene; 4-THNC, 1,2-dihydroxy-5,6,7,8-tetrahydroquinophthylene; ERT, estrogen replacement therapy; P450, cytochrome P450; quinone methide, 4-alkyl-2,5-cyclohexadien-1-one; o-quinone, 3,5-cyclohexadien-1,2-dione; DT-diaphorase, NAD(P)H:quinone oxidoreductase; SOD, superoxide dismutase; DES, diethylstilbestrol; (E)-4,4'-(1,2-diethyl-1,2-ethenediyli)biphenyl.

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implicated as the ultimate carcinogens. Redox cycling between the catechols and their o-quinones generates reactive hydroxyl radicals which causes oxidation of the purine/pyrimidine residues of DNA (10). o-Quinones are also Michael acceptors and they could be responsible for alkylation of DNA which has been detected by 3H-post-labeling methods (11). Alternatively, we have shown that additional reactive intermediates can be produced from isomerization of the catechol estrogen o-quinones to highly electrophilic p-quinone methides (12,13), which could be the ultimate DNA alkylating agents.

The relative rates of 2- and 4-hydroxylation in baboon liver microsomes has been examined for estrone, 17β-estradiol, equilin and equilenin (14, Table I). Interestingly, the ratio of 2:4 hydroxylation was found to vary dramatically with substrate; thus increasing unsaturation in the B ring leads to a change in metabolism from predominantly 2-hydroxylation for estrone to exclusively 4-hydroxylation for equilin. This is particularly significant since 4-hydroxyestrone (4-OHE) was found to be a renal carcinogen in the male Syrian golden hamster (15) whereas 2-hydroxyestrone (2-OHE) was not carcinogenic in this animal model. Further, in all susceptible tissues to tumor induction by 17β-estradiol (i.e. hamster kidney, mouse uterus and rat pituitary), 4-OHE formation predominates, whereas in tissues resistant to estrogen carcinogenesis (i.e. liver), 2-hydroxylation predominates (16). Finally, it has also been shown with in vitro models that the 4-hydroxyquinol (4-OHEN) o-quinone increases the amount of oxidative damage to DNA by 50% compared with control levels (17). These preliminary studies show the importance of fully examining the effect of structural changes in the B ring on quinoid formation from these estrogens.

NAD(P)H:quinone acceptor/oxygen reductase (DT-diaphorase) is generally known for its ability to detoxify p-quinones by catalyzing their two-electron reduction to hydroquinones using either NADH or NADPH as the reducing cofactor (18,19). Presumably, DT-diaphorase could catalyze a similar two-electron reduction of o-quinones to catechols, although, to the best of our knowledge, this reaction has not been systematically studied. In the case of some antitumor agents, it has been shown that DT-diaphorase can catalyze bioreductive activation (18). For example, the p-quinone antitumor agent, streptonigrin, is efficiently reduced by DT-diaphorase to the hydroquinone that auto-oxidizes rapidly generating two equivalents of superoxide (20). As elevated levels of DT-diaphorase have been detected in breast tumors (21), DT-diaphorase-mediated generation of reactive oxygen species through redox-cycling of catechol estrogen o-quinones may represent a carcinogenic mechanism. Alternatively, for catechol estrogens that do not auto-oxidize to o-quinones, DT-diaphorase-catalyzed reduction of the o-quinone should represent a detoxification mechanism.

In the present study, we examined how aromatization of the B ring in 4-OHEN affects the formation and reactivity of the o-quinone. Interestingly, 4-OHEN-o-quinone is much more redox-active and longer-lived than the catechol estrone-o-quinones, which suggests that the mechanism(s) of toxicity of the former could be quite different. We hypothesized that 4-OHEN-o-quinone-mediated cytotoxicity primarily involves the formation of reactive oxygen species rather than alkylation by quinoids. To confirm this theory, we conducted experiments to evaluate the role of reductive enzymes and cofactors in mediating 4-OHEN-o-quinone-catalyzed formation of reactive oxygen species to further investigate the cytotoxic mechanism of this estrogen metabolite. The AB ring analog of 4-OHEN, 4-NC, is a metabolite of the environmental pollutant naphthalene (22), showed similar effects on NAD(P)H oxidation, oxygen consumption and cytotoxicity. These data give strong evidence that bioreductive redox-cycling of 4-OHEN-o-quinone plays a major role in cellular damage, whereas with 2-OHE- and 4-OHE-o-quinones, other cytotoxic pathways likely contribute.

**Materials and methods**

### Caution

All quinones used in this study and the catechol estrogen o-quinones in particular were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens (23).

All chemicals were purchased from Aldrich (Milwaukee, WI), Fisher Scientific (Itasca, IL) or Sigma (St Louis, MO) unless stated otherwise. 2-OHE and 4-OHE were either purchased from Sigma (St Louis, MO) or synthesized as described previously (24). Both 2- and 4-THNC were synthesized as described previously (25,26). Recombinant human DT-diaphorase was obtained from expression of the DT-diaphorase coding region isolated from the H460 non-small cell lung cancer cell line in Escherichia coli as described previously (27). The specific activity was 625 ìmol of 2,6-didehydrophthalidohalphenol reduced/min per mg protein. All reductase activity was NAD(P)H-dependent and could be inhibited by dicumarol. Recombinant rat P450 reductase was purchased from Pan Vera (Madison, WI). The specific activity was 56.6 ìmol cytochrome C reduced/min per mg protein. Male Sprague–Dawley rats (180–200 g) were obtained from Sasco Inc. (Omaha, NE). Microsomes were prepared from the livers of male Sprague–Dawley rats (180–200 g) as described previously (28).

### Synthesis of 4-OHEN and 4-OHEN-o-quinone

4-OHEN was synthesized by treating equilin (Sigma) with Fremy’s salt in a similar manner to the procedure described in References 8 and 29. We used equilin as the starting material instead of equilenin (Steraloids, Inc., Wilton, NH) because of the expense of the latter, and we have found that the harsh synthetic conditions cause aromatization of the B ring in equilin leading to formation of 4-OHEN exclusively. Briefly, 500 mg Fremy’s salt in 10 ml H2O was added to 300 mg equilin in 100 ml acetone and 40 ml H2O and stirred for 10 min at 25°C. Fremy’s salt (500 mg) was repeatedly added (five times) until TLC no longer showed the existence of equilin. The mixture was extracted four times with 100 ml chloroform. The chloroform extracts were combined and washed with 30 ml cold H2O, 20 ml 1 N HCl, and then another 30 ml cold H2O. The solution was stirred with a solution of 5 g KI in 5 ml H2O and 10 ml acetic acid for 30 min then washed with 2×20 ml 10% sodium thiosulfate solution. The solution was then washed with 20 ml H2O and 20 ml saturated aqueous NaCl, and dried over MgSO4. The solvent was evaporated in the presence of 100 mg ascorbic acid (to prevent formation of 4-OHEN-o-quinone). The residue was dissolved in ethyl acetate and purified through ascorbic acid coated silica gel using a 10–30% gradient of ethyl acetate/hexane as the mobile phase. The product obtained was dissolved in ethyl acetate and washed with water. After the solvent was evaporated, the residue was crystalized in ethyl acetate/hexane. Proton NMR assignments

### Table I. Estrogens present in Premarin and ratio of 2:4 hydroxylation of the A ring

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>% of totala</th>
<th>Ratio of 2-OH:4-OHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estriol</td>
<td>42</td>
<td>9:1</td>
</tr>
<tr>
<td>8-Dehydroestriol</td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>Equilin</td>
<td>17</td>
<td>2:4:1</td>
</tr>
<tr>
<td>17α-Dehydroequilin</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>Equilenin</td>
<td>4.3</td>
<td>&lt;0.3:1</td>
</tr>
<tr>
<td>17α-Dehydroequilenin</td>
<td>3.4</td>
<td>ND</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>1.5</td>
<td>16:1</td>
</tr>
<tr>
<td>17β-Dehydroequilin</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>17β-Dehydroequilenin</td>
<td>0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

aMost are present as sulfate esters. From Reference (8).

bFrom Reference (14).

Not determined.

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Kinetic studies

The auto-oxidation rate of 4-OHEN was determined by monitoring the appearance of the o-quinone chromophore \((\lambda_{\text{max}} = 392 \text{ nm})\) spectrophotometrically. To determine the rate of disappearance of 4-OHEN-o-quinone, incubations (15 ml) were carried out at pH 7.4, 37°C and aliquots (500 µl) were quenched with perchloric acid (50 µl/mL). Aliquots of the supernatant (100 µl) were analyzed directly by HPLC with a 4.6×150 mm Ultrasphere C-18 column (Beckman) on a Shimadzu LC-10A gradient HPLC equipped with UV detector. SPD-10AV UV/VIS photodiode array detector, and SPD-10AV UV detector set at 280 nm. The mobile phase consisted of 10% methanol in 0.25% perchloric acid/0.25% acetic acid (pH 3.5) at 1.0 ml/min for 2 min and increased to 90% CH₃OH over 28 min. Under these conditions, 4-OHEN-o-quinone has a retention time of 17 min.

Cell cytosol preparation

Cell cytosol from cultured UIC-SO-MEL-2 (human melanoma) cells was obtained from two to five 75-cm² cell culture flasks. Cells were grown to 80% confluence and detached by trypsinization. The cells were pelleted by centrifugation at 4°C in 15 ml conical tubes. The cell pellets were washed twice with PBS followed each time by centrifugation. The cell pellets were suspended in ice-cold buffer containing 25 mM Tris–HCl and 125 mM sucrose (pH 7.4). The suspensions were homogenized for 2 min on ice and centrifuged at 100,000 g for 30 min at 4°C to yield a clear cytosolic fraction. DT-diaphorase activity was determined as described previously (31).

Oxidation of NADPH

Reduction of o-quinones was monitored by UV-visible spectrophotometry using a Hewlett-Packard HP4542 diode array spectrophotometer. 4-OHEN (30 µM) was added to 50 mM potassium phosphate buffer (pH 7.4) containing 0.7 mg/ml BSA and allowed to auto-oxidize to the o-quinone. 4-NC-o-quinone (Aldrich) was added as a solution in dimethylsulfoxide. 4-OHE-o-quinone was prepared by mixing catalyzed oxidation in acetonitrile as described previously (32) and introduced as a concentrated acetonitrile solution to the buffer. The acetonitrile was removed under nitrogen using an N-Evap Nitrogen concentrator. NADH (0.2 mM) was added and its oxidation monitored at 340 nm. Anaerobic reactions were performed by deoxygenating the solutions through three successive cycles of freezing, evacuation under vacuum, refilling with argon, and thawing. Measurements were carried out in argon purged cuvettes via syringe.

Concentrations of NADH were determined based on literature extinction coefficients (33). Reactions were run in the presence and absence of dicumarol (20 µM).

Reduction of o-quinones with purified P450 reductase or with UIC-SO-MEL-2 cytosol were determined in a similar fashion by monitoring NADPH oxidation spectrophotometrically at pH 7.4, 25°C. With rat liver microsomes, incubations containing microsomal protein were conducted at 37°C in 50 mM potassium phosphate buffer (pH 7.4, 1 ml total volume) (34). 4-NC-o-quinone was added as a solution in DMSO (1 µl of 100 mM). 4-OHEN was also added in DMSO (1 µl of 100 mM) and allowed to auto-oxidize to the o-quinone prior to addition of microsomes and NADPH. For control incubations, heat inactivated microsomes were used. The reactions were initiated by the addition of NADPH and the rate of NADPH oxidation was monitored at 340 nm.

Oxygen uptake

Oxygen uptake was measured in 600 µl reactions using a Clark-type polarographic micro oxygen probe (Yellow Springs Instrument Company, Yellow Springs, OH) at 25 or 37°C. Air-saturated potassium phosphate buffer (50 mM pH 7.4) was used as 100% oxygen. Substrates were introduced as described above. Oxygen-uptake was monitored over 20 min and converted to µM oxygen/min.

Evaluation of the cytotoxic potential of catechols in cultured UIC-SO-MEL-2

Assays were performed by procedures developed by the National Cancer Institute (35) as described previously (36,37). Briefly, the cells were closed-cap cultured in MEMH (Life Technologies, Grand Island, NY) supplemented with penicillin, streptomycin, fungizone and 10% fetal bovine serum (FBS). The media was changed 24 h before beginning cytotoxicity assays in order to maintain logarithmic growth. The cells were harvested by trypsinization, washed twice with PBS and resuspended in 100 µl of 100 mM) and allowed to auto-oxidize to the o-quinone chromophore (30). The test samples were assayed in triplicate and final concentrations ranged from 0.03–150 µg/ml. For each catechol, attempts were made to treat the cells with doses that were 2.5- to 6.25-fold above and below the estimated ED₅₀ value. Each assay included negative controls (cells treated with DMSO only) that were used to define 100% cell viability. The catechols did not interfere with the attachment of the cells to the plates as determined by similar ED₅₀ values for cells treated immediately upon plating as compared with cells allowed to attach for 1 h prior to addition of test compound. The plates were incubated for 3 days. Following incubation, the cells were fixed with trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound dye was liberated with 0.1 M Tris base, and the absorbance at 515 nm was measured with a microtiter plate reader. The ED₅₀ values were obtained by regression and linear estimation analysis. All assays were performed 3–6 times.

Instrumentation

HPLC experiments were performed on the above mentioned Shimadzu HPLC system. Peaks were integrated with Shimadzu EZ-Chrom software and a 486-33 computer. ¹H NMR spectra were obtained with a Varian XL-300 spectrometer at 300 MHz and CI/EI mass spectra were obtained with a Finnigan MAT 90 magnetic sector mass spectrometer.

Results

Formation and reactivity of 4-OHEN-o-quinone

In this study, we synthesized one of the metabolites of equilenin, 4-OHEN, and examined how aromatization of the B ring affects formation and reactivity of the o-quinone. Although the synthesis of 4-OHEN has been previously reported (8,29), our synthesis is much more economical since the starting material (equilenin) is 95% (4-OHEN, MH⁺). The appearance of 4-OHEN during CI-MS is probably due to reduction of the o-quinone in the mass spectrometer (30).
monitored by HPLC since the o-quinone is stable for at least 1 day in strong acid. At 37°C, pH 7.4 this o-quinone has a half-life of 2.3 h compared with 12 min for 4-OHE-o-quinone and 47 s for 2-OHE-o-quinone (12). At present it is not known what the products of 4-OHEN-o-quinone decomposition are; however, it is likely that the o-quinone isomerizes to quinone methides similar to reactions of 4-OHE- and 2-OHE-o-quinones (12,13).

**o-Quinone mediated oxidation of NAD(P)H**

As mentioned previously, it has been shown that DT-diaphorase can catalyze bioreductive activation (18). In this study, we incubated the o-quinones with and without DT-diaphorase and followed the disappearance of the NADH chromophore at 340 nm. As observed previously with 4-OHE-o-quinone (38) and 4-NC-o-quinone (39), 4-OHEN-o-quinone was spontaneously reduced by NADH. However, unlike 4-OHE-o-quinone, which is not a substrate for DT-diaphorase (38), the rate of reduction for both naphthyl o-quinones was increased 13-fold for 4-OHEN-o-quinone and 22-fold for 4-NC-o-quinone when DT-diaphorase was included in the incubation (Figure 2D, Table II). In the presence of dicumarol, a potent inhibitor of DT-diaphorase (33), the rate enhancement for 4-OHEN-o-quinone was abolished. Dicumarol also inhibited 4-NC-o-quinone mediated NADH oxidation although the rate was only decreased 7-fold. Both naphthyl catechols formed proved to be unstable in the presence of oxygen, and reduction of the o-quinones by DT-diaphorase was accomplished by continuous oxidation of NADH (Figure 2). After NADH was completely consumed the o-quinone chromophore rapidly appeared at 410 nm. Molecular oxygen was found to play a predominant role in the auto-oxidation of 4-OHEN as hypoxic conditions inhibited the rate of DT-diaphorase mediated NADH oxidation by a factor of 10 (Table II).

In agreement with previous work (38), we have found that 4-OHE-o-quinone is rapidly reduced by NADH (1.5 ± 0.1 mM NADH/min); however, unlike 4-OHEN-o-quinone, once all of the 4-OHE-o-quinone is reduced to the catechol, auto-oxidation does not take place and the reaction terminates (Figure 2C). Including DT-diaphorase in the incubation did not enhance the reduction rate, which confirmed previous studies (38). When tyrosinase (Figure 2D) or microsomes (data not shown) are included with NAD(P)H and 4-OHE-o-quinone, a redox couple is created, and NAD(P)H is rapidly consumed followed by appearance of the o-quinone (λ<sub>max</sub> = 420 nm). NADH-mediated reduction of both 4-OHE- and 4-OHEN-o-quinones can be completely abolished by addition of GSH, which suggests that GSH must be depleted in vivo prior to the onset of oxidative stress.

The reduction of the o-quinones by UIC-SO-Mel-2 cytosol was quantified by following oxidation of NADPH in the presence and absence of dicumarol. This particular cell line was chosen because of the high DT-diaphorase activity (1.3 µmol/min per mg of protein) relative to other tumor cell lines (i.e. DT-diaphorase activity for 16 tumor cell lines ranged from 0.08–1.5 µmol/min per mg of protein, 40). As observed for experiments with the purified enzyme, dicumarol decreased the rate of metabolism of 4-OHEN-o-quinone by a factor of five (Table II). Although the rate of reduction of 4-NC-o-quinone was enhanced by melanoma cytosol, dicumarol only inhibited the reaction by 23%. It appears that other cellular reductases are equally effective in metabolizing 4-NC-o-quinone. The NADH rate of reduction was 2–4-fold higher than studies discussed above due to the presence of BSA required to stabilize DT-diaphorase activity. Nucleophilic amino acid residues on BSA likely scavenge some of the naphthyl o-quinones resulting in an apparent decreased rate of NADH oxidation. These data indirectly suggest that DT-diaphorase was partially responsible for the enzyme-catalyzed reduction of 4-OHEN-o-quinone in melanoma cytosol; however, NAD(P)H alone appears to be sufficient for the metabolism of 4-NC-o-quinone.

Similar results were obtained in experiments with purified P450 reductase; the rate of NADPH oxidation was increased 10-fold for both 4-OHEN-o-quinone and 4-NC-o-quinone. In the presence of rat liver microsomes, NADPH oxidation was enhanced 2-fold for both naphthyl-o-quinones (Table II). Interestingly, when superoxide dismutase was included in the microsomal incubations, NADPH oxidation was enhanced an additional 2-fold for 4-OHEN-o-quinone.

**o-Quinone mediated oxygen consumption**

Since the oxidation of NADH could be inhibited by decreasing oxygen concentration (Table II), it is likely that molecular oxygen is responsible for auto-oxidation of the catechol and semi-quinone radical. Reduction of oxygen should produce superoxide which, in theory, could be detected by superoxide dismutase inhibition of succinyllated cytochrome C reduction (41). Unfortunately, superoxide dismutase also enhances the auto-oxidation rate of the catechols (discussed above) and the catechols reduced succinyllated cytochrome C directly (data not shown). As a result, superoxide formation could not be quantified using this spectrophotometric assay and we measured total oxygen consumption using the Clark type oxygen electrode instead (Table III). Experiments with purified DT-diaphorase showed a 5-fold increase in oxygen consumption for 4-OHEN-o-quinone and a 14-fold stimulation for 4-NC-o-quinone. Dicumarol was virtually 100% effective at restoring oxygen consumption rates to the NADH only catalyzed rate.

Metabolism of 4-OHEN-o-quinone by melanoma cytosol in the presence of NADPH also showed substantial oxygen uptake. Melanoma cytosol-mediated oxygen consumption was inhibited by dicumarol, which again suggested that DT-diaphorase was responsible for the activation of 4-OHEN-o-quinone. Experiments with 4-NC-o-quinone and melanoma cytosol also showed considerable oxygen uptake; however, oxygen consumption was not affected by dicumarol within experimental uncertainty. We also measured 4-OHE-o-quinone-mediated oxygen uptake in melanoma cytosol. Since this o-quinone does not auto-oxidize, it requires an oxidative enzyme to complete the redox couple and this is reflected in the small amount of oxygen uptake observed; 4-OHE-o-quinone consumes 0.61 µM O<sub>2</sub>/min, which is 11-fold less than 4-OHEN-o-quinone.

P450 reductase also stimulated oxygen consumption with both 4-OHEN- (5-fold) and 4-NC-o-quinone (4-fold) and the oxygen consumption rate could be further enhanced with superoxide dismutase. Microsomal incubations with NADPH and 4-OHEN-o-quinone only enhanced oxygen uptake 2-fold relative to heat-inactivated microsomes. Superoxide dismutase stimulated the rate for both naphthyl o-quinones an additional 2-fold, which is consistent with the rate enhancement detected with the NADPH oxidation experiments.

**Cytotoxicity studies of catechol estrogens in human melanoma cells**

Studies conducted with human melanoma cells displaying high levels of DT-diaphorase activity demonstrated that all of...
the catechols tested had some cytotoxic activity. The catechols caused cell death, not just inhibition of growth as ED50 values for cells treated for 1 h were comparable to data obtained after the usual 3-day incubation (data not shown). The catechol estrogens were divided into two groups based on both their cytotoxicity and the chemistry of their o-quinones (Table IV). 4-OHEN and 4-NC (group 1) are particularly potent cytotoxins, likely due to rapid auto-oxidation of the catechols to long-lived, redox-active o-quinones. Group 2 (Table IV) contains the endogenous estrone catechols and their AB ring analogs (i.e. 2-THNC and 4-THNC). In general, these catechols do not auto-oxidize to o-quinones, are not substrates for DT-diaphorase, and were much less toxic compared with the naphthyl catechols. Finally, inhibition of DT-diaphorase activity with dicumarol did not show any decrease in catechol-mediated toxicity for either 4-OHEN or 4-NC.

Discussion
Catechol estrogen-o-quinones have been implicated as the ultimate metabolites responsible for estrogen carcinogenesis although the nature of the lesion(s) and its mechanism of formation is not known. In this study, we have shown that formation of the o-quinone from the Premarin metabolite 4-OHEN occurs readily in aqueous solution without the need for enzymatic/chemical catalysis. Increasing pH enhances the auto-oxidation rate of 4-OHEN suggesting that the endogenous estrone catechols and their AB ring analogs (i.e. 2-THNC and 4-THNC). In general, these catechols do not auto-oxidize to o-quinones, are not substrates for DT-diaphorase, and were much less toxic compared with the naphthyl catechols. Finally, inhibition of DT-diaphorase activity with dicumarol did not show any decrease in catechol-mediated toxicity for either 4-OHEN or 4-NC.

Fig. 2. UV/VIS spectral analysis of NADH oxidation by 4-OHEN-o-quinone and 4-OHE-o-quinone. (A) Reactions contained 0.5 µg DT-diaphorase/ml, 0.7 mg/ml BSA, 0.2 mM NADH, 0.03 mM 4-OHEN-o-quinone, pH 7.4 phosphate buffer, 25°C. Scans every 60 s. (B) As in (A) without DT-diaphorase. (C) As in (A) with 4-OHE-o-quinone. Scans every 2 s. (D) Reaction contained 0.1 mM 4-OHE, 0.2 mM NADH, 50 µg/ml tyrosinase, pH 7.4 phosphate buffer, 25°C. Scans every 60 s.
of three determinations. contrast, superoxide dismutase can enhance the auto-oxidation of NADH to NADPH, which can stimulate the NADPH oxidation rate and oxygen consumption (38). Similarly, with DES-p-quinone, superoxide dismutase lowered reactive oxygen species formation to basal levels in hamster kidney microsomes (46). In contrast, superoxide dismutase can enhance the auto-oxidation rate of redox-labile hydroquinones and catechols (47). As a result, the SOD-mediated rate enhancement observed with 4-OHE-quinone-induced single-strand DNA breaks (48) nor did it influence hydrogen peroxide production (38). Similarly, with DES-p-quinone, superoxide dismutase stimulated both NADPH oxidation and oxygen uptake by 4-OHEN-quinone in rat liver microsomes. Depending on the reduction potential of the quinone/semi-quinone radical couple, superoxide dismutase can either stimulate or inhibit the formation of reactive oxygen species (46). For 4-OHE-p-quinone, the presence of superoxide dismutase in microsomal incubations abolished hydrogen peroxide production (38). Similarly, with DES-p-quinone, superoxide dismutase lowered reactive oxygen species formation to basal levels in hamster kidney microsomes (46).

### Table II. Rate of NAD(P)H oxidation by 4-OHEN- and 4-NC-o-quinones

<table>
<thead>
<tr>
<th>Condition</th>
<th>4-OHEN µM/min</th>
<th>4-NC µM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT-Diaphorase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4±0.1</td>
<td>6.7±0.2</td>
</tr>
<tr>
<td>NADH</td>
<td>58±5</td>
<td>150±7.0</td>
</tr>
<tr>
<td>NADH + DTD</td>
<td>3.2±0.3</td>
<td>21±2.0</td>
</tr>
<tr>
<td>NADH (An aerobic)</td>
<td>0.88±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>NADH + DTD (An aerobic)</td>
<td>6.2±0.7</td>
<td>ND</td>
</tr>
<tr>
<td>UIC-SO-MEL-2 Cytosol&lt;sup&gt;g&lt;/sup&gt;</td>
<td>11±0.3</td>
<td>9.3±0.4</td>
</tr>
<tr>
<td>NADPH</td>
<td>12±0.9</td>
<td>12±0.1</td>
</tr>
<tr>
<td>NADPH + cytosol</td>
<td>26±5.0</td>
<td>31±1.0</td>
</tr>
<tr>
<td>NADPH + cytosol + Dicumarol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7±0.8</td>
<td>24±3.0</td>
</tr>
<tr>
<td>P450 Reductase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.0±0.01</td>
<td>5.4±0.2</td>
</tr>
<tr>
<td>NADPH + P450 Reductase</td>
<td>58±2.0</td>
<td>54±2.0</td>
</tr>
<tr>
<td>NADPH + boiled P450 Reductase</td>
<td>6.2±0.01</td>
<td>5.8±0.02</td>
</tr>
<tr>
<td>Rat liver microsomes&lt;sup&gt;f&lt;/sup&gt;</td>
<td>40±4.0</td>
<td>39±2.0</td>
</tr>
<tr>
<td>NADPH + microsomes</td>
<td>59±3.0</td>
<td>74±4.0</td>
</tr>
<tr>
<td>NADPH + boiled microsomes</td>
<td>35±4.0</td>
<td>41±2.0</td>
</tr>
<tr>
<td>NADPH + microsomes + SOD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>126±7.0</td>
<td>91±1.1</td>
</tr>
<tr>
<td>NADPH + boiled microsomes + SOD</td>
<td>91±3.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reactions contained 0.5 µg DTD/ml (sp. act. = 625 µmol of 2,6-dichlorophenolindophenol reduced/min per mg protein), 0.7 mg/ml BSA, 0.2 mM NADH, 0.03 mM quinone, pH 7.4 phosphate buffer, 25°C. The values represent averages ± SD of three determinations.

<sup>b</sup>20 µM.

<sup>c</sup>Not determined.

<sup>d</sup>Reactions contained 72 µg/ml cytosol, 0.2 µg NAD(P)H, 50 µM quinone, pH 7.4 phosphate buffer, 25°C. DT-diaphorase activity in cell cytosol was 1.3 ± 0.15 µmol/min per mg protein.

<sup>e</sup>Reactions contained 5 µg P450 reductase/ml (sp. act. = 56.6 µmol cytochrome c reduced/min per mg protein), pH 7.4 phosphate buffer, 0.03 mM quinone, pH 7.4 phosphate buffer, 25°C. The values represent averages ± SD of three determinations.

<sup>f</sup>Reactions contained 0.4 mg/ml microsomal protein, 0.3 mM NADPH, 0.1 mM quinone, pH 7.4 phosphate buffer, 37°C.

<sup>g</sup>SOD from bovine liver, 0.1 mg/ml.

### Table III. Oxygen consumption by 4-OHEN- and 4-NC-o-quinones

<table>
<thead>
<tr>
<th>Condition</th>
<th>4-OHEN µM</th>
<th>Oxygen/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT-Diaphorase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±0.08</td>
<td>4.4±0.90</td>
</tr>
<tr>
<td>NADH + DTD</td>
<td>15±0.40</td>
<td>60±3.0</td>
</tr>
<tr>
<td>NADPH + DTD + Dicumarol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8±0.08</td>
<td>3.8±0.30</td>
</tr>
<tr>
<td>UIC-SO-MEL-2 Cytosol&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.7±0.03</td>
<td>35±1.7</td>
</tr>
<tr>
<td>NADPH + cytosol</td>
<td>1.7±0.10</td>
<td>25±1.5</td>
</tr>
<tr>
<td>NADPH + cytosol + Dicumarol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33±0.90</td>
<td>34±1.8</td>
</tr>
<tr>
<td>P450 Reductase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.8±0.90</td>
<td>8.3±0.30</td>
</tr>
<tr>
<td>NADPH + boiled P450 Reductase</td>
<td>42±1.0</td>
<td>48±1.3</td>
</tr>
<tr>
<td>Rat liver microsomes&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17±1.5</td>
<td>25±0.60</td>
</tr>
<tr>
<td>NADPH + boiled microsomes</td>
<td>7.3±0.60</td>
<td>15±0.80</td>
</tr>
<tr>
<td>NADPH + microsomes + SOD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32±0.70</td>
<td>56±2.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reactions contained 0.83 µg DTD/ml (sp. act. = 625 µmol of 2,6-dichlorophenolindophenol reduced/min per mg protein), 0.7 mg/ml BSA, 0.2 mM NADH, 0.03 mM quinone, pH 7.4 phosphate buffer, 25°C. The values represent averages ± SD of three determinations.

<sup>b</sup>20 µM.

<sup>c</sup>Reactions contained 33 µg/ml cytosol, 0.2 mM NADPH, 30 µM quinone, pH 7.4 phosphate buffer, 25°C. DT-diaphorase activity in cell cytosol was 1.3 ± 0.15 µmol/min per mg protein.

<sup>d</sup>Reactions contained 5 µg P450 reductase/ml (sp. act. = 56.6 µmol cytochrome c reduced/min per mg protein), pH 7.4 phosphate buffer, 0.03 mM quinone, 0.2 mM NADPH, 25°C. The values represent averages ± SD of three determinations.

<sup>e</sup>SOD from bovine liver, 0.1 mg/ml.

<sup>f</sup>Reactions contained 0.4 mg/ml microsomal protein, 0.3 mM NADPH, 0.1 mM quinone, pH 7.4 phosphate buffer, 37°C.

It has been shown that 4-OHE-o-quinone is not a substrate for DT-diaphorase although it is reduced by cytochrome P450 reductase and spontaneously by NAD(P)H alone (38). Dicumarol, a potent inhibitor of DT-diaphorase, had no effect on the 4-OHE-o-quinone-induced single-strand DNA breaks in MCF-7 cells (48) nor did it influence hydrogen peroxide production in experiments with purified DT-diaphorase (38). In contrast, the synthetic estrogen, diethylstilbestrol (DES), does undergo DT-diaphorase-mediated two-electron reduction of the catechol instead of a detoxification mechanism for 4-OHEN-p-quinone oxidation to yield benzo[a]pyrene-7,8-dione (44,45).

Table II shows that 4-OHE-o-quinone is a substrate for DT-diaphorase although it is reduced by cytochrome P450 reductase and spontaneously by NAD(P)H alone (38). Dicumarol, a potent inhibitor of DT-diaphorase, had no effect on the 4-OHE-o-quinone-induced single-strand DNA breaks in MCF-7 cells (48) nor did it influence hydrogen peroxide production in experiments with purified DT-diaphorase (38). In contrast, the synthetic estrogen, diethylstilbestrol (DES), does undergo DT-diaphorase-mediated two-electron reduction and it has been suggested that this pathway offers protection from reactive oxygen species formed through one-electron redox-cycling of the DES-p-quinone (49). In support of this, DT-diaphorase reduced the levels of superoxide by 73% in incubations with DES-p-quinone, NADPH, and kidney microsomes from hamsters (49). On the other hand, two-electron reduction by DT-diaphorase appears to be a cytotoxic pathway instead of a detoxification mechanism for 4-OHEN-o-quinone. The rate of reduction of 4-OHEN-o-quinone was increased at least 13-fold in the presence of DT-diaphorase (Table II). Once NADH is consumed however, the catechol auto-oxidized rapidly to the o-quinone. NADH consumption was accompanied by dicumarol-sensitive oxygen uptake, both with the purified enzyme and with cytosol from human melanoma cells with high levels of DT-diaphorase activity.

In this investigation, we confirmed that 4-OHE-o-quinone was rapidly reduced by NAD(P)H; however, this o-quinone...
Contribution from microsomal P450 reductase and/or other varied by a factor of 17, and yet the catechols were equally.

Competing mechanisms for estrogen.

Fig. 3. Data from Reference (25).

d Data from Reference (12).

b Three concentrations of Dicumarol were used (37, 75, 149 µM).

We have estimated the half-life at 1.5 h. It dimerizes to another

a Results are the average ± SD of at least three determinations.

Dicumarol c 6.9 µM). The results were not statistically different from experiments without dicumarol at any concentration. The data represents the average ED50 ± SD of the three concentrations of dicumarol.

We have estimated the half-life at 1.5 h. It dimerizes to another o-quinone as an example.

-quinones were the most toxic compounds substrates for DT-diaphorase. All are efficiently reduced by

-quinone. These data suggest that None of the group 2 catechols auto-oxidize nor are they reduction of estrogen-

-quinone-mediated damage since

methides and it is quite possible that a similar reaction occurs to the cell membrane to inhibit DT-diaphorase since the cell incubation media contains 10% FBS and dicumarol is highly protein bound. Alternatively, it is conceivable that a significant contribution from microsomal P450 reductase and/or other reductases occurs as DT-diaphorase is inhibited. For example, dicumarol does not protect hepatoma cells from polycyclic aromatic hydrocarbon-o-quinones (50) which could imply they are not substrates for DT-diaphorase or one electron reductases occurs as DT-diaphorase is inhibited. For example, dicumarol does not protect hepatoma cells from polycyclic aromatic hydrocarbon-o-quinones (50) which could imply they are not substrates for DT-diaphorase or one electron reductase cycles catalyzed by P450 reductase, cytochrome b5 reductase, or mitochondrial oxidoreductase take over as DT-diaphorase is inhibited (39, 51). These data along with the above experiments on NAD(P)H oxidation and oxygen consumption with purified DT-diaphorase, P450 reductase, melanoma cytosol, and microsomes strongly suggest that the cytoxic mechanism for 4-OHEN-o-quinone involves consumption of reducing equivalents (NADH/NADPH) as well as formation of superoxide and other reactive oxygen species leading to oxidative stress (Figure 3). Analogous results have been reported for several of the polycyclic aromatic hydrocarbon-o-quinones (39, 45, 50–52), which suggests that similarities in structure between 4-OHEN-o-quinone and these PAH metabolites may explain their proposed comparable cytotoxic/mutagenic mechanisms (53).

None of the group 2 catechols auto-oxidize nor are they substrates for DT-diaphorase. All are efficiently reduced by NAD(P)H however, and it is quite possible that along with enzyme-catalyzed (i.e. tyrosinase, P450, peroxidases) or metal ion oxidation, NADH/NADPH could complete the in vivo redox cycle. If the group 2 o-quinones are generated using tyrosinase, for example, we have found that NADH is completely consumed (Figure 2D), similar to what is observed with the non-enzymatic reaction with 4-OHEN-o-quinone (Figure 2B). Also, it has been shown that Cu(II) oxidizes 2-hydroxyestradiol resulting in significant oxygen uptake and DNA strand breaks (54). Interestingly, incubation of 4-OHE but not 2-OHE, with microsomes, NADPH and DNA induced 8-hydroxylation of guanine bases (17). This is particularly significant since only 4-OHE is carcinogenic in the hamster kidney (15), which is likely due to the longer lifetime of its o-quinone.

In previous work, we showed that the major products of decomposition of 4-OHE- and 2-OHE-o-quinones are quinone methides and it is quite possible that a similar reaction occurs with 4-OHEN-o-quinone. For the estrone catechols, the rate of isomerization of the o-quinones to the quinone methides varied by a factor of 17, and yet the catechols were equally.

Table IV. Cytotoxic potency of catechol estrogens versus reactivity of o-quinones in human melanoma cells

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>ED50 (µM)a</th>
<th>t1/2 of o-quinone (min, 37°C, pH 7.4)</th>
<th>DT-diaphorase substrates</th>
<th>Catechols auto-oxidation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-OHEN</td>
<td>7.8±2.5</td>
<td>138</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4-OHEN + Dicumarolb</td>
<td>8.2±4.3</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>4-NC</td>
<td>7.5±1.3</td>
<td>NDc</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4-NC + Dicumarolc</td>
<td>6.9±1.9</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-OHE</td>
<td>34±14 0.7d</td>
<td>?</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2-THNC</td>
<td>73±18</td>
<td>5.4e</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4-OHE</td>
<td>38±5.6</td>
<td>12d</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4-THNC</td>
<td>64±4.2</td>
<td>17d</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

a Results are the average ± SD of at least three determinations.
b Three concentrations of Dicumarol were used (37, 75, 149 µM). The results were not statistically different from experiments without dicumarol at any concentration. The data represents the average ED50 ± SD of three concentrations of dicumarol.
c We have estimated the half-life at 1.5 h. It dimerizes to another o-quinone as an example.
d Data from Reference (12).
e Data from Reference (25).

Fig. 3. Competing mechanisms for estrogen o-quinone toxicity using 4-OHEN-o-quinone as an example.

Table IV. Cytotoxic potency of catechol estrogens versus reactivity of o-quinones in human melanoma cells

<table>
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<tr>
<th>Estrogen</th>
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<th>Catechols auto-oxidation?</th>
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<tr>
<td>4-OHEN</td>
<td>7.8±2.5</td>
<td>138</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4-OHEN + Dicumarolb</td>
<td>8.2±4.3</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>4-NC</td>
<td>7.5±1.3</td>
<td>NDc</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4-NC + Dicumarolc</td>
<td>6.9±1.9</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2-OHE</td>
<td>34±14 0.7d</td>
<td>?</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2-THNC</td>
<td>73±18</td>
<td>5.4e</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4-OHE</td>
<td>38±5.6</td>
<td>12d</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4-THNC</td>
<td>64±4.2</td>
<td>17d</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
cytotoxic within experimental uncertainty (Table IV). The most likely explanation for this effect is a change in cytotoxic mechanism from primarily o-quinone-mediated initiation of cell death (i.e. for 4-OHE) to a bioactivation pathway based on both o-quinone and p-quinone methide formation (i.e. for 2-OHE). The initially formed o-quinone is likely responsible for GSH depletion since GSH reacts very rapidly with o-quinones forming conjugates (55). However, once GSH is depleted the 2-OHE-o-quinone rapidly isomerizes to quinone methides that could alkylate a variety of intracellular sites. In support of this, incubation of the 2-OHE-o-quinone with deoxypurine nucleosides did not give o-quinone trapped adducts; rather the o-quinone rapidly isomerized to the quinone methide, which then alkylated the nucleosides at the exocyclic amino groups of guanine and adenine (56). In contrast, reaction of 4-OHE-o-quinone with deoxyguanosine gave an N7-o-quinone-deoxyguanosine adduct (56). The slower rate of isomerization of 4-OHE-o-quinone to the quinone methide implies that the redox/alkylation chemistry of the o-quinone is responsible for damage. However, since 2-OHE is equally potent in melanoma cells, even though its o-quinone is 17-times shorter lived, quinone methide formation may also play a role in cytotoxicity.

In summary, on the basis of these data it may be concluded that the involvement of o-quinones in catechol estrogen toxicity depends on a combination of the rate of formation of the o-quinone, the rate of disappearance of the o-quinone, and the electrophilic/redox reactivity of the quinoids. The formation of reactive oxygen species is likely the cytotoxicity mechanism for the highly redox active estrogen, 4-OHEN. It should be noted that equilenin or 17-dehydroequilenin are the major urinary (57) and biliary (58) metabolites of equilin and it is quite possible that 4-OHEN quinoids are formed from these estrogens as well. For 2-OHE-o-quinone, which rapidly isomerizes to quinone methides, alklylation of cellular macromolecules by these potent electrophiles may also contribute to cellular damage. 4-OHE may also cause toxicity by all of the mechanisms outlined in Figure 3; however, redox-cycling by this o-quinone of intermediate reactivity may be more important than quinone methide alklylation reactions. All of these critical factors are closely linked to structure as well as microenvironment, and the details are just beginning to be elucidated. Given the direct link between excessive exposure to estrogens, metabolism of estrogens, and increased risk of breast cancer, it is crucial that factors that affect the formation, reactivity and cellular targets of estrogen quinoids, be explored.

Acknowledgements
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References
Bioreductive activation of estrogen-α-quinones


