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Mechanistic investigations on the antioxidant action of a neuroprotective estrogen derivative

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Abstract

Antioxidant action is an important component of the complex neuroprotective action of estrogens. Combining theoretical prediction and subsequent experimental confirmation by chemical and *in vitro* paradigms, this study focused on the mechanistic aspects of hydroxyl-radical scavenging by 17 β -butoxy-1,3,5(10)-estratrien-3-ol, a synthetic derivative of 17 β -estradiol with increased potency to inhibit lipid peroxidation and reduced affinity to estrogen-receptors compared to the endogenous hormone. In the process that acts as a “chemical shield,” the phenolic A-ring turns into 10 β -hydroxy-17 β -butoxy-1,3,5(10)-estratrien-3-one, a non-aromatic *para*-quinol, upon capturing hydroxyl-radicals, which results in the complete loss of estrogen-receptor affinity and antioxidant activity. However, the parent compound is apparently recovered in brain tissue from this *para*-quinol via enzyme-catalyzed NAD(P)H-dependent reductive aromatization without causing oxidative stress. Taken together, our report argues for a previously unrecognized antioxidant cycle for synthetic estrogen-derived compounds.

Keywords

17 β -Butoxy-1,3,5(10)-estratrien-3-ol; Antioxidant neuroprotection; Chemical shield; Steroidal *para*-quinol; Hydroxyl radical; Oxidative stress

1. Introduction

Oxidative damage by reactive oxygen species (ROS) to neuronal cells, accumulation of iron in specific brain areas, and inflammatory processes are considered major pathological aspects of aging and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (PD and AD, respectively) [1–3]. ROS are free radicals and molecules, e.g., hydrogen peroxide (H₂O₂), that have high propensity to produce free radicals under specific conditions. Free radicals have unpaired electron(s) and in order to reach a more stable energy level, they easily pick up electron(s) (or hydrogen atom) from another molecule that, in turn, converts those molecules into free radicals, thus, setting up a chain reaction. Once the process is started, it

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can cascade, finally resulting in tissue and cell damage eventually leading to cell death and diseases [4]. Therefore, antioxidants that mainly work as chemical electron sinks and quench the free-radical chain reaction has been implicated as potential therapeutic agents to fight against the detrimental effects of brain aging and neurodegenerative diseases [5,6].

It has been well-established that estrogens influence memory and cognition [7,8], decrease the risk and/or delay the onset of neurological diseases (e.g., AD and PD), and attenuate the extent of cell death that results from a variety of insults involving ROS-induced oxidative stress implicated among others in stroke or neurotrauma [9,10]. Various *in vitro* and *in vivo* models show that estrogens are capable to protect neurons in a concentration range of 0.1 nM to 50 μ M suggesting the involvement of different modes of action [11–15]. The complex events by which estrogens orchestrate neuronal survival have not been clearly understood [16]. While “classical,” nuclear estrogen-receptor (ER) dependent genomic actions seem to be plausible, it has also been established that the involvement of ERs is not necessary for neuroprotection [17]. There has been mounting evidence that estrogens enhance cell survival by suppressing the neurotoxic stimuli partly *via* their antioxidant activity [18,19]. Free-radical scavenging is believed to be a significant contributor to the rapid ER-independent neuroprotective actions of estrogens and related compounds [20–22]. The antioxidant property of these compounds is due to the free phenolic hydroxyl group on the A- ring of the steroid [23]. Structure-activity relationship studies have clearly shown that this group is the only structural requirement to achieve cell survival upon oxidative insults [17,24]. Blocking of the phenolic hydroxyl group, e.g., by ether formation completely eliminates neuroprotection [22,25]. The direct oxyradical-scavenging by phenolic antioxidants has been thought to cause interruption of free-radical chain reactions such as lipid peroxidation by rapidly donating the hydrogen atom of the phenolic OH to radicals. Reduction of phenoxyl radicals by intracellular reductants (e.g., ascorbate, thiols) and by enzymes recycles the phenolic antioxidants [26,27]. Alternatively, additional non-radical products may also form [28].

Hormonal effects, including feminization and the potential stimulation of estrogen-sensitive malignancies, have blemished convincing *in vitro* and *in vivo* neuroprotection data on estrogens. Estrogen-derived synthetic neuroprotectants that retain the positive effects of estrogens but diminish undesirable side effects have been explored to overcome this problem [17]. 17-Alkyl ethers of 17 β -estradiol [25] such as 17 β -butoxyestra-1,3,5(10)trien-3-ol (17OBu-E2, **1**, Fig. 1) is a representative compound in this regard, because of its significantly reduced ER-binding affinity and increased potency compared to 17 β -estradiol (E2) to protect cultured HT-22 neurons against glutamate-induced oxidative stress principally by non-genomic mechanisms [25]. The selection of, e.g., 17OBu-E2 (**1**) as an estrogen derivative for *in vitro* neuroprotection experiments involving HT-22 cells and glutamate-induced oxidative stress is also advantageous, since it permits the studies to focus on non-genomic mechanisms such as the antioxidant action without interference from nuclear ER-dependent genomic mechanisms. In addition, the introduction of this D-ring substitution is expected to have weak, if any, influence on the chemistry of the phenolic A-ring of the steroid, which has been presumed to play an important role in the neuroprotective activity of estrogens by exerting an antioxidant action [13,17]. Here we report the theoretical prediction and the subsequent experimental confirmation of (bio)chemical processes by which **1** may soak up the most toxic ROS, the hydroxyl-radical ($\cdot$ OH) produced by Fenton reaction [29] and argue for the existence of a previously unrecognized antioxidant cycle that maintains a “chemical shield” raised by lipophilic, neuroprotectant E2 derivatives.

2. Materials and methods

2.1. Instruments and materials

Chemicals were purchased from TCI America (Oregon, USA). A CEM (Matthews, NC) Discover monomode microwave apparatus operating at a frequency of 2.45 GHz with a continuous irradiation power from 0 to 300 W was used for microwave-assisted organic synthesis (MAOS). Temperature was measured by infrared detection with continuous feedback temperature control, and maintained at a constant value by power modulation. The closed-vessel reaction was performed under controlled pressure in a glass tube (capacity 10 mL) sealed with a septum. After the irradiation period, the reaction vessel was cooled rapidly to ambient temperature by compressed air. NMR spectral data were recorded in CDCl₃ on a Varian Mercury-400BB (Palo Alto, CA) instrument at 400 MHz instrument using tetramethylsilane as an internal standard. Mass spectra (MS) were obtained by using liquid chromatography (LC) coupled with atmospheric-pressure chemical ionization (APCI) on a quadrupole ion trap instrument (LCQ, Thermo Fisher, San Jose, CA). Melting point (uncorrected) was determined on a Fisher-Johns apparatus.

A brain-homogenate was prepared from ovariectomized (OVX) female rats (220–250 g body weight) purchased from Charles River Laboratories (Wilmington, MA). After killing by cervical dislocation, the brain was immediately removed, weighed and transferred to a 15-ml glass Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ) placed in an ice bath. After addition of an ice-cold phosphate-buffered saline solution (PBS, volume in ml equivalent to four times of the weight of the brain in grams), brain homogenate was prepared by running the ball-shaped teflon pestle of the grinder, with the steel shaft connected to a motor-driven overhead stirrer (Wheaton), for 80 s at 1500 rpm. The brain homogenate was used immediately for the in vitro studies

2.2. Computational studies

Calculations were done after performing full geometry optimization (RMS gradient <0.1 kcal/[Å · mol]) and energy minimization by using the PM5 semi-empirical method with open-shell wave functions [unrestricted Hartree-Fock (UHF) approach], implemented in the BioMedCACHé (Fujitsu, Beaverton, OR) molecular modeling program (version 6.1.12.34).

2.3. Synthesis

17β-Butoxy-1,3,5(10)-estratrien-3-ol (**1**, 17OBu-E2, Fig. 1) was prepared from 3-*O*-benzyl-17β-estradiol with sodium hydride/butyl bromide, followed by the removal of the *O*-benzyl protecting group *via* catalytic transfer hydrogenation as reported earlier [25]. 10β-Hydroxy-17β-butoxy-1,4-estradiene-3-one (17OBu-E2-quinol, **2**, Fig. 1) was obtained by MAOS [30]. Briefly, to 100 mg (300 μmol) of **1** dissolved in 3 ml acetic acid, 200 mg (450 μmol) lead(IV) acetate was added [31]. The reaction vessel was irradiated at 40 °C, with an irradiation power of 150 W, for 20 min. Then the solution was concentrated under a nitrogen stream and the residue was dissolved in 3 ml methanol and CH₃OK (15 %, w/v) was added to adjust the pH to 9. This solution was irradiated again for 5 min at 40 °C to hydrolyze the acetyl quinol. The solution was concentrated again and glacial acetic acid was added to adjust the pH to 5. Upon adding ice cold water, the precipitate obtained was filtered off and washed thoroughly with water and purified by column chromatography [silica gel, hexane:ethyl acetate 4:1, (v/v) eluent]. 17OBu-E2-quinol (**2**), a white solid, was obtained with 54% yield, m.p. 52–56 °C; R_f = 0.65 (hexane : ethyl acetate 4:1, v/v); APCI-MS: *m/z* [M+H]⁺ 345; ¹H-NMR δ (ppm) having diagnostic value: 7.08 (d, *J* = 10.2 Hz, 1H, H1); 6.17 (dd, *J* = 10.2 & 1.6 Hz, 1H, H2); 5.99 (d, *J* = 1.7 Hz, 1H, H4); 3.41 (m, 2H, OCH₂ of butyl); 3.27 (t, *J* = 8.4 Hz, 1H, 17α); 0.84 (t, *J* = 7.4 Hz, 3H, CH₃ of butyl). ¹³C-NMR δ (ppm): 185.6; 165.7; 150.7, 128.1;

122, 9; 88.7; 70.4; 69.9; 54.5; 50.1; 43.2; 37.5; 35.0; 33.2; 32.3; 32.0; 28.2; 28.0; 23.8; 23.6; 22.5; 19.4. 19.2. Anal. Calc. $C_{22}H_{30}O_2 \times 0.5 H_2O$: C, 78.76, H, 9.31; Found C, 78.63, H, 9.40.

2.4. Fenton reaction

17OBu-E2 (**1**, 100 μ M) was incubated in an aqueous medium containing $FeSO_4$ (1.8 mM dissolved in pH 3 sulfuric acid) and H_2O_2 (36 mM in water). Aliquots were taken at 2.5, 10, 30 and 60 min time points and the solutions were extracted with ethyl acetate (3×0.5 mL). The combined organic extract was washed acid free with water, dried over sodium sulfate, and the solvent was evaporated under a nitrogen stream. Samples were dissolved in LC mobile phase and analyzed by LC/APCI-MS.

2.5. In vitro metabolism studies

In vitro formation of **2** from **1** was determined upon incubating **1** (100 μ M) at 37 °C in freshly prepared OVX rat brain homogenate (20%, w/v) in 0.1 M phosphate buffer at pH 7.4. To the aliquots taken at different time points, ice-cold glacial acetic acid (50 μ L) and ethyl acetate (3×0.5 mL) were added, the mixture was vortexed for 1 min and centrifuged at 10,000 rpm for 5 min. The organic layer was removed and the solvent was evaporated at room temperature under a nitrogen stream. Each experiment was performed in triplicate. Tissue-free incubations were done by dissolving NAD(P)H (1 mM) and **2** (100 μ M) in 0.1 M phosphate buffer. Control incubations were performed in 0.1 M phosphate buffer only. The sample residues were dissolved in the LC mobile phase and analyzed by LC/APCI-MS. Compound identification was based on retention time (RT), APCI and tandem mass spectra (MS/MS) with synthetic compound (**2**) as reference.

In vitro reductive regeneration of **1** from **2** was determined analogously NAD(P)H-dependent conversion of *para*-quinol **2** to the phenolic parent compound (**1**) was determined after incubating 100 μ M *para*-quinol and 1 mM NAD(P)H in phosphate buffer/acetonitrile (50:50, v/v) at 37°C for 5 min.

2.6. LC-MS analyses

LC separation was done using a Supelco (Bellefonte, PA) 5 cm $\times$ 2.1 mm i.d. Discovery HS C-18 reversed-phase column with 0.25 ml/min flow rate using water/acetonitrile/acetic acid (20:79:1, v/v) as a mobile phase. The sample residues were dissolved in 40 μ L of mobile phase, respectively, and 5 μ L of the solution was injected for analysis. MS/MS product-ion scans were obtained after collision-induced dissociation with helium as the target gas. Compound identification was based on RT, APCI mass spectra and MS/MS with authentic compounds as references [32]. Quantitative analyses were done by LC/APCI-MS/MS and calibration with solutions of known concentrations of the analytes (**1** or **2**). As an internal standard, 3-methoxy-1,3,5(10)-estratrien-17 β -ol (1 μ M) [25] was added before each sample extraction.

2.7. Induction of hydrogen peroxide production

Prooxidant activity of **1** and **2** in OVX brain homogenate was evaluated according to a previously published method based on the production of H_2O_2 production at 37 °C [33]. Briefly, in brain homogenate (0.2% w/v in 0.1M PBS, pH 7.4, final volume of 100 μ L) 100 μ M test compounds **1**, **2** or 3,4-*ortho*-quinone of estrone as positive control, NAD(P)H (200 μ M) and sodium azide (20 μ M) at 37°C for 30 minutes in a shaking water bath. Then, 2 mL of a color developing solution (100 μ M xylénol orange, 250 μ M ferrous ammonium sulfate, and 100 μ M sorbitol in 25 mM sulfuric acid) was added, and the mixture was allowed to stand at room temperature for 45 min before spectrophotometric analysis. Absorbance was recorded at 560 nm on a Shimadzu UV250-1PC (Columbia, MD) instrument.

Quantification of H_2O_2 was done by calibrating with serially diluted H_2O_2 standard solutions. Rates of H_2O_2 production were expressed in $\text{nmol}\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$ (mean $\pm$ standard error of the mean, SEM). The protein content of the brain homogenates was determined by a dye-binding assay [34] using bovine serum albumin as a reference.

2.8. Estrogen-receptor (ER) binding

Affinities to ERs were evaluated by using an enzyme fragment complementation (EFC) method described in the HitHunter® EFC Estrogen Chemiluminescence Assay kit (Fremont, CA). Compounds (10 pM to 10 μM) were incubated with 5 nM recombinant ER α or ER β (Panvera, Madison, WI) and estradiol-conjugated enzyme donor for 1.5 hours. The enzyme acceptor was then added for another 1.5 hours after which the chemiluminescence substrate was added for another hour. Relative luminescence was determined using a Biotek FL600 plate reader (Biotek Instruments, Winooski, VT). Sigmoidal standard curves were created by GraphPad Prism (version 3.02 for Windows, GraphPad Software, San Diego, CA) using a four-parameter logarithmic curve to determine the concentration to reach 50% inhibition (IC_{50}).

2.9. Inhibition of lipid peroxidation

The capacity of **1** to inhibit lipid peroxidation was studied in comparison to E2 according to published methods [35,36]. Briefly, incubation mixtures consisted of 2 mL of the compound (at varying concentrations), 2.05 mL of 2.5% (v/v) linoleic acid in ethanol, 4 mL of 0.05 M phosphate buffer (pH 7.0) and 1.95 mL water, in a vial with a screw cap. Control experiments were also conducted using 2.05 mL ethanol without a compound. The incubations were carried out in the dark at 40°C for nine days. Then, the samples were analyzed using both the ferric thiocyanate (FTC) [37] and the thiobarbituric acid reactive species (TBARS) methods [38].

For the FTC method, 0.1 mL incubation mixture and, then, 0.1 mL of 30% (w/v) aqueous ammonium thiocyanate were added into 9.7 mL of 75% (v/v) aqueous ethanol. Thirty seconds after the addition of the ammonium thiocyanate, 0.1 mL of 0.02 M FeCl_2 in 3.5% (v/v) HCl was added and the absorbance of the solution was measured at 500 nm 3 min later. For the TBARS method, 0.4 mL 20% (w/v) trichloroacetic acid and 0.4 mL 0.67% (w/v) aqueous thiobarbituric acid were added to 0.2 mL of incubation mixture. After keeping at 90 °C for 10 minutes, the sample was allowed to cool and a supernatant was obtained by centrifugation at 10,000 rpm for 40 minutes. The absorbance of the supernatant was measured at 532 nm. The percentage of inhibition was calculated as $(1 - A/A_0) \times 100$; where A was the absorbance of the solution containing the inhibitor and A_0 represented the absorbance of the control. IC_{50} values were determined by non-linear fitting to sigmoid dose-response profiles with the Scientist for Windows program (version 2.0, MicroMath, Salt Lake City, UT).

2.10. In vitro neuroprotection studies

All studies were done on mouse clonal hippocampal HT-22 cells. The cells were cultured in Dulbecco's Modified Eagle's media supplemented with 10% fetal bovine serum under the usual conditions. Experiments were done in 96-well culture plate containing approximately 5,000 cells/well as determined by a Neubauer hemacytometer. The cells were incubated for 24 h before the test compounds were added. 17OBu-E2 (**1**) and its quinol (**2**) were dissolved in absolute ethanol and diluted with the culture media to a final concentration of 0.01, 0.1, 1.0, 10 and 100 μM in their respective wells. Sodium glutamate (20 mM) in a solution of phosphate buffer was added together with the test compounds (co-treatment) or 5 h later (pre-treatment). Then, the cells were incubated for 24 h. Cell survival was quantified by the robust Calcein AM assay [39] in a phosphate buffer solution.

2.11. Data analysis

Data are expressed as mean $\pm$ SEM. Statistical evaluations were done by one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's test (comparison to a single control group). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Computational studies

Theoretical calculations on the phenoxyl radical derived from **1** indicated that susceptibility for radical reactions, such as a radical exchange causing an interruption of free-radical chain reactions, was highest at the C-10 (tertiary) carbon. Based on this assessment, we predicted a hydroxyl radical ($\bullet\text{OH}$) radical attack to occur according to the simplified mechanisms shown in Fig. 1. Calculated heats of formation (ΔH_f) have also indicated that localizing the radical site formally at the C-10 tertiary carbon may be considered the most stable radical intermediate ($-104.4 \text{ kcal}\cdot\text{mol}^{-1}$, the lowest among the calculated ΔH_f values for possible isomeric radicals), while the C-2/C-4 radical forms obtained by a tautomeric H-shift are considerably less stable [40]. ΔH_f values of 10 β -hydroxy-17 β -butoxy-1,4-estradien-3-one (**2**, Fig. 1) and the isomeric 10 α -hydroxy-17 β -butoxy-1,4-estradien-3-one were $-155.7 \text{ kcal}\cdot\text{mol}^{-1}$ and $-151.1 \text{ kcal}\cdot\text{mol}^{-1}$, respectively, indicating the preferential formation of the β -isomer. Reaction trajectory calculations from the radical intermediate for the recombination with $\bullet\text{OH}$ ($\Delta H_f = 8.9 \text{ kcal}\cdot\text{mol}^{-1}$ [41]), an exothermic reaction ($\Delta H = -60.2 \text{ kcal}\cdot\text{mol}^{-1}$) based on the ΔH_f values of the reactants and products according to Hess's Law [42], revealed no transition states. The conclusions that could be drawn from a computational study for the formation of 10 β , 17 β -dihydroxy-1,4-estradien-3-one from E2 by the mechanism shown for **2** in Fig. 1 were essentially the same as described above in this section (However, the predicted heat of reaction was $\Delta H = -57.7 \text{ kcal}\cdot\text{mol}^{-1}$, which indicated that the radical recombination leading to the formation of the E2-derived *para*-quinol could be slightly less exothermic than the formation of **2** out of phenoxyl radical derived from **1** and $\bullet\text{OH}$).

The logarithms of the *n*-octanol/water partition coefficients calculated by the atomic typing scheme of BioMedCACHe (ClogP) for **1** and **2** were 5.49 and 3.15, respectively. The latter lipophilicity-indicating measures were considerably higher than those of the corresponding parent steroids E2 and 10 β , 17 β -dihydroxy-1,4-estradien-3-one (ClogP of 4.01 and 1.67, respectively).

3.2. Microwave-assisted organic synthesis (MAOS)

The 17OBu-E2-derived *para*-quinol, 10 β -hydroxy-17 β -butoxy-1,4-estradiene-3-one (17OBu-E2-quinol, **2**), as authentic reference compound, was synthesized by MAOS in acetic acid at 40 °C within 25 min. A rapid hydrolysis of the intermediate *para*-quinol acetate was also done under microwave irradiation. Our approach [30] significantly reduced the reaction time and the amount of lead(IV) acetate conventionally used [31] providing, therefore, an increased throughput and a "greener" chemistry to obtain synthetic estrogen-derived *para*-quinols.

3.3. Fenton reaction

When 17OBu-E2 (**1**) was exposed to $\bullet\text{OH}$ generated by chemical Fenton reaction, we found that **1** rapidly (with second-order rate constant of approx. $15 \text{ M}^{-1}\cdot\text{s}^{-1}$) converted to one major product that showed an increase of 16 in molecular weight according to its APCI mass spectrum [m/z ($\text{M}+\text{H})^+$ 345] obtained after LC separation (Fig. 3), implicating incorporation of a single oxygen into **1** [$(\text{M}+\text{H})^+$: m/z 329]. Based on detailed LC/APCI-MS analyses and coelution/ comparison (t_R , MS and MS/MS) with the synthetic standard prepared from **1** (see previous

subsection), this product was identified as 10 β -hydroxy-17 β -butoxyestra-1,4-diene-3-one (**2**, Fig. 1); the corresponding *para*-quinol derived from **1**.

3.4. In vitro metabolism studies

To probe whether **2** also arises under biological Fenton reaction, we incubated **1** in brain homogenate of OVX rats for 5 min and confirmed the formation of **2** [at an approximate initial rate of $2.1 \pm 0.4 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$] as the principal $\cdot\text{OH}$ -scavenging product by LC-APCI-MS analyses. Since *para*-quinols (similarly to cyclohexadienones) can be converted chemically to the corresponding phenols [43], we also studied whether biological reducing agents such as NAD(P)H would also regenerate the synthetic estrogen derivative (**1**) from its *para*-quinol (**2**) by reductive aromatization. We not only confirmed that **2**, indeed, smoothly converted chemically to **1** upon treatment with zinc/acetic acid [31], but the formation of **1** ($2.4 \pm 0.08 \text{ } \mu\text{M}$) could also be detected by LC/APCI-MS (Fig. 3, $t_R = 4.9 \text{ min}$), when **2** ($100 \text{ } \mu\text{M}$) was incubated in the presence of 1 mM NAD(P)H for 5 min. When OVX brain homogenate (20 % w/v) was also used, the **2** $\rightarrow$ **1** conversion was found to be approximately 8-times faster; [rate of formation $\approx 210 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$], implicating that enzymes in the brain further catalyze the recovery of **1** from its radical scavenging product.

3.5. Hydrogen peroxide production

It is imperative to probe whether the reductive recovery of **1** from **2** *via* an enzymecatalyzed NAD(P)H-dependent reduction in the brain would produce oxidative stress similarly to that of redox cycling between catechol estrogens-derived quinones and their corresponding semiquinones [44]. Such redox cycling is believed to be responsible, in part, for the carcinogenicity of estrogens [45]. The profoundly prooxidant estrone-3,4-quinone [33] was used as positive control in this experimental paradigm. The measured hydrogen peroxide production (given in $\text{nmol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$) in OVX rat brain homogenate clearly showed that the *para*-quinol to phenol (**2** $\rightarrow$ **1**) conversion does not produce oxidative stress compared to that of the control, while estrone-3,4-quinone produced approximately a 5-fold increase in H_2O_2 production (Fig. 4).

3.6. Receptor binding and inhibition of lipid peroxidation

As shown in Table 1, we have confirmed that **2**, as expected, had no intrinsic affinity to ERs ($\text{IC}_{50} > 10 \text{ } \mu\text{M}$), while the parent phenolic A-ring compound (**1**) showed competitive binding to ER- α and ER- β (with IC_{50} values of 12 and 15 nM, respectively), albeit with significantly less affinity compared to E2 (whose IC_{50} values were 1.3 and 0.7 nM for ER- α and ER- β , respectively). The IC_{50} values for the inhibition of lipid peroxidation determined by both the FTC and TBAR methods predicted that **1** was more potent [$4.1 \pm 0.8 \text{ } \mu\text{M}$, (FTC) and $2.7 \pm 0.7 \text{ } \mu\text{M}$, (TBARS), respectively] than those of the parent E2 [$11.9 \pm 1.7 \text{ } \mu\text{M}$, (FTC) and $3.9 \pm 0.6 \text{ } \mu\text{M}$, (TBARS), respectively]. On the other hand, estrogen-derived *para*-quinols (including **2**) did not directly inhibit lipid peroxidation neither in the FTC- nor in the TBARS-based *ex vivo* models used in our study [46].

3.7. In vitro neuroprotection studies

Reductive recovery of **1** from its non-phenolic *para*-quinol (**2**) was also indicated by *in vitro* neuronal survival data (Fig. 5). When cells were exposed to glutamate-induced oxidative stress in the presence of varying concentrations of the test compounds, **1** and **2** provided protection at a concentration of 10^{-7} M and $\geq 10^{-6} \text{ M}$, respectively. However, 5-h pretreatment of the cultured HT-22 cells with **2** (and, thus, “allowing” the cultured cells to convert the *para*-quinol precursor to the phenolic A-ring steroid **1** before applying the oxidative challenge) significantly improved cell survival compared to co-treatment, and the shift in the dose–response curve compared to the parent phenolic compound (**1**) diminished.

4. Discussion

Considered the most toxic ROS, $\cdot\text{OH}$ is an indiscriminant oxidant that reacts at near-diffusion limited rates with a wide variety of biological macromolecules [47] and has been directly implicated in the development and/or progression of numerous neurodegenerative processes and neurotraumas [5]. Several compounds, including estrogens, have been shown to reduce oxidative stress on neurons [5,6,13,15,17]. In addition to binding to ERs in the cellular membrane, cytoplasm and nucleus [48], estrogens may also interact with lipid-transport proteins [49]. Because of their lipophilicity, they also concentrate in lipid-rich regions of cells such as cellular membranes and affecting and, thereby, have been shown to affect membrane fluidity [50]. Therefore, it is likely that estrogens act *in vivo* as highly localized antioxidants [28], and estrogen derivatives with enhanced lipophilicity ($\log P$) are expected to increase antioxidant potency compared to the parent steroids. Here, we focused on the chemistry of antioxidant neuroprotection potentially provided by a lipophilic synthetic E2 derivative (**1**, with $\text{Clog}P$ of 5.46 versus $\text{Clog}P$ of 4.01 for E2) against $\cdot\text{OH}$ attack. This compound was chosen because of its reduced ERs-binding (Table 1), increased potency to inhibit lipid peroxidation (Table 1) and robust *in vitro* protection of HT-22 cells against glutamate insults [25] when compared to those of E2. Our theoretical calculations predicted that the capture of $\cdot\text{OH}$ by the phenoxyl-radical obtained from **1** (in a radical-exchange reaction such as chain-braking of lipid peroxidation [28]) results in a significant change in the aromatic A-ring to yield the principal molecular (non-radical) product. Specifically, the A-ring turns into a non-aromatic *para*-quinol bearing a hydroxyl group on the 10 (tertiary) position of the steroid (**2**, Fig. 1.). Because of the large steric congestion on the α -side of the steroid structure, formation of the 10β -isomer was found to be strongly favored thermodynamically. This was also reflected by the lower ΔH_f of 10β -hydroxy- 17β -butoxy-1,4-estradien-3-one (**2**, $-155.7 \text{ kcal}\cdot\text{mol}^{-1}$) versus the isomeric 10α -hydroxy- 17β -butoxy-1,4-estradien-3-one ($-151.1 \text{ kcal}\cdot\text{mol}^{-1}$). Therefore, a central hypothesis of our study was that formation of **2** upon $\cdot\text{OH}$ exposure played an important role in the neuroprotective antioxidant action of **1**. As an analytical standard and test compound for *in vitro* studies, **2** was prepared by MAOS [30] that has attracted considerable attention for the rapid synthesis of a variety of organic compounds [51].

An unequivocal experimental conformation of **2** as the principal non-radical product upon $\cdot\text{OH}$ attack was done by LC-APCI-MS analyses (Fig. 2). The significant chemical change in the A-ring of the steroid after the completion of this radical-scavenging process is also clearly expressed by the loss of ER-binding affinities and lack of antioxidant potency of **2** when compared to those of **1** (Table 1).

We have also unambiguously confirmed the *in vitro* formation of **2** in a biological medium (in OVX rat brain homogenate) that exposed the substrate (**1**) to the radical product of the Fenton reaction. It is important to recognize that this “chemical shield” erected by **1** and leading to the conversion to *para*-quinol strips the A-ring of the steroid from the quintessential functional group (the phenolic hydroxyl group) required for antioxidant neuroprotection. However, we have shown that, similarly to cyclohexadienones [43], *para*-quinols can be converted back to their phenolic precursors by reductive aromatization. Specifically, the neuroprotective **1** is recovered by a NAD(P)H-dependent enzyme-catalyzed reaction without causing oxidative stress (Fig. 4). As such, a true antioxidant cycle exists between the phenolic A-ring synthetic estrogen (**1**) and its corresponding *para*-quinol (**2**) during $\cdot\text{OH}$ detoxification (Fig. 6).

The regeneration of **1**, which is a more potent inhibitor of lipid peroxidation (Table 1) based on its higher lipophilicity (with $\text{Clog}P$ of 5.46) than the parent estrogen (E2, $\text{Clog}P = 4.01$), from **2** also implicates that the latter may be used as a biochemical precursor (prodrug) of **1**. In addition to the lack of intrinsic estrogenicity (Table 1), pharmaceutically relevant

physicochemical properties of **2** such as decreased lipophilicity (hydrophobicity) [52] may also be considered more favorable than those of **1**.

The existence of the antioxidant cycle and the prodrug mechanism [conversion of *para*-quinol (**2**) to phenolic estrogen derivative (**1**)] were also confirmed by the protection of cultured HT-22 cells against glutamate-induced oxidative stress in the presence of **2** and using **1** as positive control (Fig. 5). The observation that dosing with a non-phenolic *para*-quinol protected hippocampus-derived HT-22 neurons against glutamate-induced oxidative stress despite its lack of activity as an antioxidant supported our hypothesis for its reductive conversion to a neuroprotective phenolic A-ring steroid (Fig. 3). The shift in the dose–response curve between the parent estrogen derivative (**1**) and its quinol (**2**) in our studies was due to the treatment schedule. When compound **2** was added simultaneously with the start of the oxidative insult, the neuroprotective compound **1** evolved in time through activation from **2** as its precursor. Until an adequate concentration of **1** is reached during this activation period, oxidative stress eventually kills a fraction of the cells. On the other hand, a 5-h pretreatment of the cultured HT-22 cells with **2** before the oxidative stressing improved cell survival, and the shift in the dose–response curve compared to the parent phenolic compound practically disappeared (Fig. 5).

In conclusion, we presented the first (bio)chemical evidence of a “chemical shield” provided by a neuroprotective estrogen derivative. In this process an antioxidant cycle exists between the synthetic phenolic A-ring estrogen **1** and its corresponding *p*-quinol **2** (Fig. 6). The latter may also be used as precursors (prodrugs) for the highly lipophilic parent steroids. These findings may lay the foundation for a rational design of novel neuroprotective agents sought to improve therapeutic safety compared to estrogens.

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Fig. 1.

A predicted mechanism of $\bullet\text{OH}$ capture leading to the formation of 17OBu-E2-quinol (2) from 17OBu-E2 (1). The structure in the middle displays radical frontier densities on heavy atoms (C and O) for the intermediate obtained by semi-empirical quantum-chemical calculation (PM5, CAChe 6.1.12.34, Fujitsu), which indicates a preferential capture of $\bullet\text{OH}$ by the C-10 of the steroid. (For atoms with radical frontier densities <0.001 , values are not shown.)

Fig. 2. LC-MS analysis of the product formed from 17β-butoxy-1,3,5(10)-estratriene-3-ol (17OBu-E2) upon exposure to $\cdot\text{OH}$ generated by the Fenton reaction. (a) Extracted ion-current chromatogram (m/z 345) and APCI mass spectrum from the major LC peak of the reaction product at RT: 2.3 min; (b) Extracted ion-current chromatogram (m/z 345) and APCI mass spectra are from the major LC peak at RT: 2.3 min for synthetic 10β-hydroxy, 17βbutoxyestra-1,4-dien-3,17-dione (17OBu-E2-quinol).

Fig. 3. Reductive regeneration of 17OBu-E2 (**1**) from 17OBu-E2-quinol (**2**) upon incubation with NADPH. (a) The chromatographic traces displayed are the selected-ion monitoring (SIM) of m/z 345 for **2** and the SIM of m/z 329 for **1**. (b) The peak at RT: 4.9 min is unequivocally identified, based on coelution with an authentic reference compound and identical APCI, MS/MS (shown in chart c together with the origin of the major fragments observed), as **1**.

Fig. 4. Effect of compounds on H₂O₂ production in rat brain homogenate. Like 17OBu-E2 (**1**, open bar), 17OBu-E2-quinol (**2**, shaded bar) does not induce oxidative stress. Estrone-3,4-quinone [estra-1,5(10)-dien-3,4,17-trione, checkered bar], a profound pro-oxidant, was used as a positive control. (n = 3, *: P<0.05).

Fig. 5.

In vitro neuroprotection of hippocampus-derived HT-22 neuronal cells against glutamate-induced oxidative stress (with 20 mM Glu) by the non-phenolic 17OBu-E2-quinol (**2**, shaded bars), which is augmented upon pretreatment (closed bars), compared with that of the phenolic A-ring parent compound 17OBu-E2 (**1**, open bars) ($n = 6-20$, *: $P < 0.05$).

Fig. 6.
Hydroxyl-radical scavenging antioxidant cycle for 17OBu-E2 (1).

Table 1

ER-binding and inhibition of lipid peroxidation of the compounds.

Compound	IC ₅₀ for ER Binding (nM)		IC ₅₀ for Inhibition of Lipid Peroxidation (μM)	
	ER α	ER β	FTC Method	TBARS Method
1,3,5(10)-Estratriene-3,17 β -diol (E2)	1.3	0.7	11.9	3.9
17 β -butoxy-1,3,5(10)-estratriene-3-ol (17OBu-E2, 1)	12.3	15.5	4.1	2.7
10 β -Hydroxy-17 β -butoxy-1,4-estradiene-3-one (17OBu-E2Q, 2)	> 10,000	> 10,000	n.i.	n.i.