Non-Feminizing Estrogens Do Not Exhibit Antidepressant-like Activity

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ABSTRACT

In this exploratory study, we performed an evaluation of non-feminizing estrogens as lead compounds for the safe treatment of menopausal symptoms. Despite confirming an enhancement of antioxidant potency as a consequence of increased lipophilicity of the prototype structures, our analyses have revealed serious shortcomings regarding pharmaceutically important properties and drug-likeness. In addition, our assessment in an animal model of estrogen deprivation has confirmed that genomic mechanisms are required for the alleviation of menopause-associated depression. Therefore, non-feminizing estrogens are not suitable to fulfill their implicated premise to address unmet needs to treat neurological and psychiatric conditions associated with estrogen deprivation of the brain.

Keywords: Estrogens, Non-feminizing, Drug-likeness, Antidepressant, Porsolt swim test

Abbreviations: Ada-E2: 2-(1-Adamantyl)-estra-1,3,5(10)-triene-3,17β-diol; Ada-E1: 2-(1-Adamantyl)-3-hydroxyestra-1,3,5(10)-trien-17-one; ADMET: Absorption, Distribution, Metabolism, Elimination and Toxicity; ANOVA: Analysis of Variance; APCI-MS: Atmospheric Pressure Chemical Ionization Mass Spectrometry; E2: Estra-1,3,5(10)-triene-3,17β-diol (17β-estradiol); E1: 3-Hydroxyestra-1,3,5(10)-trien-17-one (estrone); ER: Estrogen Receptor; FTC: Ferric Thiocyanate; HPβCD: 2-Hydroxypropyl-β-cyclodextrin; i.v.: Intravenously; logP: Logarithm of n-Octanol/Water Partitioning Coefficient (P); logS: Logarithm of Water-Solubility; OVX: Ovariectomized; PST: Porsolt Swim Test; s.c.: Subcutaneously

INTRODUCTION

The most potent human estrogen, 17β-estradiol (E2, **Figure 1**), elicits broad-spectrum neuroprotection in various *in vitro* and *in vivo* models [1]. As such, E2 is capable of protecting neurons *via* a variety of mechanisms, including attenuation of oxidative stress and stabilization of mitochondrial potential [2,3]. Besides extensive basic science studies, epidemiological observations also indicate the neuroprotective role of estrogens [4] and these agents have also been proven to be most effective to combat climacteric symptoms associated with natural or surgically-induced menopause [5].

However, exogenous estrogens have been surrounded by controversy owing to the unfortunate outcomes of Women's Health Initiative trial examining postmenopausal hormone therapy [6,7]. Evaluation of this trial, which relied on non-human estrogens (Premarin®) with or without a synthetic progestin (PremPro®), has further propagated the dogma that "all estrogens are created equal." However, equine estrogens used in the widely prescribed Premarin® have different absorption, distribution, metabolism elimination and toxicity (ADMET) profile than that of E2, and combination with

synthetic progestins has also been implicated as detrimental to brain health [8,9]. Additionally, even when human estrogens like E2 or estrone (E1) would have been used, any current drug delivery method produces substantial increase in circulating E2 and E1 levels and, consequently, increased risk for thrombosis, stroke and certain types of cancers [6,7].

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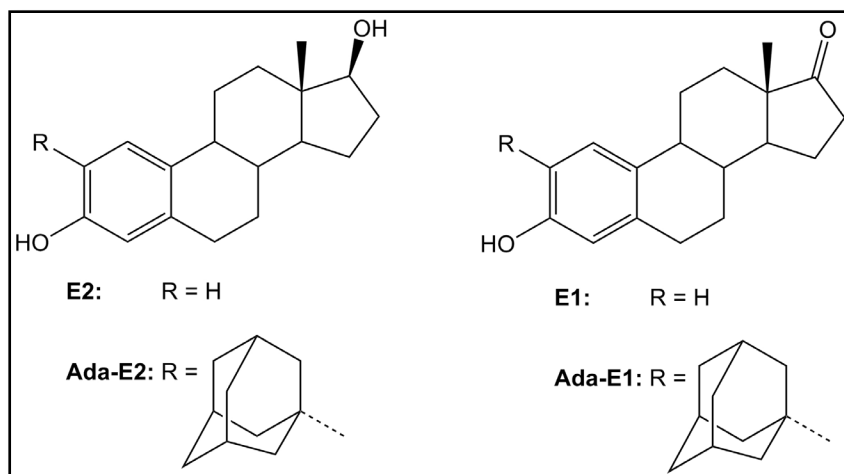
Figure 1. Chemical structure of 17β-estradiol (E2), estrone (E1) and their corresponding 2-adamantyl derivatives: Ada-E2 and Ada-E1.

One approach that has been attempting to avoid these caveats promotes the use of so-called non-feminizing estrogens possessing minimal, if any, affinity to the classical nuclear estrogen receptors (ERs) ERα and ERβ. Therefore, they eliminate genomic actions, including peripheral feminization by the hormone [10,11]. It has been known that manipulation of ER-binding of an estrogenic compound can be easily done by isomerization of and/or by introducing rather bulky substituents at strategically selected positions on the steroidal skeleton [12]. Due to synthetic simplicity, 2- and 4-substituted E2 and E1 derivatives have been mostly studied, although almost exclusively *in vitro* in various cell culture models. While these studies obviously do not require drug delivery and formulation considerations, *in vivo* applications of these agents could be problematic [13] owing to their high lipophilicity and water-insoluble nature, especially if repeated dosing is needed. Systemic toxicity upon repeated dosing may be a plausible outcome. Therefore, and perhaps it is not surprising that, only limited number of publications involving the *in vivo* use of these agents has been published [13,14]. Nevertheless, their translational potential in terms of representing safe alternatives of E2 in neuroprotection or for the treatment of climacteric symptoms has been proposed repeatedly [15, 16].

The effect of estrogen deprivation in the brain and the usefulness of exogenously provided E2 in various centrally-mediated and estrogen-responsive human maladies have been extensively studied in animal models [1]. Dramatic drops in brain estrogen levels due to natural or surgically-triggered reproductive aging are associated with increased incidence and symptomology of various neurological conditions, including anxiety and depression [17]. In this exploratory lead compound evaluation, we aimed at addressing the utility of two frequently cited non-feminizing estrogens, specifically 2-adamantyl-17β-estradiol (Ada-E2, **Figure 1**) and 2-adamantylestrone (Ada-E1, **Figure 1**)

[15,16], in a well-studied animal model of depression precipitated by estrogen deprivation [18]. Depression is highly relevant in terms of estrogen deprivation, as basic science and clinical studies convincingly show the therapeutic role of E2 in the management of this disorder [19].

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Luis, MO, USA). Ada-E2 and Ada-E1 were prepared from the corresponding estrogens according to Lunn et al. [20]. Briefly, E2 or E1 (1 mmol) and 1-adamantanol (1.05 mmol) were added to 20 mL of dry hexane followed by drop wise addition of 0.5 mL of $BF_3 \cdot Et_2O$ under ice cooling. The cooling was removed and the stirring continued overnight. Then, the reaction mixture was poured onto crashed ice and the obtained precipitate was filtered off, washed with water and dried. Column chromatographic purification was done on silica gel, using hexane:ethyl acetate 4:1 (v/v) eluent. Ada-E2: white solid, m.p. 180-182°C. APCI-MS: $(M+H)^+$ at *m/z* 407. R_f=0.85 (hexane:ethyl acetate, 3:1, v/v). Ada-E1: white solid: m.p. 169-170°C. APCI-MS: $(M+H)^+$ at *m/z* 405. R_f=0.8 (hexane:ethyl acetate, 3:1, v/v).

ER-binding and antioxidant potency

ER-binding affinities have been determined previously [4,21]. Antioxidant potencies were measured by the ferric thiocyanate (FTC) method adopted from literature [22].

Animals

Ovariectomized (OVX) young adult CD-1 mice (30 ± 4 g body weight) were purchased from Harlan Laboratories (Indianapolis, IN, USA). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center before the initiation of the studies. Four animals were

housed per cage in a room conditioned to 21-23°C with normal day/night cycles and were provided with free access to food and water. Each animal was tested only once.

Porsolt swim test (PST)

Mice were divided into six animals per treatment group. Test agents were dissolved either in corn oil vehicle or in 30% w/v aqueous 2-hydroxypropyl-β-cyclodextrin (HPβCD) similarly to our earlier studies [14,23,24]. The well-known antidepressant amitriptyline, as a reference standard, was used at 15 mg/kg dose, while the ER antagonist fulvestrant (ICI 182,780) was used at 4 mg/kg dose [25]. The control groups received vehicle only. Test compounds in corn oil vehicle were administered subcutaneously (s.c.), while those in HPβCD were given intravenously (i.v.). Each group of animals was treated daily for five consecutive days. Behavioral studies for antidepressant-like activity were evaluated 30 min after the last injection, as reported before [25]. The immobility time (in seconds, defined as the duration of floating motionless after the cessation of struggling and making only movements necessary to keep the head above the water) was recorded for 6 min simultaneously by a trained observer who was blinded to the treatment in question.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error (SEM), and statistical evaluations were done by one-way ANOVA. Two-group comparisons employed *post hoc* Tukey tests. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Owing to the superior performance in cell-culture experiments and some estrogen-responsive animal models, non-feminizing estrogens have been promoted as potential alternative to a safe chronic E2 therapy [15,16]. However, obstacles for the pharmaceutical development of these non-feminizing estrogens have never been considered. In **Table 1**, we summarized important descriptors of our test agents in this regard. As prototypical non-feminizing estrogens, we selected two frequently used derivatives containing the bulky adamantyl group on C2 of the A-ring of the steroid (Ada-E2 and Ada-E1; **Figure 1**), because they manifest an essentially complete loss of ER binding affinities. Ada-E2 and Ada-E1 exhibit significantly increased antioxidant potency compared to their unsubstituted counterparts due to steric and/or electronic effect of Ada. Direct free radical scavenging antioxidant effect is an important characteristic of estrogens that significantly contributes to the overall neuroprotection exerted by these agents [21].

Introduction of the bulky Ada to the already lipophilic E2 and E1 **(Table 1)** brought about further increase in the lipophilicity (logP) by >2 log units. Considering also that the compounds are water-insoluble (refer to logS values in **Table 1**), it is understandable why there *in vivo* application

is problematic. In fact, previously we have showed that Ada-E1 was ineffective in an animal model of stroke, when administered s.c. in corn oil [13] and formulation as a water-soluble inclusion complex in HPβCD was necessary to reduce ischemic volume by this non-feminizing estrogen. Although we have successfully formulated E2 and its lipophilic derivative with cyclodextrins for studies involving animal models [23,24], this requirement may be an obstacle for pharmaceutical development. These two shortcomings of Ada-E2 and Ada-E1 were probably the most profound contributors to their unfavorable drug-likeness score by Osiris Property Explorer [26] used for the evaluation **(Table 1)**. On the other hand, their experimentally measured antioxidant potencies indicated an improvement over the corresponding parent compounds, which could be linked to improved stroke protection in previous animal studies upon proper formulation of such a lipophilic agent [13]. Our quantitative structure- activity relationship study also supported that increase in logP enhances antioxidant effect of estrogen-derived synthetic steroids and their analogs [12]. However, Ada-E2 and Ada-E1 may be typical examples of using lipophilicity to build potency into lead molecules, which is commonly associated with the attrition of lead compounds showing promise based on exploratory hypotheses and limited *in vitro* or *in vivo* experiments [27]. Therefore, lead rescue through an approach with proven effectiveness to reduce lipophilicity with the concomitant increase of water-solubility [25,28] would only be justified for truly valuable candidates manifesting shortcomings in this regard.

Mood disorders such as depression are significant climacteric maladies [19]; therefore, addressing the potential of non-feminizing estrogens to remedy these conditions should be paramount to meaningful lead evaluation for the management of climacteric symptoms affecting the CNS [15]. In this context, we conducted such an evaluation using an established animal model involving depression-like behavior [18], as summarized in **Figure 2**. (The PST was validated with amitriptyline, a tricyclic drug that is used clinically to treat depression). While E2 and E1 did show significant reduction of immobility time, Ada-E2 and Ada-E1 failed to manifest activity in this paradigm. We also verified our earlier result that the brain-penetrating ER-antagonist fulvestrant blocked E2's effect in the PST [25]; therefore, the antidepressant-like activity required target engagement with the cognate receptors of the hormone in the brain. Accordingly, non-feminizing estrogens such as Ada-E2 and Ada-E1 are *not* appropriate for the management of climacteric symptoms that manifest through ERs. In addition to depression, vasomotor symptoms of the menopause particularly hot flushes have been also known to associate with estrogen deficiency affecting the stimulation of ERα in the hypothalamus, the thermoregulatory center of the body [29]. Therefore, alleviation of additional profound

climacteric maladies by non-feminizing estrogens will also   be unlikely.

Table 1. Physicochemical properties, drug-likeness, estrogen receptor (ER) binding affinities and antioxidant properties of human estrogens and their 2-adamantyl-subtituted derivatives.

Compound	Molecular Mass	logP	logS	Drug-likeness Score[a]	ER-binding Affinity[b] (IC$_{50}$, nM)		Antioxidant Potency (IC$_{50}$, μM)[c]
					ERα	**ERβ**	
E2	272.1	3.88	-4.02	0.09	1.3	0.7	11.8 ± 1.6
E1	270.0	4.02	-4.07	-0.41	6.1	3.5	17.7 ± 2.3
Ada-E2	406.2	6.01	-6.73	-1.23	>10,000	>10,000	1.5 ± 0.1
Ada-E1	404.2	6.15	-6.78	-1.72	>10,000	>10,000	5.3 ± 1.2

[a] *Drug-likeness scores, along with logP and logS values, were calculated through the Osiris Property Explorer [26]. Negative values indicate unfavorable drug-likeness.*
[b] *Measured by competitive radioligand-binding assay [21]*
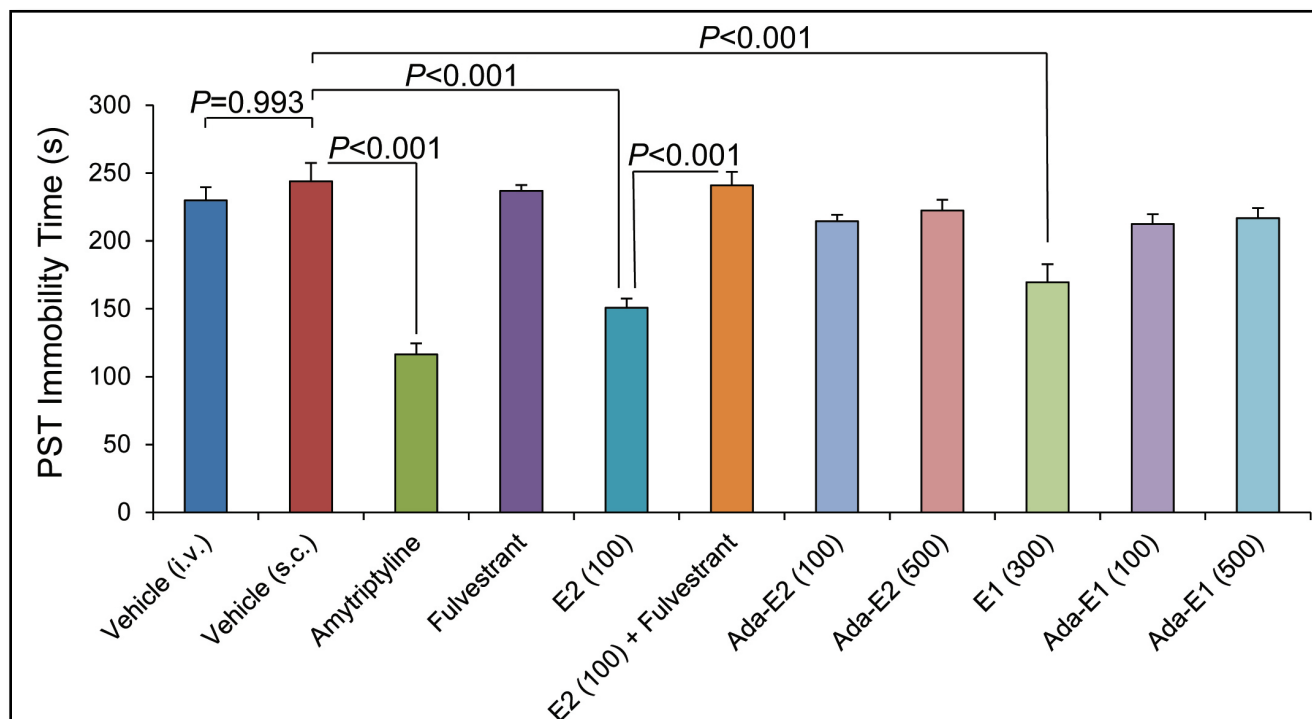[c] *Determined experimentally by the FTC method [12,22]*

Figure 2. Evaluation of E2, E1, Ada-E2 and Ada-E1 (doses given in parentheses as μg/kg body weight) for antidepressant-like activity in the PST using OVX young adult CD1 mice. Displayed data are means ± SEM (N/group=6 except for s.c. vehicle, where N/group was 12). One-way ANOVA: $F_{(10,61)}$=15.0, $P<0.001$.

CONCLUSION

In conclusion, our lead evaluation has confirmed both genomic and non-genomic mechanisms are required for broad-spectrum estrogen neuro-protection and treatment of menopausal symptoms. Therefore, non-feminizing estrogens are not suitable to fulfill their overall premise. In addition, our analyses have revealed serious shortcomings regarding pharmaceutically important properties and drug-likeness of prototypical lead compounds. On the other hand, our

recently published brain-selective estrogen therapy promises to provide full benefits of the hormone's activity through both genomic and non-genomic mechanisms with a concomitant improvement in drug-like properties and, also, fully avoiding peripheral impacts that leads to feminizing effects [25]. Consequently, the creation of novel non-feminizing estrogens as lead compounds has lost it impetus in the context of drug discovery and development.

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