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A Novel Mechanism of Non-feminizing Estrogens in Neuroprotection

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Abstract

Estrogens are potent and efficacious neuroprotectants both *in vitro* and *in vivo* in a variety of models of neurotoxicity. We determined the structural requirements for neuroprotection in an *in vitro* assay using a panel of more than 70 novel estratrienes, synthesized to reduce or eliminate estrogen receptor (ER) binding. We observed that neuroprotection could be enhanced by as much as 200-fold through modifications that positioned a large bulky group at the C2 or C4 position of the phenolic A ring of the estratriene. Further, substitutions on the B, C or D rings either reduced or did not markedly change neuroprotection. Collectively, there was a negative correlation between binding to ERs and neuroprotection with the more potent compounds showing no ER binding. In an *in vivo* model for neuroprotection, transient cerebral ischemia, efficacious compounds were active in protection of brain tissue from this pro-oxidant insult. We demonstrated that these non-feminizing estrogens engage in a redox cycle with glutathione, using the hexose monophosphate shunt to apply cytosolic reducing potential to cellular membranes. Together, these results demonstrate that non-feminizing estrogens are neuroprotective and protect brain from the induction of ischemic- and Alzheimer's disease (AD)-like neuropathology in an animal model. These features of non-feminizing estrogens make them attractive compounds for assessment of efficacy in AD and stroke, as they are not expected to show the side effects of chronic estrogen therapy that are mediated by ER actions in the liver, uterus and breast.

Key Works

Estrogens; estradiol; non-feminizing estrogens; structure-activity relationships; redox cycling

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1. Introduction

1.1 Need for non-feminizing estrogens

Menopause, in which a human female transitions to reproductive senescence, occurs during the fifth decade of life among women (Timiras et al., 1995). This transition is characterized by depleted ovarian follicles, declines in naturally circulating levels of sex hormones, such as estrogens and progesterone, and a dysregulation of gonadotrophin feedback loops marked by increasing levels of follicular stimulating hormone and lutenizing hormone (Rannevik *et al.*, 1995). Further, menopause is associated with hot flashes, urogenital atrophy, cognitive decline (specifically learning and memory), and other symptoms that reduce quality of life (Freedman, 2002; Sherwin & Henry, 2008). To alleviate these symptoms, estrogen-containing hormone therapy (HT) is given. Premarin® (conjugated equine estrogens), a purified pregnant mare urine compound first developed by Wyeth, is the most widely used estrogen-based menopausal HT in North America (Hersh *et al.*, 2004) although a plethora of other estrogen-containing treatment options exist as well (Sood *et al.*, 2014). Despite these treatments showing attenuation of undesirable menopausal symptoms (Sood *et al.*, 2014) and possible protection against brain aging and injury (Engler-Chiurazzi *et al.*, 2016a), recent comprehensive studies have demonstrated that in older women, chronic exposure to feminizing estrogens alone or in combination with a synthetic progestin leads to an increase in pro-thrombotic and pro-mitotic side effects (Manson *et al.*, 2003; Wassertheil-Smoller *et al.*, 2003; Anderson *et al.*, 2004). These chronic toxicities of feminizing estrogens are mediated by the effects of persistent estrogen exposure in estrogen responsive tissues, like the liver, uterus, and breast. This, combined with the controversial findings of the Women's Health Initiative regarding the potential increased risk for adverse outcomes among reproductively senescent women taking HT has spurred an intense debate as to whether estrogen-containing HTs should continue to be administered for treatment of menopausal symptoms and brain aging. Given that approximately half of the aging adult population is female, there is an important medical need to develop novel treatments for the menopausal transition and aging processes with a more acceptable risk-to-benefit ratio.

1.2. Neuroprotective effects of estrogens

We were among the first research groups to document the beneficial actions of estrogens on the central nervous system (reviewed in Engler-Chiurazzi *et al.*, 2016b). We first demonstrated potent neuroprotective activity of the feminizing estrogen, 17β-estradiol (17β-E2), in 1994 (Bishop & Simpkins, 1994). Since then, the neuroprotective effects of feminizing estrogens have been confirmed using neuronal cultures and primary cells against a variety of toxicities including serum deprivation (Green *et al.*, 1997a; Green *et al.*, 1997b), β-amyloid toxicity (Green *et al.*, 1998; Pike, 1999), and oxidative stress (Behl *et al.*, 1995; Goodman *et al.*, 1996; Sawada *et al.*, 1998; Sawada *et al.*, 2000)), among others, in hippocampal, amygdala, cortical and mesencephalic neurons (for review, please see Green & Simpkins, 2000; Garcia-Segura *et al.*, 2001; Lee & McEwen, 2001). Similarly, *in vivo* feminizing estrogens have been shown to enhance cognitive outcomes (Engler-Chiurazzi *et al.*, 2016a). As well, in *in vivo* animal models of brain injury, feminizing estrogens impart protection in models of cerebral ischemia (Simpkins *et al.*, 1997; Dubal *et al.*, 1998; Yang *et al.*, 2000), following kainic acid treatment (Azcoitia *et al.*, 1998), and in contusion injury

models (Nakamizo *et al.*, 2000; Gatson *et al.*, 2012). Indeed, in stroke, the protective effects of estrogens are seen in a variety of models including transient and permanent middle cerebral artery occlusion models (Simpkins *et al.*, 1997; Alkayed *et al.*, 1998; Dubal *et al.*, 1998; Perez et al., 2005b), global forebrain ischemia models (Sudo *et al.*, 1997), photothrombotic focal ischemia models (Fukuda *et al.*, 2000), and glutamate-induced focal cerebral ischemia models (Mendelowitsch *et al.*, 2001). The protection afforded by estrogens is seen in rats, mice, and gerbils (Simpkins *et al.*, 1997; Culmsee *et al.*, 1999; Chen *et al.*, 2001) and in adult and middle-aged female rats, as well as in reproductively senescent female rats (Wise *et al.*, 2001). This protection is seen even in the presence of diabetes and hypertension (Carswell *et al.*, 2000; Toung *et al.*, 2000). Similarly, the neuroprotective effects of estrogens are observed against subarachnoid hemorrhage (Yang *et al.*, 2001). Finally, the neuroprotective actions of estrogen are also seen in males (Hawk *et al.*, 1998; Toung *et al.*, 1998). Collectively, potent estrogen protection in these model systems suggest that this steroid hormone may play an important role in preserving neurons in the face of a variety of insults and represents an important therapeutic target for alleviating brain aging and disease. However, given the potential for undesirable peripheral activity of feminizing estrogens, the development of novel estrogen analogues that act in brain but not on reproductive organs represents a promising future therapeutic option for the treatment of brain aging.

1.3. Discovery of neuroprotection by non-feminizing estrogens

In the process of conducting controled studies for the neuroprotective effects of 17β-E2, we discovered that 17α-estradiol (17α-E2) was as potent as 17β-E2 in protection of neurons from toxicity (Green *et al.*, 1997a). 17α-E2 is a weak diastereomer of 17β-E2 and despite the fact that 17β-E2 binds avidly to estrogen receptors (ERs) α and β and activates tissues in a hormonally-responsive manner, 17α-E2 is biologically weak at both receptors. We went on to show that the enantiomer of 17β-E2 (*ent*-17β-E2), which has identical physiochemical properties as 17β-E2 except for interactions with other stereospecific molecules such as ERs is potently neuroprotective (Green *et al.*, 2001). *ent*-17β-E2 is reported to interact only weakly with ERs (Chernayaev *et al.*, 1975; Payne & Katzenellenbogen, 1979) and lacks estrogenic effects on reproductive tissues in rodents (Terenius, 1968; 1971). Importantly, although *ent*-17β-E2 exerts only slight anti-uterotrophic activity and can antagonize the uterotrophic effects of 17β-E2 (Edgren & Jones, 1969; Terenius, 1971), *ent*-17β-E2 is still a potent neuroprotectant (Green *et al.*, 2001). These collective findings suggest that neuroprotective effects of estrogens do not necessarily require action at the ER.

2. Structure-activity relationship among estrogens

In view of the observation that many of chronic estrogen treatment side-effects are likely due to peripheral effects of orally administered estrogen preparations acting via known ERs (Dubey *et al.*, 2005; Maki, 2006; Salpeter *et al.*, 2006; Coker *et al.*, 2009; Resnick *et al.*, 2009), we sought to determine if non-feminizing estrogens could have the beneficial effects of estrogens on brain protection, without the negative peripheral side effects of traditional feminizing preparations. We undertook a series of studies to define the structure-activity relationship among estrogen-like compounds for both neuroprotection and ER binding,

based on our and other's observations of a disparity between ER binding and neuroprotection (Behl *et al.*, 1997; Green *et al.*, 1997b; Moosmann & Behl, 1999; Green *et al.*, 2001). We initially assessed over 70 synthetic estrogens to determine the structure activity relationship among the compounds.

We defined that the minimal structural requirement for neuroprotection by estrogens is the steroid A ring. Estrogens are the only class of steroids that have a phenolic A ring (Figure 1). Any modifications that eliminated the phenolic nature of the A ring completely eliminated neuroprotective activity. These modifications included saturation of the A ring or creation of a covalent bond with substituents through the 3 carbon oxygen.

Modifications of the D ring had little effect on the neuroprotective activity, including changing the orientation of the 17 carbon hydroxyl group (as is the case with 17α-E2), eliminating the 17 carbon hydroxyl group (estratriene-3-ol), or opening the D ring (Perez *et al.*, 2005a). The addition of polar groups in the B and C rings tended to reduce neuroprotective activity of estrogens indicating that the center of the molecular requires sufficient lipophilicity for neuroprotective activity (Perez *et al.*, 2005a).

Finally, we observed a marked enhancement in neuroprotection with the addition of non-polar group to the 2 and/or 4 carbons of the A ring (Perez *et al.*, 2005a). One particularly potent non-feminizing estrogen is ZYC-26, which has a large adamantly group on the 2 carbon and a methyl group on the 4 carbon (Figure 2).

When compounds were categorized by their binding affinity to ERα, we observed the expected positive correlations with ERβ binding (Figure 3) but a negative correlation with neuroprotective activity and lipid peroxidation (Figure 3).

Collectively these structure-activity relationships argue for a neuroprotective mechanism(s) that do not require action at the classical ERs. As such, we began a program of research to determine the mechanism(s) by which non-feminizing estrogens are potently neuroprotective.

3. Mechanism of non-feminizing estrogen neuroprotection

Two observations suggested a potential mechanism by which non-feminizing estrogens could be potently neuroprotective. First, the need for the estrogen molecule to avoid polar groups on the B and C rings suggested that their interaction with lipid membranes was a critical component of their neuroprotective activity. Given the high lipophilicity of estrogens, most estrogens are associated with lipid membranes. Indeed, we have shown that estrogens insert into lipid bilayers and that the presence of an adamantyl group at the C-2 position affects the position of the A ring 3-hydroxyl group such that it is possible to detect an orientation that would bring it into close proximity to the double bonds of membrane lipids; a position that is optimal for terminating lipid peroxidation (Cegelski *et al.,* 2006).

Second, we demonstrated that 17β-E2, 17α-E2, and estratriene-3-ol all synergizes with the aqueous soluble antioxidant, glutathione to result in a 400-fold enhancement of the neuroprotective activity of both feminizing and non-feminizing estrogens (Green *et al.*,

1998). This suggests that lipid resident estrogens can interact with soluble cytosolic antioxidants to halt an oxidative/inflammatory cellular cascade and achieve neuroprotection.

We then assessed the possible redox cycling of estrogens with glutathione using human erythrocytes (Dykens *et al.*, 2004). Human erythrocytes (RBCs) lack mitochondria and nuclei, making them a useful model to assess estrogens for assessing the redox potential of non-feminizing estrogens. In this model, the hexose monophosphate shunt (HMS) generates NADPH, which serves to reduce glutathione (GSH) from oxidized glutathione (GSSG), allowing us to test the extent to which estrogens enhance the activity of this redox cycle. With H_2O_2 exposure, the HMS is activated. In the presence of either 17β-E2 or 17α-E2, an approximate doubling in HMS activity was observed (Figure 4). The potent neuroprotectant, ZYC-3 increased HMS activity about 3-fold, but the inactive compound, ZYC-23 did not affect HMS activity (Figure 4). These results indicate that estrogens are able to tap the large reducing potential of the HMS, and through NADPH-induced reduction of GSSH to GSH, and able to apply this reducing potential to lipid membranes using estrogens as a mediator.

4. Summary and Conclusions

The present series of studies provide evidence that synthetic estrogens that do not interact with ERs are potent neuroprotectants, likely working through a redox cycle that involves glutathione and the HMS. The neuroprotection depends on a phenolic A ring and potency is enhanced through aliphatic substituents on the 2 and/or 4 carbons of the estrogen molecule. These compounds avoid ERα and ERβ, and as such are candidates for chronic therapy aimed at preserving the brain from insults sustained by diseases, like AD, or more acute traumas, like stroke. However, of important clinical significance is that, despite the strong supportive evidence for their neuroprotective actions, because these non-feminizing estrogens impart these effects independent of the classical ERs, the known peripheral benefits of estrogen-containing HTs, including on urogenital tract, bone, and cardiovascular tissues when administered near to the time of menopause initiation (Freedman, 2002; Gambacciani & Levancini, 2014; Hale & Shufelt, 2015), may not be observed. Thus, in conclusion, non-feminizing estrogens represent a novel and effective therapeutic intervention approach for the prevention of injury-induced neuropathology.

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Highlights

- Estrogen neuroprotection can be independent of estrogen receptors
- The phenolic A ring of estrogen molecule is essential to its neuroprotective activity
- Allophalic substitutions at the 2 and 4 carbon of the A ring enhance neuroprotective potency
- Non-feminizing estrogens represent a novel target for post-menopausal brain aging
- Yet, with these agents, peripheral estrogenic benefits will not be observed

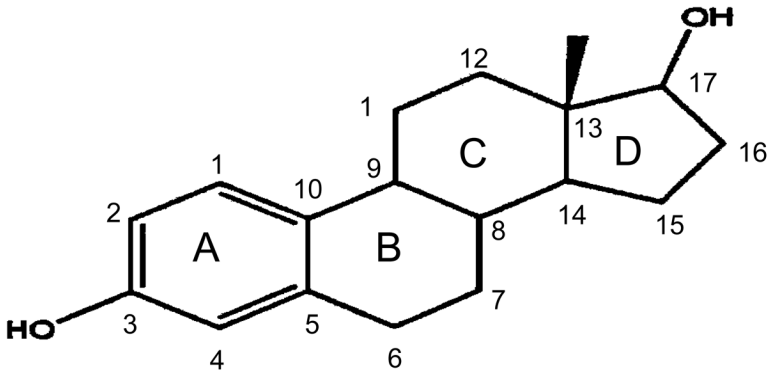

Figure 1.
Structure of 17 β-E2. Letters denote the 4 rings of the molecule and numbers indicate the carbons positions.

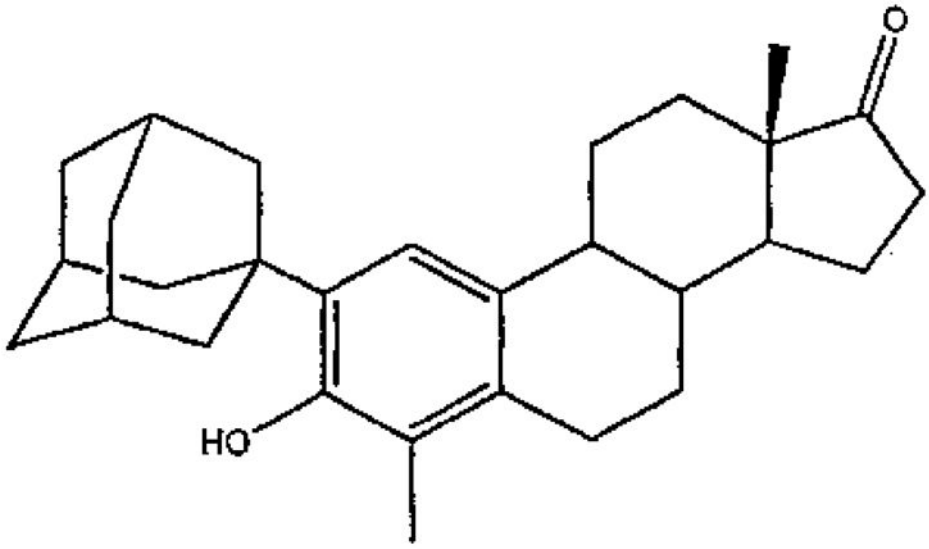

Figure 2.
Structure of 2-(1-Adamantyl)-4 methyl-3-hydroxyestra-1, 3, 5(10)-trien-17-one (ZYC-26).

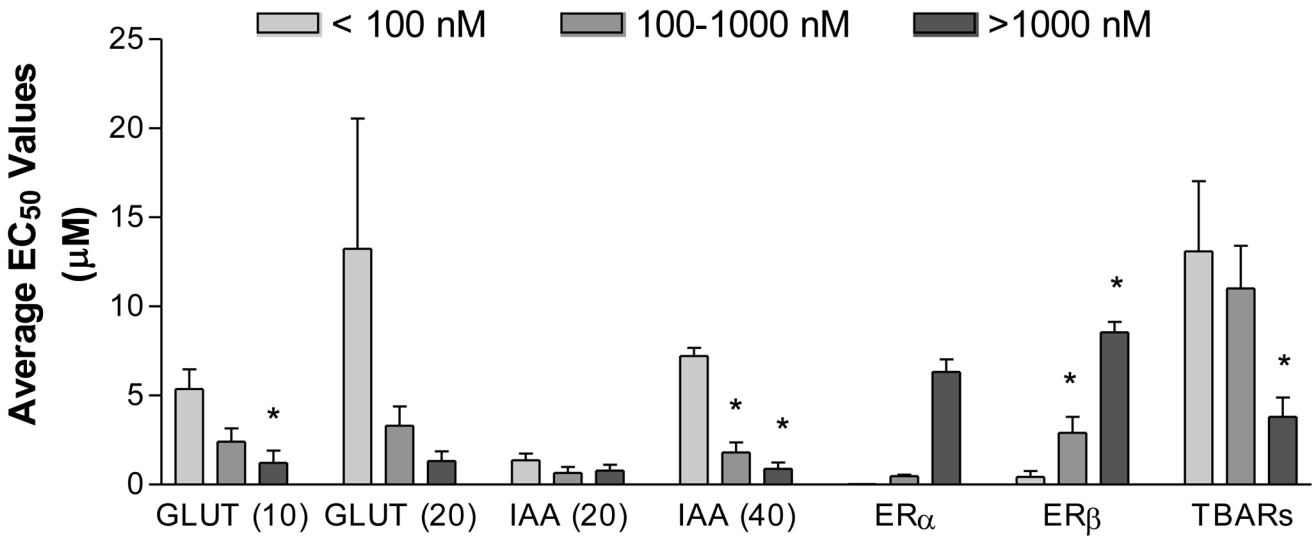

Figure 3.

Relationship among ER binding, neuroprotective activity and lipid peroxidation among estrogens. All activities were categorized by ERα binding affinity and we observed a marked negative relationship between ERα binding and neuroprotection in 4 assays of neurotoxicity. GLUT indicated glutamate toxicity at 10 and 20 mM concentrations of glutamate. IAA indicated indolacetic acid at 20 and 40 mM concentrations (IAA). TBARs indicated thiobarbituric acid reactive substances (TBARs). Reproduced from Perez et al., 2006, with permission.

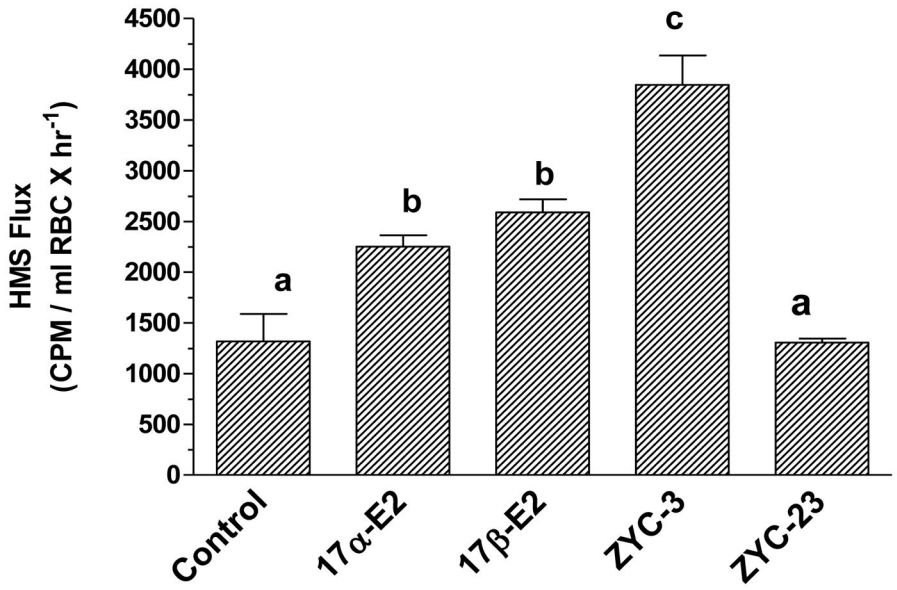

Figure 4.

Carbon flux through the hexose monophosphate shunt in normal human erythrocytes (RBCs). In the absence of an oxidative insult, the compounds have no effect on HMS flux. However, exposure to 10 mM H_2O_2 significantly increases HMS flux in untreated control RBCs. Treatment with the indicated compounds at 1 mM for 10 min prior to addition of 0.3 mCi 14C-U-glucose results in varying increases in HMS flux, with 17β-E2 and 17α-E2, showing comparable responses. ZYC-3 significantly increased HMS flux over the estrogens, and ZYC-23, a non-neuroprotective negative control, yields HMS flux rates indistinguishable from untreated controls. Means not significantly different at $P < 0.05$ (Bonferroni), share superscripts; ANOVA $F = 22.18$, * $P < 0.0001$. Adapted and reprinted with permission from Dykens et al., 2004