



Review

Non-feminizing estrogens: A novel neuroprotective therapy

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ABSTRACT

While the conflict between basic science evidence for estrogen neuroprotection and the lack of effectiveness in clinical trials is only now being resolved, it is clear that strategies for estrogen neuroprotection that avoid activation of ERs have the potential for clinical application. Herein we review the evidence from both in vitro and in vivo studies that describe high potency neuroprotection with non-feminizing estrogens. We have characterized many of the essential chemical features of non-feminizing estrogens that eliminate or reduce ER binding while maintaining or enhancing neuroprotection. Additionally, we provide evidence that these non-feminizing estrogens have efficacy in protecting the brain from AD neuropathology and traumatic brain injury. In conclusion, it appears that the non-feminizing estrogen strategy for neuroprotection is a viable option to achieve the beneficial neuroprotective effects of estrogens while eliminating the toxic off-target effects of chronic estrogen administration.

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1. Introduction

A number of recent reviews have been published that describe the potential strategies for using estrogen-like compounds to achieve neuroprotection without the side effects of chronic estrogen treatment (Simpkins et al., 2011, 2012, 2013). The present review differs from these recent reviews in that we describe the potential mechanisms of non-feminizing estrogens and focus on

two distinct indications for non-feminizing estrogens which have not been previously reviewed: neuroprotection in Alzheimer's disease (AD) and in traumatic brain injury (TBI).

2. Background

The risk of cerebrovascular stroke and AD increases with age (Roger et al., 2011). Even after adjusting for the longer lifespan of women, the incidence of Alzheimer's disease is higher in women (Hy and Keller, 2000), and AD pathology is more likely to be expressed as dementia in women than men (Barnes et al., 2005). Similarly, midlife (45–54 years) and elderly (>85 years) women

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are more likely to sustain a stroke than age-matched men (Appelros et al., 2009; Persky et al., 2010; Petrea et al., 2009; Towfighi et al., 2007). Declines in estrogen levels following menopause have been proposed as one possible explanation for the increased risk of stroke and AD in women. Numerous experimental, epidemiological, and prospective studies provide support for estrogens as neuroprotective agents. This view, however, was challenged in 2002 when the Women's Health Initiative (WHI) study was ended early due to findings of increased risks for cardiovascular disease, stroke, blood clots, breast cancer, and dementia in women on estrogen therapy (ET). Subsequent reevaluations of the WHI suggest the effects of ET depend on the post-menopausal stage, extent of existing pathology and the route of estrogen administration (Dumas et al., 2008; Grodstein et al., 2006; Harman, 2004; Hodis et al., 2003; Manson et al., 2007; Salpeter et al., 2006; Sontag et al., 2004). Whereas ET may provide protective effects for younger women or those receiving early postmenopausal treatment, if taken by women significantly past menopause or by women with pre-existing pathology, ET may be ineffective or even worsen pathology (Coker et al., 2009; Dubey et al., 2005; Maki, 2006; Resnick et al., 2009). The results of the WHI are in polar opposition to the numerous experimental studies showing ET to be protective in numerous neuropathologies, including stroke, AD, and Parkinson's disease (PD). The current challenge is to identify and target the sites responsible for estrogen's neuroprotective effects while minimizing off-target effects associated with deleterious outcomes.

The role of estrogens is not limited to maintenance of female reproductive function. Estrogens influence many physiological and at times pathological processes in a variety of tissues and systems, including the cardiovascular, immune, and nervous system (Gustafsson, 2003) and thereby account for many of the gender differences and the influence of post-menopausal ET on a variety of physiological processes.

Estrogens can exert their effects subsequent to binding with intracellular or plasma member estrogen receptors (ER). There are three known subtypes of ERs: ER α , ER β , and G-coupled protein receptor 30 (GPR30). Upon binding to an ER, there are two pathways through which estrogens are known to signal: the classical/genomic pathway or the non-genomic pathway (Hall et al., 2001; Heldring et al., 2007). In the classical pathway, estrogen binds to ER α or ER β , and the ligated ER can form a homodimer (ER α /ER α or ER β /ER β) or heterodimer (ER α /ER β) and translocate into the nucleus. Once in the nucleus, the ligated ER dimer can bind to DNA at promoter sequences containing an estrogen response element (ERE). The ligated ER dimer bound to an ERE can recruit transcription factors or other co-regulator proteins, ultimately regulating chromatin structure and the transcription of downstream genes. Estrogens can also recruit transcription factor complexes to the nucleus and influence gene transcription downstream of promoters that lack an ERE (Kushner et al., 2000). Estrogen has been shown to regulate a number of neurotrophic genes, such as brain-derived neurotrophic factor (BDNF), neutrophins 3 and 4, and nerve growth factor (Sohrabji et al., 1995; Jezierski and Sohrabji, 2000). In addition, estrogen has also been shown to regulate a number of genes involved in neuroprotection, including anti-apoptotic proteins, such as Bcl-2 (Singer et al., 1998; Alkayed et al., 2001) and Bcl-x (Stoltzner et al., 2001), and caspase inhibitors (Zhang et al., 2001). As such, it is clear that estrogens can protect neurons through a number of classical/genomic mechanisms. Unfortunately, these same ER-mediated signaling pathways also regulate gene involved in the chronic toxicities of estrogens, including those of clotting protein and cell cycles in transformed, estrogen-responsive cells (Holst et al., 2007).

Estrogens also elicit a cellular response that can occur within minutes, entirely too rapid to be attributed to genomic mechanisms. A ligated ER dimer can remain in the cytoplasm and

function as a signaling molecule through activation of protein kinases and phosphatases. There are several signaling pathways that the ligated ER can affect the expression of neurotrophic and/or neuroprotective genes, including the mitogen-activated protein kinase (MAPK) cascade and the cyclic-AMP response element binding protein (CREB) pathway (Singh et al., 1999; Manavathi and Kumar, 2006; Singer et al., 1999; Bryant et al., 2005; Carlstrom et al., 2001). Both pathways regulate the expression of neurotrophic and neuroprotective genes.

A novel ER subtype, GPR30, has been shown to mediate estrogenic signaling. GPR30 is localized to the plasma membrane and was identified following evidence showing estrogenic action in cells lacking ER α and ER β (Filardo et al., 2000). GPR30 activation by estrogen results in increased intracellular calcium, synthesis of phosphatidylinositol 3,4,5-triphosphate (PI3), and activation of MAPK (Filardo, 2002; Prossnitz et al., 2008; Prossnitz and Maggolini, 2009; Revankar et al., 2005). GPR30 was also shown to be neuroprotective through the activation of neurotrophic genes, such as nerve growth factor, and anti-apoptotic proteins, such as Bcl-2 (Kanda and Watanabe, 2003).

Estrogens may signal using genomic, non-genomic, or a combination of both mechanisms, leading to different processes depending on the specific mechanism utilized. Also, ER α , ER β , and GPR30 are differentially expressed across tissues, and the distribution and amount of each type of receptor in a tissue can dictate the tissue's response to estrogen. In addition, the population of co-regulator proteins and transcription factors within a cell can determine the genomic effects of estrogens (Nilsson et al., 2001).

Several strategies have been taken to optimize ET for use in post-menopausal women by capitalizing on the actions of estrogens that alleviate post-menopausal symptoms, while minimizing the negative side effects in peripheral tissues. One such strategy is the use of selective estrogen receptor modulators (SERM). SERMs are synthetic ER ligands that bind to and serve as an agonist for one ER subtype, while antagonizing or not affecting the other ER subtype. Many SERMs are designed to function as an ER β agonist and ER α antagonist, due to the negative effects believed to be mediated by ER α (Brinton, 2004; Shelly et al., 2008). One factor complicating the use of SERMs in neuroprotection is that, although ER α was associated with an increased risk of breast cancer in ET, ER α appears to mediate the neuroprotective action of estrogens under some conditions (Elzer et al., 2010).

A similar, yet alternative strategy to SERMs on which our lab is focused is the use of synthetic estrogen-like compounds, coined non-feminizing estrogens that do not bind to ER α , ER β or GPR30. Evolutionary evidence suggests that estrogens serve to maintain reproductive function, independent of ER activation (Keay et al., 2006). Like SERMs, non-feminizing estrogens seek to capitalize on the neuroprotective action of estrogens and minimize negative side effects associated with ER binding and activation. As such, non-feminizing estrogens have therapeutic potential for use in neuropathologies in both men and women.

3. In vitro estrogen structure–neuroprotective activity relationship

17 β -Estradiol (17 β -E2) is the most potent naturally occurring activator of genomic ER-dependent signaling. Minor modifications to the structure of 17 β -E2 can partially or completely eliminate ER-binding. For example, 17 α -estradiol (17 α -E2), a diastereomer of 17 β -E2 produced naturally in ungulates binds to both ER α and ER β with a 40-fold lower affinity than 17 β -E2 (Green et al., 1997a, 2001). Despite the lower affinity for ER α and ER β , 17 α -E2 was as potent as 17 β -E2 in neuroprotection (Perez et al., 2005; Green et al., 1997b). This finding led to the investigation of

whether estrogen-like compounds, with a structure similar to 17 β -E2 that lacked the ability to bind to ER α and ER β , would function in neuroprotection (Green et al., 1998).

Green et al. (1997b) and others (Behl et al., 1997) determined that an intact phenolic A-ring was critical for neuroprotection (Fig. 1). All modifications to the hydroxyl group at the 3-carbon resulted in total loss of neuroprotection, confirming that the phenolic nature of the A-ring is critical for neuroprotection. We also confirmed that three rings of the steroid nucleus are also necessary for neuroprotective activity. Given this, we synthesized estrogen-like compounds that retained the structure necessary for neuroprotection, but lacked ER binding.

In general, polar substituents as well as large substituents diminish ER binding. However, the ligand-binding pockets of ER α and ER β are flexible and can accommodate estrogens of different shapes and sizes, making it difficult to assess which modifications to the steroid scaffold abolish ER binding (Tamrazi et al., 2003). Nevertheless, we synthesized a library of estrogen-like compounds and measured their affinity for ER α and ER β , and simultaneously evaluated their neuroprotective function. Additions to the A-ring greatly reduced binding affinity for ER α and ER β . We (Perez et al., 2005) also confirmed that adding bulky methyl groups at the 2- or 4-carbon of the A-ring abolished ER binding (Miller et al., 1996). Addition of a hydroxyl group to the B- and C-rings completely disrupted binding to either ER.

Over 70 compounds (ZYC) were tested for their ability to protect murine hippocampal cells (HT22) against glutamate and iodoacetic acid (IAA) toxicity (Perez et al., 2005). Table 1 summarizes the EC₅₀ (IC₅₀) values for neuroprotection, ER binding, and protection against lipid peroxidation.

Addition of electron donating substituents to the steroid scaffold increases the redox potential of the phenoxy radical, generating more potent anti-oxidants, and thereby, enhancing the potential for neuroprotection. Addition of electron donating substituents to the A-ring stabilized the phenoxy radical. Compounds with these modifications were more potent than 17 β -E2 in protecting HT22 cells from both glutamate and IAA toxicity. Based on the finding that additions to the 2- or 4-carbons of the A-ring diminished ER binding, our primary strategy was to replace hydrogen at the 2- and 4-carbons of the A-ring with electron donating substituents (Fig. 2). Addition of an adamantyl group to carbon 2 (ZYC-3) increased neuroprotection compared to parent compounds 17 β -E2 and estrone (E1). Di-substitution with an adamantyl group to the 2-carbon of the A-ring and a methyl group to the 4-carbon (ZYC-26) increased potency to a greater extent than the mono-substituted ZYC-3. When two groups flanked the 3-OH in the 2- and 4-carbons (ZYC-26), neuroprotection was enhanced, with approximately 9- and 4-fold reductions in EC₅₀ values for protection against glutamate and IAA toxicity, respectively. Di-substitution with an adamantyl group to the 2-position of the A-ring and an alkyl group to the 3-carbon (ZYC-23) failed to protect against neurotoxicity, confirming our finding that the phenolic hydroxyl group is crucial for neuroprotection. However, repositioning the hydroxyl in the 2-carbon of the A-ring (ZYC37, not shown) was

protective against glutamate and IAA toxicity, although less potent than molecules with an intact 3-OH. These results indicate that the location of the hydroxyl phenol group necessary for neuroprotection is not restricted to the 3-position of the A-ring.

Introducing conjugated double bonds into the B- and/or C-rings of the steroid scaffold is another approach used to stabilize the phenoxy radical. ZYC compounds with conjugated B-rings were roughly 180-fold more potent than 17 β -E2 against both glutamate and IAA toxicity. ZYC compounds with a conjugated C-ring were approximately 490-fold more potent than 17 β -E2. Polar groups attached to the B- or C-rings reduced the compounds ability to protect against neurotoxicity. D-ring substituents enhanced lipophilicity of the compound, but also decreased potency against neurotoxicity.

In addition to protection from glutamate and IAA toxicity, we have also shown that non-feminizing estrogens are protective in a model of Friedreich's ataxia (FRDA) (Richardson et al., 2011). In this model, human FRDA skin fibroblasts are treated with compounds that increase their susceptibility to oxidative stress through inhibition of glutathione (GSH) synthesis. Our results indicate that in human FRDA skin fibroblasts, 17 β -E2 was protective against oxidative stress across a wide range of concentrations. ZYC-26, which lacks ER binding, was also potently cytoprotective. We do not believe that the protective effect of ZYC-26 is mediated by GPR30, as the GPR30 agonist, G1, was unable to protect cells from oxidative stress, and the protective action of E2 was not blocked by the GPR30 antagonist, G15 (Richardson et al., 2011).

4. Non-feminizing estrogens and AD

Accumulating in vitro evidence suggests estrogens are an important mediator of neuroprotection from amyloid- β peptide (A β). Estrogen's neuroprotective effects against A β -induced toxicity (Fitzpatrick et al., 2002; Marin et al., 2003) are mediated in part by estrogen's effects on A β levels. Estrogen can directly regulate A β levels by decreasing the production of A β and increasing A β degradation. A β is a 36–43 amino acid peptide generated following sequential proteolytic cleavage of the amyloid precursor protein (APP) by β - and γ -secretases. A β formation is precluded by α -secretase cleavage of APP within the A β domain. Estrogen decreases A β formation by enhancing the non-amyloidogenic APP processing, by upregulating α - and γ -secretase, and decreasing the amyloidogenic APP processing by downregulating β -secretase (Green and Simpkins, 2000; Manthey et al., 2001; Nord et al., 2010; Xu et al., 1998). In addition, estrogen stimulates A β degradation by upregulating A β degradation enzymes, such as insulin-degrading enzyme (IDE) and neprilysin, and increasing the internalization and phagocytosis of A β by microglia (Liang et al., 2010; Zhao et al., 2011).

Estrogen's neuroprotective effects may also be due to its regulation of tau phosphorylation, an early pathological signature of AD. 17 β -E2 increased total tau and decreased both naturally occurring and induced tau phosphorylation in a human neuroblastoma cell line (SH-SY5Y) and primary cultures of newborn male or female rat cerebral cortical neurons (Alvarez-de-la-Rosa et al., 2005). The decrease in tau phosphorylation is likely due to estrogen's effect on tau kinases, including glycogen synthase kinase-3 β (GSK3 β) and protein kinase A (PKA). 17 β -E2 prevented forskolin-induced overactivation of PKA in HEK293 cells expressing tau441 and prevented the resulting PKA-induced tau hyperphosphorylation (Liu et al., 2008). Similarly, estrogen's direct interactions with GSK3 β lowered its activity, leading to decreases in tau phosphorylation (Goodenough et al., 2005) at tau sites associated with neuropathology (Ishizawa et al., 2003). Given recent findings that pharmacological interventions targeting only A β were ineffective in phase

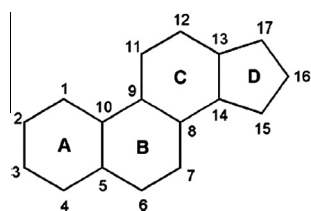


Fig. 1. The chemical structure of steroids with the 4 rings indicated with letter and the carbons numbered numerically.

Table 1
EC₅₀ (IC₅₀) values for non-feminizing estrogen analogues.

	GLUT (10)	GLUT (20)	IAA (20)	IAA (40)	ER (α)	ER (β)	TBARS
ZYC1	1.025	1.571	0.253	1.72	3.987	4.058	4.922
ZYC2	9.021	120.00	0.006		12.675	165.8	16.075
ZYC3	0.15	0.366	0.216		10,000	10,000	1.126
ZYC5	0.123	0.121	0.12	0.152	10,000	10,000	1.391
ZYC14	0.215	0.24	0.183	0.382	2263	2461	1.033
ZYC15	0.047	0.803	0.646	1.2	10,000	10,000	1.945
ZYC16	0.882	0.86	2.25	1.912	10,000	10,000	3.648
ZYC17	0.244	0.27	0.68	0.828	10,000	10,000	4.913
ZYC18	0.057	0.921	0.264	1.044	2384.5	10,000	2.307
ZYC19	0.184	0.272	0.099	0.302	784.1	10,000	2.488
ZYC20	0.134	0.273	0.908	1.134	5089	10,000	1.905
ZYC21	0.122	0.245	0.108	0.282	2855	4456	1.264
ZYC22	0.03	0.03	0.084	0.358	10,000	10,000	1.827
ZYC23	np	np	np	np	10,000	10,000	np
ZYC24	0.665	0.768	1.132	3.928	209.45	259.95	7.028
ZYC25	0.238	0.17	0.159	0.359	10,000	10,000	1.375
ZYC26	0.012	0.037	0.164	0.28	10,000	10,000	0.708
ZYC28	0.497	0.568	0.458	1.268	61.12	533.9	7.88
ZYC33	0.094	0.127	0.046	0.086	10,000	10,000	1.208
ZYC34	0.121	0.19	0.046	0.219	1460	88.01	0.664
ZYC37	11.75	8.228	1.836	4.25	4.88	7.48	11.024
ZYC38	0.133	0.213	0.021	0.035	296.4	10,000	0.895
ZYC39	0.18	0.368	0.049	0.282	633.2	10,000	1.577
ZYC40	0.443	0.524					1.901
ZYC41	11.3		1.573	1.341	34.27	1050	6.291
ZYC42	1.167	1.51	0.051	0.942	248.6	323.6	21.89
ZYC43	0.73	1.511	1.393	1.449	8729	7938	4.506
ZYC44	0.363	0.399	0.051	0.167	2637.5	10,000	0.408
ZYC45	0.684	0.975	0.239	0.446	800.2	2176	2.214
ZYC46	0.866	0.465	0.069	0.155	735	10,000	2.13
ZYC47	0.139	0.121	0.017	0.088	8831	10,000	1.415
ZYC48	0.179	0.527	0.176	1.599	877.5	5208	3.12
ZYC49	np	np	np	np	10,000	10,000	np
ZYC50	0.111	0.139	0.081	0.235	202.7	769.2	2.156

np (no protection); 1000 (over 10 μ M required for binding, or no binding activity).

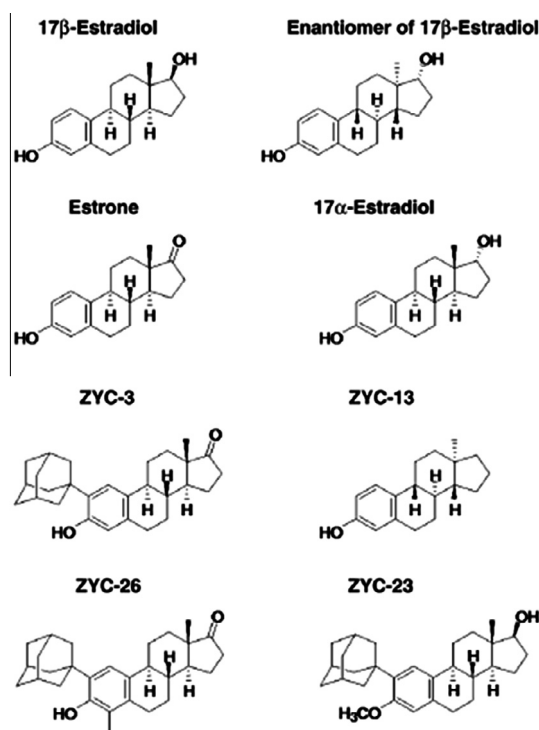


Fig. 2. The chemical structure of 17 β -estradiol, estrone, and a variety of non-feminizing estrogens for which structure-neuroprotective activity relationship studies have been conducted. Reproduced from Yi et al. (2011) with permission.

III clinical trials (see Aisen, 2009 for review), therapeutics with multi-target actions, including alterations in tau phosphorylation, warrant further investigation.

Evidence also suggests estrogen analogues with reduced or no ER binding capabilities also provide neuroprotective effects. 17 α -E2, a weak estrogen receptor agonist, was as effective as 17 β -E2 in protecting estrogen-responsive SK-N-SH cells against the cytotoxic effects of serum deprivation (Green et al., 1997a). ZYC-3, an estrogen analogue with no binding affinity for either ER α or ER β , was 10-fold more potent than 17 β -E2 against glutamate-induced cytotoxicity (Liu et al., 2002). Similarly, pretreatment of primary cultures of rat cortical neurons with genistein, a phytoestrogen, prevented the increase of peroxide levels, glutathione oxidation, mitochondrial aggregation, and cell death induced by A β (Valles et al., 2008).

In PC12 cells, which contain ERs, 17 β -E2 attenuated the cell death induced by the carboxy-terminal (CT) fragment of APP, which contains the A β sequence (Chae et al., 2001). This protective effect was ER-dependent; tamoxifen, an ER antagonist, blocked the protective effect of 17 β -E2. Interestingly, 17 α -E2 did not attenuate CT-induced cell death in PC12 cells (Chae et al., 2001).

5. Non-feminizing estrogens as anti-oxidants

Oxidative stress has been implicated in a number of neurodegenerative diseases, including PD, AD, and stroke. Neuronal membranes are rich in unsaturated fatty acids, and are therefore, highly susceptible to lipid peroxidation by reactive oxygen species (ROS). Clinical interventions based on reducing lipid peroxidation have been implicated for at least some neurodegenerative diseases (Barnham et al., 2004). Estrogens, as well as non-feminizing

estrogen analogues, are lipophilic, allowing them to cross the blood–brain barrier and embed into neuronal membranes. It is therefore reasonable to conclude, based on the findings that non-feminizing estrogens prevented lipid peroxidation in HT22 cells (Perez et al., 2005), that non-feminizing estrogens may have therapeutic anti-oxidant potential in neurodegenerative diseases. However, higher concentrations of non-feminizing estrogens were required to prevent lipid peroxidation than were needed for neuroprotection, indicating that reducing oxidative stress is likely not the only mechanism by which non-feminizing estrogens are neuroprotective.

As previously mentioned, neurodegenerative diseases are associated with increased oxidative stress through generation of ROS, including hydrogen peroxide (H₂O₂). In the presence of excess H₂O₂, hydroxyl radical is produced via the Fenton reaction. Prokai et al. (2003) demonstrated that estrogen, specifically estrone (E1), is extremely efficient in scavenging hydroxyl radical, confirming the anti-oxidant action of estrogens. More importantly, this research showed that estrogens can undergo enzyme-dependent redox cycling following their oxidation by hydroxyl or lipoxyl radicals to be reduced back to the parent estrogen, allowing a single estrogen molecule to scavenge multiple hydroxyl/lipoxyl radicals. This estrogenic recycling provides a unique mechanism that can account for an increased ability to scavenge free radicals, thereby preventing lipid peroxidation and other damage due to ROS.

6. Non-feminizing estrogens as L-type calcium channel modulators

Sarkar et al. (2008) demonstrated that estrogens directly potentiate neuronal L-type voltage-gated calcium channels (VGCC) through an ER-independent mechanism. This work was confirmed in primary rat cortical neurons, hippocampal neurons, and HEK-293 cells transfected with neuronal L-type VGCC. At 10 pM, a concentration 500 times lower than the EC₅₀ for ER α or ER β , 17 β -E2 increased intracellular calcium levels. ZYC-26, that neither binds ER α or ER β also increased calcium influx, and the actions of both 17 β -E2 and ZYC-26 were not antagonized by the ER antagonist, ICI-182,780. Furthermore, despite the fact that HEK-293 cells lack both ER α and ER β , HEK-293 transfected with L-type VGCC responded to 17 β -E2.

Previous studies had hypothesized that 17 β -E2 increased intracellular calcium through activation of protein kinases, PKA, PKC, and CamKII (Kamp and Hell, 2000; Hudmon et al., 2005); however, pharmacological inhibitors of neither PKC nor CamKII inhibited L-type VGCC potentiation by 17 β -E2 or ZYC-26. In contrast, the rapid intracellular influx was shown to be a result of estrogens directly binding to the dihydropyridine site of the L-type VGCC. L-type VGCCs play a role in neuronal growth, protection, and synaptic plasticity, confirming that ZYC-26 is a potent neuroprotective molecule, independent of its role as an anti-oxidant.

7. In vivo evidence of neuroprotection

Experimental studies suggests the increased risk for AD observed in postmenopausal women may be due to an increase in A β levels, an effect observed in multiple animal models. For example, depletion of estrogens, through bilateral ovariectomy, increased A β levels in guinea pigs (Petanceska et al., 2000). Similarly, A β production and plaque deposition was increased in estrogen-depleted mice expressing the Swedish form of APP (APP_{SWE}) an effect reversed by 17 β -E2 administration (Levin-Allerhand et al., 2002). In Tg2576 and Tg2576 \times PS1 mice, 17 β -E2 administration reversed the increase in soluble A β accumulates

observed following estrogen-depletion (Zheng et al., 2002). Crossing APP23 mice with aromatase gene knockout mice, which prevents the conversion of androgen into estrogen, increased A β production and early-onset plaque deposition (Yue et al., 2005). The increase in A β is presumably due to an increase in A β production; the level and activity of β -secretase in aromatase gene knockout mice is increased (Yue et al., 2005) and estrogen administration decreases the amyloidogenic processing of APP, as evidenced by an increase in α -secretase cleaved soluble APP (sAPP α) (Levin-Allerhand et al., 2002), the production of which precludes the formation of A β (Porayette et al., 2009).

The neuroprotective effects of estrogens are not limited to effects on A β . In a mouse model expressing both APP and tau mutations (3 \times Tg-AD), depletion of estrogens exacerbated A β and tau accumulation and memory loss. Administration of 17 β -E2 to estrogen-depleted mice prevented this worsening (Carroll et al., 2007). The estrogen-dependent decrease in tau phosphorylation can occur through a direct decrease in tau kinase activity (Wen et al., 2004) and by inhibiting c-Jun N-terminal protein kinase (JNK) and dickkopf-1 (Dkk1) and activating the Wnt/ β -catenin signaling pathway (Zhang et al., 2008).

Non-feminizing or synthetic estrogen analogues can also exert neuroprotective effects via ER-independent pathways. In estrogen-depleted APP_{SWE} transgenic mice, 17 α -E2 decreased the amyloidogenic processing of APP, leading to a reduction in A β levels, to a greater extent than 17 β -E2 (Levin-Allerhand et al., 2002). The beneficial effects of 17 α -E2 on A β levels were observed without the detrimental, superphysiological uterotrophic response observed following 17 β -E2 treatment (Levin-Allerhand et al., 2002). Similarly, in estrogen-depleted APP_{SWE} \times PS1 mice, 17 α -E2 attenuated the loss of neurons in CA1 and reduced microglial activation in the hippocampus (Manaye et al., 2011). When injected directly into the hippocampus of young, estrogen-depleted rats, the non-feminizing synthetic estrogen analogues, ZYC-5 and ZYC-13, increased cognitive performance to a similar extent as 17 β -E2 (Walf et al., 2011), suggesting non-feminizing estrogens may have functional effects on memory in addition to their neuroprotective effects. This cognitive enhancement was observed despite no appreciable binding of ERs by ZYC-5 and ZYC-13 in the hippocampus (Walf et al., 2011).

Given the now known link between ischemic stroke and AD (Wen et al., 2004; Li et al., 2013), the effects of non-feminizing estrogens on ischemia outcome is relevant. Neuroprotective effects of non-feminizing estrogens are observed in models of ischemia. Lesion volume is reduced equally by 17 β -E2 and 17 α -E2 (Simpkins et al., 1997). Similarly, we observed that lesion volume was significantly reduced by pretreated with ZYC-3 immediately before middle cerebral artery occlusion (MCAO) in estrogen-depleted rats (Liu et al., 2002). The beneficial effects of ZYC-3 were not limited to neuroprotection. ZYC-3 also provided beneficial vasoactive effects; ZYC-3 significantly increased cerebral blood flow CBF in both the nonischemic and ischemic side within 30 min after reperfusion (Liu et al., 2002). In addition, a single dose of selective GPR30 agonists, G1 and STX, administered immediately after ischemia in middle-aged, estrogen-depleted female rats significantly increased the number of surviving CA1 pyramidal neurons at 7 days post stroke (Etgen et al., 2011). Together, these studies provide further support for the development of non-feminizing estrogens as neuroprotective agents.

8. Non-feminizing estrogens and TBI

To determine the effects of non-feminizing estrogens on TBI, we used a severe TBI model that is highly reproducible and in which there is a significant increase in inflammation, oxidative stress,

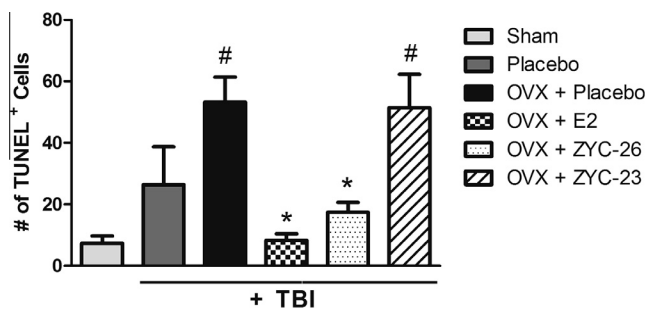


Fig. 3. Effects of 17 β estradiol, ZYC-26 and ZYC-23 on TUNEL positive cell number in the cortex in mice subjected to blunt TBI. 17 β Estradiol, ZYC-26 and ZYC-23 were administered intravenously at a dose of 100 μ g/kg, 30 min before contusion and animals were sampled 24 h later. # Indicates a $p < 0.05$ vs. sham; * Indicates a $p < 0.05$ vs. OVX + Placebo and OVX + ZYC-23.

and neuronal cell death (Gatson et al., 2012). The C57 BL/6 female mice were anesthetized with isoflurane (3%) and placed in an adapted nosecone device. Following the craniotomy procedure, a cortical contusion was delivered to the right hemisphere via a vertically directed pneumatic cylinder. The impact device is well described and consists of a 3-mm flat tip impounder that delivers a velocity of 3 m/s to a depth of 1.2 mm (severe brain injury). Following injury, the skin was closed with surgical wound clips. Control and sham (craniotomy only) animals were also included as controls. In order to determine if non-feminizing estrogens are neuroprotective in a model of TBI, we subjected mice to TBI at 30 min after administration of 17 β -E2, the neuroprotective ZYC-26 and the inactive ZYC-23, each administered at a dose of 100 μ g/kg iv in hydroxypropylcyclodextrin (HPCD).

After TBI, mice received a transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation. The brains were removed and paraffin-embedded. The brains were sectioned (5 μ m sections) using the Thermo Scientific Rotation Microtome. To quantify positive staining, the Zeiss Imager A.2 and Axiovision software was used to determine positive staining in the cerebral cortex of the TBI animals. Coronal brain slices were collected from a rostral to caudal direction starting at the epicenter of the injury zone. The brain sections (5 μ m) spanned a total of 12 microscope slides. A total of 36 slices (3 slices per slide) were collected per brain. Staining from one-in-twelve series of sections were counted.

We observed that ovariectomy significantly increase the number of TUNEL+ cells, suggesting that endogenous estrogens exert a protective effects from brain damage of TBI (Fig. 3). Also, ovariectomized mice treated with 17 β -E2 showed an amelioration of the damaging effects of TBI, as did the non-feminizing estrogen, ZYC-26 (Fig. 3). In contrast, the inactive ZYC-23 failed to protect ovariectomized mice for the effects of TBI (Fig. 3). These data are the first evidence of which we are aware for a neuroprotective effect of non-feminizing estrogens in TBI. Future studies are needed to determine whether acute administration of ZYC-26 following TBI will also be protective.

9. Conclusions/future directions

Non-feminizing estrogens have been shown to be protective in models of oxidative stress, cerebral ischemia, AD, and TBI, and the protective action of these compounds is independent of activation of the known ERs, ER α , ER β , and GPR30. Reevaluations of the WHI suggest that activation of ERs in peripheral tissues, such as the breast and uterus, may account for the negative side effects associated with ET. By avoiding activation of known ERs, non-feminizing estrogens may provide an ET strategy with minimal side effects.

Current evidence suggests that estrogenic action on osteoblasts and osteoclasts is mediated by ER α , and ER α KO mice display patterns of bone loss similar to post-menopausal women. While there is accumulating evidence supporting the neuroprotective role of non-feminizing estrogens, it is currently not known if non-feminizing estrogens can relieve post-menopausal symptoms, such as osteoporosis and hot flashes. As such, they may not be a replacement for current ET. To determine if non-feminizing estrogens offer an alternative to current ET, future studies are needed testing these compounds in animal models for osteoporosis and hot flashes. Nonetheless, non-feminizing estrogens have therapeutic potential in the prevention/treatment of neurodegenerative diseases.

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