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The Assessment of Non-Feminizing Estrogens for Use in Neuroprotection

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Abstract

The menopause is associated with a precipitous decline in circulating estrogens and a resulting loss of the neuroprotective actions of this steroid hormone. In view of the results of the Women's Health Initiative and the preceding knowledge that orally administered estrogens has a variety of adverse side effects, likely through actions on peripheral estrogen receptor alpha (ER α), we initiated a program of research to synthesis and assess a group of non-feminizing estrogens that lack ability to interact with ERs but retain much of the neuroprotective action of feminizing estrogens. This program of research is aimed at the identification of compounds which do not stimulate ERs but are potentially neuroprotective *in vitro* and in animal models of neuronal cell death. We discovered that the most effective non-feminizing estrogens were those with large bulky groups in the 2 and/or 4 carbon of the phenolic A ring of the steroid. These compounds were 8- to 114-fold more potent than 17 β -estradiol (β E2), but lacked ER binding capacity *in vitro* and feminizing effects *in vivo*. The success of this program of research suggests that strategies to optimize non-feminizing estrogens for use in postmenopausal women can be successful.

Introduction

In early 2002, the Women's Health Initiative (WHI) study was prematurely ended due to results showing increased risks for cardiovascular disease, stroke, blood clots, breast cancer, and dementia in women on estrogen therapy. Since that time, the scientific community has been struggling to reconcile the numerous epidemiological, experimental, and clinical studies showing hormone therapy (HT) to be protective against a wide variety of pathological diseased states such as Alzheimer's disease (AD) and Parkinson's disease (PD), cerebrovascular stroke, and cardiovascular diseases. Interestingly, recent reevaluations of the WHI, other clinical trials and cohort studies, such as the Nurses' Health Study, suggest that effects of HT are dependent on menopausal stage and the extent of preexisting neurodegenerative or cardiovascular disease at the onset of HT (Dumas et al., 2008;

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Grodstein et al., 2006; Harman, 2004; Hodis et al., 2003; Sontag et al., 2004). Specifically, emerging evidence indicates that younger (perimenopausal or early postmenopausal) women derive neuro- and cardiovascular protection from HT, whereas the initiation of such therapy in older individuals who are likely to have extensive preexisting conditions may be ineffective or even detrimental (Coker et al., 2009; Dubey et al., 2005; Maki, 2006; Manson et al., 2007; Resnick et al., 2009; Salpeter et al., 2006).

These later analyses of the WHI's data agree with the hypothesis that female sex hormones are potent, centrally active molecules that protect women's health. One important aspect of these hormones, specifically estrogens, is their neuroprotective activity. Sex differences in the incidence and outcome of stroke in human subjects suggest that hormonal factors may influence stroke occurrence and damage (Finucane et al., 1993; Paganini-Hill and Henderson, 1996). The severity of ischemic damage in spontaneously hypertensive rats is dependent upon estrogen status, i.e., rats subjected to MCAO during proestrous periods showed less damage than rats injured during metestrus times (Carswell et al., 2000a; Carswell et al., 2000b). While a few studies have shown neutral or negative effects of estrogens (Bingham et al., 2005; Harukuni et al., 2001; Santizo et al., 2002), exogenously administered 17 β -estradiol (β E2) is also able to reduce ischemic lesions in animals subjected to middle cerebral artery occlusion (Dubal et al., 1998; Shi et al., 2001; Simpkins et al., 1997a; Yang et al., 2000); estradiol-mediated protection against cerebral ischemia is seen in young (Liao et al., 2001; Simpkins et al., 1997a), middle-aged (Dubal and Wise, 2001), and diabetic rats (Toung et al., 2000), as well as in mice (Dubal et al., 2001) and gerbils (Shughrue and Merchenthaler, 2003). Injury due to MCAO is decreased in rats with either pretreatment with physiological levels of E2 (Dubal et al., 1998) or with pharmacological post-treatment methods (McCullough et al., 2001; Yang et al., 2000). The pathological mechanisms activated during stroke include oxidative stress, free radical activity, excitotoxicity, inflammatory response, mitochondrial dysfunction, and apoptosis, all of which are antagonized by estrogens. Moreover, estrogens have been shown to be potent neuroprotectants against *in vitro* models of neurotoxicity, including serum deprivation, oxidative stress, amyloid β peptide (A β) induced toxicity and glutamate-induced excitotoxicity (Green and Simpkins, 2000). However, the mechanisms by which estrogens are protective remain elusive. It is clear that the effects of estrogens are complex and multifaceted. The mechanisms of the neuroprotective actions of estrogen are characterized by classical estrogen receptor (ER)-dependent genomic and non-genomic actions, the latter of which is expressed by rapid interactions with neuronal membranes and intracellular signal transduction pathways. In addition, estrogens have also been shown to have an intrinsic antioxidant structure that lies in the phenolic ring of the compounds, which provide the antioxidant/redox cycling activity in neurons (Prokai et al., 2003a; Prokai et al., 2003b).

It is postulated that many of chronic HT side-effects are most likely due to peripheral effects of orally administered estrogen preparations acting via known ERs (Coker et al., 2009; Dubey et al., 2005; Maki, 2006; Resnick et al., 2009; Salpeter et al., 2006). Several strategies have been undertaken to capitalize on the potent neuroprotective effects of estrogens, while avoiding the side effects of chronic peripheral ER activation. First, selective estrogen receptor modulators are compounds that act as agonists on one ER and antagonists at the other ER (Zandi et al., 2002). This strategy has attempted to enhance ER β agonist activity while antagonizing ER α (Brinton, 2004; Shelly et al., 2008). The rationale for this approach is that chronic activation of ER α , which is highly expressed in breast, uterus and liver, may account for the now known side effects of chronic estrogen administration to post-menopausal women.

An alternative strategy that our laboratory is actively pursuing is the use of estrogen analogues that do not have ER binding capabilities. This strategy is based on the seminal

finding that the ER- α/β ligand β E2 and its enantiomer α E2, which binds to these ER-isoforms with an approximately 40-fold lower affinity (Perez, 2005), were equally potent in protecting neuronal cells and the effects were not blocked by an estrogen antagonist (Green et al., 1997a). This observation prompted a closer evaluation of the structural requirements for neuroprotection among steroids (Behl et al., 1997; Green et al., 1997b), which suggested that estrogen analogues with little ER binding affinity were just as protective as β E2; therefore, we reasoned that a substantial portion of the neuroprotective activity of estrogens was ER-independent.

***In Vitro* Screening of Non-Feminizing Estrogen Analogues**

Many of the neurotoxic insults that are antagonized by estrogens involve an oxidative stress component, and it has been well established that estrogens possess antioxidative properties. We (Green et al., 1997b) and others (Behl et al., 1997; Moosmann and Behl, 1999) have determined that the phenolic nature of the estradiol molecule is essential for neuroprotection. Therefore, we synthesized estrogen analogues to performed structure-activity relationship studies using rationally designed compounds in 17 β -estradiol-neuroprotection assays. Over 70 compounds in HT-22 (murine hippocampal) cells were tested for their ability to inhibit cell toxicity against glutamate and iodoacetic acid using the fluorescent calcien-AM viability assay. The EC₅₀ (or IC₅₀) values for neuroprotection, ER binding, and protection against lipid peroxidation were determined to ascertain potency comparisons with β E2, which is shown in Table 1 modified from Perez et al. (2005b) (representative chemical structures seen in Fig. 1).

Neuroprotective estratrienes that have electron donating constituents increases the redox potential of the phenoxy radical, providing better neuroprotective properties. The donated hydrogen radical can quench free radicals formed in oxidative stress conditions. Bulky substituents added to the A-ring and conjugated bonds introduced into the B and C rings accomplished this goal by increasing phenoxy radical stability by resonance stabilization.

Compounds with modifications to the phenolic A-Ring (Perez et al., 2005b) have been evaluated. A-ring derivatives with electron donating constituents that stabilized the phenoxy radical were more potent than β E2 in protecting these cells from oxidative stress-induced toxicity. Our primary synthetic strategy was to replace the hydrogen with a bulky alkyl group at the 2- or 4- positions of the A-ring. For those compounds that included the addition of a single group to the 2-carbon position of the A-ring (ZYC3) or 4-carbon position of the A-ring (ZYC16), there was an increase in potency by approximately ~ 2 – 170 fold as compared to the parent compound, β E2. When two groups flanked the 3-OH position (ZYC26), neuroprotection was enhanced, with approximately 9- and 4-fold decrease in EC₅₀ values for protection against neurotoxicity, respectively.

However, C3 methoxy ether analogues of the addition of a single group to the 2-carbon position of the phenolic A-ring that lack an additional phenol substituent (ZYC23), failed to protect cells against toxicity induced cell death. Further, negative controls included replacing the hydroxyl group at the 3-position of the A-ring with a CH₂OH or carboxylic acid group (PS3 or PS4, respectively), which abolished the neuroprotective effects of β E2. Extending the length of the steroid by adding a (4-hydroxyphenyl)ethyl group to the 2-position of the A-ring (ZYC46) increased neuroprotective properties. Removing one of the two hydroxyl groups had no effect on neuroprotection against glutamate, but decreased potency in the IAA model by approximately 300-fold. Displacing the phenolic hydroxyl group from the steroid backbone (ZYC28), thus extending the length of the molecule, was more protective than β E2 against toxicity. Moreover, relocating the hydroxyl group of the phenolic A-ring from the 3-position to the 2-position (ZYC37) decreased the protection

against glutamate cytotoxicity, but not against IAA toxicity. Switching the hydroxyl group to the 2-position and adding bulky groups (1-adamantyl (ZYC38) or *tert*-butyl (ZYC39)) to the 3-position greatly improved neuroprotective potencies of the parent compound (Perez et al., 2005b).

Polar groups attached to the B- and C-rings disrupted estrogen's ability to protect cells from oxidative stress. These hydroxyl groups, which impart hydrophilicity to the molecule, added to the middle of the structure influence the way these steroids fit into the center, hydrophobic lipid bilayer and thus its ability to react with lipid oxidation events. Fifteen compounds with modifications to the B or C ring of the estratriene backbone were evaluated. In all cases, when hydroxyl groups were added to the B- (E2540) and C-rings (E1240), whether above or below the plane of the ring), neuroprotection was completely abolished. Opening of the B-ring (ZYC6) causes decreased rigidity of the molecule, but had no effect on the ability of β E2 to protect against IAA-induced cell death and decreased β E2's ability to protect against glutamate induced cell death by approximately 6-12-fold. Producing a nonplanar conformation, such that the A- and B-rings lie perpendicular to the C- and D-rings (ZYC9), had no effects on neuroprotection. Introducing conjugated double bonds into the B- or C-rings of estratrienes is another way to increase the stability of the phenoxy radical (ZYC10). Compounds with these modifications were approximately 180- and 490-fold more potent than β E2 against glutamate and IAA toxicity, respectively. Steroids having an aromatic B-ring, similar to conjugated equine estrogens, with intact phenolic hydroxyl groups (E380) were more potent against IAA toxicity, but performed equally as well against glutamate toxicity as compared to β E2 (Perez et al., 2005b).

D-ring substituent also decreased the lipophilicity; however, the addition of norpregna, norcholesta, benzoate (ZYC30) and methyl-ethers to the 17-position did not result in better neuroprotection, instead decreased the potency of the parent compound. Introduction of the side chain found in 25-hydroxycholesterol (ZYC29), a 2-hydroxy-1-methylethyl side chain (ZYC36), pentanyl groups to the C-16 position (ZYC2 or ZYC4) did not enhance neuroprotection, possibly due to the fact that the pentanyl group lies orthogonal to the A-,B-,C-,D-rings and impede the ability of the estratriene to situate in the center of the lipid membrane. However, complete removal of a 17-substituent enhanced neuroprotection (Perez et al., 2005b). The combination of the 17 β -hydroxyl and 13 α -methyl groups induces a boat conformation for the C-ring (PS1). The combination of the 17 α -hydroxyl and 13 α -methyl groups does not change the chair configuration of the C-ring, but the D-ring is orthogonal and above the A-,B-,C-rings (PS2). These changes reduced neuroprotective potencies of β E2.

These results indicate that protection against toxicity requires a phenolic hydroxyl group as the 3-O-methyl congener of ZYC-5 and the O-methyl congener of ZYC50 proved ineffective. The hydroxy-phenol group does not have to be part of the A-ring since the addition of a hydroxyl phenol group to the 3-position was equally or more protective against glutamate and/or IAA-induced toxicities. This molecule remains planar and can "fit" into the membrane. Repositioning the hydroxyl in the 2-carbon of the A-ring (ZYC37) was still, albeit less, protective against glutamate-induced toxicity but a better protector against IAA-induced toxicity. This and the fact that the nonsteroidal compounds with phenolic groups are still protective demonstrate that the position of the hydroxy phenol group is not restricted to the 3-position of the steroid backbone.

Green (1997a) found that nanomolar concentrations were neuroprotective against serum deprivation in SK-N-SH neuroblastoma cells. In this case estrogens may work as neurotrophic agents. However, in concert with glutathione, estrogens were also potent neuroprotectants (in the nanomolar range) against A β (25–35) toxicity in SK-N-SH (Gridley

et al., 1998) and HT-22 cells (Green et al., 1998). Using the same paradigm as Behl's group (1997), we found that β E2 was protective against glutamate toxicity at 10 μ M concentration. All of the structure-activity studies found that the phenolic A-ring was an absolute requirement for neuroprotection, as compounds such as mestranol and quinestrol did not have protect effects. That EC₅₀ values seen with some of the more potent compounds were in the nanomolar range is the key for therapeutic purposes.

Other steroids (progesterone, DHEA, testosterone) and non-steroidal hormones (melatonin, etc) have been shown to be neuroprotective in various injury paradigms. Green et al. (1997b) did not find progesterone to be protective against serum deprivation in SK-N-SH cells up to 200 nM. Behl et al. (1997) did not find 10 μ M progesterone (P4) to be neuroprotective against glutamate insult in HT-22 cells. On the other hand, Gursoy et al. (2001) found optimal protection with 500 nM pregnanolone (a steroid with a hydroxyl group in the A-ring, but not a hydroxy phenol group) and no protection at 10 μ M dose against glutamate insult in HT-22 cells. However, P4 has been found to be protective in acute global cerebral ischemia (Cervantes et al., 2002; Gonzalez-Vidal et al., 1998), excitotoxicity in primary hippocampal cells (Goodman et al., 1996), and traumatic brain injury (Jiang et al., 1996; Roof and Hall, 2000a; Roof and Hall, 2000b). P4's neuroprotective abilities in traumatic brain injury may be due to its effects on edema. Progesterone derivatives have been shown, like E2, to elicit fast nongenomic events. There have also been several studies on the androgens, testosterone and DHEA, on neuroprotection. Some have determined a deleterious effect, while others a neuroprotective effect of these various hormones (Cardounel et al., 1999), (Kimonides et al., 1998). Validation for neuroprotection by testosterone, however, necessitates the use of aromatase inhibitors as it is a precursor to estradiol (Veiga et al., 2003). Other estrogen-like substances, such as conjugated equine estrogens (Zhao et al., 2003), phytoestrogens (Gelinas and Martinoli, 2002; Roth et al., 1999; Wang et al., 2001), and estrogen metabolites (2-OH-estradiol) (Teepker et al., 2003) were also found to be neuroprotective, although not as potent as the compounds screened in our studies. In summary, critical feature of the estradiol structure and stabilization of the phenoxy radical state has been identified that increased the potency against oxidative stress damage; thus, advancing our understanding of estrogen-mediated neuroprotection.

Estrogen-Receptor Binding of Non-Feminizing Estrogen Analogues

Competition binding experiments revealed that β E2 bound to ER α and ER β with EC₅₀ values of 3.04 and 4.51 nM, respectively (Table 1). DES, as expected, bound slightly better and had EC₅₀ values of 2.62 nM for ER α and 1.84 nM for ER β . Estrone, α E2, and *ent*-E2 bound to these receptors with less affinity (Table 1).

Reductions made to the steroid structure (ZYC1, ZYC 10, ZYC27, E380) did not drastically change the affinity of the estratrienes for the estrogen receptors, exceptions being with ZYC12 and E400 (Table 1). Additions to the A-ring drastically affected binding to the estrogen receptors. Additions to the 2- and 4-positions (ZYC 22, ZYC25, ZYC26) completely abolished the affinity of estratrienes for both estrogen receptors. Adamantyl groups added to the 2 position (ZYC3, ZYC5, and ZYC35), likewise, abolished binding to the estrogen receptors. Methylpropenyl, methylpropyl, tert-butyl additions to the 2-position of the A ring greatly reduced the binding affinity for the receptors. Midsize additions (tert-butyl) to the 2-position and norpregna additions to the other end of the estratriene (ZYC47) further reduced the binding capacity. While bulkier additions (methyl propenyl (ZYC16) and methyl propyl (ZYC17)) to the 4-position also completely inhibited estrogen receptor binding, smaller additions (methyl, ZYC24) were approximately 69 and 58-fold less effective in binding to the estrogen receptors as compared to β E2.

Hydroxyl additions to the B- and C-rings in the alpha or beta positions inhibited binding activity. Hydroxyl groups in the β configuration to the C11 position and of the estradiol derivative (E2560) bound 10-fold less to ER α than β E2, while the estrone derivative (E1240) bound with even less affinity.

Changes to the planarity of the steroid ring structure also affected the affinity of these compounds for estrogen receptors. Opening the ring structure at the 9-position (ZYC6 and ZYC7) diminished the affinity of the steroid for both estrogen receptors; the estrone derivative (ZYC6) bound with less affinity than the estradiol derivative (ZYC7). The nonsteroidal compounds (ZYC51-59) bound with negligible affinity.

Anti-Oxidative Properties of Non-Feminizing Estrogen Analogues

Estratrienes are potent lipid antioxidants. This is a well-known property as previous studies have determined the radical scavenging, iron chelating, and total anti-oxidative properties of various estratrienes (Mooradian, 1993; Romer et al., 1997a; Romer et al., 1997b). We have confirmed other reports that showing that 2- and 4-additions to the phenolic A-ring are potent antioxidants (Miller et al., 1996). Further, they are able to do so in a similar pattern to their ability to protect HT-22 cells from oxidative stress-induced cell death.

In the DCF assay, levels of reactive oxygen species (ROS) were detected by the direct bolus addition of hydrogen peroxide and an enzyme-mediated method. Glucose oxidase (GO) and hydrogen peroxide-mediated increases in ROS reached a plateau. Additional glucose (substrate for GO) was not co-administered with the enzyme, and this might account for the plateau seen. There could also be a self-quenching effect. β E2 was not able to significantly decrease ROS when HT-22 cells were insulted with glucose oxidase. When higher concentrations of estratrienes were used, we were able to see a scavenging effect in SK-N-SH cells.

In a cell free system, we have shown that estratrienes are able to inhibit iron-induced lipid peroxidation (Perez et al., 2005b). The scavenging abilities for soluble ROS (DCF assay) were not robust; however, the lipid-based antioxidant model (TBARs assay) proved to be a better measure of antioxidative abilities of estratrienes. This is contrary to a report showing that antioxidant capacities of various estrogenic structures were found to be dependent upon the aqueous or lipophilic nature of the assay system (Ruiz-Larrea et al., 2000). In the aqueous-based antioxidant assay, phenolic estrogens performed better than catecholestrogens and diethylstilbestrol in quenching the chromogenic radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS $\bullet$ +). On the other hand, in the lipid-based antioxidant system catecholestrogens were able to regenerate α -tocopherol for oxidative stress-treated LDL while phenolic estrogens could not. Nevertheless, we confirmed that β E2 is able to inhibit lipid peroxidation in rat brain homogenates with an IC₅₀ of 19.83 μ M, which is comparable to the IC₅₀ seen by other groups (IC₅₀ = 21 μ M) (Hall et al., 1991).

Catecholestrogens and DES have been shown to increase the rate of Fe (III) reduction in aqueous solutions, while β E2 and E1 did not have an effect (Ruiz-Larrea et al., 1995). If lipid peroxidation is based on Fe (III), this corroborates our findings that DES was better able to decrease MDA formation compared to E2 and E1: DES (6.7) < E2 (19.8) < E1 (80.8). It remains to be determined whether the more potent estratrienes will be as effective as catecholestrogens. Iron metabolism, that is redox iron cycling, is complex; it has been shown that a Fe(II)/Fe(III) has to be at a 1:1 ratio for optimally initiating peroxidation events. We added ferric ions at a 50 μ M concentration and show that estratrienes were able to inhibit lipid peroxidation events at doses below this iron load. While chelation has been

shown to occur with steroidal molecules at a 3:1 stoichiometry (Ryan et al., 1993); many of our compounds were effective at low micromolar concentrations.

The lipophilicity of estratrienes leads to their accumulation in the hydrophobic plasma membranes and affects membrane fluidity (Dicko et al., 1999; Liang et al., 2001) and increased lipid ordering (Liang et al., 2001). Estrogen- localization to this lipid environment places the steroids at the key site of peroxidation events, and with their antioxidant properties allows prevention of oxidative damage. The lipid membrane is also the site of various signal transduction processes including PI3K signaling and phosphatidylserine flipping in apoptosis. Also, estratrienes could situate themselves in membranes of different compositions and subcellular locations (plasma membrane of different cell types, mitochondrial membranes, etc).

Oxidative stress is of major pathophysiological relevance for a variety of process including ischemia-reperfusion injury, neurodegeneration, cardiovascular diseases, atherosclerosis, and cataractogenesis. Because an imbalance in the production and elimination of ROS exists in many disease processes, it would be imperative to reestablish the imbalance by increasing the elimination process or preventing ROS production. There are two ways to combat this: enzymatically and non-enzymatically. The use of antioxidants that penetrate the blood-brain barrier could alleviate oxidative damage in the brain. Estrogens are known antioxidants and with its hormone structure can easily cross the blood brain barrier.

***In Vivo* Assessment of Non-Feminizing Estrogen Analogues**

Numerous published data show that both endogenous and exogenous β E2 administration attenuates infarct volume and are neuroprotective against the pathological events seen in experimental animal models of cerebral ischemia (Alkayed et al., 1998; Dubal et al., 1998; Green et al., 1996; Green et al., 1997a; Green et al., 1997b; Green et al., 1998; Green and Simpkins, 2000; Green et al., 2001; Gridley et al., 1997; Simpkins et al., 2005; Sudo et al., 1997; Yang et al., 2000). From our *in vitro* screening, we selected several of the estratrienes for *in vivo* assessment against a routinely used model of cerebral ischemia, transient MCA occlusion, in ovariectomized rats. To date, we have assessed eight compounds in this model, including β E2, estrone, α E2, *ent*-E2, enantiomer of 17-desoxyestradiol (ZYC-13), 2-(1-adamantyl) estrone (ZYC-3), 2-(1-adamantyl)-4-methylestrone (ZYC-26), and 3-O-methyl analog of (17 β)-2-(1-adamantyl)estradiol (ZYC-23) (Figure 1). Consistent with our *in vitro* studies, β E2, estrone, α E2, *ent*-E2, enantiomer of 17-desoxyestradiol (ZYC-13), 2-(1-adamantyl) estrone (ZYC-3), and 2-(1-adamantyl)-4-methylestrone (ZYC-26) exert potent neuroprotective effects in focal cerebral ischemia/reperfusion injury (Fan et al., 2001; Green et al., 2001; Liu et al., 2002; Simpkins et al., 1997b; Simpkins et al., 2004).

Subcutaneously implantation of α E2 24 hours before MCA occlusion showed neuroprotective effects. The survival rate of vehicle-treated animals was 54%, whereas the survival rate of α E2-treated animals was 71% (Simpkins et al., 1997b), even though α E2 binds to ER-isoforms with an approximately 40-fold lower affinity (Perez et al., 2005a). Vehicle-treated rats showed the expected ischemic lesion; with the maximum ischemic area ($24.1 \pm 2.4\%$) occurring in the slice obtained 9 mm posterior to the olfactory bulb and a smaller lesion occurring in slices obtained from more rostral and more caudal areas. Animals pretreated with α E2 exhibited smaller ischemic areas in all slices evaluated. The area under the ischemic lesion curve for the vehicle-treated and α E2-treated groups was $8.1 \pm 0.8\%$ and $3.7 \pm 1.3\%$, respectively, with a mean ischemic area of $14 \pm 1.5\%$ for vehicle-treated group and $6.3 \pm 2.3\%$ for α E2 group.

Two hour pretreatment with E1 before the onset of transient MCA occlusion significantly reduced infarct volume by 53% compared with vehicle control following 24-h reperfusion in

ovariectomized rats. The quinol-conjugated E1 was equipotent with the parent estrogen in reducing lesion.

Ent-E2, which has shown to be less than one-eighth as active as β E2 in competitive bind assays for human recombinant ER α and ER β , is as effective as β E2 in reducing infarct size after MCA occlusions. There was no evidence of metabolic conversion of *ent*-E2 to β E2 in ovariectomized rats (Simpkins et al., 2004). In addition, there were no effects of *ent*-E2 on physiological parameters before, during, or after MCA occlusion (Simpkins et al., 2004), which suggests that non-feminizing enantiomers of estrogens can be potentially neuroprotective in animal models for human ischemic damage.

ZYC-3 and ZYC-26 were ineffective in protecting against the insult when administered in corn oil. The inability of these analogues to protect against cerebral ischemia when administered subcutaneously via corn oil is most likely due to their high lipophilicity. In fact, the logarithm of the 1-octanol/water partition coefficient (log P) estimated by an atom fragment method (Ghose and Crippen, 1987) indicate that ZYC-3, for example, has a log P of 6.83 compared with 4.01 for E2. The extremely high lipophilicity indicates that ZYC-3 (or ZYC-26) essentially accumulates in a lipid environment and therefore, remains in the injection site. The lack of effects of ZYC-3 via subcutaneous injection with corn oil as vehicle is probably due to the inappropriate formulation method. Therefore, to achieve a rapid delivery of ZYC-3 (or ZYC-26) into the circulation, it was complexed with aqueous 30% 2-hydroxypropyl- β -cyclodextrin solution. With this formulation, neuroprotective and vasoactive effects were clearly manifested (Simpkins et al., 2004).

ZYC-3 showed no binding to either ER α or ER β in competitive binding assays but was 6-times more potent than β E2 in a test for neuroprotection against glutamate toxicity. Further, in our *in vivo* model of transient MCA occlusion, ZYC-3 performed better than β E2 without affecting physiological parameters (Liu et al., 2002). The placement of a bulky adamantyl group in the 2-position of the A-ring of the estrone eliminates estrogenicity, enhances antioxidant potential (Perez et al., 2005b), and enhances the potent neuroprotective activity of estrogens against ischemic brain damage. ZYC-26 has an adamantyl moiety on the 2-carbon and a methyl group on the 4 carbon of estrone. ZYC-23 is similar, but the phenolic nature of the A-ring is eliminated through O-methylation of the 3-carbon, a chemical change that completely eliminate neuroprotection of estrogens (Simpkins et al., 2004). Neither of these compounds binds to either estrogen receptor. Similar to our *in vitro* study, ZYC-26 reduced lesion volume by ~68%. As expected, no protective effects of ZYC-23 were seen *in vitro* or after transient MCA occlusion.

That ZYC-3 and ZYC-26 are non-feminizing is supported by several observations. *In vitro*, we observed no binding of this estratriene to either ER α or ER β , even at concentrations as high as 10 μ M. Further, 1 day of exposure to estrone increase uterine weights by 71%, while ZYC-26 caused no change in uterine weights in that time. The mitogenic effect of estrogen is the primary cause of uterine weight increase, an effect that is mediated through ER α (Ing and Ott, 1999). Despite this lack of interaction with cognate ERs, ZYC-26 is potentially neuroprotective. These data argue that the observed neuroprotection is not mediated through known ERs. These data are similar to our observations that other non-feminizing analogues of estratrienes are potent neuroprotectants, including 2-(1-adamantyl)-estradiol (Liu et al., 2002), the enantiomer of estradiol (Green et al., 2001), and the enantiomer of 17-desoxyestradiol (Fan et al., 2001).

Summary

We are actively pursuing the optimization of estrogen analogues that do not have ER binding capabilities, but which are more potent neuroprotectant than β E2. This program has demonstrated that estrogen analogues with large bulky groups at the 2 and/or 4 carbon of the phenolic A ring eliminate ER binding but enhance neuroprotective potency in cell culture screening systems. We cannot rule out the possibility that these analogues might bind to membrane bound estrogen receptors (GPR30), and we are unaware of any studies which have assessed the stereospecificity of β E2 binding GPR30. These A-ring derivatives work in part by increased electron donating capacity that stabilized the phenoxy radical. Our estrogen analogues are also potent neuroprotectants in an animal model of cerebral ischemia. Studies are in progress to understand the mechanism by which these analogues are neuroprotective. The discovery of these non-feminizing estrogens opens the possibility of making these compounds more “drug-able” and thereby producing non-feminizing estrogens that can achieve the beneficial effects of estrogens without the unwanted side-effect related to interaction with ERs. Such an achievement could markedly change our approach to postmenopausal HT, as well as treatment of brain injuries.

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Fig. 1.

Fig. 2.

TABLE 1

EC₅₀ (IC₅₀) VALUES FOR NON-FEMINIZING ESTROGEN ANALOGUES

Compound	Glut (10mM)	Glut (20 mM)	IAA (20 μ M)	IAA (40 μ M)	ER α Binding	ER β Binding	TBARs
ZYC1	1.025	1.571	0.253	1.72	3.978	4.058	4.922
ZYC2	9.021	10.00	0.006	8.259	126.75	165.8	16.075
ZYC3	0.159	0.365	0.216		10000	10000	1.126
ZYC4	9.207	17.14	196.2	0.052	43.57	31.41	10.171
ZYC5	0.023	0.121	0.120	0.152	10000	10000	1.391
ZYC6	17.61	13.69	2.708	8.483	1698	10000	17.079
ZYC7	8.582	14.73	2.405	1.729	133.5	242.1	5.332
ZYC9	1.956	1.315	5.020	5.296	480.65	204.2	29.465
ZYC10	0.488	0.851	0.296	0.679	16.32	8.138	3.132
ZYC11	4.627	7.031	0.296	0.679	107.0	64.49	26.115
ZYC12	0.548	1.953	1.290	4.440	73.96	53.35	4.763
ZYC13	0.239	1.173	0.191	0.129	25.30	18.70	9.167
ZYC14	0.215	0.240	0.183	0.382	2263	2461	1.033
ZYC15	0.047	0.803	0.646	1.200	1649	10000	1.495
ZYC16	0.882	0.860	2.250	1.912	10000	10000	3.648
ZYC17	0.244	0.270	0.680	0.828	10000	10000	4.913
ZYC18	0.568	0.921	0.264	1.044	2384.5	10000	2.307
ZYC19	0.184	0.272	0.099	0.302	784.1	10000	2.488
ZYC20	0.132	0.273	0.908	1.134	5089	10000	1.905
ZYC21	0.122	0.245	0.108	0.282	2855	4456	1.264
ZYC22	0.030	.030	0.084	0.358	10000	10000	1.827
ZYC23	NP	NP	NP	NP	10000	10000	NI
ZYC24	0.665	0.768	1.132	3.928	209.45	259.95	7.028
ZYC25	0.238	0.147	0.159	0.359	10000	10000	1.375
ZYC26	0.012	0.037	0.164	0.280	10000	10000	0.708
ZYC27	0.992	1.195	0.541	1.932	14.28	11.97	3.279
ZYC28	0.497	0.568	0.458	1.268	61.12	533.9	7.88
ZYC29	6.730	8.252	1.576	7.525	10.97	17.42	10.47

Compound	Glut (10mM)	Glut (20 mM)	IAA (20 μ M)	IAA (40 μ M)	ER α Binding	ER β Binding	TBARs
ZYC30	8.290	9.033	0.591	8.814	26.95	16.84	14.89
ZYC33	0.0938	0.1273	0.046	0.086	10000	10000	1.208
ZYC34	0.121	0.19	0.046	0.219	1460	88.01	0.664
ZYC35	3.203	3.896	1.021	1.623	5.53	14.31	7.806
ZYC36	6.883	6.627	1.195	N/A	5.55	2.64	17.445
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	10000	0.895
ZYC39	0.180	0.368	0.049	0.282	633.2	10000	1.577
ZYC40	0.524	0.443					1.901
ZYC41	11.30		1.573	1.341	34.27	1050	6.291
ZYC42	1.167	1.510	0.051	0.942	248.5	323.6	21.89
ZYC43	0.730	1.511	1.393	1.449	8729	7938	4.506
ZYC44	0.363	0.399	0.051	0.167	2637.5	10000	0.408
ZYC45	0.684	0.975	0.239	0.446	800.2	5176	2.214
ZYC46	0.866	0.465	0.069	0.155	7305	10000	2.130
ZYC47	0.139	0.121	0.017	0.088	8831	10000	1.415
ZYC48	0.179	0.527	0.176	1.599	877.5	5208	3.120
ZYC49	NP	NP	NP	NP	10000	10000	NI
ZYC50	0.111	0.139	0.081	0.235	202.7	769.2	2.156
ZYC51	0.653	1.769	0.446	0.651	1649	4702	1.521
ZYC52	2.439	4.101	0.057	0.286	4258	1143	16.59
ZYC53	14.68	143.2	0.072	0.375	10000	10000	NI
ZYC54	5.138	7.621	0.210	0.918	10000	10000	1.580
ZYC55	0.913	0.926	0.246	0.458	10000	10000	1.369
ZYC56	3.550	4.59	0.313	1.717	445.3	354.1	18.60
ZYC57	NP	NP	0.165	1.311	593.9	10000	20.19
ZYC58	1.655	2.922	0.163	0.829	10000	10000	2.564
ZYC59	0.765	0.924	0.162	0.337	752.6	263.9	7.398
α E2	3.102	5.739	3.375	161.7	16.46	83.75	
β E2	1.364	1.978	2.902	4.704	3.041	4.512	19.83
DES	3.772	14.10	0.769	3.802	2.615	1.84	6.882

Compound	Glut (10mM)	Glut (20 mM)	IAA (20 μ M)	IAA (40 μ M)	ER α Binding	ER β Binding	TBARs
E1	3.029	8.920	2.092	1609.2	13.35	30.92	80.79
E1-quinol					10000	10000	
EntE2	0.936	1.166	7.110	35.01	24.65	5.635	23.95
E380	1.277	1.980	0.159	1.230	5.912	2.372	6.725
E400	1.651	1.866	0.126	1.245	117.5	52.37	20.88
E430	NP	NP	7.923	33.47	1880	10000	NI
E1240	NP	NP	NP	NP	785.5	1197	NI
E2540	NP	NP	NP	NP			NI
E2550	NP	NP	NP	NP	953.4	260.3	NI
E2555	NP	NP	NP	NP	1513	10000	NI
E2560	NP	NP	NP	NP	33.06	261.7	NI
PS1	13.08	13.84	17.45	2.547	29.04	12.22	9.701
PS2	10.15	16.21	278.7	1662	27.65	33.09	8.951
PS3	NP	NP	NP	NP	692.2	615.4	NI
PS4	NP	NP	NP	NP	2862	10000	NI

NP (no protection); NI (no inhibition); 10000 (over 10 μ M required for binding, or no binding activity)