Estrogen-Like Compounds for Ischemic Neuroprotection

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Abstract—We have synthesized a library of estrogen analogues, including enantiomers of estradiol and A-ring substituted estrogens. These compounds have reduced or no binding to either estrogen receptor-α or estrogen receptor-β, exhibit enhanced neuroprotective activity in in vitro models, and are potent in protecting brain tissue from cerebral ischemia/reperfusion injury. These potent, nonfeminizing estrogen analogues are prime candidates for use in stroke neuroprotection. *(Stroke. 2004;35[suppl 1]:2648-2651.)*

Key Words: estrogens ■ estrogen receptors ■ neuroprotection ■ stroke

There are >750,000 new strokes per year in the United States. Unfortunately, there are few available options for the treatment of stroke-related brain damage. Efforts to develop effective therapies for acute ischemic stroke achieved several important successes during the past decade, the greatest of which is thrombolysis. However, with the restrictive 3-hour therapeutic window for recombinant tissue plasminogen activator in stroke therapy, only a small number of acute stroke patients are estimated to receive this intervention. Even with the patient educational efforts, institutional initiatives and the advanced techniques of diffusion and perfusion magnetic resonance imaging, only 10% of the acute stroke patients are candidates for the intervention.

We first demonstrated that estrogens are potent neuroprotectants and are very effective against ischemia-induced brain damage. Also, sex differences in the incidence and outcome of stroke suggest that hormonal factors may influence the development and outcome of stroke. Recently, estrogens have been found to be associated with a decreased risk and delayed onset of stroke and enhanced recovery from traumatic and chronic neurological and mental diseases. Various lines of clinical and experimental evidence have shown that both endogenous and exogenous estrogens exert neuroprotective effects. Protective effects of estrogens have been widely reported in different types of neuronal cells against different toxicities, including serum deprivation, oxidative stress, amyloid β peptide (Aβ)-induced toxicity, and excitotoxicity.

The neuroprotective effects of estrogens have been demonstrated in a variety of models of acute cerebral ischemia, including transient and permanent middle cerebral artery (MCA) occlusion models, global forebrain ischemia models, photothrombotic focal ischemia models, and glutamate-induced focal cerebral ischemia models. The protective effects of estrogens have been described in rats, mice, and gerbils. Estrogen-induced neuroprotection has been demonstrated in adult and middle-aged female rats, as well as in reproductively senescent female rats. These effects of estrogens have been shown despite the presence of diabetes and hypertension. The neuroprotective effects of estrogens have also been demonstrated against subarachnoid hemorrhage, a highly prevalent form of stroke in females. Finally, the neuroprotective action of estrogen is not limited to the female; estrogen protection is also seen in males. Collectively, these results indicate that estrogens could be valuable candidates for brain protection during acute stroke in males and females.

Concentrations of estrogens ranging from low-physiological to high-pharmacological have been shown to produce protective effects in stroke models. When estradiol is administered at low physiological levels soon before the onset of an ischemic event, no protection is seen. In contrast, neuroprotective effects of estradiol were clearly demonstrated with the acute treatment at the time of or just before an ischemic event, as well as after its onset. The therapeutic window of estrogens at the dose of 100 μg/kg lasts up to 3 hours after insult, and this therapeutic window can be extended to up to 6 hours after ischemic insult with doses of 500 to 1000 μg/kg. This long postevent efficacy of estrogens is promising, because the therapeutic window for estrogen neuroprotection could be insult severity-dependent and could be different between different species. It has been shown that the infarct penumbra, which is the infarct area that can be protected, develops over a longer period in human subjects than in rodent, suggesting that estrogens may have an even longer therapeutic window in human subjects.

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Clinical studies are at odds with these consistent animal studies. Both the Women’s Health Initiative (WHI) and the WEST trials failed to show a beneficial effect of estrogen therapy on stroke, and the WEST trials demonstrated an increase in fatal strokes in subjects using estrogen therapy. The differences between animal and clinical studies are many but may relate to the clinical study designs. Both clinical studies used continuous oral estrogen exposure, whereas the animal studies used a single dose or a short period of dosing of estrogen that was timed with the experimentally induced stroke. Continuous oral estrogen therapy in women is well known to increase clotting factor and to reduce antithrombotic enzymes. Thus, the published continuous estrogen therapy trials are not a test of the ability of acutely administered estrogens to prevent brain damage from strokes.

Structure–Activity Relationship for Estrogen Analogues and Neuroprotection

Using our library of >70 newly synthesized estrogen analogues, we conducted dose-response assessments to determine ED₅₀ for neuroprotection against 2 different toxins. Here, we report on some of the compounds (structures depicted in Figure 1). We observed that estrogens that are modified to enhance their redox potential also have 2 related and useful effects on the activity of the compounds. First, estrogenicity is reduced or eliminated; second, the neuroprotective activity of the compounds is increased, in some cases as much as 50 times that of 17β-estradiol (17β-E2) (Figure 2).

Comparison of Estrogens and Nonfeminizing Estrogen Analogues on Stroke Neuroprotection Using an In Vivo Model

From these compounds, we selected several for in vivo assessment against a routinely used model of cerebral ischemia, transient MCA occlusion, in ovariectomized rats. To date, we have assessed 8 compounds in this model, including 17β-E2, estrone, 17α-E2, the enantiomer of 17β-E2 (ent-E2), the enantiomer of 17-desoxyestradiol (ZYC-13), 2-(1-adamantyl) estrone (ZYC-3), and 2-(1-adamantyl)-4-methylestrone (ZYC-26) (Figure 1). Data for 3 of these novel estrogen analogues are provided.

Ent-E2 was assessed for ER binding and was shown to be less than one-eighth as active as 17β-E2 in competitive bind assays for human recombinant estrogen receptor-α (ERα) (Figure 2B) and ERβ (data not shown). Despite this low affinity for binding to either ER, ent-E2 was as effective as 17β-E2 in preventing glutamate-induced cell death in HT-22 cells (Figure 2) and in reducing infarct size after MCA occlusions (Figure 3). We found no evidence of metabolic conversion of ent-E2 to 17β-E2 in ovariectomized rats. Finally, we observed no effects of ent-E2 on physiological parameters before, during, or after MCA occlusion. These data suggest that nonfeminizing enantiomers of estrogens can be potently neuroprotective in vitro and in animal models for human ischemic damage.

ZYC-3 showed no binding to either ERα or ERβ in competitive binding assays (Figure 2) but was 6-times more potent than 17β-E2 in a test for neuroprotection against glutamate toxicity (Figure 2). Further, in an assessment of brain protection from transient MCA occlusion, ZYC-3 performed better than 17β-E2 (Figure 4) without affecting physiological parameters. The placement of a bulky adamantyl group in the 2 position of the A-ring of the estrone eliminates estrogenicity, enhances antioxidant potential (un-
published observations), 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 we have previously shown to completely eliminate neuroprotection of estrogens.34 Neither of these compounds bound to either estrogen receptor (Figure 2). Consistent with our previous study,35 estrone significantly decreased ischemic lesion volume by 47% (Table). Similar to our in vitro study, ZYC-26 reduced lesion volume by 68% (Table). As expected, no protective effects of ZYC-23 were seen in vitro (Figure 2) or after transient MCA occlusion (Table).

Estrone treatment significantly induced uterine hypertrophy at 24 hours after treatment (Table). Consistent with our ER binding assay in vitro, neither estrogen analogue showed feminizing effects in vivo (Table).

**Conclusion and Future Studies**

Collectively, these studies demonstrate several principles that are useful in the further discovery of novel drugs based on the potent neuroprotective effects of estrogens. First, estrogenticity can be reduced or eliminated from the estrogen molecule through chiral changes in the steroid, as was performed with 17β-E2, ent-E2, and ZYC-13, or by adding bulky groups to the 2 and/or 4 position of the A-ring, as is performed for ZYC-3 and ZYC-26. Second, in vitro neuroprotection screen-
ing assays are effective in selecting compounds of potential use in an animal model for cerebral ischemia. Finally, chemical modifications that eliminate estrogenicity while enhancing neuroprotection allow for brain protection without the side effects associated with chronic hormone use.\textsuperscript{56,63} This would allow for treatment of women and men with these estrogen analogues.

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