Neuroprotective Effects of a Novel Non–Receptor-Binding Estrogen Analogue
In Vitro and In Vivo Analysis

Ran Liu, MD; Shao-Hua Yang, MD; Evelyn Perez, BS; Kun Don Yi, MS; Samuel S. Wu, PhD; Kathleen Eberst, BS; Laszlo Prokai, PhD; Katalin Prokai-Tatrai, PhD; Zu Yun Cai, PhD; Douglas F. Covey, PhD; Arthur L. Day, MD; James W. Simpkins, PhD

Background and Purpose—Although estrogens are neuroprotective, hormonal effects limit their clinical application. Estrogen analogues with neuroprotective function but lacking hormonal properties would be more attractive. The present study was undertaken to determine the neuroprotective effects of a novel 2-adamantyl estrogen analogue, ZYC3.

Methods—Cytotoxicity was induced in HT-22 cells by 10 mmol/L glutamate. 17β-Estradiol (E2) or ZYC3 was added immediately before the exposure to glutamate. Cell viability was determined by calcein assay. The binding of E2 and ZYC3 to human α (ERα) and β (ERβ) estrogen receptors was determined by ligand competition binding assay. Ischemia/reperfusion injury was induced by temporary middle cerebral artery occlusion (MCAO). E2 or ZYC3 (100 μg/kg) was administered 2 hours or immediately before MCAO, respectively. Infarct volume was determined by 2,3,5-triphenyltetrazolium chloride staining. Cerebral blood flow was recorded during and within 30 minutes after MCAO by a hydrogen clearance method.

Results—ZYC3 significantly decreased toxicity of glutamate with a potency 10-fold that of E2. ZYC3 did not bind to either ERα or ERβ. Infarct volume was significantly reduced to 122.4 ± 17.6 and 83.1 ± 19.3 mm³ in E2 and ZYC3 groups, respectively, compared with 252.6 ± 15.6 mm³ in the ovariectomized group. During MCAO, both E2 and ZYC3 significantly increased cerebral blood flow in the nonischemic side, while no significant differences were found in the ischemic side. However, E2 and ZYC3 significantly increased cerebral blood flow in both sides within 30 minutes after reperfusion.

Conclusions—Our study shows that ZYC3, a non–receptor-binding estrogen analogue, possesses both neuroprotective and vasoactive effects, which offers the possibility of clinical application for stroke without the side effects of estrogens. It also suggests that both the neuroprotective and vasoactive effects of estrogen are receptor independent. (Stroke. 2002; 33:2485-2491.)

Key Words: cerebral blood flow ■ estrogens ■ ischemia ■ neuroprotection ■ receptors, estrogen ■ reperfusion injury

Neuroprotective agents and strategies have been studied for years and appear to be effective in a variety of stroke models. One of the major focuses in the last decade has been related to the activities of estrogens. Sex differences in the incidence and outcome of stroke suggest that hormonal factors may influence the development and outcome of stroke.1,2 Estrogens have been found to be associated with a decreased risk, delayed onset, and progression of stroke and enhanced recovery from numerous traumatic and chronic neurological and mental diseases.3 Various lines of clinical and experimental evidence have shown that both endogenous and exogenous estrogens exert neuroprotective effects.3,4 The protective effects of estrogens have been widely reported in different types of neuronal cells against various toxicities, including serum deprivation, oxidative stress, amyloid β peptide, and excitotoxicity.5 Furthermore, estrogens can decrease ischemia/reperfusion injury in animal studies.5,6 Although estrogens have been shown to be neuroprotective in both female and male rodent stroke models,7 the feminizing effects limit their clinical application. Clinical trials have...
minutes at room temperature. During the 45 minutes, the solid present in the reaction mixture was dissolved, and yellow oil formed. Crushed ice was then added while the reaction flask was shaken and swirled, and pink solid was formed. The filtered crude product was washed with water until the filtrate had a neutral pH, and the solid was dried in a vacuum oven at 50°C. The crude powder was purified by flash chromatography from contamination of other steroids (silica gel eluted with 20% ethyl acetate in hexanes). The product was recrystallized from a mixture of chloroform and isopropyl alcohol and had the following: melting point 322°C; 1H NMR (CDCl3, 300 MHz) δ 0.91 (s, 3H, Cα-CH3), 2.8 (m, 2H, C1-CH2), 4.71 (s, 1H, Cα-CH), 6.42 (s, 1H, aromatic H), 7.15 (s, 1H, aromatic H); 13C NMR (CDCl3, 300 MHz) δ 13.76, 21.47, 25.93, 26.42, 28.63, 28.95 (3×C), 31.56, 35.81, 36.56, 36.98 (3×C), 38.42, 40.70 (3×C), 44.25, 48.00, 50.35, 116.87, 124.11, 131.59, 134.00, 135.02, 152.44, 221.43.

Steroids (Steraloids, Inc) were dissolved in absolute ethanol and then diluted to appropriate concentrations in culture media or assay buffer for cell culture or receptor-binding assays, respectively. For subcutaneous injection, 17β-estradiol (E2) and ZYC3 were dissolved in absolute ethanol and then dissolved in corn oil at a concentration of 100 µg/mL. To achieve a formulation that is aqueous soluble and therefore suitable for intravenous administration, ZYC3 was dissolved in aqueous 30% 2-hydroxypropyl-β-cyclodextrin (HPβCD) solution at a concentration of 100 µg/mL.

**Ligand Competition Assay of ER Binding**

Fifty microliters of 2,4,6,7-3H-βE2 (NEN Life Science Products, Inc) and 100 µL of recombinant human ERα or ERβ (Affinity Bioreagents, Inc) were incubated in ER binding buffer (20 mmol/L Tris, 1 mmol/L EDTA, 400 mmol/L KCl, 1 mmol/L dithiothreitol, 10% glycerol, pH 7.8) for 75 minutes at 29°C either with no added steroid (total binding), 2 µL 1 mmol/L diethylstilbestrol (nonspecific binding; Steraloids), or 50 µL 0.001 to 10 nmol/L βE2 or ZYC3. Bound and unbound radioligands were separated with the use of Sephadex G25 (Amersham Pharmacia Biotech) columns (0.5-mL bed volume) with a 1-mL elution volume. Scintillation fluid was added, and counts were determined.

**Effects of E2 and ZYC3 on Glutamate Toxicity in HT-22 Cells**

HT-22 cells (Salk Institute) were maintained in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone) and 20 µg/mL gentamycin under standard cell culture conditions. HT-22 cells (passages 18 to 25) were seeded into 96-well plates at a density of 5000 cells per well. E2 or ZYC3 was added at concentrations ranging from 1 nmol/L to 10 µmol/L immediately before the addition of glutamate. Glutamate was diluted to a final concentration of 10 mmol/L in culture media.

Cells were exposed to steroid and glutamate for approximately 14 hours, then cell viability was determined by calcein AM assay (Molecular Probes). Wells were rinsed with PBS, after which 25 µmol/L calcein AM in PBS was added. After incubation at room temperature for 15 minutes, fluorescence was determined (excitation 485, emission 530). All data were normalized to percent kill.

**Experimental Animals**

Female Sprague-Dawley rats (weight, 250 g; Charles River, Wilmington, Mass) were acclimatized for 3 days before surgery. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO). All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Ischemic stroke was induced by MCAO as described before. Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was maintained at 37.5 ± 0.5°C during the procedure. The left femoral artery was cannulated and connected to a blood pressure monitor for mean arterial blood pressure monitoring. The left MCA was occluded by a 3-0 monofilament suture introduced via the
internal carotid artery. After 1 hour, the suture was withdrawn for reperfusion. Blood samples were taken before, 30 minutes during, and 30 minutes after MCAO. Physiological parameters were measured by an ISTAT clinical analyzer.

Animals were decapitated 24 hours after reperfusion. Brains were harvested, and 7 slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Slices were incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C and then fixed in 10% formalin. The stained slices were photographed and subsequently measured for ischemic lesion volume (Image-Pro Plus 4.1, Media Cybernetics).

A hydrogen clearance blood flowmeter (Unique Medical Co) was used for CBF measurement. Two electrodes were stereotaxically inserted into caudate putamen of the ischemic and contralateral side (posterior bregma 0.5 mm, 4 mm lateral and 5 mm depth).

Protocol 1: Effect of ZYC3 on Ischemia/Reperfusion Injury Using Corn Oil as Vehicle
E2 and ZYC3, dissolved in corn oil, were injected subcutaneously (100 µg/kg) 2 hours before MCAO in each group, respectively. An equivalent dose of vehicle was administered subcutaneously 2 hours before MCAO in ovariectomized females as control (n=4 to 6 per group).

Protocol 2: Effect of ZYC3 on Ischemia/Reperfusion Injury Using 30% HPβCD as Vehicle
ZYC3 (100 µg/kg) in HPβCD was administered through the jugular vein immediately before MCAO (ZYC3 group; n=7). E2 (100 µg/kg) in corn oil was injected subcutaneously in ovariectomized females 2 hours before MCAO (E2 group; n=15). In controls, ovariectomized females (OVX group; n=14) were treated with equivalent volumes of corn oil (subcutaneously) at 2 hours before and 30% HPβCD (intravenously) immediately before MCAO.

Protocol 3: Effect of E2 and ZYC3 on CBF During Ischemia/Reperfusion Injury
MCAO was induced in 16 rats (OVX, n=5; ZYC3, n=6; E2, n=5) in a separate study. Mean arterial blood pressure, pH, PO2, PCO2, Na+, K+, hemoglobin, HCO3-, So2, and total carbon dioxide (TCO2) were monitored before, 30 minutes during, and 30 minutes after MCAO. E2 was dissolved in corn oil and injected subcutaneously (100 µg/kg) 2 hours before MCAO in the E2 group. In the ZYC3 group, ZYC3 (100 µg/kg) in HPβCD was administered through the jugular vein immediately before MCAO. CBF was recorded at 30 minutes during MCAO and at 5, 15, and 30 minutes after reperfusion.

Statistical Analysis
All data are presented as mean±SEM. Cell death, ischemic volumes, and physiological parameters in each group were compared by 1-way ANOVA followed by Tukey tests. For CBF we used a linear mixed model (SAS PROC mixed; SAS Institute) to evaluate mean differences in treatment for each side of the brain.14,15 The effects of treatment, time, and treatment by time interaction were considered fixed, while the response of animals was treated as random. This model took into consideration repeated-measures data (multiple CBF measurements were made for each rat), the inhomogeneous variance of CBF at 4 different time points, and the variation among rats. Linear contrast tests were used to distinguish treatment groups at specific time points. The difference for each comparison was considered significant at the P<0.05 level.

Results
Ligand Competition for Estrogen Receptor Binding
In competition binding experiments, ZYC3 showed very low affinity for both ERα and ERβ within the indicated concentrations (Figure 2).

Effect of E2 and ZYC3 on Ischemia/Reperfusion Injury Using Corn Oil as Vehicle
E2 and ZYC3, dissolved in corn oil, were injected subcutaneously (100 µg/kg) 2 hours before MCAO in the E2 group. In the ZYC3 group, ZYC3 was not neuroprotective when injected subcutaneously 2 hours before MCAO in each group, respectively (Figure 4).

Effect of E2 and ZYC3 on Glutamate Toxicity in HT-22 Cells
The EC50 values for neuroprotection against a 10-mmol/L glutamate challenge for ZYC3 and E2 were 0.16 and 1.90 µmol/L, respectively, suggesting that ZYC3 was 10-fold more potent than E2. With exposure of HT-22 cells to 10 mmol/L glutamate for 14 hours, 1 µmol/L ZYC3 ameliorated glutamate toxicity by 70%, while 1 µmol/L E2 decreased cell death by 10% (Figure 3).

Effect of E2 and ZYC3 on Ischemic Lesion Volume
ZYC3 was not neuroprotective when injected subcutaneously 2 hours before MCAO with corn oil used as vehicle. The lesion volumes were 238.1±23.9, 128.5±24.3, and 274.2±31.4 mm3 in OVX, E2, and ZYC3 groups, respectively (Figure 4).

Figure 2. Competition binding curve of ZYC3 and E2 for human recombinant ERα and human recombinant ERβ. No binding affinity of ZYC3 to either ERα or ERβ was indicated.

Figure 3. Concentration-response curves for E2 and ZYC3 on glutamate toxicity in HT-22 cells. *P<0.05 vs 17βE2 (E2) group.
Effect of E2 and ZYC3 on Physiological Parameters and CBF

Physiological parameters are shown in the Table. There were no significant differences among groups at comparable experimental time points. Regional CBF decreased to 8.5 ± 1.3, 6.3 ± 1.4, and 7.4 ± 1.4 mL/min per 100 g tissue during MCAO in the ischemic side in the ZYC3, E2, and OVX groups, respectively. However, both E2 and ZYC3 significantly increased CBF in the nonischemic side during MCAO, which was 91.6 ± 10.7 and 82.7 ± 11.7 mL/min per 100 g tissue, respectively, compared with 48.0 ± 11.7 mL/min per 100 g tissue in the OVX group. E2 and ZYC3 significantly increased CBF in both sides within 30 minutes after reperfusion (Figure 5).

Discussion

A large number of reports have shown that E2, naturally occurring estrogen, exerts neuroprotective effects in a variety of in vitro and in vivo model systems. Several synthesized estrogen analogues have also been reported to possess neuroprotective properties. The most essential structural motif that elicits estrogenic activity is a phenol that is relatively unhindered, attached to a rather bulky hydrophobic structure. Additionally, the neuroprotective function of estrogens has been shown to be related to the phenolic A ring of the steroid. Adamantyl modulation of phenol has been reported previously. In the present study an electron-donating, adamantyl moiety was introduced at the C-2 position on the A ring of the steroid. We anticipated that the bulky adamantyl group would decrease binding of the steroid to ERs. It was also anticipated that the bulky group would increase the ability of the steroid to scavenge free radicals derived from the reactive oxygen species. The effects of this novel analogue on ischemia/reperfusion injury were determined in both a neuronal cell culture model and a rodent MCAO model.

Oxidative stress is implicated in a number of neurological disorders, including stroke. HT-22 is an immortalized mouse hippocampal neuronal cell line, which lacks inotropic gluta-
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Figure 5. Effects of E2 and ZYC3 on CBF. Both E2 and ZYC3 significantly increased CBF in the nonischemic side during MCAO. E2 and ZYC3 significantly increased CBF in both sides within 30 minutes after reperfusion. *P<0.05 vs OVX group.

Exogenous glutamate blocks cysteine uptake in HT-22 cells via inhibition of the glutamate-cysteine antiporter, resulting in decreases in intracellular cysteine, which is 1 of the 3 peptides of glutathione. Depletion of glutathione leads to accumulation of reactive oxygen species and Ca\(^{2+}\) influx, ultimately resulting in cell death. The present study showed that both E2 and ZYC3 treatment ameliorated glutamate toxicity to HT-22 cells. With the free radical–scavenging adamantyl group, ZYC3 was more potent and efficacious than E2. Furthermore, because ZYC3 had very low affinity to either of the 2 known ERs and HT-22 cells lack functional ERs, classic ER-independent neuroprotective actions of E2 were suggested.

Consistent with our in vitro studies, both E2 and ZYC3 exert neuroprotective effects in focal cerebral ischemia/reperfusion injury. For the in vivo study, ZYC3 was ineffective in protecting against the insult when administered in corn oil. The logarithm of the 1-octanol/water partition coefficient (log P) estimated by an atom fragment method indicated that ZYC3 has a log P of 6.83 compared with 4.01 for E2. The extremely high lipophilicity indicated that ZYC3 should essentially accumulate in a lipid environment and would therefore remain in the injection site. The lack of effects of ZYC3 could be attributed to the inappropriate formulation method. To achieve a rapid delivery of ZYC3 into the circulation, ZYC3 was complexed with HP\(\beta\)CD. With this formulation, neuroprotective and vasoactive effects were clearly manifested.

Neuroprotective effects of E2 have been indicated at a very large range of concentrations in both in vitro and in vivo studies. In in vitro studies, the effective concentrations for E2-mediated neuroprotection range from low nanomolar (approximately 0.1 nmol/L) to high micromolar (approximately 50 \(\mu\)mol/L) concentrations. High physiological concentrations (low nmol/L) were sufficient to attenuate toxicity in a variety of cell types, while significantly higher pharmacological concentrations (low \(\mu\)mol/L) were required to lessen glutamate toxicity in HT-22 cells. In in vivo studies, neuroprotective properties of E2 have been observed in both low physiological and high pharmacological concentrations. A myriad of factors contribute to the vast differences in neuroprotection of E2 concentrations in vitro. In cell culture studies, differences in cell type, culturing conditions, cell density, and media components may contribute to the wide range of protective concentrations. Other factors that may also alter the potency of E2 neuroprotection include type of insult, severity of insult, and time of beginning of treatment. In the present study 10 mmol/L glutamate was used as insult. The neuroprotective potency of E2 was 1.9 \(\mu\)mol/L, which is consistent with previous studies.

Many actions of estrogens are mediated by the binding of the steroid to the nuclear ERs, and the binding of the steroid-receptor complex to the ER response element thereby activates transcriptional events. However, it has become evident that estrogens exert activities in neurons independent of the activation of the classic ERs. Several lines of evidence suggest that the neuroprotective effects of estrogens do not require ER-dependent gene transcription. First, several non–receptor-binding estrogen analogues such as the enantiotomer of E2 have been shown to exert neuroprotective efficacy as potent as E2. Second, ER antagonists do not attenuate the protective action of E2 in all models of neurotoxicity. Third, neuroprotection of estrogens can occur in the presence of mRNA or protein synthesis inhibitors. Furthermore, E2 has been shown to activate a signal transduction pathway involving mitogen-activated protein kinase within minutes of E2 treatment, which is too rapid to involve genomic ER actions. Taken together, the present study suggests that many of the neuroprotective effects of estrogens are mediated through nongenomic pathways independent of classic ERs.

Vasoactive effects of estrogens in the central nervous system have been suggested. Estrogens can increase CBF during global ischemia and restore postischemic pial microvascular dilatation. Estrogens can increase CBF during early reperfusion in an ischemia/reperfusion model. In the present study rapid vascular responses were observed in both E2 and ZYC3 treatment groups. Our study indicated that vasoactive effects of estrogens were not through the classic genomic pathway, which is consistent with previous studies. Because the vascular response of estrogen was determined in the core area of the ischemia, which was not protected by either E2 or ZYC3, the association between neuroprotective properties and vasoactive activity of estrogens cannot be determined in the present study.

Although classic ER-independent neuroprotective effects are suggested by the present study, evidence for ER-dependent action has also been reported. Estrogens have been shown to activate the phosphatidylinositol-3-kinase pathway in vitro, which has been proposed to mediate inhibition of...
apoptosis and support neuronal survival. This activation can be inhibited by ER antagonists.\textsuperscript{42,43} However, whether this pathway is necessary and sufficient to mediate the neuroprotective effects of estrogens in vivo still remains to be elucidated. It has also been suggested that ER$\alpha$, but not ER$\beta$, was critical for the neuroprotective properties of estrogen in one ER$\alpha$ and ER$\beta$ knockout (aERKO, $\beta$ERKO) study,\textsuperscript{44} while another study did not support this conclusion.\textsuperscript{45} The differences between the ERKO studies could have resulted from the different estrogen concentrations used in the 2 treatment regimens or from the different MCAO models, permanent versus transient, used in these 2 studies. Higher doses of E2 have been demonstrated to exert neuroprotective effects in aERKO mice.\textsuperscript{46} These higher doses of E2 could be the result of the elevated levels of androgen in aERKO mice,\textsuperscript{47} since androgen has been shown to exacerbate ischemia/reperfusion injury.\textsuperscript{48}

In summary, our in vitro and in vivo study suggests that this novel estrogen analogue exerts both neuroprotective and vasoactive effects. The low affinity of binding of ZY3C to the classic ERs and rapid cerebrovascular action of this estrogen derivative indicate that the neuroprotective and vasoactive effects of estrogens are mediated through nongenomic pathways independent of classic ERs. This non–ER-binding estrogen analogue could be applied in situations in which the feminizing effects of estrogens are undesirable and to avoid other receptor-dependent side effects of estrogens.

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References


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