Synthetic Combined Superoxide Dismutase/Catalase Mimetics Are Protective as a Delayed Treatment in a Rat Stroke Model: A Key Role for Reactive Oxygen Species in Ischemic Brain Injury

KEITH BAKER, CATHERINE BUCAY MARCUS, KARL HUFFMAN, HENRY KRUJK, BERNARD MALFROY and SUSAN R. DOCTROW
Eukarion, Inc., Bedford, Massachusetts
Accepted for publication September 8, 1997

ABSTRACT
Stroke is a severe and prevalent syndrome for which there is a great need for treatment, including agents to block the cascade of brain injury that occurs in the hours after the onset of ischemia. Reactive oxygen species (ROS) have been implicated in this destructive process, but antioxidant enzymes such as superoxide dismutase (SOD) have been unsatisfactory in experimental stroke models. This study is an evaluation of the effectiveness of salen-manganese complexes, a class of synthetic SOD/catalase mimetics, in a rat focal ischemia model involving middle cerebral artery occlusion. We focus on EUK-134, a newly reported salen-manganese complex demonstrated here to have greater catalase and cytoprotective activities and equivalent SOD activity compared with the previously described prototype EUK-8. The administration of EUK-134 at 3 hr after middle cerebral artery occlusion significantly reduced brain infarct size, with the highest dose apparently preventing further infarct growth. EUK-8 was also protective but substantially less effective. These findings support a key role for ROS in the cascade of brain injury after stroke, even well after the onset of ischemia. The enhanced activity of EUK-134 suggests that, in particular, hydrogen peroxide contributes significantly to this injury. Overall, this study suggests that synthetic SOD/catalase mimetics might serve as novel, multifunctional therapeutic agents for stroke.

Stroke is a syndrome characterized by the rapid onset of neurological impairment due to interruption of blood flow to the brain, most often caused by a thrombus formed in an artery or the lodging of an embolus from a distant site. It is the third leading cause of death in the United States and considered the most common cause of disability in adults (Camarata et al., 1994; Ziven and Choi, 1991). There has been some progress in decreasing the incidence of stroke through preventative measures such as control of hypertension. However, once a stroke occurs, no pharmacological treatment has been available to prevent subsequent brain damage, although some experimental drugs with potential promise are emerging; these include the thrombolytic agent tissue plasminogen activator, which has shown recent clinical success for a certain subset of stroke patients (Barinaga, 1996).

An alternative or complementary approach to thrombolytic therapy would be the administration of agents capable of protecting ischemic neurons. It is now generally believed that irreversible brain damage occurs over a period of hours after the stroke. During this time, a cascade of cellular and biochemical events, triggered by the initial vascular insult, leads to the production of neurotoxic mediators and, ultimately, destruction of brain tissue. Much research has focused on understanding this cascade of damage and identifying key steps for possible intervention (for a review, see Ziven and Choi, 1991). Such work has led to experimental therapeutic strategies aimed at, for example, reducing the entry or consequences of excess cytosolic calcium (Choi, 1988a, 1988b; Meldrum, 1989; Olney, 1986; Sauter and Rudin, 1991) or blocking receptor activation by the potentially excitotoxic neurotransmitter glutamate (Choi, 1995; Goldberg et al., 1987; Nakanishe, 1992; Simon et al., 1984). One other important class of therapeutic targets for stroke are ROS, such as the superoxide ion, hydrogen peroxide and the hydroxyl radical, which are thought to play a key role in tissue damage associated with a wide variety of diseases (for a review, see Halliwell and Gutteridge, 1989).

In stroke, damaging ROS might arise from several sources, such as production by infiltrating activated leukocytes (Clark...
et al., 1993; Connolly et al., 1996; Matsuo et al., 1994), generation in neurons as a direct consequence of excitotoxic stimulation (Dugen et al., 1995; Lafon-Cazal et al., 1993) and enzymatic formation during, when it occurs, reperfusion of ischemic tissue (Granger, 1988). The implicated involvement of ROS in ischemic brain injury suggests that therapeutic strategies directed against certain ROS might be of value in stroke treatment. In support of this concept, transgenic mice overexpressing Cu/Zn SOD showed reduced brain edema and tissue injury when subjected to focal brain ischemia (Kinouchi et al., 1991). Several studies have investigated the protective effects of exogenously administered SOD (Chan, 1992; He et al., 1993; Imaizumi et al., 1990; Tagaya et al., 1992; Uyama et al., 1990) or SOD and catalase combinations (Armstead et al., 1991; Liu et al., 1989) in experimental stroke models. Generally, these enzymes show modest protective effects when treatment is administered before ischemia, but little to no protection in a delayed-treatment protocol (Tagaya et al., 1992). The possibility that the in vivo activity of antioxidant enzymes would be hindered by low plasma stability or inadequate delivery to the site of injury has led several investigators to use, albeit with limited success, liposome-entrapped (Chan, 1992; Imaizumi et al., 1990) or polymer-conjugated (Armstead et al., 1991; He et al., 1993; Liu et al., 1989) antioxidant enzymes.

As we previously reported, salen-manganese complexes are low-molecular-weight synthetic compounds that exhibit both SOD and catalase activities, catalytically destroying both superoxide and hydrogen peroxide, respectively (Baudry et al., 1993; Doctrow et al., 1996; Gonzalez et al., 1995). Several properties of these molecules—including their catalytic mechanism of action, activity against at least two distinct ROS and synthetic, nonproteinaceous nature—might make them useful not only as potential therapeutic agents but also in elucidation of the role of oxygen radicals in pathologies associated with diseases such as stroke. Indeed, a prototype salen-manganese complex, EUK-8, shows efficacy in several models for ROS-associated disease in, for example, protecting pulmonary function in a stringent porcine model of adult respiratory distress syndrome (Gonzalez et al., 1995). EUK-8 also is protective in several models for neurological disease in preserving synaptic function in hippocampal slices subjected to anoxia/reoxygenation (Musleh et al., 1994), protecting striatal dopaminergic neurons in two mouse models for Parkinson’s disease (Doctrow et al., 1996), preventing paralysis in mouse experimental autoimmune encephalomyelitis (Malfroy et al., 1997) and protecting organotypic hippocampal cultures from toxicity by the β-amylloid peptide (Bruce et al., 1996). Although previous studies have focused on EUK-8, new analogs with improved properties have been developed. One of these compounds, EUK-134, is introduced in the present study and shown to be significantly more active than the prototype EUK-8 with respect to catalytic, cytoprotective and in vivo properties.

Because of their demonstrated ability to protect neuronal and other tissue against a variety of severe oxidative insults, we hypothesized that salen-manganese complexes might be of value in preventing brain damage after stroke. The objective of the present study, therefore, was to evaluate the ability of these compounds, specifically EUK-8 and EUK-134, to protect brain tissue in a rat focal cerebral ischemia model. In particular, the study emphasizes the effectiveness of the compounds when administered several hours after ischemia because of the potential clinical applicability of such a delayed-treatment regimen.

Methods

Materials. Chemicals for the synthesis of salen-manganese complexes were obtained from Aldrich Chemical (Arlington Heights, IL). Human dermal fibroblasts were obtained from American Type Culture Collection (Rockville, MD), and cell culture medium components were obtained from BioWhittaker (Walkersville, NY). The XTT reagent was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Male Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) or Harlan Sprague-Dawley (Indianapolis, IN). Injectable anesthetics, culture materials and gel foam were obtained from Henry Schein (Reno, NV). MK-801 hydrochloride was obtained from Research Biochemicals (Natick, MA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Synthesis and characterization of salen-manganese complexes. EUK-8 was prepared according to the procedure of Bouchez et al. (1974), which was modified to produce EUK-134. The bis(salicilaldehyde)ethylene diamine (salen-H₂) substituted ligands were prepared by the addition of 1 equivalent of ethylenediamine in absolute ethanol to a solution of 2 equivalents of the substituted aldehyde (salicylaldehyde or o-vanillin for EUK-8 or EUK-134, respectively) in absolute ethanol (0.05–0.2 M solution). The precipitate was filtered, washed with ethanol and air-dried to give the desired product in 79% to 96% yield. Structures of the ligands were confirmed by proton NMR with a Bruker ARX 400-MHz instrument (B. Bruker Instruments, Inc., Billerica, MA). One equivalent of solid manganese(II) acetate tetrahydrate was added to a stirred suspension of 1 equivalent of the ligand in 95% ethanol (0.025–0.03 M) at ambient temperature or reflux, and the reaction was then stirred for 1 to 2 hr. The dark-brown solutions were dried under a stream of air. The crude product, a brown solid, was washed with acetone, filtered and air-dried. These acetate complexes were converted to the corresponding chlorides through treatment of an aqueous solution (0.03–0.06 M) of the acetate, warmed to 50°C, with 5 equivalents of KCl dissolved in distilled water. A brown precipitate formed immediately. The suspension was cooled in an ice/water bath and then filtered; the brown solid was washed with water and acetone. All products were obtained as hydrates in 66% to 78% yield. Elemental analyses of the final products (Canadian Microanalytical Services, Delta, BC, Canada) were consistent with the reported structures.

Catalase assay. Catalase activity was assessed using a Clark-type polarographic electrode to measure conversion of hydrogen peroxide to oxygen as described previously (Gonzalez et al., 1995) in reaction mixtures consisting of 50 mM sodium phosphate, pH 8.1, 0.9% sodium chloride, 10 μM salen-manganese complex and 10 mM hydrogen peroxide maintained at 25.0 ± 0.1°C. Oxygen concentration was monitored at 1-sec intervals, and the average base-line oxygen concentration (−2.5 × 10⁻⁴ M), calculated from readings obtained for 25 sec before the initiation of the catalase reaction, was subtracted from all values. Initial rates were calculated by linear regression using data from the first 5 sec of the reaction. As an indication of the total amount of substrate converted, the total amount of oxygen generated was calculated based on the maximal oxygen concentration achieved in the reactions, which level off before consumption of all substrate.

Cytoprotection assay. Human dermal fibroblasts (American Type Culture Collection) were grown to confluence on 96-well plates in culture medium consisting of Dulbecco’s modified Eagle’s medium (4.5 g of glucose/liter) with 10% calf serum and antibiotics. To induce oxidative toxicity, cells were incubated with culture medium containing 0.02 unit/ml glucose oxidase for 18 hr in the presence or absence of test substances (either salen-manganese complex or bovine liver catalase), as indicated in the figure legend. After the incubation...
period, cell layers were washed with phosphate-buffered saline and fresh medium lacking glucose oxidase, and test substances was added. Cell viability was then assessed using the XTT reagent according to the manufacturer’s instructions, with absorbance read at 490 nm with a microplate reader (model 3550, BioRad, Hercules, CA). Cell viability was also confirmed by visual inspection of the monolayers under a phase contrast microscope. Salen-manganese complexes, used under these conditions, did not interfere with XTT-associated color development.

**MCA-o surgery and treatment administration.** All studies were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and under the auspices of Eukarion’s Animal Care and Use Committee. The MCA-o surgical procedure was performed on 300- to 350-g male Sprague-Dawley rats using a previously described protocol (Bartus et al., 1994a), which was an adaptation of the method of Chen et al. (1986) except that the position of occlusion of the parietal branch of the left MCA was modified slightly to exacerbate the infarction. Thus, the parietal branch of the MCA was permanently occluded immediately after the bifurcation, using a single 10-0 suture, including within the occlusion any large arterial branches that may bifurcate from the parietal branch within 2 mm of the frontal/parietal bifurcation. After the MCA-o procedure, both common carotid arteries were clamped for 60 min to temporarily interrupt collateral flow to the ischemic region (Chen et al., 1986). Throughout the surgery, rats were placed on a circulating-water heating pad to regulate body temperature, which was monitored rectally, and any animal not maintaining a temperature between 96° and 99°F was excluded from the study. Where indicated, rats also received a bolus tail vein injection of test substance or vehicle while under light anesthesia with inhaled ether. Treatment, either test substance or vehicle, was assigned to each animal in a randomized fashion after completion of surgery, such that the investigator performing the surgery was unaware of the ultimate treatment. The vehicle for all such test substances was sterile 0.9% saline. Unless otherwise indicated, rats were killed at 21 hr after MCA-o for analysis of brain infarct volumes. For the time course study summarized in figure 3, sacrifice death occurred at the indicated times of 3, 6 or 21 hr after MCA-o. For the study summarized in figure 5, rats were killed at 72 hr after MCA-o where indicated. In all cases, rats were killed by carbon dioxide asphyxiation and decapitated immediately before processing of tissue for analysis.

**Brain infarct volume determination.** To visualize infarcted and uninfarcted tissue, the brains were removed, placed in a chilled stainless-steel cutting block and sectioned coronally into 2-mm slices. The sections were stained with TTC as described previously (Bartus et al., 1994a). To quantify infarcted and uninfarcted tissue, the six sections for each brain were photographed on slide film using a 35-mm camera with a close-up lens. Photographs were made from both frontal and posterior views. These slides were projected with a photographic enlarger, and outlines of the uninfarcted (red) and infarcted (white or pink) areas of the face of each section were drawn onto paper. These outlines were scanned with a Hewlett-Packard Deskscan, and the files imported into an image analysis program (NIH Image, version 1.4), enabling the area of each region to be measured in pixels. The pixel areas were converted to mm² based on the final image enlargement, and volumes were determined as follows: Total area = infarcted area + uninfarcted area, calculated for each face of a given section; Average area X = (area Xfrontal + area Xposterior)/2, calculated for a given section or region thereof; and Volume X (mm³) = (average area X mm² x 2.0 mm), calculated for a given section or region thereof. Total brain volume and infarct volume were obtained by summing total volumes and infarcted region volumes, respectively, for the six sections that composed each brain. (Total brain volumes did not differ significantly among the various groups.) Photography, drawing and image analysis were conducted under blinded conditions. Differences among group means were assessed by a one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons (Kramer, 1956).

**Results**

**Catalytic and in vitro cytoprotective properties of the salen-manganese complexes EUK-8 and EUK-134.** Although previously described pharmacology studies conducted with salen-manganese complexes used EUK-8, the present study focuses primarily on a new analog, EUK-134, which is described here for the first time. As shown in figure 1, EUK-134 has a structure analogous to that of EUK-8 but with the substitution of methoxy substituents for hydrogen at the 3-position of each salen ring.

The catalase activities of the two compounds were compared by monitoring their ability to convert hydrogen peroxide to oxygen under standard reaction conditions described in Methods. As shown in figure 2A, EUK-134 is a more active catalase than EUK-8, as is reflected in both the initial reaction rate and the total amount of substrate consumed. Under these reaction conditions, the initial reaction rates were 234 ± 29 and 114 ± 9 μM oxygen produced/min for EUK-134 and EUK-8, respectively. The total amount of substrate consumed was 85 ± 4 and 24 ± 1 nmol for EUK-134 and EUK-8, respectively. The latter property is believed to reflect the relative resistance of these compounds to inactivation by hydrogen peroxide (Doctrow et al., 1996). In contrast to their different catalase activities, the two compounds exhibited equivalent SOD activities, ~10³ SOD units/μmol in a standard SOD assay system (Baudry et al., 1993).

The salen-manganese complexes were evaluated for their ability to protect human fibroblasts against cytotoxicity by glucose and glucose oxidase, an enzyme-substrate system that generates hydrogen peroxide (Bentley, 1963). In this experimental model, the glucose and glucose oxidase treatment was completely lethal to the cells during an 18-hr incubation. Bovine liver catalase was completely protective at 290 units/ml (fig. 2B) and suboptimally protective at 29 units/ml of bovine liver catalase (fig. 2B). As figure 2B also shows, EUK-134, consistent with its enhanced catalase activity, had significantly greater cytoprotective activity in this model than did EUK-8. At 80 μM, EUK-134 was equally as protective as the 290 units/ml of bovine liver catalase. EUK-8 was much less efficacious than EUK-134, with only partial protection exhibited at the highest concentration tested (80 μM here and up to 200 μM in other experiments).

**Fig. 1.** Structures of the salen-manganese complexes EUK-8 and EUK-134.
bovine liver catalase. EUK-8 and EUK-134, both salen-manganese complexes, were also present, as indicated. Absorbance indicates cell viability as assessed with the XTT reagent, with appropriate controls as described in Methods.

Protective effects of EUK-134 and EUK-8 in an in vivo stroke model. EUK-134 and EUK-8 were next evaluated for their neuroprotective efficacy in an adaptation (Batist et al., 1994a) of a well-characterized rat model for focal cerebral ischemia (Chen et al., 1986) involving occlusion of a branch of the MCA. Among the variety of experimental stroke models available, this system is well suited for our study because it involves induction of a substantial, reproducible cortical infarct that develops over a period of hours after the ischemic event. The controlled time course and reproducibility of the model provide a reliable postischemic “window of opportunity” in which to evaluate a method of pharmacological intervention. The intended strategy for testing the salen-manganese complexes was to use a treatment time that was substantially later than the induction of ischemia yet was a time at which there still was a significant amount of potentially salvageable brain tissue. To choose such a treatment time, it was necessary to first characterize the time course of infarct formation in the model. To accomplish this, the MCA-o procedure was performed, and rats were killed at 3, 6 and 21 hr after surgery for analysis of infarct volume. The results (fig. 3) showed that only minimal cortical infarction had developed by 3 hr after MCA-o. By 6 hr, the infarcted region was much larger and not statistically different from that observed at 21 hr. Thus, 3 hr post-MCA-o was selected as the time at which to administer salen-manganese complexes.

To evaluate the ability of salen-manganese complexes to protect against focal cerebral ischemia, EUK-134 or EUK-8 in 0.9% saline was administered to rats as a single intravenous bolus injection at 3 hr after MCA-o. Rats were then killed at 21 hr to assess infarct volume, as described in Methods. A control group was treated identically, except that it received only vehicle. The results are summarized in figure 4. Rats treated with EUK-134 at doses of 0.5 and 5.0 μmol/kg (0.25 and 2.5 mg/kg, respectively) had infarct volumes that were significantly less than those of vehicle-injected rats. At 5.0 μmol/kg, EUK-134 reduced the infarct volume by ~90% compared with that of the vehicle controls. This degree of protection is consistent with the compound preventing further growth of the infarct because the final infarct volume was equivalent to that found in untreated rats killed at 3 hr after MCA-o (fig. 3). As figure 4 also shows, EUK-8 afforded some protection in this model at 5.0 μmol/kg (1.9 mg/kg) but was significantly less effective than EUK-134.
To further examine the robustness of the protection by EUK-134, rats were treated with vehicle or EUK-134 (2.5 mg/kg) at 3 hr after MCA-o, as shown for figure 4, but were allowed to live for 72 hr before death. As figure 5 shows, after 72 hr, the EUK-134-treated group still exhibited a substantial degree of protection (P < .01 vs. vehicle), and the mean infarct volume did not differ significantly (P > .05) from that observed in EUK-134-treated rats at 21 hr. The vehicle-injected rats exhibited large infarcts at 72 hr and showed no evidence of enhanced TTC staining, as would indicate, for example, glial proliferation into the infarcted tissue. The mean infarct volume of vehicle-injected rats at 72 hr did not differ significantly from that observed at 21 hr.

Because stroke models in the literature vary widely and are therefore difficult to compare directly with one another, a well known agent that had shown efficacy in other published stroke studies, although reportedly not at late treatment times, was included in this study as a reference compound; MK-801, a noncompetitive antagonist for N-methyl-D-aspartate receptors (Wong et al., 1986), was evaluated using an identical protocol to that used for the salen-manganese complexes. A group (n = 9) of rats treated at 3 hr after MCA-o with MK-801 at 4 mg/kg (12 μmol/kg) exhibited a mean infarct volume of 95.2 ± 15.8 mm³, a value not differing significantly (P > .05) from that of the vehicle group. Higher doses of MK-801 were poorly tolerated by the animals.

Discussion

The study findings demonstrate that the synthetic SOD/catalase mimetics show substantial neuroprotective effects in this rat focal ischemia model using a stringent post-treatment strategy. The degree of protection observed with EUK-134 at the highest dose is consistent with an apparent prevention of further infarct growth beyond the 3-hr postocclusion treatment time. As is important to note, this efficacy was obtained with a single intravenous injection of a relatively low dose of salen-manganese complex.

We know of no other comparable experimental therapeutic strategy producing a protective effect of this magnitude in a focal ischemia model at 3 hr after occlusion. However, the wide variety of experimental stroke models and different routes of test substance administration hinder direct comparisons between this study and other reports in the literature. Thus, the well known noncompetitive N-methyl-D-aspartate receptor antagonist MK-801 (Wong et al., 1986) was included in the present study as a reference compound. The ineffectiveness of MK-801 at 3 hr after MCA-o in our experiments is consistent with other reports in the literature. In various focal ischemia models, MK-801 treatment has been reported to reduce infarct volume by ~30% to 50% when administered before ischemia (Beilinberg and Beck, 1991; Hattfield et al., 1992; Pan et al., 1995). In one study investigating delayed treatment, MK-801 was found to exhibit some protection when administered up to 1 hr after ischemia but not at later times (Hattfield et al., 1992).

Another class of compounds, calcium-dependent protease (calpain) inhibitors, have also been reported to show significant protection in delayed-treatment regimens. Two published studies with calpain inhibitors used a stroke model that is very similar to ours (Bartus et al., 1994a), although it has more complicated, sustained dosing regimens. One of these agents, AK-295, reduced infarct volume by ~32% when continuously infused intra-arterially from 1.25 until 21 hr after MCA-o (Bartus et al., 1994b). A second calpain inhibitor, AK-275, infused supracortically rather than via the bloodstream, reduced infarct volume by ~75% when administered continuously from 3 until 21 hr after MCA-o (Bartus et al., 1994a). These studies, together with our present findings, implicate both ROS and calcium-activated proteases as promising “downstream” targets for intervention at relatively late treatment times. Both approaches appear to prevent a very high degree of brain damage discernible with TTC staining. However, more subtle forms of neuronal damage, in particular functional impairment, have not been addressed in these studies (or, indeed, in any study using standard experimental stroke models). Future research investigating, for example, the functional neuroprotection achievable with SOD/catalase mimics, calpain inhibitors or the two approaches in synergy would be of great interest.

Although both salen-manganese complexes significantly reduced ischemic brain injury in these experiments, EUK-134 was at least 1 order of magnitude more potent than EUK-8. It is possible that the enhanced activity of EUK-134 is related to its greater catalase activity because the two molecules have equivalent SOD activities. The dismutation of superoxide yields the ROS hydrogen peroxide, which is itself toxic. In certain experimental systems, SOD has been found to be either ineffective or deleterious, whereas EUK-8 was protective (Doctrow et al., 1996), implying that catalase activity might be an important element of the protective activity of salen-manganese complexes. The difference in efficacy between EUK-8 and EUK-134 in our experiments may also relate to pharmacokinetic factors such as, for example, in vivo stability or delivery to the brain. Such potential differences are not, however, readily predicted by physicochemical properties because, for example, EUK-134 and EUK-8 are quite similar with respect to solubility, hydrophobicity and stability in solution (data not shown). Substantial additional structure-reactivity data would, of course, be required to

![Fig. 5. Effect of delayed treatment with EUK-134 on infarct volume assessed 3 days after MCA-o. Rats were subjected to the MCA-o protocol and treated with EUK-134 (5 μmol/kg) or vehicle at 3 hr after occlusion as described for figure 4. Where indicated, animals treated with vehicle alone (n = 3) or EUK-134 (n = 4) were killed at 72 hr after MCA-o, and infarct volumes were assessed as described in Methods. The figure shows the infarct volumes (mean ± S.E.M.) of these two groups in comparison with identically treated groups killed at 21 hr after MCA-o (these latter data are from the experiment summarized in figure 4). a Significantly different from vehicle-treated group killed at 21 hr (P < .01). b Significantly different from vehicle-treated group killed at 72 hr (P < .01). The two vehicle-treated groups did not differ from each another, nor did the two EUK-134-treated groups (P > .05).](https://jpet.aspetjournals.org/doi/abs/10.1124/jpet.1998.219.5.72)
prove a close relationship between catalase activity and protection against ischemia-induced brain injury. However, these observations support continued investigation of an hypothesized association between ischemic brain injury and hydrogen peroxide.

These results, especially the complete degree of protection afforded by the highest dose of EUK-134, further substantiate the hypothesized involvement of ROS in the cascade of brain damage that follows stroke. ROS may be produced from several sources in ischemic brain, including infiltrating activated leukocytes (Clark et al., 1993; Connolly et al., 1996; Matsuo et al., 1994) and enzymatic production during reperfusion (Granger, 1988). Perhaps the most interesting source, however, is supported by observations that ROS are generated in neurons stimulated with excitatory amino acids (Dugan et al., 1995; Lafon-Cazal et al., 1993). The implication is that ROS may be key contributors to excitotoxicity, a process that has long been regarded as mediating ischemic brain injury (Ziven and Choi, 1991).

Previous studies have shown that antioxidant enzymes have some protective effects in experimental stroke models when administered before ischemia (Armstead et al., 1991; Chan, 1992; He et al., 1993; Imaizumi et al., 1990; Liu et al., 1989; Tagaya et al., 1992; Uyama et al., 1990) but not in a delayed-treatment protocol (Tagaya et al., 1992). It has not necessarily been clear whether the relatively modest effectiveness of the enzymes and their inactivity in delayed-treatment studies are due to their inaccessibility to brain tissue or to a relative unimportance of ROS in the later progression of neuronal injury. However, our observation that combined SOD/catalase mimetics protect brain tissue when administered after several hours of ischemia suggests that ROS do indeed play a key role relatively late in the cascade of ischemic brain injury. This has important implications for the design of new therapeutic agents for stroke, lending further support to the rationale of developing antioxidant molecules and other strategies aimed at combating oxidative damage for clinical use in acute brain injury (Braughler and Hall, 1989; Ziven and Choi, 1991).

Overall, this study suggests that salen-manganese complexes such as EUK-134 or EUK-8 would be of potential clinical value in stroke, their particular ability to protect brain tissue even when administered hours after the induction of ischemia suggests that their effectiveness might persist over the relatively long “therapeutic window” required for a clinical application. In addition to their potential neuroprotective effectiveness as demonstrated in this experimental model, SOD/catalase mimetics might potentially be administered in conjunction with recently approved thrombolytic stroke therapies (Barinaga, 1996; Ziven and Choi, 1991) to minimize any oxidative reperfusion-associated injury that might theoretically occur on clot dissolution. In summary, should further preclinical and clinical studies continue to support their development, combined synthetic SOD/catalase mimetics, as exemplified by EUK-134, might fulfill a unique, multifunctional role in the treatment of stroke.


**Send reprint requests to:** Dr. Susan R. Doctrow, Eukarion Inc., 6F Alfred Circle, Bedford, MA 01730. E-mail: s.doctrow@eukarion.com