Ebselen, a Seleno-Organic Antioxidant, Is Neuroprotective After Embolic Strokes in Rabbits: Synergism With Low-Dose Tissue Plasminogen Activator

Paul A. Lapchak, PhD; Justin A. Zivin, MD, PhD

Background and Purpose—It has been proposed that antioxidants and spin-trap agents may be neuroprotective after acute ischemia stroke. Although the antioxidant ebselen is currently in clinical trials, little is known about the effectiveness of ebselen, which has glutathione peroxidase–like and anti-inflammatory properties in embolic stroke models. Therefore, we determined the effects of ebselen when administered alone or with the thrombolytic tissue plasminogen activator (tPA), the only Food and Drug Administration–approved pharmacological agent for the treatment of stroke.

Methods—Male New Zealand White rabbits were embolized by injection of a suspension of small blood clots into the middle cerebral artery via a catheter. Five minutes after embolization, ebselen (10 to 50 mg/kg) was infused intravenously. Control rabbits received infusions of the vehicle required to solubilize ebselen. In additional rabbits, ebselen (20 mg/kg) was administered 60 minutes after embolization, either alone or in combination with tPA (0.9 or 3.3 mg/kg tPA). Behavioral analysis was conducted 24 hours after embolization, allowing determination of the effective stroke dose (P50) or clot amount (mg) that produces neurological deficits in 50% of the rabbits.

Results—A drug is considered neuroprotective if it significantly increases the P50 compared with the vehicle-treated control group. The P50 of controls 24 hours after embolization was 1.35±0.30 mg. Rabbits treated 5 minutes after embolization with 10, 20, or 50 mg/kg ebselen had P50 values of 2.12±0.56, 2.82±0.75 (P<0.05), and 0.49±0.54 mg, respectively. A significant neuroprotective effect was observed with the 20-mg/kg dose, but not if there was a 60-minute delay before administration (P50=1.69±0.32 mg). When tPA (3.3 mg/kg) was infused 60 minutes after embolization and ebselen (20 mg/kg) was injected at either 5 (P50=2.98±0.18 mg) or 60 (P50=3.60±0.79 mg) minutes, there was no additional neuroprotective effect compared with tPA alone (P50=3.38±0.55 mg). However, if ebselen (20 mg/kg) was administered concomitantly with low-dose tPA (0.9 mg/kg) 60 minutes after embolization, the P50 was 3.52±0.73 mg (P<0.05), indicating a synergistic effect of the drug combination because neither alone was effective (P50=1.69±0.32 and 1.54±0.36 mg, respectively).

Conclusions—This study indicates that ebselen may be neuroprotective when administered shortly after an embolic stroke, but the time- and dose-response analyses suggest that it has a narrow therapeutic window. Nevertheless, ebselen may be beneficial if administered concomitantly with a thrombolytic because it significantly enhanced the neuroprotective activity of low-dose tPA. (Stroke. 2003;34:2013-2018.)

Key Words: ischemia ■ neuroprotection ■ reperfusion ■ thrombolytic therapy ■ tissue plasminogen activator ■ rabbits

Ebselen [2-phenyl-1,2-benziselenazol-3(2H)-one] is a selenium-based antioxidant compound with numerous pharmacological activities,1 including neuroprotection mediated by REDOX reactions,2 reduced inflammatory mechanisms,1 scavenging of reactive nitrogen species including peroxynitrite,3–5 and inhibition of apoptotic mechanisms.6 More important, recent studies using models of neurodegenerative diseases have shown that ebselen can modify N-methyl-d-aspartate (NMDA) receptor function, reducing the devastating effects of NMDA receptor activation.2 Additionally, ebselen neutralizes free radicals produced by NMDA receptor stimulation7 and reduces lipoperoxidation mediated by glutamate-induced excitotoxicity.8 This represents an important property of ebselen because free radicals, particularly reactive oxygen species, can accumulate to such a degree as to cause a biochemical chain of events that ultimately lead to cell death.9

Preliminary clinical trials have shown that ebselen may be useful in the treatment of acute ischemic stroke. The first report of a placebo-controlled, double-blind clinical trial showed that there was a significantly better outcome in ebselen-treated patients at 1 month but not 3 months after

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From the University of California San Diego, Department of Neuroscience, La Jolla; and VASDHS and Veterans Medical Research Foundation, San Diego, Calif.

Correspondence to Dr Paul A. Lapchak, University of California San Diego, Department of Neuroscience, MTF 316, 9500 Gilman Dr, La Jolla, CA 92037–0624. E-mail plapchak@ucsd.edu

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treatment.\textsuperscript{10} A second report concluded that ebselen slightly reduced cerebral infarct volume, but the magnitude of the neuroprotection was not statistically significant.\textsuperscript{11} In fact, a good outcome was seen in only $\approx 15\%$ of patients. Therefore, although the results are promising, ebselen alone may not be an optimal treatment to achieve significant clinical improvement in patients with severe acute ischemic stroke. This is not surprising given that, with the exception of thrombolysis, monotherapy for stroke has proven unsuccessful (see reviews\textsuperscript{12–14}).

The main objective of the present study was to assess the neuroprotective profile of ebselen in a rabbit model of embolic stroke that reproduces many facets of human stroke.\textsuperscript{15–17} The rabbit small clot embolism model (RSCEM) uses administration of sized blood microclots to induce strokes and behavioral deficits that can be quantified, resulting in random infarcts throughout the brain.\textsuperscript{15,16} Moreover, the RSCEM is useful in testing the effects of drugs, whether alone or in combination with the only Food and Drug Administration–approved therapy for stroke, the thrombolytic tissue plasminogen activator (tPA). This makes the RSCEM ideally suited to assess whether the combination of 2 drugs (ebselen and tPA) with radically different mechanisms of action interact to enhance behavioral outcome.

Methods

The procedures used in this study were approved by the Department of Veterans Affairs and the subcommittee on animal studies at the VA San Diego Healthcare System (VASDHS). Throughout the study, the health status of the rabbits was closely monitored by the veterinarian and staff on duty at the VASDHS.

Male New Zealand White rabbits were anesthetized with halothane (5% induction, 2% maintenance by face mask); the bifurcation of the right carotid artery was exposed; and the external carotid artery was ligated just distal to the bifurcation, where a catheter was inserted anteriorly into the common carotid and secured with ligatures. The incision was closed around the catheter with the distal ends left accessible outside the neck; the catheter was filled with heparinized saline and plugged with an injection cap. Rabbits were allowed to recover from anesthesia for a minimum of 3 hours until they awoke and behaved normally.

For the RSCEM, microclots were prepared from blood drawn from a donor rabbit and allowed to clot at 37°C as described in detail previously.\textsuperscript{15,16} To assess the hemorrhage incidence with low- (0.9 mg/kg) and high- (3.3 mg/kg) dose tPA, we used the rabbit large clot embolism model (RLCEM). For RLCEM experiments, blood clots were prepared and injected as described previously.\textsuperscript{15,18}

Drug Administration

Drugs were given intravenously starting 5 or 60 minutes after embolization. Ebselen (10, 20, or 50 mg/kg), a gift from DAIICHI Pharmaceuticals or purchased from Alexis Biochemical, was suspended in 50% $\beta$-hydroxypropyl cyclodextrin (Cerestar Inc) for injection. tPA (3.3 or 0.9 mg/kg) was given 60 minutes after embolization, with 20% as a bolus injection over 1 minute, followed by the remainder infused over 30 minutes.\textsuperscript{16} Genentech, Inc supplied tPA lyophilized in 50-mg configurations containing 50 mg tPA (29 million IU), 1.7 mg L-arginine, 0.5 g phosphoric acid, and <4 mg Polysorbate 80, the same formulation used clinically, that was then reconstituted with sterile water (1 mg/mL).

For combination studies, ebselen (20 mg/kg) was infused beginning 5 or 60 minutes after embolization, and tPA was given (3.3 or 0.9 mg/kg) beginning 60 minutes after embolization.

Rabbits were observed continuously for a minimum of 2 hours after embolization and treatment; neurological function was scored than and again at 24 hours after embolization. For the 24-hour score, an observer who was naive to the treatments did the end-point analysis.

RSCEM Analyses

For the RSCEM, a quantal dose-response data analysis technique was used as described previously\textsuperscript{15,16} and reviewed in detail by Zivin and Wand.\textsuperscript{17} A wide range of clot doses were used, resulting in normal and abnormal animals, with small numbers of microclots causing no grossly apparent neurological dysfunction and large numbers of microclots invariably causing encephalopathy or death. Using a simple dichotomous rating system with a reproducible composite result and low interrater variability (<5%), a naive observer rated each animal as either behaviorally normal or abnormal. Abnormal rabbits include those with $\geq 1$ of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of sensation, and occasionally paraplegia. Using the system, we are unable to detect small changes in improvement over the duration of the study, which may be considered a type 2 error because of reduced power to detect small changes. However, the model and analysis are well suited to detect robust changes after pharmacological intervention. Moreover, because of the use of a fully randomized design, multiple groups of rabbits can be directly compared. The effective stroke dose or $P_{50}$ value was then calculated as the amount of microclots that produce neurological dysfunction (impairment) in 50% of the rabbits within a specific treatment group.

RLCEM Analyses

Embolized rabbits that died before euthanasia were included in the study. Their brains were fixed and sectioned as described below. Surviving animals were euthanized 48 hours after embolization. Their brains were removed, immersion fixed in 4% paraformaldehyde for at least 5 days, and then examined by an observer blinded to the treatment groups. The right middle cerebral artery also was examined for the presence of emboli; surface blood vessels were stripped from the cerebral hemispheres; and the cerebellum was removed from the brain stem. Hemispheres and brain stem were cut into seven 5-mm-thick coronal slices, each having 2 faces, for a total of 14. The presence and size of each hemorrhage and infarct were noted as described previously.\textsuperscript{15,16} Hemorrhage size was recorded as the number of section faces showing hemorrhage, whereas infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the sections. Finally, the total radioactivity in brain slices, cerebellum, and brain stem, as well as surface vessels from the right hemisphere, was measured with a gamma counter. The amount of radiolabel in the tissues and right hemisphere vessels was compared with label in the blood clot at embolization. If <10% of the counts were in the brain and vessels, it was assumed that the labeled blood clot did not reach the brain,\textsuperscript{15,16} and data from these animals were excluded from further analyses.

For all experiments in this study, rabbits were randomly allocated into treatment groups before the embolization procedure, with concealment of the randomization guaranteed by the use of an independent third party. The randomization sequence was not revealed until postmortem analyses were complete.

Statistical Analysis

For the RSCEM, a separate curve was generated for each treatment tested. The $t$ test was used for comparison between groups, with the Bonferroni correction used for multiple comparisons when appropriate. The RLCEM data were analyzed with the $\chi^2$ test for hemorrhage/infarct rate and analysis of variance when relevant.

Results

Neuroprotection by Ebselen

Quantal dose-response analysis was used to determine whether ebselen was neuroprotective after an embolic stroke and whether
there was a significant behavioral difference between vehicle- and ebselen-treated rabbits. Quantal response curves were constructed from the raw data presented in Tables 1 and 2 for the 20-mg/kg dose of ebselen and for the vehicle-treated group (Figure 1). When administered starting 5 minutes after embolization, ebselen (20 mg/kg) significantly (P<0.05) increased the P50 by 107% compared with the vehicle-treated controls (1.35±0.30 mg) to 2.82±0.75 mg, indicating a neuroprotective effect of the drug at this dose (Figure 1 and Tables 1 and 2). No significant differences between vehicle and the 10- or 50-mg/kg doses of ebselen were observed (Figure 2 and Table 3), with P50 values of 2.12±0.56 and 0.49±0.54 mg, respectively.

**Ebselen and tPA Combination**

These experiments assessed the effects of treating embolized rabbits with ebselen and tPA. Ebselen (20 mg/kg)
was given starting 5 or 60 minutes after embolization, with tPA (3.3 mg/kg) injection starting 60 minutes after embolization. With tPA alone, a significant increase in the P50 (P50/H11005 = 3.38/H11006 0.55 mg) was noted. When the 2 drugs were administered in combination, there was no additional behavioral improvement: the P50 values for the combination groups were 2.98/H11006 ± 0.18 and 3.60/H11006 ± 0.79 mg for the 5- and 60-minute ebselen treatment, respectively (Table 3). That the combination did not significantly increase P50 values compared with monotherapy suggests that a ceiling effect caused by the high dose of tPA and/or the short delay (5 minutes) between embolization and ebselen treatment may have occurred but does not exclude the possibility of a positive result if a lower dose of tPA and a longer delay (60 minutes) for ebselen treatment were used. To test this hypothesis, a suboptimal dose of tPA (0.9 mg/kg) that by itself did not significantly increase the P50 value (1.54±0.35 mg) was used in combination with ebselen (20 mg/kg) and given 60 minutes after embolization, which also did not significantly increase the P50 value (1.65±0.32 mg). A statistically significant increase (P<0.05) in the P50 value (3.52±0.73 mg) was observed with this combination (Table 3 and Figure 3), suggesting a synergistic effect of the drugs on behavioral improvement.

Table 3. Effects of Treatments on Behavioral Outcome in Embolized Rabbits

<table>
<thead>
<tr>
<th>Drug Treatment and Time</th>
<th>P50 (Mean±SEM)</th>
</tr>
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<tbody>
<tr>
<td>5 min postembolization</td>
<td></td>
</tr>
<tr>
<td>Vehicle (n=16)</td>
<td>1.35±0.30</td>
</tr>
<tr>
<td>Ebselen (10 mg/kg) (n=13)</td>
<td>2.12±0.56</td>
</tr>
<tr>
<td>Ebselen (20 mg/kg) (n=8)</td>
<td>2.82±0.75*</td>
</tr>
<tr>
<td>Ebselen (50 mg/kg) (n=13)</td>
<td>0.49±0.54</td>
</tr>
<tr>
<td>60 min postembolization</td>
<td></td>
</tr>
<tr>
<td>Vehicle (n=24)</td>
<td>1.53±0.21</td>
</tr>
<tr>
<td>Ebselen (20 mg/kg) (n=16)</td>
<td>1.69±0.32</td>
</tr>
<tr>
<td>tPA 3.3 mg/kg (n=11)</td>
<td>3.38±0.55*</td>
</tr>
<tr>
<td>tPA 0.9 mg/kg (n=13)</td>
<td>1.54±0.36</td>
</tr>
<tr>
<td>Ebselen (20 mg/kg) 5 min + tPA (3.3 mg/kg) 60 min (n=12)</td>
<td>2.98±0.18*</td>
</tr>
<tr>
<td>Ebselen (20 mg/kg) 60 min + tPA (3.3 mg/kg) 60 min (n=12)</td>
<td>3.60±0.79*</td>
</tr>
<tr>
<td>Ebselen (20 mg/kg) 60 min tPA (0.9 mg/kg) 60 min (n=16)</td>
<td>3.52±0.73*†</td>
</tr>
</tbody>
</table>

*P<0.05 compared with vehicle; †P<0.05 compared with tPA (0.9 mg/kg) or ebselen (20 mg/kg).

Figure 1. Percentage of rabbits behaviorally abnormal or dead as a function of microclot weight (mg) injected into the carotid artery system. Dashed curve (left) shows that 50% of vehicle-treated control rabbits will be abnormal or dead 24 hours after injection of 1.35±0.30 mg (P50) of clots. Solid curve (right) indicates that when ebselen (20 mg/kg) is given 5 minutes after embolization, P50 is significantly increased to 2.82±0.75 mg (P<0.05).

Figure 2. Dose-response analysis for the effect of ebselen (10 to 50 mg/kg) on behavioral outcome (P50) measured after embolism. Ebselen (20 mg/kg) increased P50 (P<0.05), but other doses did not (P>0.05).

Figure 3. Percentage of rabbits behaviorally abnormal or dead as a function of microclot weight (mg) injected into the carotid artery system. Drugs were given 60 minutes after embolization. Dashed curve (left) shows that 50% of vehicle-treated control rabbits treated with 1.53±0.21 mg clots (P50) will be abnormal or dead 24 hours after injection. Solid curve overlapping the vehicle curve shows that tPA (0.9 mg/kg) administered 60 minutes after embolization results in P50 of 1.54±0.36 mg (P>0.05). Dotted/dashed curve shows that ebselen (20 mg/kg) is ineffective when administered 60 minutes after embolization, but when combined with tPA (0.9 mg/kg) also given 60 minutes after embolization (far right curve), the P50 value is significantly increased to 3.52±0.73 mg (P<0.05).
of combination drug therapy may be necessary to reduce the devastating neurological and behavioral deficits of acute ischemic stroke. Because of the observed synergistic effects of ebselen with tPA, ebselen or other neuroprotective small-molecules like NXY-0599 may be prime candidates for combination therapy with tPA or second-generation thrombolytics like Tenecteplase or microplasmin.15,21 Future placebo-controlled, double-blind clinical trials are needed to test the hypothesis that combination therapies will result in better outcomes for stroke patients.

Conclusions
Ebselen is neuroprotective because it significantly improved behavioral rating scores in a rabbit embolic stroke model. The limited dose and time of administration range indicate that ebselen has a narrow therapeutic window. However, our results showing a synergistic effect of ebselen plus tPA indicate that ebselen may be administered concomitantly with lower-dose thrombolytics to produce significant neuroprotection and behavioral improvements while reducing the incidence of thrombolytic side effects (ie, hemorrhage rate).

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References
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