Neuroprotective Effects of the Spin Trap Agent Disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-Oxide (Generic NXY-059) in a Rabbit Small Clot Embolic Stroke Model

Combination Studies With the Thrombolytic Tissue Plasminogen Activator

Paul A. Lapchak, PhD; Dalia M. Araujo, PhD; Donghuan Song, MD; Jiandong Wei, MD; Justin A. Zivin, MD, PhD

Background and Purpose—It has been proposed that the novel spin trap agent disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (NXY-059) may be useful in the treatment of ischemic stroke. However, there is little information concerning the neuroprotective properties of NXY-059 when administered after an embolic stroke. Moreover, there is no information available concerning the combination of NXY-059 with the only Food and Drug Administration–approved pharmacological agent for the treatment of acute stroke, the thrombolytic tissue plasminogen activator (tPA). Thus, we determined the effects of NXY-059G, a generic form of NXY-059, on behavioral outcome after an embolic stroke when administered alone or in combination with tPA.

Methods—Male New Zealand White rabbits were embolized by injecting a suspension of small blood clots into cerebral circulation via a carotid catheter. NXY-059G (100 mg/kg) was infused intravenously 5 minutes or 3 hours after embolization, whereas control rabbits received infusions of the saline vehicle. In tPA studies, the thrombolytic was administered intravenously starting 60 minutes or 3 hours after embolization (3.3 mg/kg). In combination studies, NXY-059G was given 5 minutes after embolization, followed by the administration of tPA beginning either 60 minutes or 3 hours after embolization. Behavioral analysis was conducted 24 hours after embolization.

Results—In the vehicle control group, the ES50 value (calculated as the amount of microclots [milligrams] that produce neurological dysfunction [impairment] in 50% of the rabbits within a specific treatment group) measured 24 hours after embolism was 1.04 ± 0.31 mg, and this was increased by 153% to 2.54 ± 0.72 mg if NXY-059G was administered beginning 5 minutes after embolization. However, if NXY-059G was administered beginning 3 hours after embolization, the ES50 was 2.01 ± 0.40 mg. The rabbits treated with tPA alone had an ES50 of 2.64 ± 0.66 or 0.63 ± 0.35 mg if tPA administration started 60 minutes or 3 hours after embolization, respectively. If tPA was administered after NXY-059G (started at 5 minutes), the ES50 values were 3.15 ± 0.50 or 2.66 ± 0.82 if tPA administration started 60 minutes or 3 hours after embolization, respectively.

Conclusions—This study suggests that NXY-059G is neuroprotective and can increase behavioral ratings if administered early after an embolic stroke. In addition, the study shows that NXY-059G can be used in combination with tPA without negative side effects. The drug combination can improve behavioral function and increase ES50 values. However, during the short time course of the behavioral analysis, the combination was not statistically better than either drug alone.

Key Words: indoles ■ ischemia ■ neuroprotection ■ nitrogen oxides ■ oxygen radical ■ reactive oxygen species ■ reperfusion ■ tissue plasminogen activator ■ rabbits

Recent reports suggest that spin trap agents such as phenyl butyl nitrone (PBN) and the related compound disodium 4-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (NXY-059) are neuroprotective in a variety of small animal stroke models. To date, all of the preclinical studies with NXY-059 have relied on occlusion of the middle cerebral artery (MCA) with filaments, microaneurysm clips, or electrocoagulation. Because of methodologies used to induce cerebral ischemia or strokes in each of the models, combination studies with tissue plasminogen activator (tPA), the only...
Food and Drug Administration–approved treatment for stroke, are precluded.

We have developed and used a rabbit embolic stroke model that reproduces many facets of human stroke.9–12 The rabbit small clot embolism model (RSCEM) uses administration of sized blood clots to induce strokes and behavioral deficits that can be measured quantitatively. Previous studies have shown that embolization with such microclots results in random infarcts throughout the brain.10,13 Moreover, the RSCEM is useful to conduct studies testing the effects of drug combinations or neuroprotective agents plus a thrombolytic.

On the basis of a recent publication,14 it appears that NXY-059 is being considered as a candidate for a clinical trial in patients with acute stroke. Studies describing the pharmacological effects of NXY-059 in combination with a thrombolytic have not been reported, and it is likely that such a combination will be used for patient management. Therefore, we determined the pharmacological effects of administration of generic NXY-059 (NXY-059G) on behavioral deficits measured in embolized rabbits in the presence or absence of tPA treatment.

Materials and Methods
Male New Zealand White rabbits weighing 2 to 3 kg were used for the study. We randomly allocated the rabbits into 7 different treatment groups before the embolization procedure. The randomization sequence was generated with the use of randomization tables, and concealment of the randomization was achieved by use of an independent third party. The randomization sequence was not revealed until all data analyses were complete.

Rabbits were anesthetized, the bifurcation of 1 carotid artery was exposed, and the external carotid was ligated just distal to the bifurcation. A catheter was inserted anterograde into the common carotid and secured with ligatures. The incision was closed around the catheter so that the distal ends were accessible outside the animal’s neck. The catheter was filled with heparinized saline and plugged with an injection cap. The animals were allowed to recover from anesthesia for a minimum of 3 hours so that they were awake and behaving normally.

To prepare small clots, blood was drawn from a donor rabbit and allowed to clot at 37°C. The clot was suspended in Dulbecco’s PBS solution containing 0.1% bovine serum albumin and fragmented with a Polytron (setting 6, 3 seconds). The fragments were sized by sequential filtration through a 240-μm screen and a 100-μm nylon net. The clots retained by the nylon net were suspended in PBS, and the suspension was washed and allowed to settle. The supernatant was washed and allowed to settle. The spin trap agent disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (NXY-059G [generic NXY-059]) was custom synthesized in our laboratories according to the synthetic scheme described by Hinton and Janzen15 and Carney.16 The proton MR spectrum at 550 Hz of the white crystalline product in deuterochloroform was consistent with disodium-[(tert-butyl-imino)methyl]benzene-1,3-disulfonate N-oxide (ie, NXY-059G).

NXY-059G Administration
NXY-059G was administered at a dose of 100 mg/kg IV infused over 30 minutes beginning 5 minutes or 3 hours after embolization. This dose of spin trap agent was chosen on the basis of our previous in vivo pharmacological studies with the use of the parent compound PBN.17

tPA Administration
The tPA regimen used in this study is as follows: 3.3 mg/kg tPA, 20% as a bolus injection given over 1 minute, followed by the remainder infused over 30 minutes.17,18 Genentech, Inc (South San Francisco, Calif) supplied tPA as a lyophilized cake 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 tPA, which is the same formulation used clinically, was reconstituted with sterile water, at a concentration of 1 mg/mL. tPA was administered beginning 60 or 180 minutes after embolization.

Combination Therapies
For combination studies, NXY-059G was administered at a dose of 100 mg/kg IV infused over 30 minutes beginning 5 minutes after embolization, and tPA was administered at a dose of 3.3 mg/kg IV (20% bolus, 80% infused over 30 minutes) beginning 60 minutes or 3 hours after embolization.

Quantal Dose-Response Analysis
For the RSCEM, we used a quantal dose-response data analysis technique. A wide range of lesion volumes was induced to generate both normal and abnormal animals. The use of ≥3 different doses of microclots generated each quantal analysis curve. We found the low end of the curve (small numbers of microclots cause no grossly apparent neurological dysfunction) and the high end (large numbers of microclots invariably cause encephalopathy or death). Each animal was rated as either normal or abnormal (including dead animals), and interrater variability was very low (<5%). Behaviorally normal rabbits did not have any signs of impairment, whereas behaviorally abnormal rabbits had loss of balance, head leans, circling, seizure-type activity, or limb paralysis. With this simple rating system, the composite result for a group of animals is quite reproducible. The ES50 values were then calculated. These parameters are measures of the amount of microclots that produce neurological dysfunction in 50% of a group of animals. A separate curve was generated for each treatment condition that we tested. The data were analyzed with the use of ANOVA with post hoc t test, which included the Bonferroni correction where appropriate.

Results
NXY-059G Administration
In the RSCEM, the ES50 in vehicle-treated control rabbits 24 hours after embolization was 1.04±0.31 mg (n=23) (Table 1, Table 2, and Figure 1). When NXY-059G was administered 5 minutes after embolization, the group ES50 was 2.54±0.72 mg (P=0.0313 compared with vehicle; n=11) when measured 24 hours after embolization. However, when NXY-059G was administered 3 hours after embolization, the ES50 was 2.01±0.40 mg (P=0.0586 compared with vehicle; n=18) (Tables 1 and 2).

tPA Administration
In the RSCEM, tPA administration 60 minutes after embolization significantly increased the ES50 value to 2.64±0.66 mg (P=0.0221 compared with vehicle; n=17)
(Table 1 and Figure 2). However, tPA was no longer effective when administered 3 hours after embolization since the ES$_{50}$ value was 0.63 ± 0.35 mg ($P = 0.4172$ compared with vehicle).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Procedure A</th>
<th>Procedure B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>ES$_{50}$, mg</td>
</tr>
<tr>
<td>Vehicle</td>
<td>23</td>
<td>1.04±0.31</td>
</tr>
<tr>
<td>NXY-059G</td>
<td>11</td>
<td>2.54±0.72*</td>
</tr>
<tr>
<td>tPA</td>
<td>17</td>
<td>2.64±0.66‡</td>
</tr>
<tr>
<td>tPA+NXY-059G</td>
<td>8</td>
<td>3.15±0.50¶</td>
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</tbody>
</table>

Table 1. Estimated ES$_{50}$ Values Measured in Embolized Rabbits

ES$_{50}$ values (expressed as milligrams of clot) were calculated as follows: in procedure A, vehicle and NXY-059G were given 5 min after embolization, while tPA was administered 60 min after embolization; in procedure B, NXY-059G and tPA alone were given 3 h after embolization, whereas in the combination drug treatment NXY-059G was given 5 min after embolization and tPA was given 3 h after embolization.

NXY-059G significantly increased the ES$_{50}$ when administered 5 min after embolization but not 3 h after embolization. Statistical comparisons: NXY-059G 5 min vs vehicle, *$P = 0.0313$; NXY-059G 3 h vs vehicle, $P = 0.0586$; NXY-059G 5 min vs NXY-059G 3 h, †$P = 0.05$. tPA significantly increased the ES$_{50}$ when administered 60 min after embolization but not 3 h after embolization compared with vehicle. tPA/NXY-059G (3 h/5 min) was statistically different from vehicle and tPA at 3 h alone. tPA 60 min vs vehicle, ‡$P = 0.0221$; tPA 3 h vs vehicle, $P = 0.4172$; NXY-059G plus tPA 60 min vs NXY-059G 3 h, $P = 0.05$; NXY-059G plus tPA 3 h vs tPA 3 h, $P = 0.0421$; NXY-059G plus tPA 60 min vs tPA 3 h, $P < 0.05$; NXY-059G plus tPA 60 min vs vehicle, $P = 0.0015$; NXY-059G plus tPA 3 h vs vehicle, **$P = 0.0375$; NXY-059G/tPA combination vs NXY-059G alone, $P > 0.05$.

**Combination Therapies**

Since NXY-059G and tPA were effective when administered 5 and 60 minutes after embolization, respectively, we wanted to determine whether the drug combination would have a syner-

Table 2. Effect of NXY-059G Treatment on Behavioral Outcome of Embolized Rabbits

<table>
<thead>
<tr>
<th>Clot Dose, mg</th>
<th>Vehicle (n=23)</th>
<th>NXY-059G (5 min) (n=11)</th>
<th>NXY-059G (180 min) (n=18)</th>
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<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td>Normal</td>
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<tr>
<td>0.14</td>
<td>1</td>
<td>0</td>
<td>0.09</td>
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<tr>
<td>0.27</td>
<td>1</td>
<td>0</td>
<td>0.25</td>
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<tr>
<td>0.28</td>
<td>1</td>
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<tr>
<td>0.36</td>
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<td>0.37</td>
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<td>1.02</td>
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<tr>
<td>1.04</td>
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</tr>
<tr>
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<td>1</td>
<td>0</td>
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<td>2.94</td>
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<tr>
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</tbody>
</table>

Behavioral results are expressed as normal or abnormal rabbits for each dose of clot, given in milligrams; n is the number of animals in each treatment group.
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Figure 1. Percentage of rabbits behaviorally abnormal as a function of the weight of clots injected into the carotid artery system. The curve on the left (solid) shows the response of vehicle-treated control rabbits to clot administration. It demonstrates that 50% of the animals treated with 1.04±0.31 mg of clots (ES$_{50}$) will be abnormal or dead 24 hours after injection of the clots. The curve in the middle (dashed) indicates that if NXY-059G is administered 3 hours after embolization, the ES$_{50}$ is increased to 2.01±0.40 mg (P<0.05). The curve on right (dotted) indicates that if NXY-059G is administered 5 minutes after embolization, the ES$_{50}$ is increased to 2.54±0.72 mg (P<0.05).

Figure 2. Percentage of rabbits behaviorally abnormal as a function of the weight of clots injected into the carotid artery system. The curve in the middle (solid) shows the response of vehicle-treated control rabbits to clot administration. It demonstrates that 50% of the animals treated with 1.04±0.31 mg of clots (ES$_{50}$) will be abnormal or dead 24 hours after injection of the clots. The curve in the middle (dashed) indicates that if tPA is administered 3 hours after embolization, the ES$_{50}$ was decreased to 0.63±0.35 mg (P>0.05). The curve on the right (dotted) indicates that if NXY-059G is administered 5 minutes after embolization and tPA is administered 3 hours after embolization, the ES$_{50}$ is significantly increased to 2.66±0.82 mg (P<0.05).

In the present study we found that the spin trap agent NXY-059G was neuroprotective if administered early after embolization. However, if this agent was administered at an extended time after embolization, the neuroprotective activity was lost. Furthermore, we found that administration of NXY-059G in combination with tPA was safe, but there was not a statistically significant synergistic effect of the drug combination.

The spin trap agent NXY-059G reduced embolism-induced behavioral deficits when administered immediately after embolization. However, if drug administration was delayed, neuroprotection was no longer observed, and the ES$_{50}$ value was not statistically different from the vehicle group. This result is in agreement with previous studies using different techniques to induce cerebral ischemia. Kuroda et al used a fixed 2-hour occlusion with cerebral reperfusion and various NXY-059 administration times. The authors show that NXY-059 could be neuroprotective if administered up to 6 hours after reperfusion if given as a loading dose (bolus) followed by long-term infusion. Marshall et al showed that administration of a loading dose of NXY-059 beginning 5 minutes after occlusion, followed by chronic infusion via a minipump for 48 hours, could significantly reduce behavioral deficits and spare neurons. Peeling et al also showed that NXY-059 was effective at improving behavioral deficits in a hemorrhagic stroke model. The differences in the results observed and the time window for neuroprotection may be related to a variety of factors such as treatment regimens, methods of MCA occlusion, and extent of ischemia induced by the occlusion.

We also found that tPA administration either alone or in combination with NXY-059G was neuroprotective if NXY-059 and tPA were administered 5 and 60 minutes after embolization, respectively. Using the 5 minutes/60 minutes administration times, we did not observe a synergistic effect of the drug combination. It is possible that synergism was not observed because the maximal neuroprotective effect (shift in ES$_{50}$) was already achieved. This may be a limitation of the RSCEM when used. However, if NXY-059G and tPA were administered 5 minutes and 3 hours after embolization, respectively, we found that the drug combination significantly increased the ES$_{50}$ value compared with tPA administration (3 hours) alone. Furthermore, the ES$_{50}$ for the drug combination at the 5 minutes/3 hours administration time was significantly different from that measured for NXY-059G or tPA when administered 3 hours after embolization. This result indicated that there was a behavioral effect of the drug combination. However, the effect was similar in magnitude to NXY-059 alone when administered 5 minutes after embolization.

Our result, showing that NXY-059G reduces embolization-induced behavioral deficits, suggests that NXY-059G scav-
Peroxides free radicals produced during and after an ischemic stroke induced by injection of blood clots. A recent publication by Maples et al. compared the biochemical properties of NXY-059 with those of PBN. Both compounds were found to trap carbon- and oxygen-centered radicals, with PBN being more effective than NXY-059. Nevertheless, both compounds were effective spin trapping free radical scavengers. Thus, the beneficial effect of NXY-059 alone or in combination with tPA may be due to mechanisms that are based on the known pharmacology of PBN since both drugs have similar structures. PBN is reported to scavenge free radicals at the blood–endothelial cell interface.21,22 The spin trap agent may exert its effects by reducing microvascular dysfunction indirectly caused by polymorphonuclear neutrophil leukocytes that give rise to free radicals.23

In the present study we studied NXY-059G at a dose of 100 mg/kg to determine whether NXY-059G alters embolism-induced behavioral deficits in our thromboembolic stroke model. In our study, after a dose of 100 mg/kg, we did not observe any obvious physical or behavioral signs of “toxicity.” This is consistent with a recent clinical study with NXY-059 that indicated that the spin trap was well tolerated in patients with acute stroke.14 Our finding that NXY-059G is neuroprotective by itself and that combining NXY-059G with tPA is safe suggests that the spin trap may be used alone or in combination with tPA to treat stroke.

In conclusion, we have shown that the spin trap agent NXY-059G may be effective as a monotherapy for stroke. In addition, the study shows that NXY-059G can be used in combination with tPA without negative side effects. Administration of NXY-059G in combination with tPA may provide additional neuroprotection in the long term since the thrombolytic may improve cerebral blood flow, whereas NXY-059G will not. Further detailed drug combination and timing studies are required to determine the optimal efficacious effect that can be achieved by the spin trap and thrombolytic.

Acknowledgments
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References
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