Pharmacological Effects of the Spin Trap Agents
N-t-Butyl-Phenylnitrone (PBN) and 2,2,6,6-
Tetramethylpiperidine-N-Oxyl (TEMPO) in a Rabbit
Thromboembolic Stroke Model
Combination Studies With the Thrombolytic Tissue Plasminogen Activator

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Background and Purpose—It has been proposed that spin trap agents such as N-t-butyl-phenylnitrone (PBN) may be useful as neuroprotective agents in the treatment of ischemia and stroke. However, to date, there is little information concerning the effectiveness of spin trap agents when administered in combination with the only Food and Drug Administration–approved pharmacological agent for the treatment of stroke, the thrombolytic tissue plasminogen activator (tPA). Thus, we determined the effects of PBN when administered before tPA on hemorrhage and infarct rate and volume. We also compared the effects of PBN with those of 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), another spin trap agent that has a different chemical structure and trapping profile, on the incidence of infarcts and hemorrhage.

Methods—One hundred sixty-five male New Zealand White rabbits were embolized by injecting a blood clot into the middle cerebral artery via a catheter. Five minutes after embolization, PBN or TEMPO (100 mg/kg) was infused intravenously. Control rabbits received saline, the vehicle required to solubilize the spin traps. In tPA studies, rabbits were given intravenous tPA starting 60 minutes after embolization. Postmortem analysis included assessment of hemorrhage, infarct size and location, and clot lysis.

Results—In the control group, the hemorrhage rate after a thromboembolic stroke was 24%. The amount of hemorrhage was significantly increased to 77% if the thrombolytic tPA was administered. The rabbits treated with PBN in the absence of tPA had a 91% incidence of hemorrhage compared with 33% for the TEMPO-treated group. In the combination drug–treated groups, the PBN/tPA group had a 44% incidence of hemorrhage, and the TEMPO/tPA group had a 42% incidence of hemorrhage. tPA, PBN/tPA, and TEMPO/tPA were similarly effective at lysing clots (49%, 44%, and 33%, respectively) compared with the 5% rate of lysis in the control group. There was no significant effect of drug combinations on the rate or volume of infarcts.

Conclusions—This study suggests that certain spin trap agents may have deleterious effects when administered after an embolic stroke. However, spin trap agents such as PBN or TEMPO, when administered in combination with tPA, may improve the safety of tPA by reducing the incidence of tPA-induced hemorrhage. Overall, the therapeutic benefit of spin trap agents for the treatment of ischemic stroke requires additional scrutiny before they can be considered “safe” therapeutics. (Stroke. 2001;32:147-153.)

Key Words: ischemia ■ neuroprotection ■ nitrogen radicals ■ oxygen radicals ■ reactive oxygen species ■ reperfusion ■ tissue plasminogen activator ■ rabbits

With the use of tissue plasminogen activator (tPA) for the treatment of thromboembolic stroke,1–3 there is reason to be concerned that thrombolysis may expose patients to secondary intracerebral hemorrhage (ICH).4,5 There is an approximately 6% incidence of subsequent symptomatic ICH, and half of these patients die.4,6,7 Currently, there are no acceptable pharmacological treatments for ICH, although surgery is sometimes used without proof of efficacy or safety.8 We have recently started to focus on development of pharmacological strategies to reduce tPA-induced ICH.

Even though thrombolytics are beneficial because they restore perfusion,9 blood flow may not resume promptly enough to prevent the onset of tissue damage, nor do thrombolytics guard against ICH.5 There is a need to develop new drugs that may be used in combination with thrombolytics to manage not only secondary tissue damage
but also hemorrhage. When tPA is administered, the clot dissolves, allowing reperfusion of the formerly ischemic brain tissue.\textsuperscript{5,10} It has been suggested that reperfusion is associated with endothelial cell damage, an increase in edema, and an increased risk of ICH.\textsuperscript{5,11–13} It is likely that free radicals are mediators of a variety of injuries after an ischemic stroke.\textsuperscript{14–16}

Much experimental stroke research has focused on developing neuroprotective agents to reduce secondary damage after the onset of ischemia. One class of compound that has received a great deal of attention is free radical spin traps.\textsuperscript{14,15,17–20} The spin trap agent N\texttextsuperscript{-}t-butyl-phenylnitrone (PBN) is reported to have multiple pharmacological activities, including the ability to trap alkoxyl radicals,\textsuperscript{21} superoxide radicals,\textsuperscript{22} and hydroxyl radicals.\textsuperscript{23,24} PBN can also decrease inducible cytochrome (cytochromeogenase-2) levels and activity, decrease inducible nitric oxide synthase, inhibit mechanisms involved in nuclear factor-\kappaB transduction, induce heme oxygenase-1, inhibit mitochondrial complex I function, enhance cholinergic function via acetylcholinesterase inhibition, and inhibit calcium channels.\textsuperscript{25–30} One or more of the actions may be involved in the pharmacological activities of PBN in vivo. PBN has been shown to be neuroprotective after middle cerebral artery occlusion,\textsuperscript{31–35} to decrease ICH in a rat collagenase model,\textsuperscript{36} and to reduce hemorrhage in a rat embolism model.\textsuperscript{37} Moreover, with the use of a variation of the rabbit thromboembolic model that we used in the present investigation, the spin trap agent MDL 101,002\textsuperscript{38} was shown to be efficacious in reducing ICH.\textsuperscript{39}

To our knowledge, few studies describing the pharmacological effects of spin traps in combination with thrombolysis have been conducted. Therefore, we determined the pharmacological effects of administration of the 2 different spin traps PBN and 2,2,6,6-tetramethylpiperidine-N\texttextsuperscript{-}oxyl (TEMPO) on hemorrhage and infarct rate in the presence or absence of tPA treatment in a thromboembolic stroke model.

Materials and Methods

One hundred sixty-five male New Zealand White rabbits weighing 2 to 3 kg were used for the study. The common carotid artery was catheterized as described previously (References 40 and 42, and D. Chapman, P. Lyden, P.A. Lapchak, S. Nunez, H. Thibodeaux, and J. Zivin, unpublished data, 2000). Emboli were prepared, and the rabbits were embolized according to the procedure of Lapchak et al.\textsuperscript{42} If the animal did not react behaviorally (nystagmus, hemiparesis, seizure) to the embolization, a second blood clot was injected in the same way 3 minutes after the first embolization. If there was no behavioral reaction to either embolization, no further blood clots were administered. Animals that had no behavioral reaction after administration of 2 clots were treated in the same manner as animals responding to emboli. Inclusion or exclusion of animals was based on the criteria described below. After the embolization process was completed, the catheter was ligated close to the neck, and the rest of the catheter and injection port was cut off.

Animals that died before euthanasia were included in the study, and the brains were fixed and sectioned as below. The surviving animals were killed 48 hours after embolization. The brains were removed and immersion fixed in 4% paraformaldehyde for at least 1 week and then examined by a blinded observer. The right middle cerebral artery of each brain was examined for the presence of emboli. The surface blood vessels were then stripped from the right hemisphere of each brain. The cerebellum was also removed from the brain stem. Hemispheres and brain stem were cut into five 5-mm-thick coronal slices, each having 2 faces. We noted the presence, location, size, and type of each hemorrhage and infarct. We recorded the size of hemorrhage as the number of sections showing hemorrhage.\textsuperscript{42–44} Infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. Three major types of hemorrhage were identified according to the grading system we used in previous studies.\textsuperscript{45–48} Hemorrhagic infarction was characterized by red speckling of an area, usually surrounded by soft, infarcted tissue. Punctate hemorrhages were isolated small red marks within the tissue that did not extend through the tissue as a blood vessel would. Parenchymatous ICH were large homogeneous masses of blood within the tissue. After evaluation for hemorrhage and infarcts, the total radioactivity in the brains was measured by placing the slices into a gamma counter. The surface vessels from the right hemisphere were placed in a separate container and counted. Then the cerebellum, each hemisphere, and brain stem were counted in separate tubes. The amount of radiolabel present in the brain (including the right hemisphere vessels) was compared with that contained in the labeled blood clot at embolization. If <10% of the counts were found in the brain and vessels, it was assumed that the labeled blood clot had not reached the brain.\textsuperscript{49} The data from these animals were excluded from further analysis.

Drug Administration

We randomly allocated 165 animals to 6 different treatment groups before the embolization procedure. Sample size was based on power analysis with a coefficient of variation of 15%, and a difference between means of 20%. It was determined that a sample size of 12 to 14 animals per group was required. Our previous experience with this stroke model indicates that we actually need an average of 20 animals per group because of premature losses caused by various preparation difficulties or deaths after embolization before treatments can be fully administered. The treatment groups were as follows: tPA (n = 51), PBN plus tPA (n = 25), vehicle control (n = 28), PBN (n = 17), TEMPO plus tPA (n = 20), and TEMPO (n = 24). The higher number of rabbits in the tPA and vehicle control groups is due to inclusion of rabbits from both groups throughout the duration of the study.

The spin trap agents PBN and TEMPO were dissolved in normal saline and administered at a dose of 100 mg/kg IV by infusion over 30 minutes starting 5 minutes after embolization. This dose of spin trap agent was chosen on the basis of previous in vivo pharmacological studies which showed that doses in the range of 100 to 150 mg/kg\textsuperscript{36,50} attenuate central nervous system neurodegeneration and hemorrhage. PBN and TEMPO were chosen as the spin traps for the present study because of the wealth of information on both compounds and their effectiveness in various central nervous system neurodegeneration, ischemia, and hemorrhage models.\textsuperscript{19,27,30} In the remaining groups of rabbits, we then administered tPA or vehicle 1 hour after embolization. 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 (References 44 and 51, and D. Chapman, P. Lyden, P.A. Lapchak, S. Nunez, H. Thibodeaux, and J. Zivin, unpublished data, 2000). Genentech, Inc (South San Francisco, Calif) supplied tPA and its vehicle. tPA was supplied 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 was reconstituted with sterile water, at a concentration of 1 mg/mL. We analyzed the data with the χ\textsuperscript{2} test corrected for multiple comparisons, using the Bonferroni technique and ANOVA when relevant. Fisher’s exact test was used as the post hoc test after ANOVA.
Figure 1. Effects of tPA, PBN, and TEMPO on hemorrhage rate in embolized rabbits. Hemorrhage rate was quantified by counting the number of macroscopically visible hemorrhages in coronal brain sections. The results are expressed as percentage of total for the experimental group. ANOVA showed that tPA and PBN administration significantly increased hemorrhage rate compared with control (P<0.001 for each group compared with control). In rabbits treated with the combination of PBN/tPA, there was a significantly lower rate of hemorrhage (P<0.05 compared with tPA alone). The TEMPO and TEMPO/tPA groups were not significantly different from the control group (P>0.05). With TEMPO/tPA, there was a trend favoring reduction of tPA-induced hemorrhage (P=0.055).

Results

Stroke Success Rate

Of 165 embolized rabbits included in the study, we found that 110 rabbits (67%) had >10% recovered radioactivity in the brain postmortem. The majority of embolized rabbits responded by behavioral manifestations, including nystagmus, pupillary dilation, hemiparesis, seizure, or brief uncoordinated jerking movements. Rabbits that did not have behavioral manifestations of embolization, but did have >10% recovered in the brain postmortem, were included in the analyses. The remaining 35% of the rabbits had ≤10% of the label present in the brain postmortem, indicating that the injected blood clot did not reach the brain. The breakdown of the excluded rabbits (n=55) is as follows: vehicle (n=7 of 28), tPA (n=16 of 51), PBN (n=6 of 17), PBN/tPA (n=9 of 25), TEMPO (n=9 of 24), and TEMPO/tPA (n=8 of 20). The rabbits that did not reach criteria were excluded from the study, and the data were not used in the final analysis. This success rate corresponds well with other studies involving this model (References 42, 45, and 52, and D. Chapman, P. Lyden, P.A. Lapchak, S. Nunez, H. Thibodeaux, and J. Zivin, unpublished data, 2000).

Hemorrhage Rate

Figure 1 shows the hemorrhage rate for the 6 groups of rabbits included in this study. The percentages of rabbits with brain hemorrhages in the 6 groups were as follows: 24% in the vehicle control group (n=21), 77% in the tPA-treated group (n=35), 91% in the PBN-treated group (n=11), 44% in the PBN/tPA-treated group (n=16), 33% in the TEMPO-treated group (n=15), and 42% in the TEMPO/tPA-treated group (n=12). Overall, there was a statistically significant difference in hemorrhage rates (Table). tPA caused significantly more hemorrhages than in the vehicle control group (P<0.01). There was also a difference in hemorrhage rate between the PBN/tPA and tPA groups (P<0.05) and PBN and control groups (P<0.05). The hemorrhage rate after PBN administration was not statistically different from that of the tPA-treated group (P>0.05) since PBN increased the hemorrhage rate by 3.75-fold compared with control. The combination of TEMPO/tPA showed a trend toward a decrease (42%) in hemorrhage rate; however, this trend did not reach statistical significance (P=0.055). TEMPO administration in the absence of tPA was similar to the control hemorrhage rate, producing a 33% incidence of hemorrhage.

Hemorrhage Volume

Figure 2 shows the number of faces with observed hemorrhage, which is a measure of hemorrhage volume. For each animal in the study, the maximum number of faces observed was 10. There were statistically significant differences among the 6 treatment groups. The control group had 2.2±0.7 faces with hemorrhage present. When PBN or TEMPO was administered, the number of faces was 3.4±0.4 and 1.8±0.2, respectively (Figure 2). Of the tPA-treated rabbits, there were 3.1±0.4, 5.1±1.0, and 1.8±0.2 faces per hemorrhage for the tPA-treated group, PBN/tPA-treated group, and TEMPO/tPA-treated group, respectively. There was a significant difference between the tPA and PBN/tPA-treated groups.

Comparison of Hemorrhage Types in tPA-and Spin Trap–Treated Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rabbits With</th>
<th>Type of Hemorrhage</th>
<th>Total Rabbits</th>
<th>% Rabbits With</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Hemorrhage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>2</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>77</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>tPA</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>15</td>
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<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>23</td>
<td>10</td>
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<tr>
<td>TEMPO</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>TEMPO/tPA</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>PBN</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>11</td>
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<td></td>
<td>1</td>
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<td>3</td>
<td>11</td>
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<tr>
<td>PBN/tPA</td>
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<td>6</td>
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<td></td>
<td>9</td>
<td>9</td>
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</tbody>
</table>

Most of the hemorrhages observed were hemorrhagic infarctions (HI), but ICH and punctate hemorrhages (PT) were also observed. Some rabbits had multiple forms of hemorrhage present in brain after thromboembolism. In the PBN-treated group, there was a trend for an increase in ICH.
The PBN/tPA-treated group was also significantly different from control (P = 0.048).

Types of Hemorrhage

The Table shows the types of hemorrhage present in each of the experimental groups. Most of the hemorrhages seen were hemorrhagic infarctions, but ICH and punctate hemorrhages were also present in each of the groups. Some of the animals had >1 type of hemorrhage present in the brain. For quantitative purposes, we treated each individual hemorrhage observed as a separate entity. Hemorrhages occurred throughout the brain and included the following structures: caudate putamen; thalamus; hippocampus; frontal, parietal, and occipital cortex; hypothalamus; suprachiasmatic area; cerebellum; pons; and midbrain. There were no apparent differences among the groups in the distribution of types or locations of hemorrhages.

Thrombolysis Rate

The combined clot lysis rate when tPA and a second pharmacological agent were administered is shown in Figure 3. We estimated thrombolytic efficacy by calculating the percentage of animals in each treatment group that had <20% of the total recovered radiolabel in the surface vessels of the right hemisphere.44 Thrombolysis was measured in 49% of the tPA-treated rabbits (Figure 3) and 5% of the vehicle control-treated rabbits. There was little measurable thrombolysis in the groups treated with PBN (9%) and TEMPO (27%) only. There were no significant differences in thrombolysis rate between the tPA (49%) and PBN/tPA (44%) or TEMPO/tPA (33%) groups.

Infarct Rate and Volume

In a subset of 4 of the experimental groups used in this study (vehicle control, tPA, PBN/tPA, and TEMPO/tPA groups), we determined whether spin trap agents affected infarct rate and volume (the number of brain slice faces with infarcts) observed in brain after a stroke. In the vehicle control and tPA-treated groups, infarcts were found in 86% (18/21) and 94% of treated rabbits (15/16). In the PBN/tPA-treated group 65% of the rabbits (11/17) had infarcts, and in the TEMPO/tPA-treated groups 83% (10/12) of the rabbits had infarcts. The drug combination did not significantly alter infarct rate. The measurement of infarct volume also showed that there were no statistically significant differences among the 4 groups. The volumes were 4.0 ± 0.5, 3.3 ± 0.6, 4.1 ± 0.9, and 4.7 ± 0.8 faces in the vehicle control, tPA, PBN/tPA, and TEMPO/tPA groups, respectively.

Discussion

In the present study we found that the spin trap agent PBN had differential effects on hemorrhage rate. PBN administration after embolization significantly increased hemorrhage rate, whereas TEMPO did not affect basal hemorrhage rate. However, if PBN was administered before tPA administration, the spin trap agent attenuated tPA-induced hemorrhage. Thus, the effects of PBN appear to be dependent on whether or not the embolized rabbits had previously received tPA.

The observation that a spin trap agent affects hemorrhage rate is in agreement with previous studies.37,39 Of importance to our study are the findings of Asahi et al.37 The researchers showed that PBN effectively decreased tPA-induced hemorrhage in a rat embolic focal cerebral ischemia model,37 an effect that they hypothesize to be mediated in part by the antioxidative actions of PBN. Moreover, Hu et al39 showed that MDL 101,002, a conformationally constrained cyclic
analogue of PBN, reduced hemorrhage rate after an embolism. However, in that study hemorrhage rate in the absence of treatment was between 55% and 77% when measured up to 72 hours after embolization. In our study basal hemorrhage rate was approximately 24% in vehicle control animals. This is in agreement with previous studies References 42 and D. Chapman, P. Lyden, P.A. Lapchak, S. Nunez, H. Thibodeaux, and J. Zivin, unpublished data, 2000). The difference in hemorrhage rate in vehicle-treated controls is most likely due to the use of a different embolism protocol. First, in the present study we used a large clot embolus (approximately 3.5 mg), whereas Hu et al used a small clot embolus (approximately 1.5 mg). Second, Hu et al simultaneously injected 2 clots, whereas in our study only a single clot was injected. If there was no behavioral response to the first clot, we then administered the second clot using a 3-minute interval. The differences in hemorrhage rate between the 2 studies may be related to the blood pressure effects of 2 simultaneous clot injections. In agreement with this hypothesis is the work of Asahi et al. The authors also suggest that blood pressure is an important correlate of tPA-induced hemorrhage. Alternatively, the difference in hemorrhage rate may be associated with the use of a small clot embolus that may have access to smaller vessels than the large clot embolus that we used in the present study.

The beneficial effect of PBN to reduce hemorrhage in tPA-treated rabbits may be due to one of a variety of mechanisms. PBN is reported to ameliorate secondary mitochondrial function, and it has been suggested that PBN may scavenge free radicals at the blood-endothelial cell interface. The spin trap agent may exert its effects by reducing microvascular dysfunction indirectly caused by polymorphonuclear leukocytes that give rise to free radicals. It is conceivable that free radicals are directly responsible for endothelial weakening and damage, resulting in increased hemorrhage.

Even though PBN increased hemorrhage rate when infused 5 minutes after embolization, it significantly reduced tPA-induced hemorrhage when tPA was administered 60 minutes after embolization. The reason for a PBN-induced increase in hemorrhage rate after a stroke is unclear. It is entirely possible that PBN may differentially affect one or more of the radicals, receptors, enzymes, or proteins that have previously been shown to be affected by PBN, resulting in hemorrhage. Our result showing that PBN reduces tPA-induced hemorrhage suggests that PBN effectively scavenges free radicals produced during and after an ischemic stroke. In contrast, TEMPO did not affect basal hemorrhage rate. The difference of drug effects may be related to the structure of the molecules and their ability to differentially affect multiple biochemical processes, in addition to their free radical scavenging activity. However, since we used only a single dose of the spin trap agents in this study, it is possible that the difference is due to drug dosing. Furthermore, some of the effects of PBN that we observed might be related to the observation that PBN decomposes to nitric oxide in aqueous solution via the intermediate compound tert-nitrosobutane. The end product of decomposed PBN, nitric oxide, has the ability to activate a variety of cellular mechanisms in brain. Further pharmacological studies are required to elucidate the mechanism(s) of action of PBN in the absence or presence of thrombolytic therapy.

In contrast to the information regarding PBN, there is a smaller literature base on the pharmacology of TEMPO. It is known that TEMPO is a cell-permeable nitroxide spin trap reputed to be a “superoxide dismutase” mimetic. TEMPO is postulated to be an electron acceptor and is reported to trap electrons from nitroxyl, hydroxyl, and superoxide radicals. The observation that TEMPO reduced tPA-induced hemorrhage may be due to the fact that TEMPO appears to trap hydroxyl and superoxide radicals, the 2 main free radicals linked to membrane damage. However, it is possible that the beneficial effects of PBN and TEMPO may be due not only to their scavenging activity but to other pharmacological activities detailed in the introduction.

In the present study we tested PBN and TEMPO at relatively high doses (100 mg/kg) to determine whether either compound was pharmacologically active against hemorrhage or infarcts in our thromboembolic stroke model. A pharmacokinetic study from Chen et al previously showed that a peripheral injection of PBN is evenly distributed among a wide range of tissues, and PBN is slowly excreted by the body. Chen et al indicate that 70% of a bolus dose of PBN is excreted by the first 3 days. In our study, after a dose of 100 mg/kg, we did not observe any behavioral signs of “toxicity.” Nevertheless, previous studies have shown that PBN can induce seizures, impair respiration, and result in abnormal blood chemistry and tissue damage when administered at a dose 10 times higher than that used in our study. Moreover, nitroxides such as TEMPO can induce hyperkinetic activity, hypoxia, seizure activity, and restlessness indicating that nitroxides can be neurotoxic. Hahn et al found that TEMPO was directly active on central nervous system neurons, where it increased spiking activity in the hippocampus. Taken together, the studies cited above and our finding that PBN increases hemorrhage rate in the absence of thrombolytic therapy indicate that under certain conditions spin trap compounds can produce adverse effects. Further in-depth preclinical testing of spin trap compounds in appropriate animals models is necessary before the compounds are used clinically.

In conclusion, we have shown that effective combination drug treatments can be developed as novel treatments for stroke. Preadministration of the spin trap agent PBN significantly reduced tPA-induced hemorrhage. However, the compound did not significantly alter infarct size or rate. Our study suggests that certain spin trap molecules may improve the safety of tPA by reducing hemorrhage. However, the development of new spin trap agents should be approached with caution, since under certain circumstances they may exacerbate the damage caused by a thromboembolic stroke.

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