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Antioxidative and thrombolytic TMP nitrone for treatment of ischemic stroke

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ABSTRACT

Ischemic stroke results from brain blood vessel blockage by thrombus, and produces neuronal cell damage and death. While thrombolytic therapy with tPA has achieved some success in clinic, the strategy of using neuroprotective agents to treat ischemic stroke has been disappointing thus far. In the present work, we synthesized TBN, a derivative of the clinically useful stroke drug TMP armed with a powerful free radical-scavenging nitrone moiety. TBN retains the thrombolytic activity of the parent TMP and possesses strong antioxidative properties. TBN demonstrates significant activity in the rat MCAo stroke model. The results suggest that design of molecules possessing both thrombolytic and neuroprotective properties may be a novel strategy for effective stroke therapeutics.

1. Introduction

Stroke is one of the most devastating diseases after heart disease and cancer in developed countries. Despite the remarkable progress achieved in the last two decades in understanding the pathophysiology of stroke, tissue-type plasminogen activator (tPA) remains the only therapy approved by the US FDA for acute ischemic stroke, which accounts for 70–85% of all stroke patients and carries a 15–33% mortality rate.

Brain tissues rely on circulating blood to deliver oxygen and other nutrients, as well as remove metabolic wastes. When a blood clot (thrombus) forms in a brain blood vessel, the delivery system to the brain may be compromised, resulting in an ischemic stroke. Therefore, the first task of ischemic stroke therapy is to remove/dissolve the blood clot, that is, thrombolytic therapy. tPA activates zymogen plasminogen into plasmin, resulting in thrombolysis. However, tPA has a narrow therapeutic window of 3 h within the occurrence of a stroke, limiting its clinical use.

An ischemic stroke results in a cascade of biochemical events producing profound cellular changes. These include a rapid decrease in ATP, calcium overload, disruption of various ion pumps, and excitotoxic changes resulting from glutamate release, acidosis, and edema.[1–3] Many of these changes are associated with increased free radical production (mostly reactive oxygen species, ROS), occurring both during ischemia and during the subsequent reperfusion stage. ROS, which have short half-lives, can be extremely detrimental to the surrounding tissue. Normal tissues have a defense system against these toxic ROS; however, ischemia either interrupts or overwhelms the protective mechanisms and allows increased ROS production in the surrounding tissues, leading to neuronal cell damage/death. For this reason, thrombolytic therapy alone is not enough to cure an ischemic stroke. Therefore, therapeutics which reduce the damage caused by ROS are needed.

Emerging treatments for acute ischemic stroke include use of thrombolytic and neuroprotective agents.[4] While thrombolytic treatments lyse blood clots to restore blood flow, neuroprotective treatments prevent cell death during and after ischemia and reperfusion. One of the most extensively studied classes of neuroprotective agents is the free radical-scavenging nitrone. Nitrones react with free radicals to form nitroxides, which act as superoxide dismutases (SOD), mimicking and catalyzing the dismutation of superoxide anions,[5–7] thereby protecting cells from free radical-mediated cell damage.

Nitrones were originally developed as free radical-trapping agents in free radical chemistry. Two decades later, it was realized that nitrones could protect biological systems from oxidative stress. Nitrones have been tested as therapeutic agents for neural and systemic dysfunctions including atherosclerosis, septicemia, stroke, and Alzheimer's disease.[8,9] For example, phenyl *tert*-butyl nitrone (PBN) was initially shown to ameliorate ischemic brain damage in a rodent model (Fig. 1).[10–12] Later experiments demonstrated that PBN markedly reduced infarct volumes in rats subjected to long periods of focal ischemia induced by middle cerebral artery (MCA) occlusion.[13] PBN was found to be effective when administered 5 h after onset of ischemia. The nitrone NXY-059 (disodium 4-[(*tert*-butylimino)methyl] benzene-1,3-disulfo-

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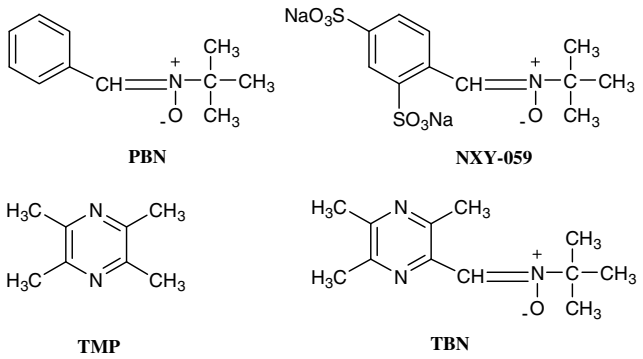


Figure 1. Structures of PBN, NXY-059, TMP and TBN.

nate *N*-oxide, Fig. 1) was shown to significantly reduce infarct volumes in animal stroke models.[14,15] Based on the impressive preclinical data, NXY-059 was evaluated in two phase III clinical trials.[16,17] Unfortunately, the SAINT II trial conducted in about 350 centers worldwide across approximately 30 countries failed to reveal any positive effects in ischemic stroke patients.[17] Although the clinical results of NXY-059 are disappointing, the concept of using neuroprotective agents for stroke therapy remains viable. For example, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, Eda), also a free radical scavenger, has been approved for treatment of patients with acute ischemic stroke in Japan[18] and China.

Based on our current knowledge of the pathophysiology of ischemic stroke, the extensive research done on thrombolytic and free radical-scavenging agents, and the shift from single thrombolytic or neuroprotective therapy toward combined therapy, we reason that a compound possessing both the thrombolytic and free radical-scavenging activity will be more efficacious than either a thrombolytic or free radical-scavenging agent alone. To our knowledge, such a dual-functional agent has not been reported in the past.

In China, *Ligusticum wallichii* Franchat (Chuan Xiong) and its main active ingredient 2,3,5,6-tetramethylpyrazine (TMP, Fig. 1) have been used for treatment of ischemic stroke for many years.[19] Although the exact mechanism(s) of action has/have not been completely understood, a variety of mechanisms has been attributed to TMPs beneficial effects in stroke patients. TMP was found to inhibit platelet aggregation[20,21], lyse blood clots,[21] block calcium entry[22,23] and scavenge ROS.[24,25] TMP has demonstrated significant activity in the animal stroke model.[26] Herein we describe the design, synthesis, and evaluation of a novel TMP nitrone, which retains the thrombolytic activity of its parent TMP while additionally armed with a powerful free radical-scavenging nitrone functionality.

2. Results

2.1. Drug design and chemical synthesis

Our goal is to design a compound possessing both the thrombolytic and free radical-scavenging activities necessary for effective stroke treatment. In addition, the compound must penetrate the blood brain barrier (BBB) readily, an important requirement for a stroke drug. Furthermore, the compound must be non-toxic at therapeutic doses. With these criteria in mind, we chose to add further free radical-scavenging ability to TMP, already an anti-stroke drug clinically used in China for many years with multiple mechanisms of action. In addition to inhibiting platelet aggregation and lysing blood clots, TMP is stable, has good aqueous solubility, and penetrates the BBB readily. The reasons to use a nitrone as the added free radical-scavenging functionality are obvious. First of all, a nitrone has powerful free radical-trapping activity; secondly, a nitrone is relatively non-toxic; finally, it also readily crosses the BBB. Thus, a TMP-nitrone conjugate satisfies all of the designing criteria.

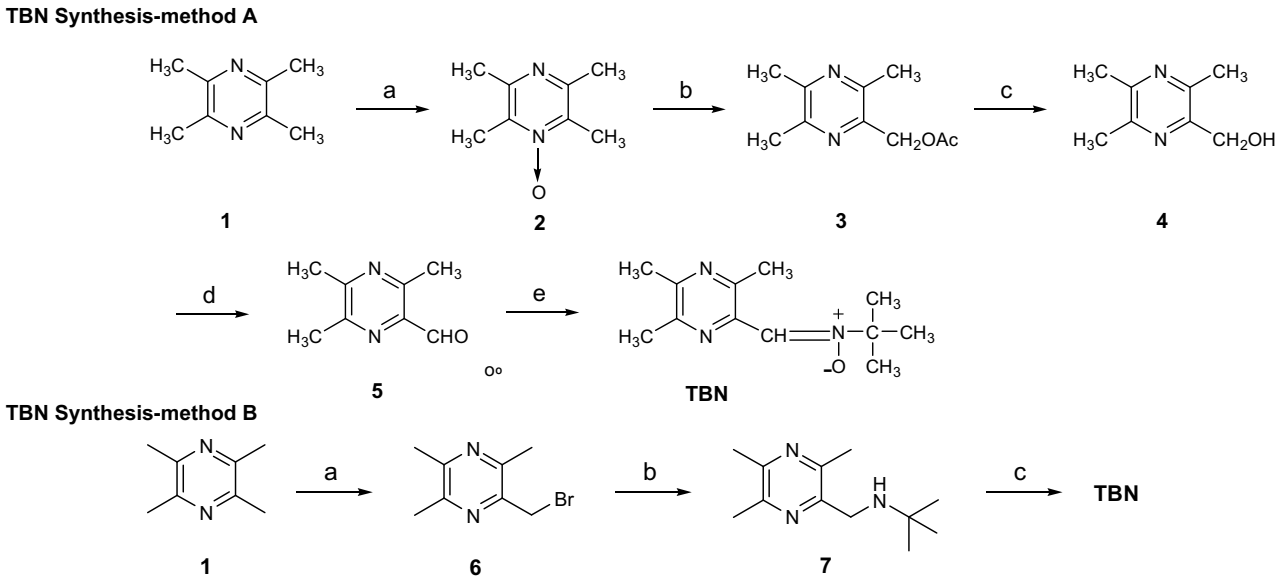
The target molecule, 2-[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN, Fig. 1), was synthesized by two methods (methods A and B, Scheme 1). In method A, the known 2-hydroxymethyl-3,5,6-trimethylpyrazine, **4**, synthesized from TMP,[27] was oxidized by MnO_2 to aldehyde **5**.[28] The latter was treated with *tert*-butyl hydroxylamine to produce TBN with an overall yield of 17.7% from TMP.[29] A short synthesis route to TBN was used in method B, where 2-bromomethyl-3,5,6-trimethylpyrazine, **6**,[30] was treated with *tert*-butylamine to afford **7**. The latter was then oxidized using H_2O_2 catalyzed by Na_2WO_4, affording TBN with an overall yield of 20.2% from TMP.[31]

2.2. Antioxidative activity assay against ROS-induced lipid peroxidation

TBN is designed to possess both the antioxidative and thrombolytic activities. We first measured its antioxidative activity. Numerous methods have been developed to measure antioxidative effects. One is the thiobarbituric acid reactive substance (TBARS) assay. Oxidation of polyunsaturated fatty acids in biological membranes often leads to the formation and propagation of lipid radicals, uptake of oxygen, and even destruction of membrane lipids. This process can lead to the production of breakdown products, mostly malondialdehyde (MDA), which are highly toxic to mammalian cells.[32] MDA reacts with thiobarbituric acid to form a fluorescent red adduct which can be measured by a spectrophotometer. The TBARS assay measures a compound's protective effect against the damaging lipid peroxidation produced by ROS rather than by a direct reaction with ROS. This method is widely used in studies of antioxidative activity of natural products in urine, plasma, and tissue homogenates. To determine the antioxidative activity of TBN, we conducted a modified TBARS assay, using linoleic acid as an oxidative substrate instead of a more commonly used biological sample.[33] At 1 mM, the antioxidative activity of TBN is similar to PBNs, but is much stronger than that of the parent TMP (Table 1). However, at 2 mM, the antioxidative activity of TBN is not only much stronger than that of TMP, but also significantly stronger than that of PBN. These results demonstrated that our goal of the drug design, that is, arming TMP with a powerful nitrone moiety, was realized. The clinically used stroke therapeutic Eda also demonstrated significant antioxidative activity in this assay. Vitamin C (Vc) was used as a positive control, which showed the strongest antioxidative activity.

2.3. TBN inhibited H_2O_2-induced cortical neuronal cell injury

After demonstrating TBNs antioxidative activity by the TBARS assay, we then examined its protective effects against H_2O_2-induced damage in cortical neuronal cells. Newborn rat cortical neurons were cultured and treated with various drugs 30 min prior to addition of H_2O_2. After 24 h of incubation, cell viability was determined (Fig. 2). Hydrogen peroxide at 0.5 mM killed approximately 40% of neuronal cells. Neither TMP nor PBN offered any protection against the H_2O_2-induced damage up to 1 mM. In sharp contrast, TBN began to show a protective effect at 10 μM and had a very good dose-response curve from 1 to 1000 μM. At 1 mM, 96 ± 3.78% of cells treated by TBN survived. Eda began to show a protective effect at 100 μM, and at 1 mM, it protected 82 ± 5.12% cells from death. The data demonstrate



Scheme 1. Synthesis of TBN. TBN synthesis-method A. Reagents and conditions: (a) AcOH, 30% H_2O_2, 70 °C, 10 h; (b) Ac_2O, reflux, 2 h ; (c) 20% NaOH, 2 h, 45% from 1; (d) MnO_2, C_2H_5OH, reflux, 2 h, 100%; (e) *tert*-butyl hydroxylamine, reflux, 39.3%. TBN synthesis-method B. Reagents and conditions: (a) NBS, benzoyl peroxide, 70 °C, 10 h, 52%; (b) *tert*-butylamine, 2–4 h, 100%; (c) Na_2WO_4, 30% H_2O_2, 2 h, 38.9%.

that TBN is at least 100-fold more effective than either TMP or PBN, and approximately 10-fold more effective than Eda in protecting cortical neuronal cells from H_2O_2-induced damage. These results further confirm that our goal of arming TMP with a powerful antioxidative nitrone moiety was achieved.

2.4. TBN inhibited ADP-induced platelet aggregation

Previously, TMP was shown to inhibit platelet aggregation in vitro.[20,21] To determine the activity for inhibition of platelet aggregation, TBN was tested in a adenosine-5′-diphosphate (ADP)-induced rabbit platelet aggregation assay in vitro following the turbidimetric method[34] with a Platelet-Aggregometer. The results are shown in Figure 3. At 2 mM, the percentages of platelet aggregation treated with Aspirin, PBN, TMP, TBN, and Eda were 24.8 ± 2.2, 33.9 ± 3.1, 36.2 ± 2.9, 30.0 ± 3.0 and 32.4 ± 3.3, respectively, while that of the sample treated with saline was 40.0 ± 3.08. Once again, TBN was more active than either TMP or PBN in inhibiting platelet aggregation. TBN was also more active than Eda (30.0% vs 32.4%).

Table 1
Protective effect against linoleic acid peroxidation*

Compound	% Inhibition of peroxidation	
	1 mM	2 mM
TMP	6.6 ± 0.01	9.0 ± 0.01
PBN	15.8 ± 0.04	25.1 ± 0.02
TBN	13.7 ± 0.02	40.0 ± 0.01
Eda	37.9 ± 0.02	43.7 ± 0.01
Vc	43.3 ± 0.01	53.2 ± 0.03

* Reaction proceeded for 15 min at 100 °C, and absorbance was determined at a wavelength of 532 nm. Data were processed statistically by a single-tail Student's *t*-test.

2.5. Antithrombotic activity determination in a rat model

After demonstrating that TBN inhibited ADP-induced platelet aggregation, we used a rat venous thrombus model to demonstrate its thrombolytic activity. TMP has shown significant thrombolytic activity in the same model.[21] In this model, TBN showed significant thrombolytic activity with a 57.61% inhibition of thrombus formation at a dose of 3.25 mg/kg (Fig. 4). TMP also demonstrated significant thrombolytic activity with a 71.2% inhibition of thrombus formation at 2 mg/kg, an equal molar dose to 3.25 mg/kg of TBN. This data is consistent with what has been reported on the thrombolytic activity of TMP, where TMP inhibited thrombus formation by approximately 50% at 1.2 mg/kg.[21]

2.6. Evaluation of protective effect in a rat middle cerebral artery occlusion (MCAo) stroke model

The rat model of transient focal cerebral ischemia produced by intraluminal occlusion of the middle cerebral artery (MCAo)

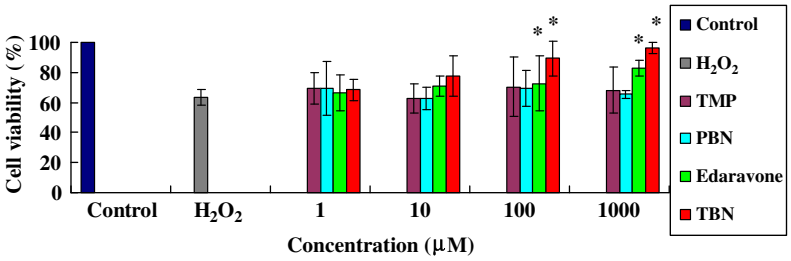


Figure 2. Protective effect against H_2O_2-induced damage in neuronal cells. Cells were treated with 0.5 mM H_2O_2 for 24 h. The results are expressed as the percentage of that of the untreated cells. Data were expressed as means ± SEM of three independent experiments. Data were processed statistically by a single-tail Student's *t*-test. $^*P < 0.05$ compared to H_2O_2 group.

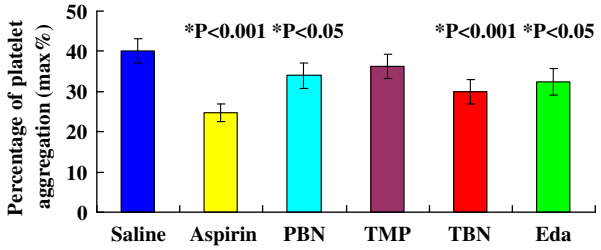


Figure 3. Prevention of platelet aggregation in vitro. Rabbit platelet suspensions were preincubated with drugs (2 mM) at 37 °C for 1 min followed by the addition of ADP (10 μM). The extent of aggregation was expressed as the percentage of the control (in the absence of drugs). Data were processed statistically by a single-tail Student's *t*-test. *Compare with the saline treated group.

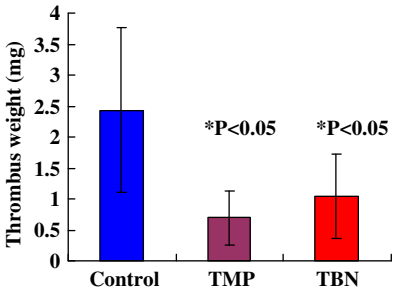


Figure 4. Thrombolytic activity in rats. Drug dose: TMP (2 mg/kg); TBN (3.25 mg/kg). The molar doses of TMP and TBN were the same. Data were processed statistically by a single-tail Student's *t*-test. Values represented means of 6 animals/group. *$P < 0.05$ versus control.

for 2 h, followed by recirculation has been widely used to evaluate protective effects of anti-stroke agents.[35] In the present work, the drugs were administered ip 1 h after the MCA occlusion, and the animals were sacrificed after 24 h of reperfusion. Morphometry and image-analysis of the brain revealed that TBN at 80 mg/kg reduced the total infarct area by 56.3% compared to the vehicle-treated controls (Fig. 5). In contrast, at equal molar doses, TMP (50 mg/kg) and PBN (65 mg/kg) only reduced the infarct area by 46.3%, and 31.1% respectively. TBN was equal to or even better than Eda in this model (56.3% vs 52.5% protection). These data showed that TBN had significant activity in protecting ischemia-induced brain damage, and had a much higher therapeutic efficacy than either TMP or PBN in the animal model.

3. Discussion

Ischemic stroke results from a blockage of blood supply to the brain by either an in situ clot (thrombus) formed inside the brain vessels or the migration of a peripheral clot (embolus) to the brain followed by an ischemic cascade of events including glutamate release and calcium overload, leading to the production of ROS. Brain tissue ceases to function if deprived of oxygen for more than 60–90 s and after a few hours will suffer irreversible injury, possibly cell death. For this reason, the first task for ischemic stroke treatment is to use thrombolytic and anticoagulant drugs to remove the blood clot, that is, thrombolysis, restoring blood circulation quickly. The lone thrombolytic drug approved in the US is tPA, which is used within 3 h of the occurrence of a stroke. However, clinical and experimental data indicate that the benefits of treatment with tPA is limited by its narrow therapeutic window. As a result, less than 5% of stroke patients may benefit from tPA treatment.[36]

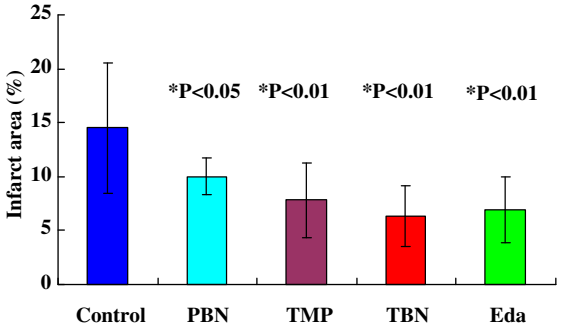


Figure 5. Protective effect in the rat MCAo stroke model. Rats were subjected to 2 h ischemia followed by 24 h of reperfusion. Brain infarction was determined by TTC stain. The percentage of infarct area in two hemispheres was calculated. Drug dose: PBN (65 mg/kg); TMP (50 mg/kg); TBN (80 mg/kg); Eda (62 mg/kg). No. of animals: PBN ($n = 3$); TMP ($n = 8$); TBN ($n = 13$); Eda ($n = 6$); Control ($n = 17$). All drugs were in equal molar doses. Data were processed statistically by a single-tail Student's *t*-test. *Compared to the control.

In the region of the brain affected by ischemia, neuronal cells in the ischemic core may die (by necrosis) but those in the other parts (penumbra) may be salvaged. Thrombolytic treatment is not intended to salvage injured neurons in the penumbra. Neuroprotective agents, used after thrombolytic treatment, are thus needed to accomplish this task. Various kinds of neuroprotective agents including glutamate antagonists, free radical scavenger-antioxidants, calcium chelators, calcium channel blockers, and GABA antagonists have been developed and tested.[37] For example, tirilazad, a lipid peroxidation inhibitor, showed beneficial effects in mortality and behavior in stroke patients.[38] Ebselen, a seleno-organic compound with antioxidant activity, significantly reduced infarct volume and improved functional outcome in acute stroke patients.[39] NXY-059, a free radical-trapping agent, showed significant effects in various animal acute ischemic stroke models, although ultimately failed in the second Phase III trial.[40] Edaravone, an antioxidant, has been approved as a stroke treatment in Japan and China (EAISG, 2003). Unfortunately, Edaravone was approved only in a few countries because of its potentially damaging effects to kidney and liver.[41] Despite the testing of more than 1000 candidates, neuroprotective agents are still rarely successful.[42]

Therapy with a combination of thrombolytic and neuroprotective agents is a rational approach based on our understanding of ischemic stroke pathophysiology. Indeed, this concept has been tried, and meaningful benefits have been observed in experimental animal models. However, these beneficial effects have not yet been translated into positive clinical results. For example, therapy combining neuroprotective agents such as the free radical scavenger Tirilazad,[43] AMPA antagonists (NBQX),[44] and NMDA antagonists (dizocilpine),[45] with tPA, extended the time window and enhanced the effect of thrombolysis after stroke.

There is no doubt that stroke is a tough disease to treat; however, great scientific progress has been achieved during the last two decades. We now have a better understanding of ischemic stroke pathophysiology than ever before. In a recent report, Young et al. pointed that stroke should be considered as a cerebral disease rather than merely a degeneration of neurons.[4] Thus, neurons and all other brain cells including astrocytes, oligodendrocytes, microglia, etc. need to be protected. To find an effective treatment for ischemic stroke beyond the current insufficient therapy, a novel compound or combination of compounds with multiple mechanisms of action needs to be developed.

TBN showed greater inhibition than either TMP or PBN against lipid peroxidation in the TBARS assay, which measured the decomposition products of lipid peroxidation by ROS, especially alkoxy

(RO•), and peroxy radicals (ROO•). In the MCAo stroke model, TMP at 20 mg/kg ip did not show activity in the TBARS assay.[46] The discrepancy between the results of these experiments may lie on the fact that we used a much higher concentration of 1 and 2 mM, but in animals, it is unlikely that 20 mg/kg of TMP will produce a 1 mM concentration in the plasma. Furthermore, we used pure linoleic acid as a substrate, which is much different than the substrates in the animal tissues. In the TBARS assay of liver and kidney tissues in streptozotocin-induced diabetic mice, TMP showed significant activity and significantly reduced the degree of lipoperoxidation at doses of 10, 25 and 50 mg/kg.[47] The purpose of using the TBARS assay was to compare the antioxidative activities of TMP and TBN to see if TBN has a stronger activity. The fact that the activity of TBN is much greater than that of TMP, but is approximately equal to that of PBN in this TBARS assay, suggests that the nitrone moiety accounts for a major part of TBNs anti-lipid peroxidation activity. These results validated the design rationale.

TMP has been shown to protect against H_2O_2-induced damages in cultured rat cortical neurons,[48] cultured PC12 cells,[25] cultured rat retinal cells,[49] and endothelial cells.[50,51] PBN has been shown to protect cultured neuronal cells from damages induced by different agents.[52–54] In the present work, it is a surprise to find that TBN is not only more effective than either TMP or PBN in protecting cortical neuronal cells from H_2O_2-induced damage, but at least 100-fold more effective than either of its parent compounds. The reason for TBNs superior protective effect is not clear at present. In the anti-lipid peroxidation TBARS assay, Eda was more potent than TBN at both 1 and 2 mM. However, TBN was approximately 10-fold more effective than Eda in protecting cortical neuronal cells from H_2O_2-induced damage. These data suggest that TBN acts by mechanisms involving more than just ROS-scavenging.

Platelets become activated and accumulate in cerebral microvessels of the ischemic penumbra after focal cerebral ischemia.[55–58] Platelet activation contributes significantly to ischemic microvascular occlusion and causes secondary injury after the initial ischemic insult. Thus, inhibition of platelet aggregation in the cerebral microvessels is important. We demonstrated that TBN is significantly more potent (Fig. 3) than TMP in inhibiting platelet aggregation, and is also more potent than the free radical scavengers PBN and Eda. Previously, TMP was found to inhibit platelet aggregation via different mechanisms.[20,59–61] Our experimental results showed that TBN retains and even improves the anti-platelet aggregation property of TMP, beneficial for treatment of ischemic stroke.

TBN is designed based on TMP, a widely used treatment for ischemic stroke in China with multiple mechanisms of action, and PBN, a well-known free radical scavenger. Like TMP, TBN significantly reduced the thrombus size in experimental animals at a very low dose of 3.25 mg/kg. Thrombolysis restores blood circulation in the damaged brain, the foremost step in ischemic stroke therapy. Consistent with our present findings, TMP has also been shown by others to have strong thrombolytic activity in animal stroke models.[21,61] It was reported that PBN had no thrombolytic activity in a rabbit thromboembolic stroke model administered at a dose of 100 mg/kg iv by infusion over 30 min while tPA showed significant activity[62]; thus, it is likely that the thrombolytic activity of TBN is attributable to the TMP part of the molecule. The results show that TBN protects against ROS-induced lipid peroxidation and neuronal cell injury, and at the same time, possesses thrombolytic activity. As proven, TBN is dual-functional. To our knowledge, this is the first anti-stroke drug with both capabilities ever reported.

TMP has other properties beneficial for stroke treatment as well. For example, TMP suppressed oxidative stress and attenuated neuronal cell death in cultures induced by the excitotoxicity of glutamate.[63,64] In the cultured glioma cells, 50 μM TMP significantly inhibited glutamate-induced increase in intracellular calcium.[65] It is known that ischemic stroke triggers glutamate release and calcium overload, leading to over production of ROS.[1–3] TMP reduced arterial resistance and increased cerebral blood flow,[66] and improved microcirculation.[67] In an *N*-formylmethionylleucyl-phenylalanine-stimulated PMN respiratory burst model, TMP exhibited significant activity in scavenging ROS produced by the induction of NADPH oxidase.[68] TBN may retain or even improve upon these beneficial properties, which remains to be demonstrated experimentally.

The MCAo focal ischemic stroke model is a widely used animal model that evaluates anti-stroke agents; however, this model is suitable to evaluate an agent's neuroprotective effect or neuron-salvaging capability, not its antithrombotic or thrombolytic activity since the cerebral artery occlusion is induced by a suture, not a blood clot. Therefore, the activity that TBN demonstrated in this MCAo model is most likely a reflection of its neuroprotective property. To evaluate the full potential of TBN as an anti-stroke agent, a better animal model is needed. Nevertheless, this model serves as a preliminary tool for evaluation of TBNs beneficial effects as a stroke therapy. Our study showed that TBN significantly decreased the focal infarct area in MCAo rats, and more importantly, was also much more efficacious than either TMP or PBN. Furthermore, TBN was better than or equal to Eda in reducing the focal infarct area. These results are in agreement with what we have found in vitro, that TBN is much more potent and effective in protecting primary cortical neurons from H_2O_2-induced damage than TMP, PBN and Eda, suggesting that the antithrombotic/thrombolytic activity of the TMP moiety of TBN provides additional therapeutic benefits against ischemic stroke. It is worthy to point out that both TMP and Eda are currently used stroke therapeutics in clinic.

4. Conclusion

We have synthesized a potential new stroke therapeutic TBN, and demonstrated that this novel compound possesses both antithrombotic/thrombolytic and neuroprotective properties. Further studies of TBNs mechanisms of action and anti-stroke profile, such as the dose-response relationship, schedule of administration, therapeutic window and toxicity are warranted. The findings of the present work support a novel strategy that the design of molecules possessing both antithrombotic/thrombolytic and neuroprotective properties may provide effective stroke therapeutics.

5. Experimental

5.1. Chemistry

Melting points were measured using a Mel-Temp (X_7L_{20}, Beijing) and are uncorrected. ^{1}H NMR spectra were recorded at ambient temperature on a 400 MHz spectrometer (AV-400, Bruker) in $CDCl_3$ or DMSO-d_6. Electrospray ionization mass spectra (ESI-MS) were obtained in the positive ion detection mode on a Finnigan LCQ Advantage MAX mass spectrometer (Applied Biosystems, 4000 Q TRAP). Elemental analysis was performed at the Experimental Center, Jinan University, Guangzhou, China, and the results were within ±0.4% of the theoretical values unless otherwise noted.

5.1.1. 2-[[(1,1-Dimethylethyl)oxidoimino]methyl]-3,5,6-trimethylpyrazine (TBN)

5.1.1.1. Method A. To aldehyde **5** (1.9 g, 0.013 mol) in methanol (200 mL) was added *tert*-butylhydroxylamine (1 g, 0.011 mol), and the solution was refluxed for 2 h. Another portion of *tert*-butyl hydroxylamine (1 g, 0.011 mol) was then added, and the solution

refluxed until aldehyde **5** was completely reacted. Solvent was removed in vacuo, and the product was extracted. The solution was dried with Na_2SO_4, and solvent removed in vacuo. The product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:1, v/v), to produce TBN as a light yellow solid (1.1 g, 39.3% yield), mp: 68–70 °C. ^{1}H NMR ($CDCl_3$, ppm): 7.82 (s, 1H), 2.47 (s, 3H), 2.50 (s, 3H), 2.52 (s, 3H), 1.63 (s, 9H). ESI-MS: 222 $[M+H]^+$, 244 $[M+Na]^+$. Anal. ($C_{12}H_{19}N_3O$) C, H, N.

5.1.1.2. Method B. To compound **6** (1.0 g, 0.005 mol) in methanol (100 mL) was added $Na_2WO_4{\cdot}2H_2O$ (0.4 g) and 30% H_2O_2 (2.5 mL), and the reaction mixture was stirred at room temperature for 2 h. The product was filtered, and solvent removed in vacuo. To the residue was added saturated $Na_2S_2O_3$ solution (8 mL). The product was extracted with ethyl acetate (3× 25 mL), and the solution was dried with Na_2SO_4. Solvent was removed in vacuo, and the product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:1, v/v), to produce TBN as a light yellow solid (0.4 g, 38.9% yield).

5.2. TBARS lipid peroxidation assay

Linoleic acid (1.5% in ethanol) was diluted with twofold of PBS (v/v, 200 mM, pH 7.4). To a test tube was added 1 mL of linoleic acid suspension, 0.2 mL of 2.5 mM $FeSO_4$ and the test sample. PBS was added to make a total volume of 2 mL. The suspension was incubated in a water bath at 37 °C for 30 min. The reaction was stopped by addition of 1 mL of 10% trichloroacetic acid (TCA) and 1.5 mL of 0.67% thiobarbituric acid (TBA). The test tube was then placed into a boiling water bath for 15 min. After cooling, the tube was centrifuged at 10,000 rpm/min for 10 min. The malondialdehyde–TBA complex with a pink color in the supernatant was detected by its absorbance at a wavelength of 532 nm, using a TU-1810S spectrophotometer (Beijing General Equipment Inc., Beijing). The percentage of inhibition ratio was calculated using the following formula: % Inhibition ratio = $[(A - A1)/A] \times 100$, where A was the absorbance of the control and $A1$ was the absorbance of test samples.

5.3. In vitro neuron protection assay

Newborn (1–3 day old) Sprague–Dawley rats were anesthetized with 10% chloral hydrate (400 mg/kg) ip Cortex was removed and placed into a vial containing 3 mL of HBBS. The tissue was cut into pieces, and 0.25% pepsin (3 mL) was added. The tissues were digested at 37 °C for 15 min under 5% CO_2. DMEM supplemented with 10% FBS was added, and the tissues were filtered. The filtrate was centrifuged for 5 min at 1000 rpm/min. The supernatant was removed, and medium was added.

Cortical neurons (9×10^4 cells/well) were placed into 96-well cell culture plates, and were incubated at 37 °C for 24 h under 5% CO_2. The medium was changed, and the cells were incubated for another 12 h. Drugs at different concentrations were added, and the cells were incubated at 37 °C for 30 min. Hydrogen peroxide were then added, and the cells were incubated for 24 h at 37 °C for 24 h under 5% CO_2. A solution of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl-tetrazoliun bromide (MTT) was added, and the cells were incubated for another 4 h before DMSO was added. After the crystals were completely dissolved (30 min), the absorbance was read at 570 nm with a spectrophotometer (Bio-Rad Model 680, Japan). The results were expressed as the percentage of the control (saline group).

5.4. Platelet aggregation assay

The turbidimetric method (Born and Cross, 1963) was used to measure platelet aggregation.[34] Blood drawn from New Zealand rabbits was placed into sterile tubes, and 3.8% buffered sodium citrate (v/v, 9:1) was added. Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 800 rpm/min for 5 min. PRP (0.3 mL) was pre-warmed at 37 °C for 1 min, and drugs were added. The final drug concentration was 2 mM. After 1 min of incubation with continuous stirring, ADP (10 μM) was added to induce aggregation. The reaction was allowed to proceed for 4 min. Light transmission through the platelet suspension was monitored by a Platelet-Aggregometer (Se-2000 Platelet-Aggregometer, Beijing) to measure platelet aggregation. Maximum change in light transmission was used as the aggregation endpoint for potency comparisons. The extent of aggregation was expressed as the percentage of the control (in the absence of drug).

5.5. Thrombolytic activity assay in rats

The thrombolytic activity of TBN was determined following a published procedure with modifications.[21] Sprague–Dawley rats (180–210 g, Sun Yat-Sun University Experimental Animal Center) were anesthetized with 10% chloral hydrate (400 mg/kg) ip. The inferior vena cava was isolated and a tight ligature was applied below the left renal vein branch. The abdomen was then closed. Drugs were administered 2 h after ligation iv via the dorsal tail vein. The abdomen was reopened after 1 h of ligation. The thrombus was removed and dried at 50 °C for 24 h. The dried thrombus was weighted. The percentage of thrombolysis was calculated using the following formula: thrombolysis (%) = $[(A - A1)/A] \times 100$, where A is the weight of the control and $A1$ is weight of drug-treated group. Due to large differences among experimental results, the surgery, drug administration and thrombus-weighting were assigned to different people, who were blinded to each other's work.

5.6. Middle cerebral artery occlusion (MCAo) stroke model

The proximal middle cerebral artery (MCA) was transiently occluded for 2 h by the widely used intraluminal-suture occlusion model.[35,69] Female Sprague–Dawley rats (210–240 g, Sun Yat-Sun University Experimental Animal Center) were anesthetized with 10% chloral hydrate (400 mg/kg) ip. The right common carotid artery (CCA) was carefully exposed. The occipital branch of the external carotid artery (ECA) was coagulated and the internal carotid artery (ICA) was isolated. A poly-L-lysine-coated nylon suture (0.32 mm in diameter) was inserted into the ECA and advanced retrogradely until the bifurcation of the CCA from where it was advanced a distance of approximately 18–20 mm into the ICA to occlude the MCA. A ligature was tied around the ICA, the incisions were closed, and the animal was extubated.

TMP (50 mg/kg), TBN (80 mg/kg), PBN (65 mg/kg), Eda (62 mg/kg) and saline were administered ip 1 h after MCA occlusion, respectively. After 2 h of MCA occlusion, the suture was withdrawn to allow reperfusion. After 24 h of reperfusion, the animals were re-anesthetized with 10% chloral hydrate (400 mg/kg) ip, and were then decapitated. The brain was quickly removed, rinsed with PBS, and stored in a refrigerator at −20 °C until it was frozen. The brain was sliced with a blade at every 2-mm from the frontal pole, and the slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC, 0.5% in normal saline) for 30 min at 37 °C. Electronic images of the sections were made with a high-resolution camera (Sony X700, Japan). The area of infarction was quantified by the Osiris 4 software, and the results were expressed as the percentage of the total brain section area.

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