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Novel multi-functional nitrones for treatment of ischemic stroke

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ABSTRACT

Ischemic stroke resulting from obstruction of blood vessels is an enormous public health problem with urgent need for effective therapy. The co-administration of thrombolytic/antiplatelet agent and neuroprotective agent improves therapeutic efficacy and agent possessing both thrombolytic/antiplatelet and antiradical activities provides a promising strategy for the treatment of ischemic stroke. We have previously reported a novel compound, namely TBN, possessing both antiplatelet and antiradical activities, showed significant neuroprotective effect in a rat stroke model. We herein report synthesis of a series of new pyrazine derivatives, and evaluation of their biological activities. Their mechanisms of action were also investigated. Among these new derivatives, compound **21**, armed with two nitrone moieties, showed the greatest neuroprotective effects in vitro and in vivo. Compound **21** significantly inhibited ADP-induced platelet aggregation. In a cell free antiradical assay, compound **21** was the most effective agent in scavenging the three most damaging radicals, namely ·OH, $O_2^{\cdot-}$ and $ONOO^-$.

1. Introduction

Ischemic stroke is one of the major causes of death behind heart diseases and cancer, and is the leading cause of adult disability in the developed countries. There are approximately 795,000 Americans each year suffering a new or recurrent stroke, killing more than 137,000 people. It is estimated that Americans will pay about $50 billion annually for stroke-related medical costs and disability.[1] The large numbers of stroke patients and great economic expenses have imposed great burden to society.

Occlusion of cerebral vessels by blood thrombus is the main cause of ischemic stroke, which subsequently leads to a cascade of damaging biologic events, including the release of excitatory amino acid, calcium influx, neuroinflammation and over production of free radicals.[2–5] There are increasing evidences showing that free radicals play a crucial role in the damaging effects caused by stroke.[5–7] Thus, agent that either lyses blood thrombus/inhibits platelet aggregation or scavenges free radicals or combination of these two effects can be useful for the treatment of stroke.[8–10]

In China, *Ligusticum wallichii Franchat* (Chuan Xiong) and its main active component, 2,3,5,6-tetramethylpyrazine (TMP), have been used for treatment of ischemic stroke for many years.[11] The precise mechanism(s) of action of TMP has/have not been completely understood. TMP inhibits platelet aggregation,[12,13] lyses blood clots,[12] blocks calcium entry[14,15] and scavenges free radicals.[16,17] TMP is neuroprotective in animal stroke models.[18]

Nitrones were originally developed as free radical-trapping agents in free radical chemistry. Two decades later, nitrones were found to protect biological systems from oxidative stress. Nitrones had been tested as therapeutic agents for neural and systemic dysfunctions including atherosclerosis, stroke, and Alzheimer's disease.[19–21] For example, *N-tert*-butyl-a-phenylnitrone (PBN), the most widely investigated nitrone, was shown to ameliorate ischemic brain damage in a rodent model.[22,23] The nitrone NXY-059 (disodium 4-[(*tert*-butylimino)methyl] benzene-1,3-disulfonate N-oxide) was shown to significantly reduce infarct volumes in animal stroke models.[24] Although the clinical results of NXY-059 are disappointing, the concept of using neuroprotective agents for stroke therapy remains viable.

For an anti-stroke therapy to be most effective, it should ideally accomplish two things. Firstly, it should dissolve the thrombus to restore blood flow or should inhibit thrombus formation; that is it should have a thrombolytic/antiplatelet function. Secondly, it should reduce the impact of biochemical changes in the brain brought on by the stroke.[25,26] Our current research seeks optimal combinations of both thrombolytic/antiplatelet and neuroprotective agents to prevent cell death and damage during and after ischemia and reperfusion. We have previously reported a multifunctional compound, TBN, a conjugate of TMP and a nitrone moiety. In our previous work, we found that TBN showed significant neuroprotective effect by scavenging free radicals and directly removing blood thrombus.[27,28] Based on the significant anti-stroke effect of TBN, we had synthesized a new series of multi-functional nitrone derivatives, and evaluated their biologic activities.

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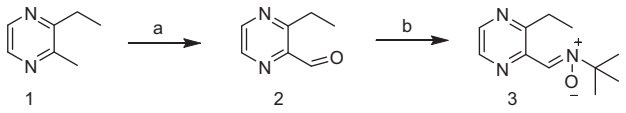


Scheme 1. Synthesis of compound **3**. Reagents and conditions: (a) SeO_2, 84 °C, 2 h, 70%; (b) *N*-*tert*-butyl-hydroxylamine, reflux, 4 h, 35%.

2. Results

2.1. Drug design and chemical synthesis

Our goal is to design compounds possessing both the antiplatelet/thrombolytic and free radical-scavenging activities. In addition, the compounds 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 points in mind, we designed and synthesized a series of new TMP analogues, most of which were armed with nitrone moiety(ies). Compounds **1**, **4**, **10**, **14** and **17** were chosen as parent compounds for their antiplatelet activities, since inhibition of platelet aggregation and thrombus formation is crucial for stroke treatments.[29] In addition, compound **14** shows thrombolytic effect in a vivo study at a dose of 2 mg/kg.[12] The purpose of the structure modification is to provide these compounds with free radical-scavenging effects, meanwhile, their antiplatelet activities can be retained. Since the nitrone moiety is the main functional group in scavenging free radicals, and it is therefore possible that a compound armed with two nitrone moieties will be more potent in scavenging free radicals than those with only one. For this reason, compound **21**, bearing two nitrone moieties, was synthesized.

The new compounds (compounds **3**, **9**, **19** and **21**) were synthesized through condensation of the corresponding aldehyde and *N*-*tert*-butyl-hydroxylamine (Schemes 1, 2, 5 and 6)[30] or oxidized by Na_2WO_4/H_2O_2 from the corresponding secondary amine (compound **13**, Scheme 3).[31] The aldehyde intermediates were synthesized by oxidation of alcohols using activated manganese dioxide or directly oxidized by selenium dioxide.[32] The corresponding secondary amines were synthesized through condensation of the bromo-substituted precursors and *tert*-butylamine.

In synthesis of compound **9**, the starting parent **4** was first transformed to compound **7**, which was then oxidized by activated manganese dioxide to produce compound **8**. Without further purification, compound **8** was allowed to react with *N*-*tert*-butyl-hydroxylamine to afford the target compound **9**.

In synthesis of compounds **3**, **19** and **21**, the formyl aldehyde intermediates were prepared through direct oxidation by selenium dioxide from their corresponding parent compounds.

In preparation of compound **13**, the parent **10** was transformed to compound **11** in the presence of *N*-bromosuccinimide and benzoyl peroxide. Compound **11** was then treated with *tert*-butylamine to produce intermediate **12**, which was then oxidized by Na_2WO_4/H_2O_2 to afford the target compound **13**.

Compound **16** was synthesized by treatment of excess 2-(bromomethyl)-3,5,6-trimethylpyrazine (compound **15**) with *tert*-butylamine (Scheme 4).

2.2. Protective effect on *t*-BHP induced cerebellar granule cell injury

The neuroprotective activities of the new compounds were investigated using rat cerebellar granule neurons (CGNs) exposed to *tert*-butyl-hydroperoxide (*t*-BHP) as an oxidant (the final concentration of *t*-BHP is 50 μM). Preparation of rat cerebellar granule neurons was performed according to a published method.[33] TMP was used as a positive control in the experiment (Fig. 1). The basal percentage of viable neurons after only *t*-BHP treated cells was 49.4%. TMP offered weak neuroprotective effect against *t*-BHP induced cell damage up to 1000 μM (58.4%). In sharp contrast, compound **16**, the TMP dimer amine, and compound **21**, bearing two nitrone moieties, afforded greater neuroprotective effect at the concentrations of 100 μM and 1000 μM. At 1000 μM, 80.6 ± 6.0% of cells treated with compound **21** survived while 75.5 ± 3.0% of cells treated with compound **16** survived. Compounds **3** and **9** were less potent while compounds **13** and **19** had no effects compared with the group treated with *t*-BHP.

2.3. Neuroprotective effects in rat transient middle cerebral artery occlusion model

After demonstrating that compound **21** showed the strongest neuroprotective effect in vitro, we then examined its neuroprotective effect in vivo. The neuroprotective effect of compound **21** was investigated in a rat transient middle cerebral artery occlusion (t-MCAO) model. The model was established according to a published method with modifications.[34] Compound **21** was administered (ip) 2 h after MCAO, and the animals were sacrificed after another 22 h reperfusion. The brain infarct size was determined by TTC staining 24 h after MCAO. Image-analysis of the brains showed that compound **21** at a dose of 110 mg/kg reduced the total infarct volume by 65.0% compared to the vehicle-treated control. The data demonstrated that compound **21** significantly protected brain tissues against ischemic damage compared to the control group (Fig. 2A). Rats received compound **21** treatment had lower neurological scores (Fig. 2B) (compound **21**: $n = 13$; control: $n = 13$).

2.4. Effect on ADP-induced platelet aggregation

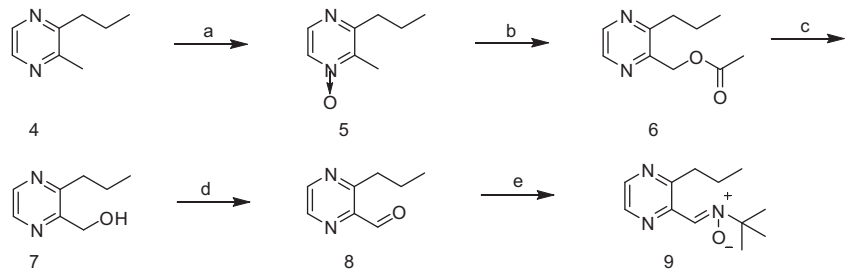
To investigate whether these new compounds retained antiplatelet activity, their effects on inhibiting platelet aggregation were studied. The turbidimetric method with a platelet-aggregometer was applied in the measurement.[35] Since aspirin was a golden standard in inhibiting platelet aggregation and TMP was effective in inhibiting platelet aggregation, aspirin and TMP were used as positive controls (Fig. 3). At 2 mM, the maximum percentage of platelet aggregation treated with aspirin, TMP, compound **3, 9, 16** and **21** were 26.6 ± 4.7%, 42.9 ± 9.2%, 33.8 ± 2.5%, 48.0 ± 12.4%, 44.3 ± 5.5% and 37.7 ± 4.0%, respectively; while that of the sample treated with saline was 57.3 ± 8.3%. The results demonstrated that compound **21** was more effective than TMP in inhibiting platelet aggregation, but less effective than aspirin.

2.5. Free radical-scavenging capacity in vitro

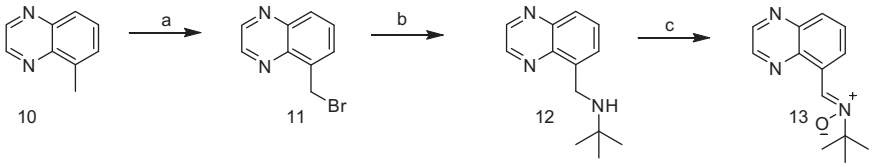
Results in Figure 4A showed that all compounds especially compound **16** were highly effective in scavenging the ·OH radical (100% at 1000 μM). Compounds **3**, **9** and **21** showed significant activity for removing $O_2^{\cdot-}$, but compounds **16**, TMP and PBN were less potent (Fig. 4B). Compound **21** potently reduced the amount of $ONOO^-$ by 31.9% at a concentration of 1 μM and 99.4% at 1000 μM. Compounds **13**, **16**, **19** and TMP had no effect. The effect of compounds **3**, **9**, and PBN were moderate (Fig. 4C). Compounds **9**, **16** and **21** remarkably decreased the free radical DPPH; while compounds **3**, **13**, **19**, TMP and PBN were only marginally effective (Fig. 4D). These results demonstrated that compound **21** had significant activities in removing all of the three most detrimental radicals.

3. Discussion

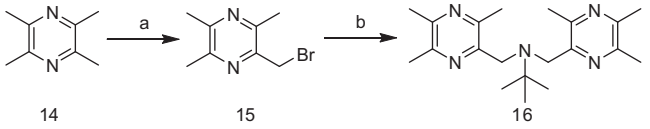
Stroke occurs as a result of an obstruction by blood clots within blood vessels supplying blood to the brain. Since thrombosis plays



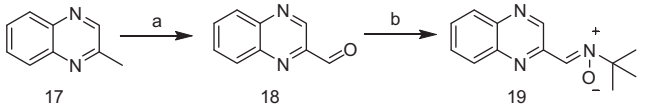
Scheme 2. Synthesis of compound **9**. Reagents and conditions: (a) AcOH, 30% H_2O_2, 70 °C, 10 h; (b) AC_2O, reflux, 12 h; (c) 20% NaOH, 5 h, 45% from **4**; (d) MnO_2, C_2H_5OH, reflux, 12 h; (e) *N-tert*-butyl-hydroxylamine, reflux, 8 h, 30% from **7**.



Scheme 3. Synthesis of compound **13**. Reagents and conditions: (a) NBS, benzoyl peroxide, 70 °C, 10 h, 40%; (b) *tert*-butylamine, 4 h, 100%; (c) Na_2WO_4, 30% H_2O_2, 2 h, 65.2%.



Scheme 4. Synthesis of compound **16**. Reagents and conditions: (a) NBS, benzoyl peroxide, 70 °C, 10 h, 52%; (b) *tert*-butylamine, 2 h, 42.5%.



Scheme 5. Synthesis of compound **19**. Reagents and conditions: (a) SeO_2, 84 °C, 2 h, 75.2%; (b) *N-tert*-butyl-hydroxylamine, reflux, 4 h, 75.2%.

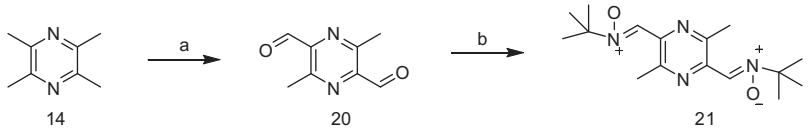
an important role in the occurrence of ischemic stroke, drugs that interfere with thrombus formation such as anticoagulants and antiplatelet aggregants are commonly employed in the management of patients with cerebrovascular disease. Considerable evidence supports the use of antiplatelet aggregants in stroke prevention and treatment.[36,37] In our present work, the newly synthesized nitrone compounds **3**, **9**, **21** and compound **16** retained the parent TMP's antiplatelet activities. The introduction of nitrone moiety(ies) did not significantly affect their antiplatelet activity (the percentages of platelet aggregation of the compounds **1**, **4** and **14** were 43.1%, 43.1% and 46.5%).[29]

In the region of the brain affected by ischemia, neuronal cells in the ischemic core may die but those in the other parts (penumbra) may be salvaged. Thrombolytic/antiplatelet agents are not intended to salvage injured neurons in the penumbra for their powerlessness in scavenging the active radical species produced by reperfusion. Neuroprotective agents such as free radical scavengers have been developed and tested for this purpose in animal models or in clinic.[37,38]

The ·OH, $O_2^{\cdot-}$ and $ONOO^-$ are the three most detrimental active species in human body. The ·OH is a very active radical with a short life. It can quickly react with cell membranes or other cell components to cause cell damage. The $O_2^{\cdot-}$ is the culprit of many other radicals. It can react with H_2O_2 to form ·OH through the Fenton reaction or react with nitric oxide (·NO) to afford $ONOO^-$, which is about 1000 times more destructive than H_2O_2. Therefore, it is important for a stroke drug to be able to neutralize all of these detrimental radicals. DPPH is a dark-colored crystalline powder composed of stable free-radical molecules. It has been used widely in laboratory as an agent to test a compound's antiradical activity.

TMP is reported to scavenge ·OH, $O_2^{\cdot-}$ and ·NO,[39,40] but its ability toward $ONOO^-$ is not reported. Nitrone is effective in scavenging ·NO, ·OH and $O_2^{\cdot-}$.[41,42] There is only indirect evidence showing that nitrone might inhibit $ONOO^-$ formation by scavenging $O_2^{\cdot-}$, but its direct activity in scavenging $ONOO^-$ was not performed in the report.[43] In the ·OH assay, the introduction of a nitrone group in compounds **3**, **9** and **21** did not significantly improve their anti-·OH activity. While, in the $O_2^{\cdot-}$ and $ONOO^-$ assays, the nitrone compounds **3**, **9** and **21** were more potent comparing with TMP. These results demonstrate that the nitrone group is essential for their activities against $O_2^{\cdot-}$ and $ONOO^-$. This conclusion is further confirmed by the fact that compound **16**, bearing no nitrone, had no activities against $O_2^{\cdot-}$ and $ONOO^-$, and compound **21**, bearing two nitrone groups, was the most powerful against $O_2^{\cdot-}$ and $ONOO^-$.

Structure–activity relationship analysis of the new compounds suggest that firstly, introduction of a nitrone group to TMP and other pyrazines retains antiplatelet activity; Secondly, a nitrone group increases antiradical activity, and especially accounts for the anti-superoxide and anti-peroxynitrite activities.



Scheme 6. Synthesis of compound **21**. Reagents and conditions: (a) SeO_2, 84 °C, 2 h, 35.6%; (b) *N-tert*-butyl-hydroxylamine, reflux, 4 h, 86.2%.

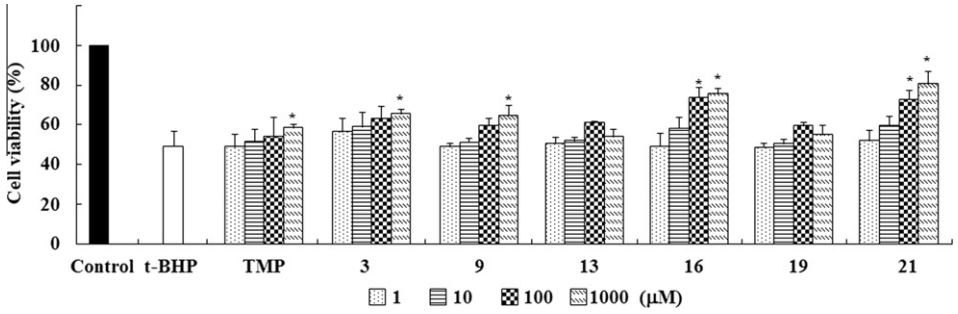


Figure 1. Protective effect against *t*-BHP-induced cell damage. The results are expressed as the percentage of that of the untreated cells. The basal percentage of viable neurons after only *t*-BHP treated cells was 49.4%. At the concentration of 1000 μM, the percentage of viable neurons was: TMP 58.4%, compound **3** 64.6%, compound **9** 66.0%, compound **13** 54.8%, compound **16** 75.5%, compound **19** 53.9%, compound **21** 80.6%. Data was performed via one-way analysis of variance, with subsequent individual comparisons performed by Scheffe's test. Data were expressed as means ± SD of three independent experiments. $^{*}P$ <0.05 compared to *t*-BHP group.

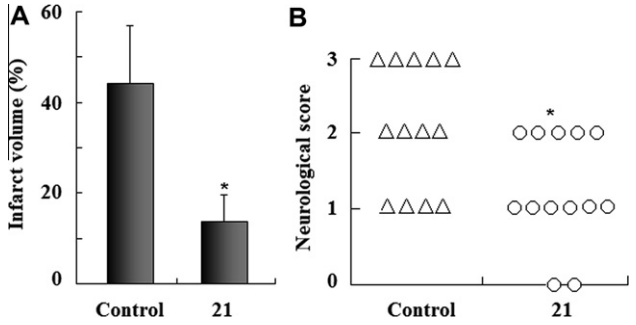


Figure 2. (A) Protective effect in the rat t-MCAO model. Rats were subjected to 2 h ischemia followed by another 22 h of reperfusion. The percentage of infarct volume in contralateral hemisphere was calculated. Compound **21**: 110 mg/kg; control group received the same volume of saline. Compound **21**: n = 13; control: n = 13. Data was performed via one-way analysis of variance, with subsequent individual comparisons performed by Scheffe's test. $^{*}P$ <0.05 compared to the control. (B) Neurological recovery score in animals treated with compound **21**. The neurological deficit scores were analyzed by Mann–Whitney nonparametric test, and the difference was considered significant at P <0.05.

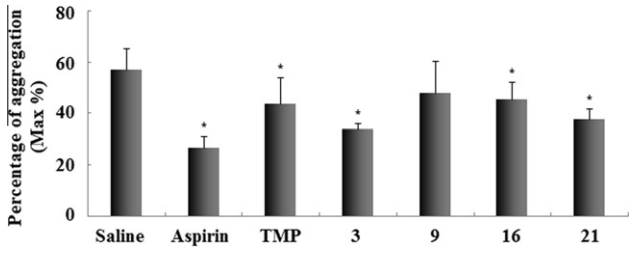


Figure 3. Prevention of platelet aggregation. Rabbit platelet suspensions were pre-incubated with each compound (2 mM) at 37 °C for 8 min followed by the addition of ADP (8 μM). The extent of aggregation was expressed as the percentage of the control (in the absence of drugs). Data was performed via one-way analysis of variance, with subsequent individual comparisons performed by Scheffe's test. Data were expressed as means ± SD of four independent experiments. $^{*}P$ <0.05 compared to saline treated group.

4. Conclusion

New pyrazine derivatives were synthesized. Among the new compounds, compound **21** showed significant neuroprotective effect in cultured cerebellar granule neurons and in rat MCAO model. Compound **21** was effective in inhibiting ADP-induced platelet aggregation and was the most powerful compound in scavenging the three most damaging radicals to brain tissues, including $^{\cdot}OH$, $O_2^{\cdot-}$ and $ONOO^-$. Compound **21** was a promising multi-functional agent for the treatment of ischemic stroke.

5. Experimental

5.1. Chemistry

Melting points were determined with an electro-thermal melting point apparatus (Electro-thermal 9100). ^{1}H NMR spectra were recorded at ambient temperature on a 300 MHz spectrometer (AV-300, Bruker) in $CDCl_3$ or DMSO-d_6. The chemical shifts values were expressed in ppm relative to tetramethylsilane as an internal standard. Electrospray ionization mass spectra (ESI-MS) was 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 of Jinan University, Guangzhou, China, and the results were within±0.4% of the theoretical values unless otherwise noted. High resolution mass spectrometry was performed in an Agilent 6210 ESI/TOF mass spectrometer. Column chromatography was carried out using ordinary silica gel (200–300 mesh). Most chemicals and solvents were analytical grade and were used without further purification.

5.1.1. 3-Ethylpyrazine-2-carbaldehyde (2)

Compound **2** was synthesized according to a report by Thomas and Nowak.[44]

5.1.2. 2-[[(1,1-Dimethylethyl)oxidoimino]methyl]-3-ethylpyrazine (3)

To compound **2** (1.3 g, 0.01 mol) in ethanol (200 mL) was added *N*-*tert*-butyl-hydroxylamine (1 g, 0.011 mol), and the solution was refluxed for 2 h. Another portion of *N*-*tert*-butyl-hydroxylamine (1 g, 0.011 mol) was then added, and the solution was refluxed until compound **2** was completely transformed. Solvent was removed in vacuo. The crude product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:1, v/v), to produce compound **3** (brown liquid, 0.7 g, 35% yield). ^{1}H NMR ($CDCl_3$): 1.21 (t, 3H), 1.54 (s, 9H), 2.74 (q, 2H), 8.06 (s, 1H), 8.51 (s, 1H), 8.56 (d, 1H). ESI-MS: 208 $[M+H]^+$.

5.1.3 3-Propylpyrazin-2-yl-methanol (7)

Compound **7** was prepared according a published method.[45]

5.1.4. 2-[[(1,1-Dimethylethyl)oxidoimino]methyl]-3-propylpyrazine (9)

Compound **7** (0.9 g, 0.006 mol) in ethanol (200 mL) was oxidized at 84 °C for 12 h by activated MnO_2 (5 g, 0.06 mol) to afford compound **8**. Without further purification, to the compound **8** in ethanol (200 mL) was added *N*-*tert*-butyl-hydroxylamine (1 g, 0.011 mol), and the solution was refluxed for 2 h. Another portion of *N*-*tert*-butyl-hydroxylamine (1 g, 0.011 mol) was then added, and the solution was refluxed until compound **8** was completely reacted. Solvent was removed in vacuo. The crude product was

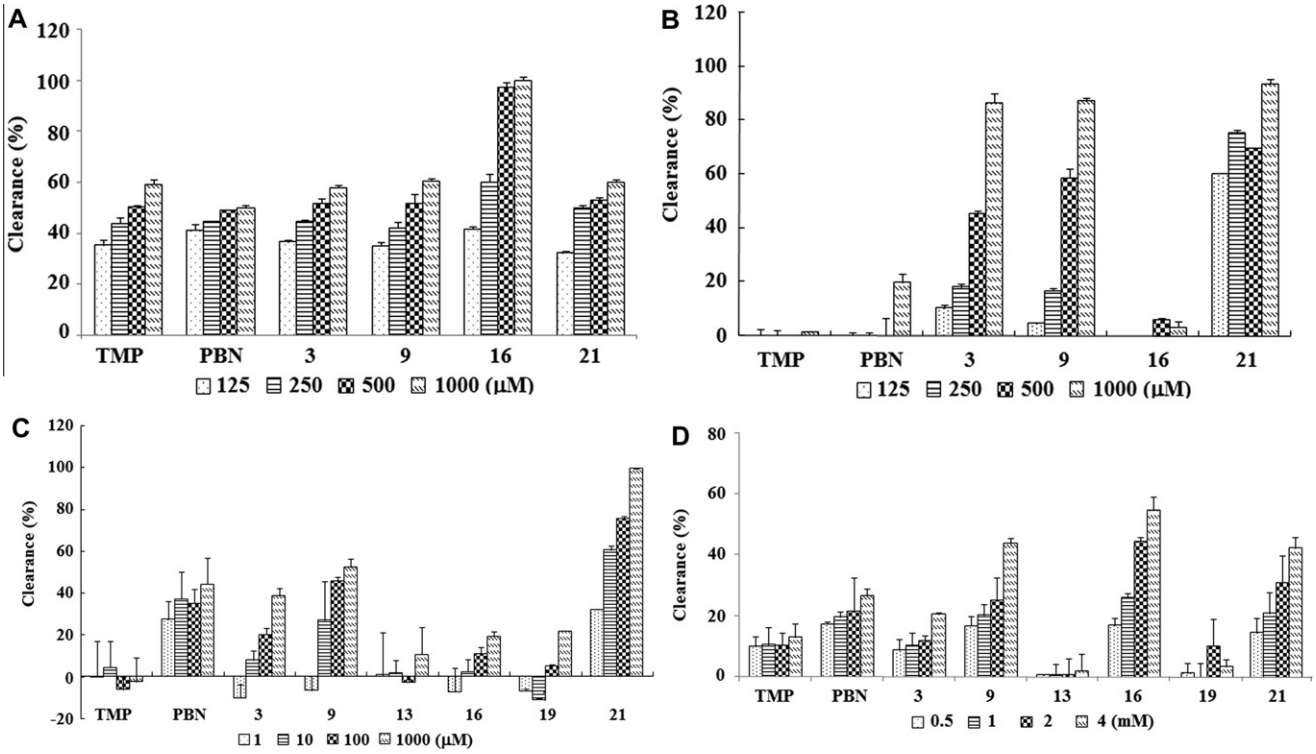


Figure 4. Free radical-scavenging effects. (A) Activity against ·OH; (B) activity against $O_2^{\cdot-}$, (C) activity against $ONOO^-$, (D) activity against DPPH. TMP and PBN were used as positive controls. Results were the mean of three independent experiments.

purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:1, v/v), to produce compound **9** (brown liquid, 0.4 g, 30% yield, two steps). ^{1}H NMR ($CDCl_3$): 0.87 (t, 3H), 1.49 (s, 9H), 1.67 (m, 2H), 2.73 (t, 2H), 7.77 (s, 1H), 8.40 (dd, 2H). ESI-MS: 222 $[M+H]^+$, 244 $[M+Na]^+$. HRMS (ESI, *m*/*z*): calcd for $C_{12}H_{20}N_3O$ 221.14848, found 221.14879.

5.1.5. 5-Bromomethyl-quinoxaline (11)

Compound **11** was synthesized as described by Berg et al.[46]

5.1.6. 5-[(1,1-Dimethylethyl)oxidoimino]methylquinoxaline (13)

To compound **11** (1.8, 0.008 mol) was added excess *tert*-butylamine and the reaction mixture was stirred at room temperature for 4 h until compound **11** was completely transformed to compound **12**. The excess *tert*-butylamine was removed in vacuo. Without further purification, to compound **13** in 20 mL methanol was added 646 mg $Na_2WO_4{\cdot}2H_2O$, 4 mL 30% H_2O_2 and the reaction mixture was stirred for another 2 h at room temperature. After that, the mixture 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 (petroleum ether/ethyl acetate 4:1) to afford compound **13** as a red solid (1.2 g, 65.2% yield), mp: 84–86 °C. ^{1}H NMR ($CDCl_3$): 1.69 (s, 9H), 7.83 (dd, 1H), 8.10 (dd, 1H), 8.80 (d, 1H), 8.87 (d, 1H), 9.19 (s, 1H), 9.96 (dd, 1H). ESI-MS: 230 $[M+H]^+$. Anal. ($C_{13}H_{15}N_3O$) C, H, N.

5.1.7. 2-(Bromomethyl)-3,5,6-trimethylpyrazine (15)

Comopund **15** was synthesized according to a published method.[27]

5.1.8. 2-Methyl-*N*,*N*-bis((3,5,6-trimethylpyrazin-2-yl)methyl)propan-2-amine (16)

To compound **15** (1.5 g, 0.007 mol) was added *tert*-butylamine (0.26 g, 0.0035 mol) and the mixture was stirred at room temperature for 5 h. After that, the reaction mixture was extracted with ethyl acetate (100 mL) and dried with Na_2SO_4. The solvent was removed in vacuo and the crude was further purified by column chromatography (petroleum ether/ethyl acetate 1:4) to provide compound **16** as a white solid (10.6 g, 42.5% yield), mp: 106–108 °C. ^{1}H NMR ($CDCl_3$): ^{1}H NMR ($CDCl_3$): 1.25 (s, 9H), 2.30 (s, 6H), 2.35 (s, 6H), 2.39 (s, 6H), 3.86 (s, 4H). ESI-MS: 342 $[M+H]^+$, 364 $[M+Na]^+$. Anal. ($C_{20}H_{31}N_5{\cdot}2.5\ H_2O$) C, H, N.

5.1.9. 2-Quinoxalinecarbaldehyde (18)

Compound **18** was synthesized as described by Dale et al.[47]

5.1.10. 2-[(1,1-Dimethylethyl)oxidoimino]methylquinoxaline (19)

Compound **18** (3.2 g, 0.02 mol) was dissolved in 250 mL ethanol. To the solution was added freshly prepared *N*-*tert*-butylhydroxylamine (2.67 g, 0.03 mol). The reaction was allowed to proceed for 4 h. After the workup, solvent was removed in vacuo and the crude product was purified by column chromatography (petroleum ether/ethyl acetate 4:1) to afford compound **19** as a red solid, (3.49 g, 75.2% yield), mp: 88–89 °C. ^{1}H NMR ($CDCl_3$): 1.70 (s, 9H), 7.77 (m, 2H), 8.03 (m, 2H), 8.14 (s, 1H), 10.49 (s, 1H). ESI-MS: 230 $[M+H]^+$. Anal. ($C_{13}H_{15}N_3O$) C, H, N.

5.1.11. 3,6-Dimethylpyrazine-2,5-dicarbaldehyde (20)

Compound **20** was synthesized as described by Schumann and Luo.[48]

5.1.12. 2,5-[[(1,1-Dimethylethyl)oxidoimino]methyl]-3,6-trimethylpyrazine (21)

Compound **20** (1.2 g, 0.007 mol) was dissolved in 200 mL ethanol and then *N*-*tert*-butyl-hydroxylamine (2.49 g, 0.028 mol) was added. The reaction was allowed to continue for 4 h at 84 °C. After that, solvent was removed in vacuo and the crude product was purified by column chromatography (petroleum ether/ethyl acetate 1:1) to provide compound **21** as a yellow solid (1.9 g, 86.2% yield), mp: 198–201 °C. ^{1}H NMR ($CDCl_3$): 1.61 (s, 18H), 2.48 (s, 3H), 2.50 (s, 3H), 7.83 (s, 2H). ESI-MS: 307 $[M+H]^+$, 329 $[M+Na]^+$. Anal. ($C_{16}H_{26}N_4O_2$) C, H, N.

5.2. In vitro neuron protection assay

Cerebellar granule neurons were prepared and cultured according to a published method.[33] Cerebellar granule neurons were placed into 96-well cell culture plates and were cultured for 7 days at 37 °C under 5% CO_2. Drugs at different concentrations were added, and the cells were incubated at 37 °C for 30 min. *t*-BHP was then added (the final concentration of *t*-BHP was 50 μM), and the cells were incubated for 4 h at 37 °C under 5% CO_2. A solution of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl-tetrazoliun bromide was added, and the cells were incubated for another 4 h before DMSO was added. After the crystals were completely dissolved, 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 group.

5.3. Neuroprotective effect in rat transient-MCAO model

5.3.1. Animal preparation for transient-MCAO model

Experimental protocols were approved by the ethical committee and conformed to internationally accept ethical standards (Guide for the Care and Use of Laboratory Animals. NIH Publication 86-23, revised 1985). Adult male Sprague–Dawley rats (Guangdong Province Animal Center) weighing 260–310 g (approximate 3 month old) were used. Animals were housed in groups of five in plastic cages under controlled temperature (20–22 °C), humidity (40 ± 60%), ventilation and lighting (12 h light/dark cycle) and were allowed free access to food and water.

The t-MCAO model was made according to a published method with modification.[34] Briefly, rats were anesthetized with 10% chloral hydrate (400 mg/kg, ip). A neck incision was made and the right common carotid artery (CCA) was carefully closed by a ligature. The occipital branch of the external carotid artery (ECA) was ligated and the internal carotid artery (ICA) was isolated. ICA was temporarily closed by a microvascular clip and a small incision was made on CCA. A poly-L-lysine-coated nylon suture (0.36 mm in diameter) was inserted into the CCA and was advanced into ICA until MCA was occluded, about a distance of approximately 18–20 mm from the bifurcation of CCA. A ligature was tied around the ICA, and then the incisions were closed. The nylon suture was withdrawn after 2 h to achieve reperfusion.

During the experiments, body temperature (rectal temperature) was monitored and adjusted with heating lamps and a heated operating mat if the temperature fluctuated beyond predetermined limits of 37 ± 1 °C. Rats were divided into drug treated and vehicle (control) treated groups. In order to ensure the reliability of the results, only rats with neurological scores of 2 or 3 were used in the experiments, and each group had a large number of animals. The failure rate of MCAO model was about 3% and the mean survival rate of the rats enrolled in the experiments after 24 h was about 90%. Neurological scores were measured 2 h after transient MCAO according to a published method.[34] After surgery, animals were allowed to recover in a warm (27 ± 3 °C), quiet environment and given free access to food and water.

5.3.2. Evaluation of infarct size by TTC staining

Rats were re-anesthetized by 10% chloral hydrate and were killed by decapitation. Brain was quickly removed and chilled in the fridge for about 30 min. Six 2-mm thick coronal slices were cut with a tissue slicer, beginning 1 mm posterior to the anterior pole. Then the slices were immersed in PBS solution containing 0.5% 2,3,5-triphenyl-tetrazolium chloride (TTC) (Sigma) at 37 °C for 30 min and fixed by immersion in 4.0% phosphate-buffered formalin solution. The sections were scanned with a digital camera (Sony W220, Japan) and the areas of infarction were quantified using an image analyzing system (Osiris 4 software, developed by Université de Genève, Switzerland). The infarct area in each slice was calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral edema. The total infarct volume was determined by summing up the infarct volume of the six sections and is presented as a percentage of the volume of the contralateral hemisphere.[49,50]

5.4. Platelet aggregation assay

Rat platelet suspensions were prepared from New Zealand rabbits as previously described.[12] Briefly, rabbits were fasted overnight with free access to tap water before blood collection. Blood was withdraw from arteria auricularis and mixed with sodium citrate (9:1, v:v). Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 800 rpm/min for 20 min. The supernatant was collected to afford PRP. The platelet poor plasma (PPP) was obtained by a further centrifugation at 3000 rpm/min for 10 min. Drugs (the final drug concentration was 2 mM) were added into PRP suspension (0.29 mL) and pre-warmed at 37 °C for 8 min. After that, ADP (the final concentration was 8 μM) was added to induce aggregation. The reaction was allowed to proceed for 5 min. Light transmission through the platelet suspension was monitored by a Platelet-Aggregometer (Sc-2000 Platelet-Aggregometer, Beijing) to measure platelet aggregation. Maximum change in light transmission was used as the aggregation endpoint for potency comparisons.

5.5. Free radical scavenging capacity in vitro

5.5.1. Determination of hydroxyl radical-scavenging activity

The newly synthesized compounds' ·OH-scavenging activity was determined in a cell-free assay following a published procedure.[51] The assay was dependent upon a compound's ability to inhibit the ·OH-dependent bleaching of *para*-nitrosodimethylaniline (*p*-NDA). *p*-NDA was prepared at 1.6 mM in 50 mM NaCl solution. ·OH was generated using the Fenton reaction (Fe_2^+/H_2O_2). $FeSO_4$ was dissolved in double-distilled water to afford a final concentration of 4.0 mM while H_2O_2 solution (7.3 mM) was freshly prepared from a 30% stock solution. The compounds were dissolved in double-distilled water and the final concentrations were 125, 250, 500 and 1000 μM. The bleaching of *p*-NDA was monitored as the loss in absorbance at 440 nm for 100 s. TMP and PBN were used as positive controls. The clearance was calculated as follows: Clearance (%) = $1-[(A_0-A_{100})/A_0] \times 100$, where $A_{C(0)}$ was the absorbance at $t = 0$ s and $A_{(100)}$ was the absorbance at $t = 100$ s.

5.5.2. Determination of superoxide anion-scavenging activity

A published procedure was used to determine these new compounds' ability to scavenge $O_2^{\cdot-}$ radicals.[52] A solution in a glass cuvette containing 1.565 mL Tris–HCl buffer (pH 8.2), 0.174 mL pyrogallic acid (0.16 mmol/L) and 1.461 mL testing sample (the final concentrations were 125, 250, 500 and 1000 μM) was prepared. The solution was mixed thoroughly and absorbance was recorded continuously for 300 s at 320 nm on a UV 22PC ultraviolet spectrophotometer. The autoxidation rate of pyrogallic acid was

presented as the change in absorbance of autoxidation products per second at 320 nm (dAs/dt). The clearance of each sample was calculated as follows: Clearance (%) = (dA/dt–dAs/dt)/dA/dt.

5.5.3. Determination of peroxynitrite radical-scavenging activity

The assay was based on the chemical interaction of amino-3-morpholinyl-1,2,3-oxadiazolium (SIN-1) and luminol, which produces blue light upon oxidation.[53] SIN-1 was used to generate $ONOO^-$. Luminol was dissolved in 5% NaOH (20 μL) and was diluted to 1 mM with 0.1 M PBS buffer (pH 7.4). SIN-1 was dissolved in 0.1 M PBS, giving a final concentration of 100 μM for this experiment. The solution contained 350 μL PBS, 50 μL luminol, 50 μL each compound (the final concentration 1, 10, 100 and 1000 μM) or PBS (control) and 50 μL SIN-1 which was added last to initiate the reaction. The reaction was allowed to continue for 1250 s. Chemiluminescence was recorded per 100 s. The clearance at peak height represented the compounds' ability to trap $ONOO^-$. The clearance was calculated according to the formula: Clearance (%) = $[(A_c–A_{sample})/A_c] \times 100$.

5.5.4. Determination of DPPH radical-scavenging activity

The validated 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method was applied to evaluate a compound's antiradical activity.[54] DPPH radical has a deep violet color in solution and become colorless or pale yellow when neutralized. To measure DPPH radical-scavenging activity, 50 μL methanolic solution of each compound was placed in 96 well plates, where 150 μL methanolic solution of DPPH (100 μM) was then added. The decrease in absorbance at 515 nm was determined continuously with a microplate reader until the absorbance stabilized (50 min). The clearance of the DPPH radical was calculated according to the formula: Clearance (%) = $[(A_{c(0)}–A_t)/A_{c(0)}] \times 100$, where $A_{C(0)}$ was the absorbance of the control and $A_{(t)}$ was the absorbance of the reaction solution at t = 50 min.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.016. These data include MOL files and InChiKeys of the most important compounds described in this article.

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