Design, Synthesis, and Biological Evaluation of Novel Tetramethylpyrazine Derivatives as Potential Neuroprotective Agents

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Oxidative stress plays a crucial role in neurological diseases, resulting in excessive production of reactive oxygen species, mitochondrial dysfunction and cell death. In this work, we designed and synthesized a series of tetramethylpyrazine (TMP) derivatives and investigated their abilities for scavenging free radicals and preventing against oxidative stress-induced neuronal damage in vitro. Among them, compound 22a, consisted of TMP, caffeic acid and a nitrone group, showed potent radical-scavenging activity. Compound 22a had broad neuroprotective effects, including rescuing iodoacetic acid-induced neuronal loss, preventing from tert-butylhydroperoxide (t-BHP)-induced neuronal injury. Compound 22a exerted its neuroprotective effect against t-BHP injury via activation of the phosphatidyl inositol 3-kinase (PI3K)/Akt signaling pathway. Furthermore, in a rat model of permanent middle cerebral artery occlusion, compound 22a significantly improved neurological deficits, and alleviated the infarct area and brain edema. In conclusion, our results suggest that compound 22a could be a potential neuroprotective agent for the treatment of neurological disease, particularly ischemic stroke.

Key words tetramethylpyrazine; nitrone; caffeic acid; free radical; neuroprotection; permanent middle cerebral artery occlusion.

Ischemic stroke, resulting mainly from the interruption of cerebral blood flow, is the second cause of morbidity and mortality worldwide. Many neuroprotective drug candidates have been tested in preclinical and clinical studies, unfortunately, none have succeeded clinically. In the past few decades, great progresses have been made in understanding the pathophysiology of ischemic stroke. A cascade of biochemical events involved in ischemic stroke produces profound cellular changes, including a rapid decrease in ATP content, disruption of various ion channels, calcium overload, glutamate release, acidosis and edema. Many of these changes are associated with excessive production of free radicals. Free radicals including reactive oxygen species (ROS) and reactive nitrogen radicals (RNS) cause mitochondrial dysfunction and subsequent cellular apoptosis. Therefore, antioxidants could be developed as neuroprotective therapies against neuronal damage because of their capability to combat free radicals.

Tetramethylpyrazine (TMP, Fig. 1) is a biologically active component of the traditional Chinese medicine Chuanxiong (Ligusticum wallichii Franchat), which is widely used in China for the treatment of cardio- and cerebro-vascular diseases clinically. Previous research found that TMP had therapeutic effects in neurodegenerative diseases, such as Parkinson’s disease (PD), Alzheimer’s disease (AD), and ischemic stroke. TMP’s precise mechanisms of neuroprotection are not completely understood, but it has been proven to be linked to its ability in maintaining mitochondrial function, scavenging free radicals, increasing transcription of redox proteins and protecting antioxidant enzyme. In addition, TMP has superior capability to enter the brain. Using a microdialysis method, Tsai et al. demonstrated that TMP effectively penetrated the blood–brain barrier (BBB), yielding a progressively higher brain/blood ratio at 10 to 120 min following intravenous administration.

Nitrones are a class of free radical-trapping agent and have been proven their therapeutic potential for providing beneficial effects in a number of diseases including stroke, AD and PD. N-tert-Butyl-a-phenylnitrone (PBN) and NXY-059 are two representative nitrone compounds. PBN reduced brain infarction in a rat model of middle cerebral artery occlusion (MCAO). NXY-059 had exhibited promising therapeutic effects when evaluated in various animal stroke models but failed its second phase III clinical trial because of its poor ability to cross the BBB. Previously, we have reported a TMP nitrone conjugate, named a-tetramethylpyrazinyl-N-tert-butyl nitrone (TBN), which readily crossed the BBB and showed potent free radical scavenging ability, protecting neurons and rat suffering ischemic stroke and PD.

Caffeic acid (Fig. 1), a hydroxyl derivative of cinnamic acid bearing a phenolic moiety, is belonging to the family of the phenyl propanoids. Caffeic acid is abundant in nature, has versatile pharmacological activities, including antioxidative stress, anti-inflammatory, anti-cancer, and anti-viral activities. Caffeic acid and its ester derivatives showed an excellent neuroprotection and therapeutic effects in various neurological diseases. The pharmacokinetics and tissue distribution study revealed that caffeic acid had weak BBB permeability and presented a low brain distribution proportion.

Fig. 1. Structures of TMP, TBN and Caffeic Acid

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Traditional drug development approach, one drug one target, is proven unsuccessful in treating complex brain disorders that involve multiple pathogenic factors. A relatively simple strategy for developing novel multi-functional drugs starts from structural combination/modification of compounds with some desirable biological properties, may lead to novel molecule with multiple properties derived from the original compounds, even possessing unique properties that are distinct from the original compounds. Since all of the three pharmacologically active compounds/functional moieties, i.e., TMP, caffeic acid and nitrone, have versatile functions beneficial to neurological diseases, we reason that compounds combining one or more of these moieties may afford new compounds with much improved efficacy. In addition, because of the superior capability penetrating BBB, TMP could be used as a carrier to deliver nitrone/caffeic acid into the brain. Thus, in this work, we designed and synthesized two series of novel TMP derivatives: (1) TMP moiety conjugated with another TMP molecule or caffeic acid; (2) TMP–caffeic acid derivatives conjugated with the potent radical scavenging nitrone moiety. Moreover, their free radical scavenging activities against ROS and RNS were tested, and their neuroprotective effects were also evaluated in oxidative stress damaged PC12 cells and in a rat model of permanent middle cerebral artery occlusion (p-MCAO).

Results and Discussion

Rational Drug Design and Synthesis Previous structure–activity relationship studies revealed that the pyrazine ring of TMP might principally determine its pharmacodynamics and the methyl groups might primarily govern its pharmacokinetics and toxicity. Substitutions of the methyl group apparently improved drug efficacy and prolonged action time. Our previous study also demonstrated that the derivative of two TMP molecules linked via a tert-butyl amine group exhibited enhanced efficacy in preventing cells against oxidative damage and in scavenging hydroxyl radicals. Our goal is to design a compound possessing multi-functions necessary for effective treatment of neurological diseases. In addition, the compound must penetrate the BBB readily, an important requirement for central nervous system drugs. Furthermore, the compound must be non-toxic at therapeutic doses. With these criteria in mind, based on the previous findings and by virtue of properties of TMP, caffeic acid and nitrone, in this work, we introduce another TMP or caffeic acid molecule(s) to TMP moiety via an ester bond or an ether bond (compounds 4, 6, 8a, b, 12a, b, Fig. 2). Since the nitrone moiety has great potential to scavenge free radicals, to further enhance the anti-radical activities, the nitrone moiety(ies) was (were) introduced into the new compounds to obtain compounds 20a, b and 22a, b (Fig. 2).

Compound 4 was obtained by treatment of compound 2 with compound 3 in a solution of NaHCO₃ (Chart 1). Compounds 2 and 3 were synthesized as previously reported. The preparation of compounds 6 was depicted in Chart 2. TMP was oxidized to afford compound 5, which was then treated with compound 3 to yield compound 6.

Synthesis of compounds 8a and b was showed in Chart 3. The commercially available caffeic acid (7a) was treated with...
acetic anhydride to afford compound 7b. Compounds 7a and b were treated with compound 3 in the presence of NaHCO₃ to afford compounds 8a and b, respectively.

Synthesis of compounds 12a and b was showed in Chart 4. TMP (1) was oxidized by SeO₂ to afford compound 9, which was reduced to compound 10 by treatment with NaBH₄. Compound 10 was brominated in the presence of PBr₃ to afford compound 11. Compound 11 was reacted with compound 7a or b to produce compound 12a or b.

The synthesis of compounds 20a and b was showed in Chart 5. The key intermediates 18a and b were prepared by treatment of compound 17 with compounds 16a or b. Compound 17 was obtained by treating compound 9 with ethylene glycol catalyzed by p-toluenesulfonic acid. The protective group of compounds 18a and b was cleaved in a solution of concentrated sulfuric acid, water and tetraphosphofuran to afford compounds 19a and b, which were then reacted with tert-butyl-hydroxylamine to produce the target compounds 20a and b.

Synthesis of compounds 22a and b was showed in Chart 6. Compounds 7a and b were treated with compound 14 in the presence of NaHCO₃ to afford compounds 21a and b. Compounds 21a and b were converted into compounds 22a and b using a similar method to that as described for the synthesis of compounds 20a and b.

**Free Radical Scavenging Activity in Vitro** A growing body of evidence suggests that free radicals play a pivotal role in cellular and tissue injury during numerous diseases, including cardiovascular²⁷) and neurodegenerative diseases.²⁸) Free radical scavengers ameliorated cell damage caused by reactive oxygen species during the development of diseases.⁹,²⁹) Because TMP, caffeic acid and nitro compound all have anti-free radical activity,⁸,¹²,³⁰) we first investigated the new compounds’ activity to scavenge diphenyl picryl hydrazyl radical (DPPH·), OH, O₂– and ONOO− in vitro. As shown in Figs. 3A–D, all the new compounds showed a radical clearance activity in a dose-dependent manner and the TMP–caffeic acid derivatives were more potent than TMP dimer derivatives.

There is a positive correlation between free radical scavenging activity and the numbers of the phenolic hydroxyl and nitro groups. As shown in Figs. 3A and D, compound 12a showed the best activity in scavenging DPPH and ONOO−, while 8a exhibited potent scavenging activity of OH and O₂− radicals (8a > 22a > 12a). However, TMP had weak activities on clearance for the tested radicals. TMP was oxidized to TMP-1, 4-((N,N')-dioxide in the presence of H₂O₂ at 70°C²⁴) or catalyzed by horseradish peroxidase at 37°C.⁹) The direct free radical scavenging ability of TMP is weak. The mechanism of anti-oxidative activity and neuroprotection effects of TMP is mostly related to its role in maintaining mitochondrial function, regulating cellular Ca²⁺ homeostasis, enhancing the expression of anti-oxidant proteins and suppressing the ROS generation in vivo.⁶,³¹) Although the anti-oxidant activity of caffeic acid derivatives had been reported, the free cinnamic acid had no activity on free radical scavenging. However, caffeic acid with phenolic hydroxyl group on the benzene ring of cinnamic acid, can scavenge free radicals directly.³²) The phenolic hydroxyl group is essential for free radical scavenging activity, and the 3-hydroxyl group is more important than the 4-hydroxyl group.³⁰)

**Protective Effects of New Compounds against Chemical Ischemic Insult Induced by Iodoacetic Acid (IAA) in Cultured PC12 Cells** IAA is an irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase. It inhibits glycolysis and triggers cellular energy metabolism deficits and induces cell injury quickly in vitro.³³) IAA was usually applied to induce chemical ischemia as a model of neuronal ischemic/hypoxic injury in vitro.³³,³⁴) We evaluated the cytoprotective effects of TMP derivatives against IAA-induced ischemic injury in PC12 cells. As shown in Fig. 4A, there was no cytotoxicity up to 10µM when cells were incubated for 24h with all compounds except compound 12b. Compounds 4, 6, 8a, 20a, b, and 22a, b were not toxic even up to
100 µM. Exposure to 30 µM IAA for 2 h induced approximate 50% reduction of cell viability. All the compounds showed good cytoprotective effect (Fig. 4B). Compounds 8a, b, 12a, 20a, b and 22a, b significantly protected PC12 cells from IAA injury at a concentration of 0.1–10 µM. Although the exact mechanism of IAA toxicity is unclear,35) neural cells exposed to IAA present with an increased production of ROS,36) leading to mitochondrial stress and DNA damage.37,38) Since TMP can maintain mitochondrial function and suppresses ROS generation,9) TMP was found to significantly prevent PC12 cells against IAA-induced neurotoxicity at 1–100 µM. However, TMP was less potent than 8a, b, 12a and 22a, b (Fig. 4B). By comparison, the compounds that combined TMP with other anti-oxidant and radical scavenging moieties were more potent than TMP and TMP dimer compounds in protecting cells against IAA injury. Moreover, compound 22a exhibited more potent cytoprotection and was less cytotoxic than other derivatives. Therefore, compound 22a was selected for further investigation.

**Compound 22a Effectively Prevents tert-Butylhydroperoxide (t-BHP)-Induced Neurotoxicity**

We have previously reported that tert-BHP, an oxidizing agent, produced a concen-
tration-dependent decrease of cell viability in PC12 cells. In the present experiments, exposure to 60 μM t-BHP for 24 h induced about 50% reduction of cell viability in PC12 cells. As shown in Fig. 5A, compound 22a concentration-dependently prevented t-BHP-induced toxicity and the maximum protection reached 96.75 ± 2.55%. Consistently, pretreatment with 22a significantly inhibited t-BHP-induced lactate dehydrogenase (LDH) release (Fig. 5B). The parent TMP at 100 μM had no obvious protection against t-BHP-induced PC12 cells injury.

To further characterize the neuroprotective effects of 22a against neurotoxicity induced by t-BHP, the impact of 22a on
Fig. 4. Neurorescue Effect of New Compounds on IAA-Induced PC12 Cell Injury

(A) Effect of new compounds on viability of PC12 cells (24 h treatment). Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 h incubation with different concentrations of compounds. (B) New compounds rescued IAA-induced PC12 cell injury. PC12 cells were exposed to IAA (30 µM) for 2 h, and the culture medium was then removed and replaced with fresh culture medium contained new compounds with different concentration. Cell viability was measured after 24 h by the MTT assay. ### p<0.001 versus control; ** p<0.05, *** p<0.001 versus IAA alone-treated cells.

Fig. 5. Compound 22a Prevents t-BHP-Induced Neurotoxicity

PC12 cells were pre-incubated with 22a or TMP at the indicated concentrations for 2 h, and were then exposed to 60 µM t-BHP. (A) Compound 22a prevented t-BHP-induced neuronal death in PC12 cells. (B) Compound 22a attenuated t-BHP-induced LDH release in PC12 cells. LDH release was measured at 24 h after t-BHP challenge. (C) Compound 22a prevented t-BHP-induced decrease in mitochondrial membrane potential in PC12 cells. (D) Compound 22a attenuated t-BHP-induced increase of ROS in PC12 cells. Data were expressed the mean±standard error of the mean (S.E.M.) from three independent experiments; ### p<0.001 versus control group; ** p<0.01 and *** p<0.001 versus t-BHP group.
mitochondria membrane potential and intracellular ROS was evaluated. As shown in Fig. 5C, compound 22a significantly prevented the decrease in mitochondrial membrane potential. Moreover, pretreatment of PC12 cells with 22a (1–100 µM) significantly prevented the increase of intracellular ROS induced by t-BHP in PC12 cells (Fig. 5D). Also, TMP at 100 µM had mild effects on the decrease of mitochondrial membrane potential and the ROS overproduction. Therefore, our result suggests that the neuroprotective effect of 22a against t-BHP neurotoxicity is partially attributed to maintaining mitochondrial membrane potential and inhibiting the ROS overproduction.

**Compound 22a Blocks t-BHP-Induced Apoptosis in PC12 Cells**

To verify whether apoptosis is involved in 60 µM t-BHP induced neuronal injury in PC12 cells, Hoechst 33342 staining was performed. As observed from phase contrast fluorescence microscopy (Fig. 6A), most nuclei in the control displayed uniform distribution of chromatin. Intense Hoechst-stained nuclei (chromatin condensation), indicating cells apoptosis, were not frequently observed in control group. However, apoptotic nuclei were observed with increased frequency in t-BHP-stimulated cells compared to control cells. Pretreatment with 22a significantly reversed the cell numbers of nuclear condensation (Fig. 6B). Furthermore, Western blot assay showed that pretreatment of 22a reversed the t-BHP-induced down-regulation of Bcl-2 expression and up-regulation of Bax expression, two apoptosis-related proteins. The Bcl-2/Bax ratio was increased to 124.6±2.4% of control by pretreatment of 22a (Figs. 6C, D).

**Effect of Compound 22a on the Phosphatidylinositol 3-Kinase (PI3K)/Akt/Glycogen Synthase Kinase-3β (GSK3β) Signaling Pathway**

In order to elucidate the signaling pathways involved in the protective effects of 22a against oxidative stress-induced cell injury, we assessed the relationship between cell viability and regulation of PI3K/Akt/GSK3β. PC12 cells were pretreated with Akt inhibitor IV (1 µM) and PI3K inhibitor LY294002 (1 µM) for 30 min prior to the addition of compound 22a for 2 h incubation, and were then exposed to 60 µM t-BHP. Pretreatment with these inhibitors could significantly attenuate the cytoprotective effect of 22a was dependent on activation of PI3K/Akt pathway. Furthermore, Western blot assay showed that pretreatment of 22a reversed the t-BHP-induced down-regulation of Akt and GSK3β phosphorylation (Figs. 7B–E). When the cells were exposed to the PI3K inhibitor, LY294002 (1 µM), 30 min prior to 22a treatment, 22a-induced phosphorylation of Akt and GSK3β were almost completely suppressed by LY294002 (Figs. 7B–E). Previous studies have demonstrated that activation of Akt promoted cell survival and provided protection against oxidative stress-induced neurotoxicity via the PI3K/Akt/GSK3β pathway.39,40

In our present study, exposure to t-BHP significantly down-regulated phosphorylation of Akt and GSK3β in PC12 cells. However, 22a pretreatment reversed down-regulation of phosphorylated Akt and GSK3β stimulated by t-BHP. Moreover, PI3K inhibitor LY29004 completely suppressed upregulation of phosphorylated Akt and GSK3β, and abolished the neuro-

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**Fig. 6. Compound 22a Blocks t-BHP-Induced Cell Apoptosis in PC12 Cells**

PC12 cells were pre-incubated with 22a or TMP at the indicated concentrations for 2 h, and were then exposed to 60 µM t-BHP. (A) Compound 22a attenuated t-BHP-induced increase of pyknotic nuclei in PC12 cells. (B) Statistical analysis of the number of pyknotic nuclei. The number of pyknotic nuclei with condensed chromatin were counted from representative Hoechst staining photomicrographs and represented as a percentage of the total number of nuclei counted. (C) Representative blots of the apoptosis-related protein Bcl-2 and Bax. (D) Densitometric analysis of the protein expression of Bcl-2 and Bax. Data were expressed the mean±S.E.M. from three independent experiments; ***p<0.001 versus control group; *p<0.05, **p<0.01 and ***p<0.001 versus t-BHP group.
protection conferred by 22a pretreatment. Collectively, our results suggest that activation of PI3K/Akt/GSK3β pathway is involved in the neuroprotective effect of 22a against t-BHP-induced PC12 cell injury.

**Effect of Compound 22a on Neurological Deficits, Cerebral Infarction and Brain Edema in a Rat Model of p-MCAO** Since compound 22a conferred potent radical scavenging and cytoprotective activities in vitro, we further evaluated its effects in a rat model of p-MCAO. Neurological deficits may persist for months or years post-stroke in those affected individuals who survived in initial cerebrovascular insult. As one of the most commonly used stroke model, the rat MCAO model produces a replicable and reliable lesion both in the cortex and striatum, and results in both motor and some cognitive deficits. As shown in Fig. 8A, all rats except sham-operated ones presented neurological deficit. However, the rats received 30mg/kg of 22a by intraperitoneal administration at 3 and 6h after p-MCAO exhibited significant improvement in neurological deficits at 24h after p-MCAO injury compared with the model group.

The size of the infarction after an ischemic insult is regarded as the most straight forward indicator of the effectiveness of a neuroprotective agent because it shows the severity of brain damage. To observe the neuroprotective efficacy of 22a...
against p-MCAO injury, the infarct area and brain edema were also evaluated (Figs. 8B, C). Infarct area of p-MCAO model group was 62.1±6.2%, while the infarct area was decreased to 49.1±8.9% in the 22a treated group. In addition, the brain edema of vehicle group was 17.1±3.2% and that of the 22a treated group was reduced to 11.5±5.4%. These data revealed that 22a significantly reduced the infarct volume and brain edema compared to p-MCAO rat. The reduced brain infarction and edema would contribute the neurobehavioral improvement exerted by 22a.

It has been reported that oxidative stress is an early and foremost pathological factor of MCAO-induced brain damage, which leads to deteriorating functional outcomes. Numerous neuroprotective agents with anti-oxidant activity have been tested on stroke animal models and in clinical stroke patients. One of the principle features of an effective stroke neuroprotective agent is its ability to penetrate the BBB to reach the site of action. TMP’s superior ability to enter the brain was documented as early as 500 years ago in the landmark traditional Chinese medicine literature. Compound 22a consisted of TMP, caffeic acid and a nitrone group, thus, it is highly likely that 22a can readily penetrate the BBB to exert potent anti-free radical activity in the experimental animal stroke model. In further studies, we will investigate the ability of compound 22a in penetrating the BBB and report the result in due course.

Taken from all the results, compound 22a consisted of TMP, caffeic acid and nitrone group exhibited much more potent radical-scavenging activity and neuroprotective effect in vitro and in cerebral ischemic stroke in vivo. In free radicals scavenging assay, compound 22a was effective in scavenging different types of free radicals, especially for peroxynitrite. We think that the improved peroxynitrite scavenging activity of compound 22a is primarily due to the introduction of nitrone group since TMP showed very weak activity in scavenging peroxynitrite in the current and our previous studies. It should be noted that compound 22a’s free radical-scavenging activity and neuroprotective effect in oxidative-induced neurotoxicity are much more potent than the parent compound TMP. However, TMP moiety has super ability to cross BBB, thus it can deliver caffeic acid and nitrone to the brain tissue, conferring neuroprotection in vivo. Therefore, the excellent bioactivities of compound 22a in vitro and in vivo are possible combinational effects of TMP, caffeic acid and nitrone.

Conclusion
In this study, we have designed and synthesized novel TMP derivatives. Among them, compound 22a, TMP conjugated with caffeic acid and tert-butylnitrone, showed potent free radical scavenging activity and neuroprotective effect against oxidative injury in vitro and cerebral ischemic stroke in vivo. Based on these findings, compound 22a may be a promising drug candidate for treating neurodegenerative disorders, including ischemic stroke in particular. Therefore, the pharmacokinetics, toxicity profile, and the BBB penetrating property of 22a warrant further evaluation.

Experimental
The details of the experimental procedures are provided in the supplementary materials, which can be found as attachment.

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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Materials
The online version of this article contains supplementary materials.

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