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

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Abstract

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 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 *t*-BHP-induced neuronal injury. Compound **22a** exerted its neuroprotective effect against *t*-BHP injury *via* activation of the PI3K/Akt signaling pathway. Furthermore, in a rat model of permanent middle cerebral artery occlusion, compound **22a** significantly improved neurological deficits, and alleviated the infract 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.

Keyword

Tetramethylpyrazine, nitrones, caffeic acid, free radical, neuroprotection, permanent middle cerebral artery occlusion.

1. Introduction

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.[1)] 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.[2)] 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.[3,4)] Previous research found that TMP had therapeutic effects in neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), and ischemic stroke.[5-8)] 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.[8,9)] In addition, TMP has superior capability to enter the brain.[10)] 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.[11)]

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.[12] PBN (N-*tert*-butyl-α-phenylnitrone) and NXY-059 are two representative nitrone compounds. PBN reduced brain infarction in a rat model of middle cerebral artery occlusion (MCAO).[13] 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.[14] Previously, we have reported a TMP nitrone conjugate, named TBN, which readily crossed the BBB and showed potent free radical scavenging ability, protecting neurons and rat suffering ischemic stroke and PD.[15,16]

Caffeic acid (**Fig. 1**), a hydroxyl derivative of cinnamic acid bearing a phenolic moiety, is belonging to the family of the phenyl propanoids.[17] Caffeic acid is abundant in nature, has versatile pharmacological activities, including anti-oxidative stress, anti-inflammatory, anti-cancer, and anti-viral activities.[18] Caffeic acid and its ester derivatives showed an excellent neuroprotection and therapeutic effects in various neurological diseases.[19] The pharmacokinetics and tissue distribution study revealed that caffeic acid had weak BBB permeability and presented a low brain distribution proportion.[20]

Traditional drug development approach, one drug one target, is proven unsuccessful in treating complex brain disorders that involve multiple pathogenic factors.[21] 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.[22] Since all of the three pharmacologically active compounds/functional moieties, i.e., TMP, cafferic acid and nitrone, have versatile functions beneficial to neurological diseases, we reason that compounds combing 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).

2. Results and Discussion

2.1. 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.[23] Substitutions of the methyl group apparently improved drug efficacy and prolonged action time.[24] 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.[25] 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-8b**, **12a-12b**, **Fig. 2**). Since the nitrone moiety has great potential to scavenging free radicals, to further enhance the anti-radical activities, the nitrone moiety(ies) was (were) introduced into the new compounds to obtain compounds **20a-20b** and **22a-22b**

(**Fig. 2**).

Compound **4** was obtained by treatment of compound **2** with compound **3** in a solution of $NaHCO_3$ (**Chart 1**). Compounds **2** and **3** were synthesized as previously reported.[26,25)] 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-b** was showed in **Chart 3**. The commercially available caffeic acid (**7a**) was treated with acetic anhydride to afford compound **7b**. Compounds **7a** and **7b** were treated with compound **3** in the presence of $NaHCO_3$ to afford compounds **8a** and **8b**, respectively.

Synthesis of compounds **12a** and **12b** was showed in **Chart 4**. TMP (**1**) was oxidized by SeO_2 to afford compound **9,** which was reduced to compound **10** by treatment with $NaBH_4$. Compound **10** was brominated in the presence of PBr_3 to afford compound **11**. Compound **11** was reacted with compound **7a** or **7b** to produce compound **12a** or **12b**.

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

Synthesis of compounds **22a** and **22b** was showed in **Chart 6**. Compounds **7a** and **7b** were treated with compound **14** in the presence of $NaHCO_3$ to afford compounds **21a** and **21b**. Compounds **21a** and **21b** were converted into compounds **22a** and **22b** using a similar method to that as described for the synthesis of compounds **20a** and **20b**.

2.2. 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 cardiovascular[27] and neurodegenerative diseases.[28] Free radical scavengers ameliorated cell damage caused by reactive oxygen species during the development of diseases.[19,29] Because TMP, caffeic acid and nitrone group all have anti-free radical activity,[9,12,30] we first investigated the new compounds' activity to scavenge DPPH•, •OH, $O_2^{\bullet-}$ 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 nitrone groups. As shown in **Figs. 3A and 3D**, compound **12a** showed the best activity in scavenging DPPH• and $ONOO^-$, while **8a** exhibit potent scavenging activity of •OH and $O_2^{\bullet-}$ 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 H_2O_2 at 70 ^oC[24] or catalyzed by horseradish peroxidase at 37 ^oC.[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 Ca^{2+} homeostasis, enhancing the expression of anti-oxidant proteins and suppressing the ROS generation *in vivo*.[9,31] 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]

2.3. Protective effects of new compounds against chemical ischemic insult induced by iodoacetic acid in cultured PC12 cells

Iodoacetic acid (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 10 μM when cells were incubated for 24 h 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 prevented 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 combing 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.

2.4. Compound 22a effectively prevents *t*-BHP-induced neurotoxicity

We have previously reported that *t*-BHP, an oxidizing agent, produced a concentration-dependent decrease of cell viability in PC12 cells.[33] 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 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 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.

2.5. Compound 22a blocks *t*-BHP-induced apoptosis in PC12 cells

To verify weather 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 and 6D**).

2.6. Effect of compound 22a on the PI3K/Akt/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** (**Fig. 7A**), suggesting that 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 neuroprotection 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.

2.7. 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.[41] 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.[42] As shown in **Fig. 8A**, all rats except sham-operated ones presented neurological deficit. However, the rats received 30 mg/kg of **22a** by intraperitoneal administration at 3 and 6 h after *p*-MCAO exhibited significantly improvement in neurological deficits at 24 h 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 infract area and brain edema were also evaluated (**Figs. 8B and 8C**). Infract area of *p*-MCAO model group was 62.1±6.2%, while the infract 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.[43] Numerous neuroprotective agents with anti-oxidant activity have been tested on stroke animal models and in clinical stroke patients.[44] 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.[10] 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 result, compounds **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.[45] 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 compounds **22a** in vitro and in vivo are possible combinational effects of TMP, caffeic acid and nirtone.

3. Conclusion

In this study, we have designed and synthesized novel TMP derivatives. Among them, compound **22a**, TMP conjugated with caffeic acid and *tert*-butyl nitrone, 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 Section

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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Figure and Chart legends

Fig. 1. Structures of TMP, TBN and caffeic acid.

Fig. 2. Structures of new compounds.

Fig. 3. Free radical scavenging effects. (A) Activity against DPPH•. (B) Activity against •OH. (C) Activity against $O_2^{\bullet-}$ (D) Activity against $ONOO^-$.

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 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<0.001 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 ± SEM from three independent experiments; ###P<0.001 versus control group; **P<0.01 and ***P<0.001versus *t*-BHP group.

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 pykonitic 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 ± SEM from three independent experiments; ###P<0.001 versus control group; *P<0.05, **P<0.01 and ***P<0.001versus *t*-BHP group.

Fig. 7. Involvement of PI3K/Akt/GSK3β pathway in cytoprotection exerted by 22a in PC12 cells. (A) Pretreatment with the LY294002 (LY29) and Akt inhibitor IV (Akt IV-i) abolished the protective effect of **22a** against *t*-BHP-induced PC12 cells damage. PC12 cells were pretreated with PI3K inhibitor LY294002 (1 μM) and Akt inhibitor IV (1 μM) for 30 min prior to the addition of compound **22a** for another 2 h co-incubation**,** and then followed by exposure to 60 μM *t*-BHP. Cell viability was measured at 24 h post *t*-BHP challenge by using the MTT assay. (B) Representative blots of p-Akt and Akt expression. (C) Densitometric analysis of the protein expression of p-Akt and Akt. (D) Representative blots of p-GSK3β and GSK3β expression. (E) Densitometric analysis of the protein expression of p-GSK3β and GSK3β. Data were expressed the mean ± SEM from three independent experiments. ###P<0.001 and ##P<0.01 versus control group; *P<0.05, **P<0.01 and ***P<0.001versus *t*-BHP group.

Fig. 8. Therapeutic effect of 22a in a rat model of *p*-MCAO. (A) Compound **22a** alleviated neurological deficits. (B) Compound **22a** attenuated infract areas. (C) Compound 22a reduced brain edema. Data were expressed the mean ± SEM, n=6 rats/group. *P<0.05 versus model group.

Chart 1. Synthesis of compound 4. Reagents and conditions: (a) $KMnO_4$, H_2O, 50 ^oC, 10 h; (b) NBS, benzoyl peroxide, r.t., overnight; (c) $NaHCO_3$, DMF, r.t., 2 h.

Chart 2. Synthesis of compound 6. Reagents and conditions: (a) 1. H_2O_2, AcOH, 70 ºC, 8 h; 2. Ac_2O, 125 ºC, 3 h; 3. NaOH, r.t., 5 h; (b) 1. NaH, THF, r.t.; 2. compound **3**, DMF, r.t., 3 h.

Chart 3. Synthesis of compounds 8a-8b. Reagents and conditions: (a) DMAP, Ac_2O, r.t., overnight; (b) compound **7a** for **8a**, or compound **7b** for **8b**, $NaHCO_3$, DMF, r.t., 2 h.

Chart 4. Synthesis of compounds 12a-12b. Reagents and conditions: (a) SeO_2, dioxane, 110 ^oC, 6 h; (b) $NaBH_4$, $(CH_2Cl)_2$, r.t., 3 h; (c) PBr_3, DCM, 0 ^oC, 3 h; (d) compound **7a** for **12a** or compound **7b** for **12b**, $NaHCO_3$, DMF, r.t., 3 h.

Chart 5. Synthesis of compounds 20a-20b. Reagents and conditions: (a) $NaBH(OCOCH_3)_3$, $(CH_2Cl)_2$, r.t., 4 h; (b) PBr_3, CH_2Cl_2, 0 ^oC, 3 h; (c) $HOCH_2CH_2OH$, toluene, TsOH, 80 ^oC, 3 h; (d) $(CH_3CH_2O)_3OP$, toluene, 110 ^oC, overnight; (e) $HOCH_2CH_2OH$, toluene, TsOH, 80 ^oC, 3 h; (f) compound **16a** or **16b**, $NaOCH_3$, CH_2Cl_2, r.t., 2 h; (g) con.HCl/H_2O/THF=2/6/7, r.t., 2 h; (h) *tert*-butylhydroxylamine, CH_3CH_2OH, r.t., 4 h.

Chart 6. Synthesis of compounds 22a-22b. Reagents and conditions: (a) compound **14**, $NaHCO_3$, DMF, r.t., 3 h; (b) *tert*-butylhydroxylamine, CH_3COCH_3, r.t., 4 h.

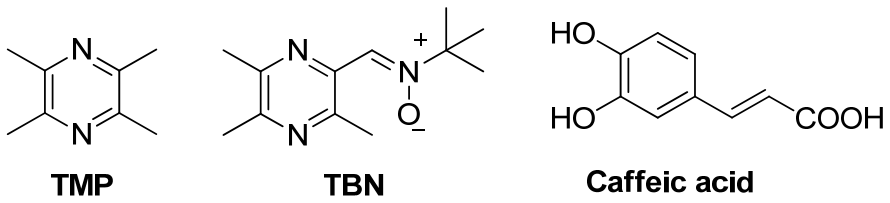

TMP
TBN
Caffeic acid

Fig.1.

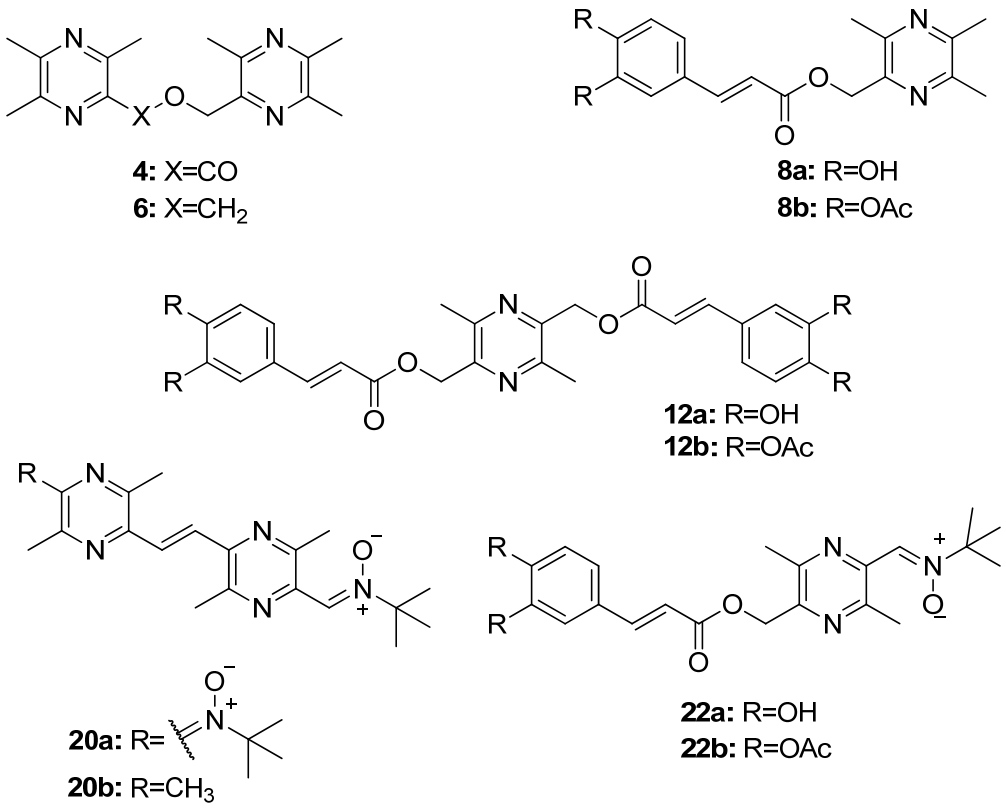

4: X=CO
6: X=CH2
8a: R=OH
8b: R=OAc
12a: R=OH
12b: R=OAc
20a: R=
20b: R=CH3
22a: R=OH
22b: R=OAc

Fig. 2.

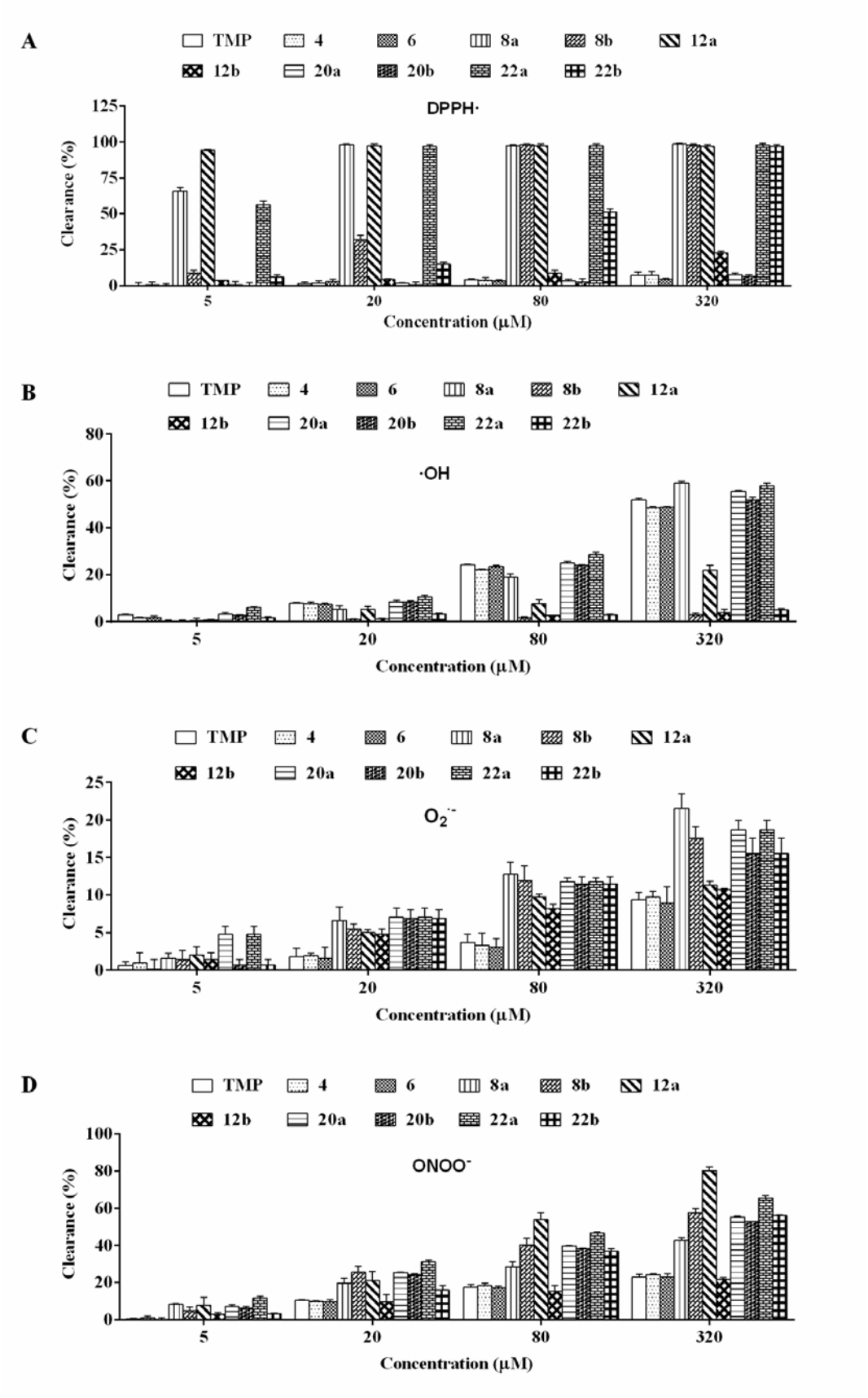

A
TMP
4
6
8a
8b
12a
12b
20a
20b
22a
22b
DPPH·
Clearance (%)
125
100
75
50
25
0
5
20
80
320
Concentration (μM)
B
TMP
4
6
8a
8b
12a
12b
20a
20b
22a
22b
·OH
Clearance (%)
80
60
40
20
0
5
20
80
320
Concentration (μM)
C
TMP
4
6
8a
8b
12a
12b
20a
20b
22a
22b
O2·-
Clearance (%)
25
20
15
10
5
0
5
20
80
320
Concentration (μM)
D
TMP
4
6
8a
8b
12a
12b
20a
20b
22a
22b
ONOO-
Clearance (%)
100
80
60
40
20
0
5
20
80
320
Concentration (μM)

Fig. 3.

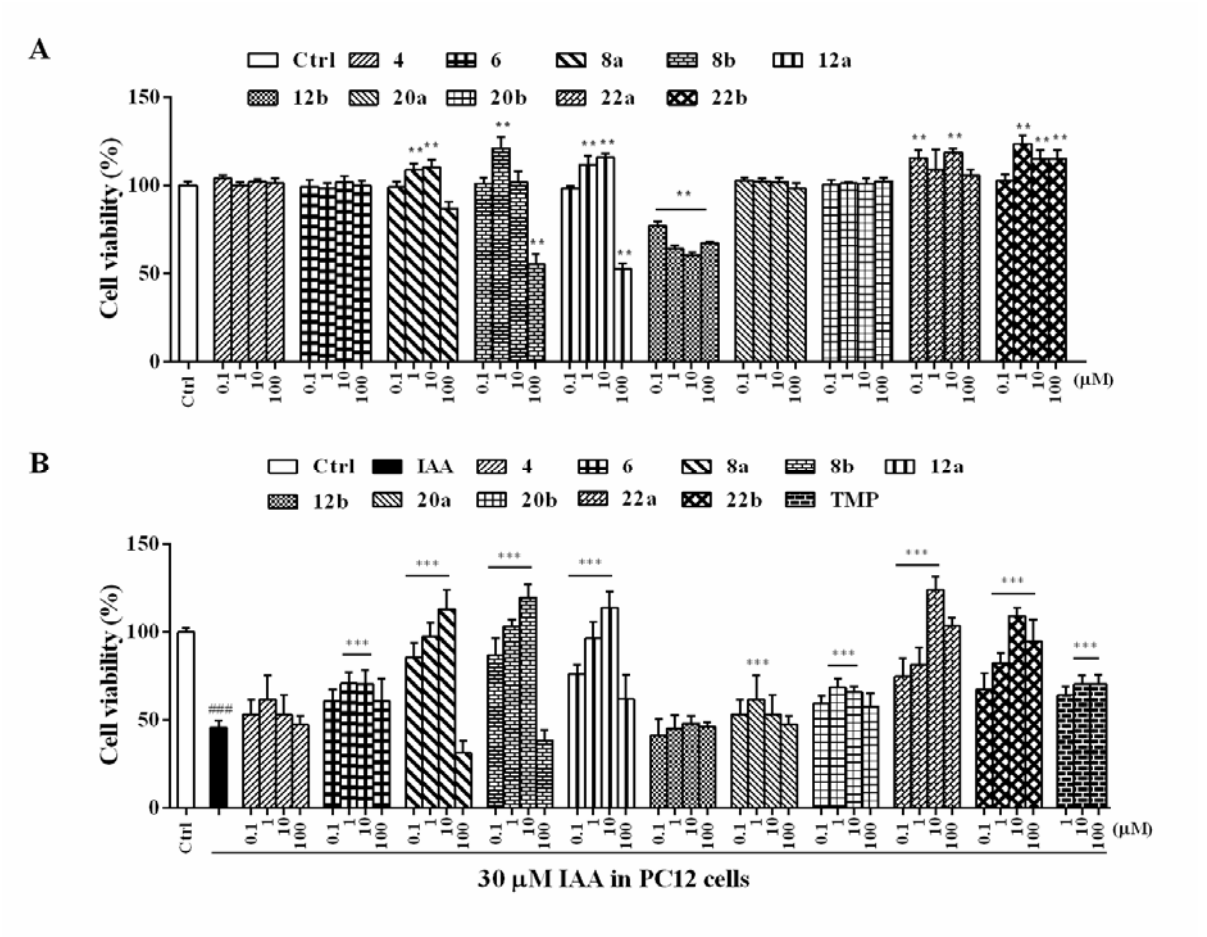

A
Ctrl
4
6
8a
8b
12a
12b
20a
20b
22a
22b
Cell viability (%)
150
100
50
0
(μM)
B
Ctrl
IAA
4
6
8a
8b
12a
12b
20a
20b
22a
22b
TMP
Cell viability (%)
###
30 μM IAA in PC12 cells

Fig. 4.

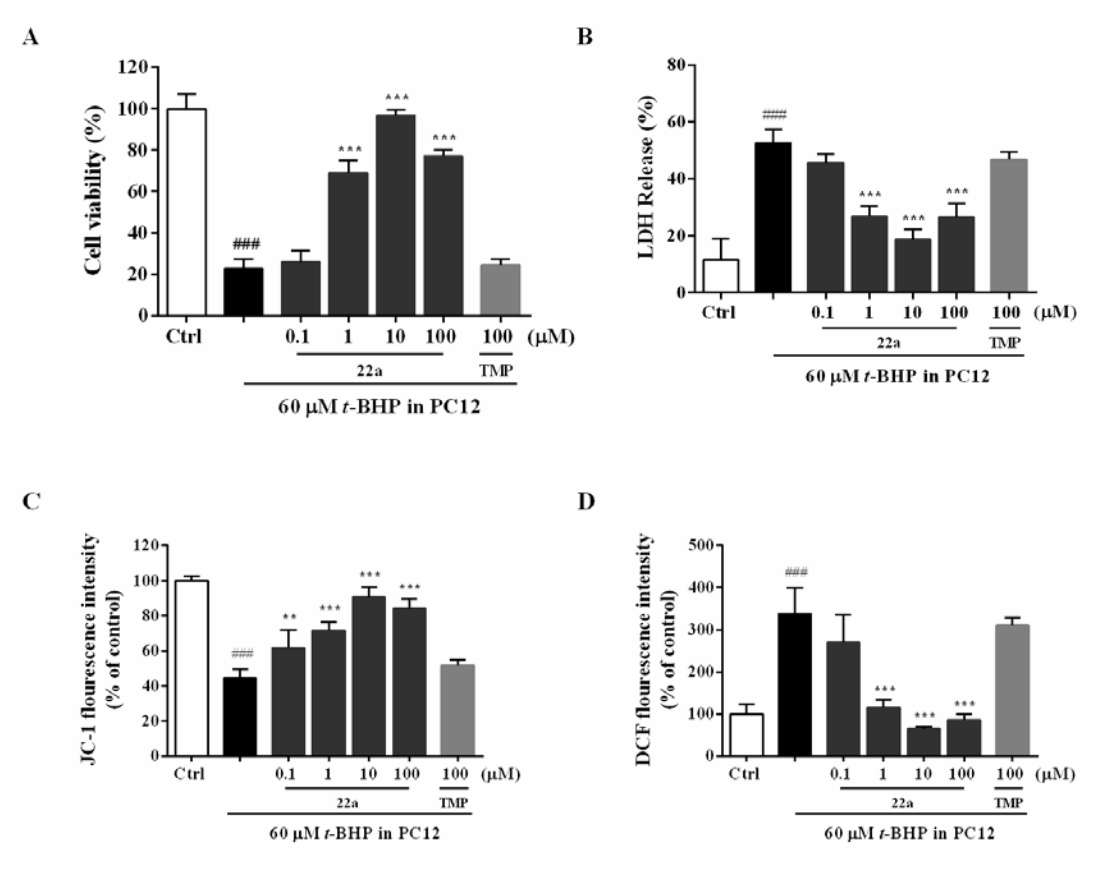

A
Cell viability (%)
120
100
80
60
40
20
0
###
***
***
***
Ctrl
0.1
1
10
100
100 (μM)
22a
TMP
60 μM t-BHP in PC12
B
LDH Release (%)
80
60
40
20
0
####
***
***
***
Ctrl
0.1
1
10
100
100 (μM)
22a
TMP
60 μM t-BHP in PC12
C
JC-1 flourescence intensity (% of control)
120
100
80
60
40
20
0
###
**
***
***
***
Ctrl
0.1
1
10
100
100 (μM)
22a
TMP
60 μM t-BHP in PC12
D
DCF flourescence intensity (% of control)
500
400
300
200
100
0
###
***
***
***
Ctrl
0.1
1
10
100
100 (μM)
22a
TMP
60 μM t-BHP in PC12

Fig. 5.

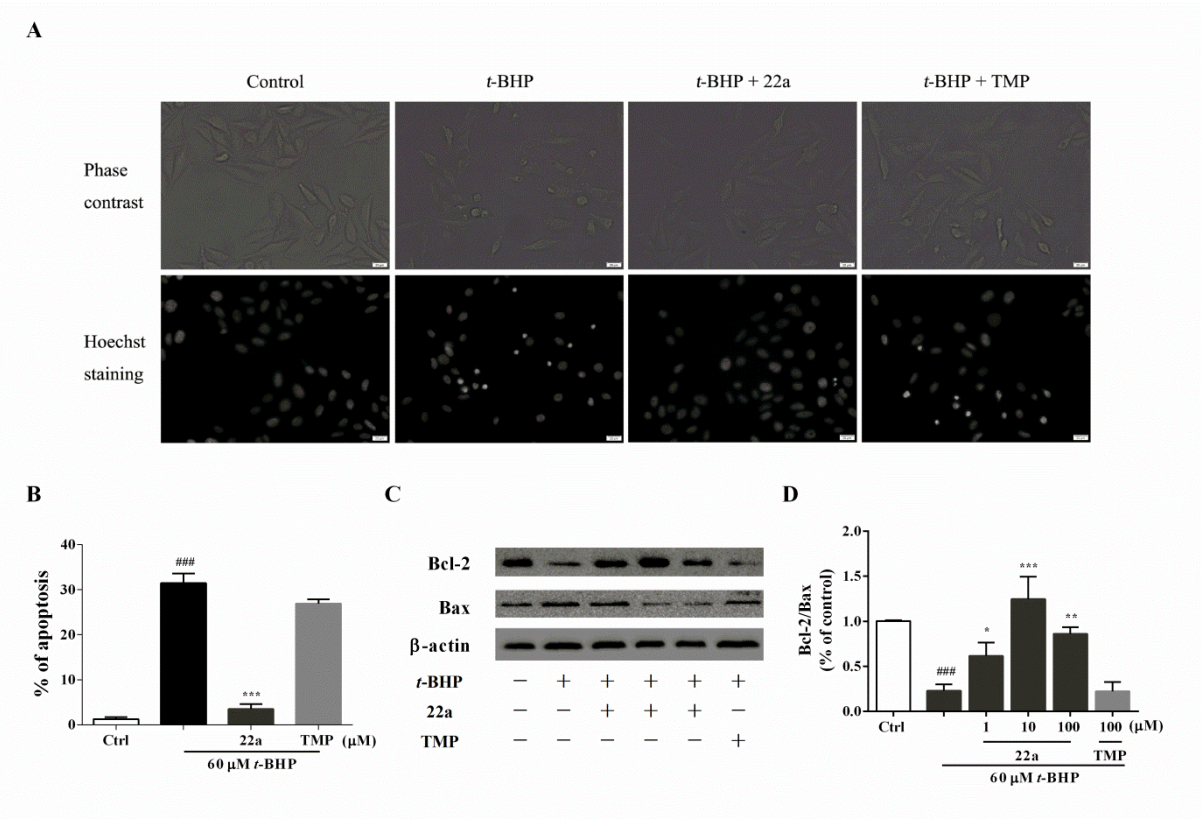

A
Control
t-BHP
t-BHP + 22a
t-BHP + TMP
Phase contrast
Hoechst staining
B
% of apoptosis
###
***
Ctrl
22a
TMP
(μM)
60 μM t-BHP
C
Bcl-2
Bax
β-actin
t-BHP
22a
TMP
D
Bcl-2/Bax (% of control)
###
*
***
**
Ctrl
1
10
100
100
(μM)
22a
TMP
60 μM t-BHP

Fig. 6.

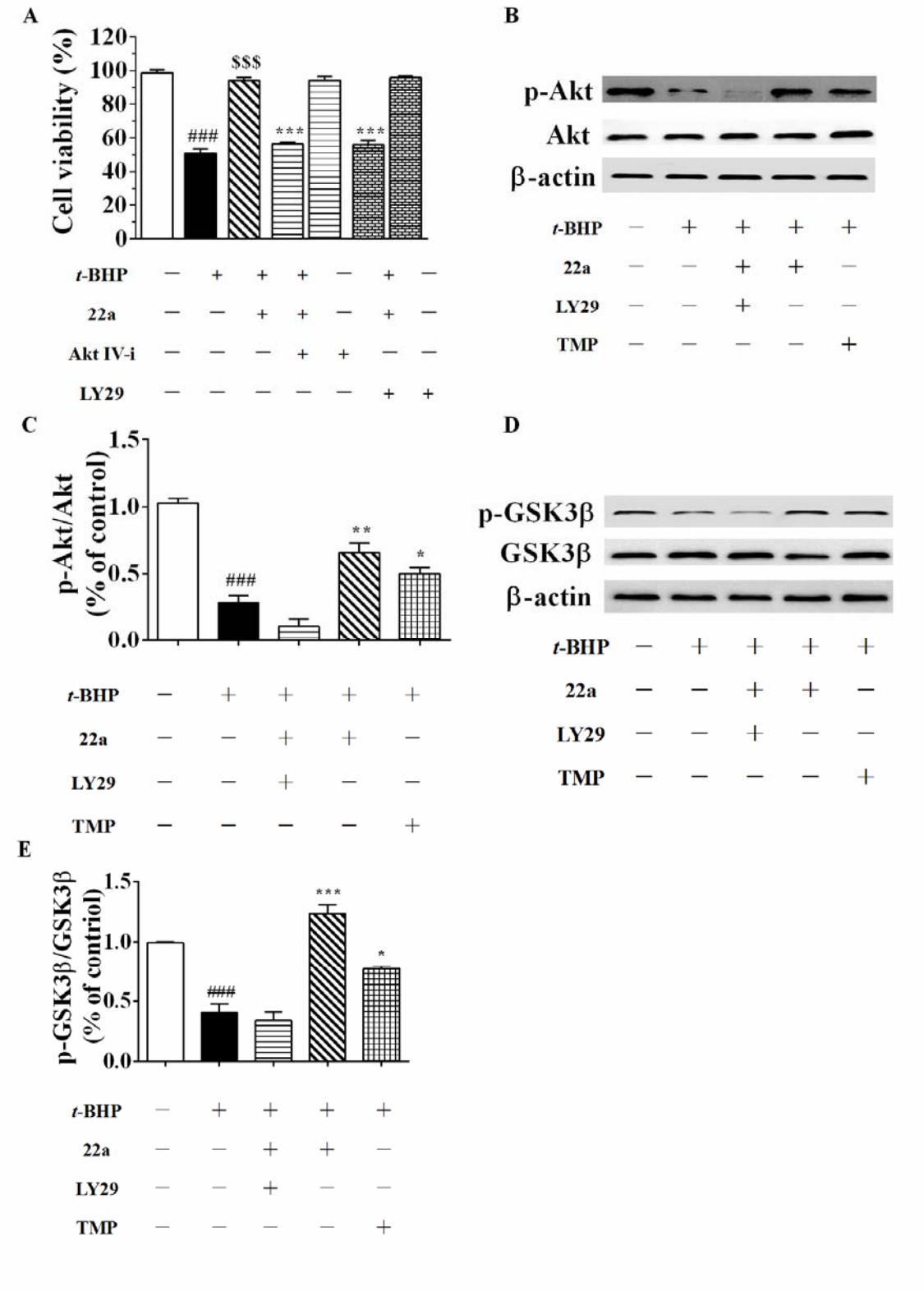

A
Cell viability (%)
120
100
80
60
40
20
0
###
$$$
***
***
t-BHP
22a
Akt IV-i
LY29
B
p-Akt
Akt
β-actin
t-BHP
22a
LY29
TMP
C
p-Akt/Akt (% of control)
1.5
1.0
0.5
0.0
###
**
*
t-BHP
22a
LY29
TMP
D
p-GSK3β
GSK3β
β-actin
t-BHP
22a
LY29
TMP
E
p-GSK3β/GSK3β (% of contriol)
1.5
1.0
0.5
0.0
###
***
*
t-BHP
22a
LY29
TMP

Fig. 7.

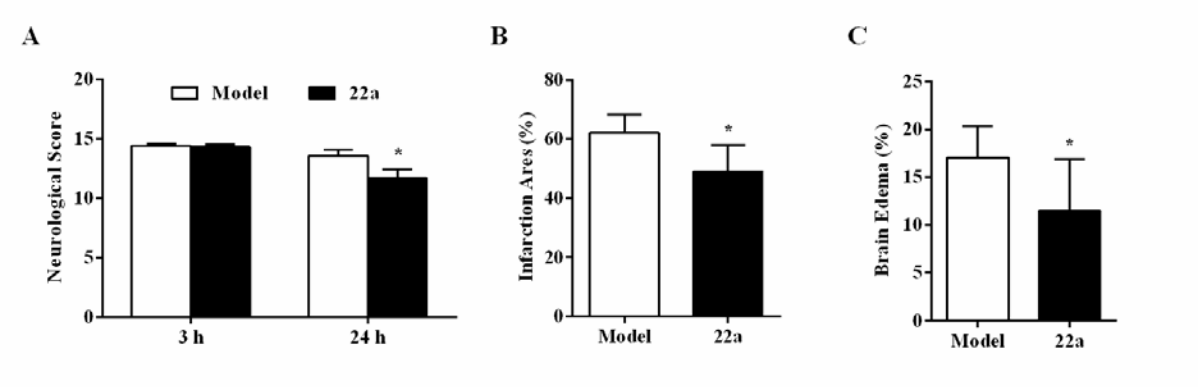

A
B
C
Model
22a
Neurological Score
20
15
10
5
0
3 h
24 h
*
Infarction Ares (%)
80
60
40
20
0
Model
22a
*
Brain Edema (%)
25
20
15
10
5
0
Model
22a
*

Fig. 8.

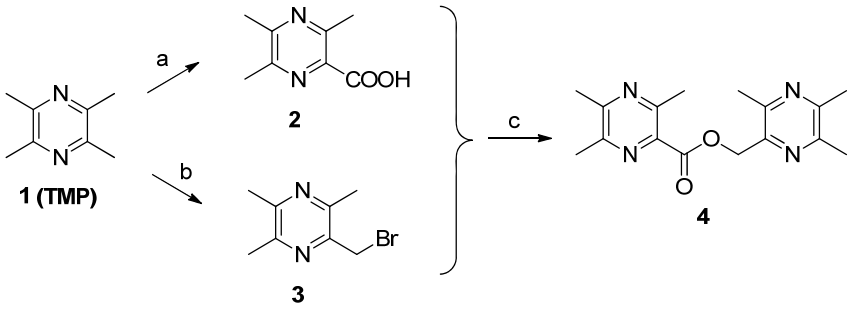


Chart 1.

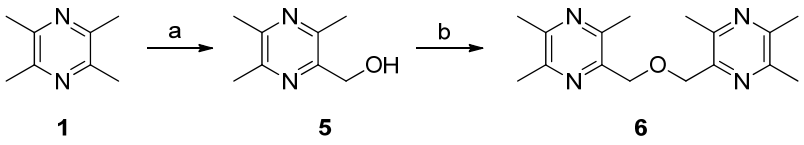
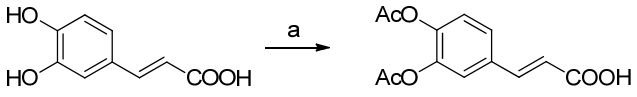
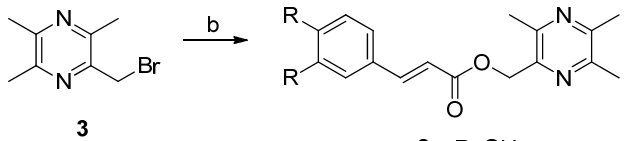


Chart 2.



Cafefic acid 7a

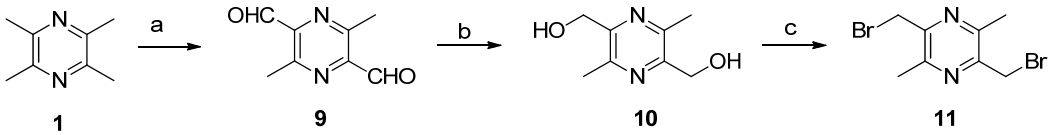
7b

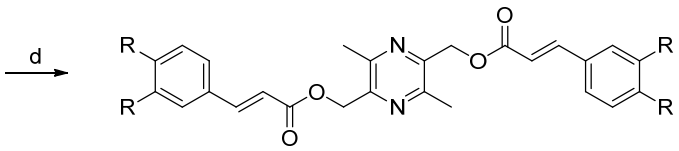


3

8a: R=OH
8b: R=OAc

Chart 3.





12a: R=OH
12b: R=OAc

Chart 4.

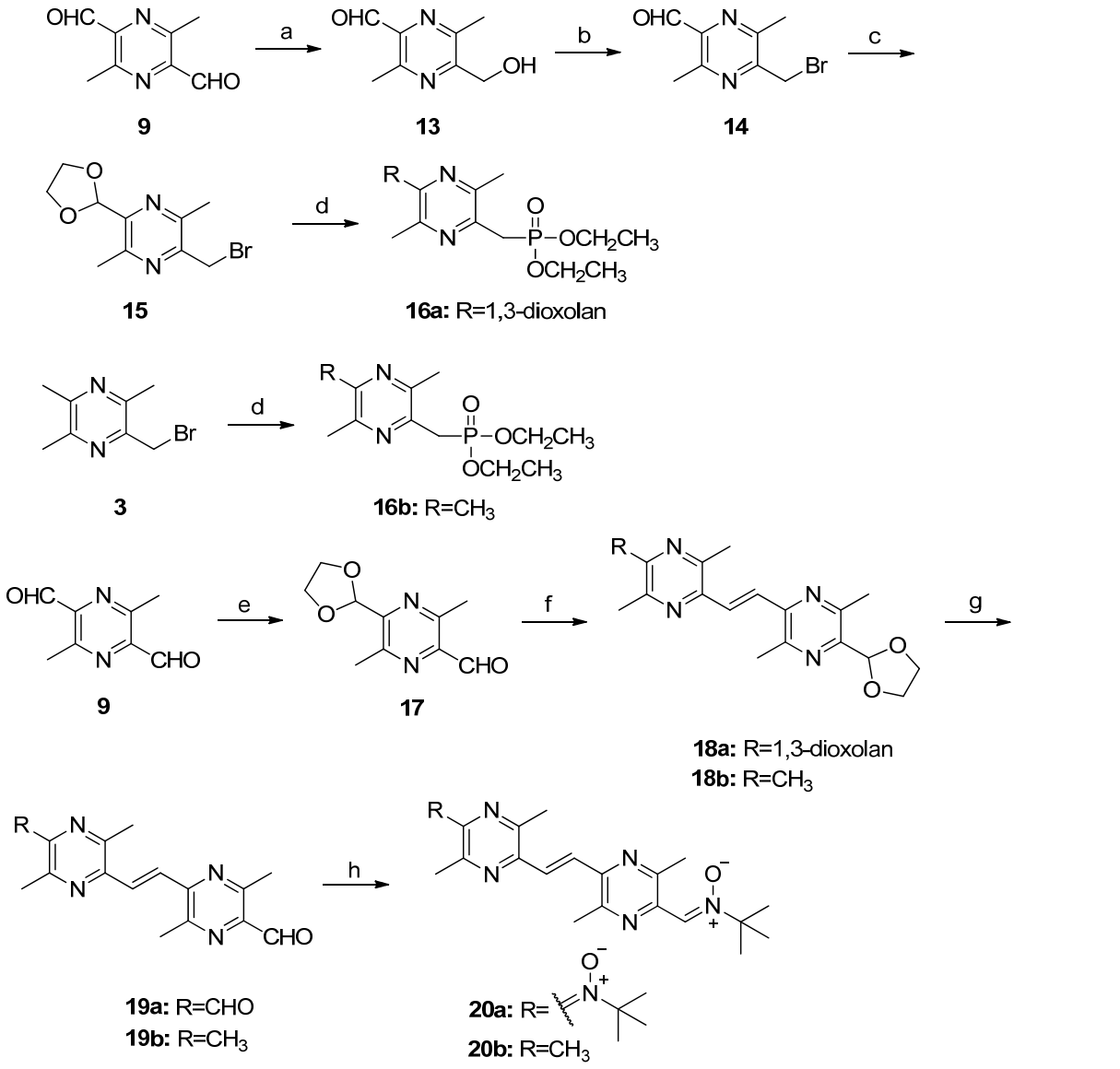


Chart 5.

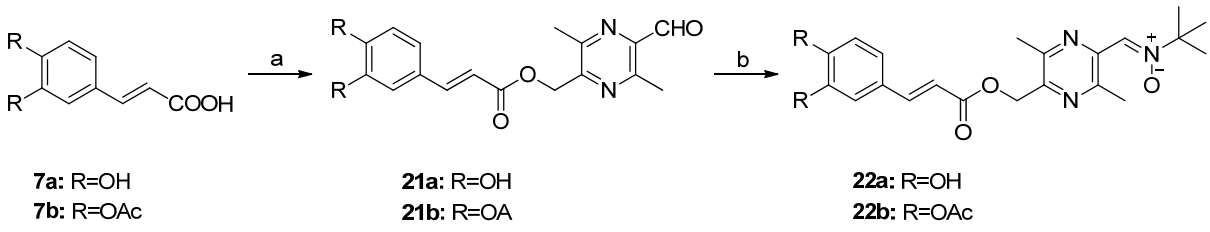


Chart 6.