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The synthesis and biological evaluation of novel gardenamide A derivatives as multifunctional neuroprotective agents†

Zuzhi Zhang,^{ab} Yujun Wang,^a Yanchun Zhang, ^{*ab} Jiaming Li,^{ab}
Weijun Huang^a and Lei Wang^{ab}

A novel series of gardenamide A derivatives was synthesized as potential anti-Alzheimer's disease agents. The neuroprotective effects of these multifunctional agents against oxygen–glucose deprivation (OGD)-induced neurotoxicity in rat cortical neurons, and hydrogen peroxide (H₂O₂)- and amyloid- β_{1-42} (A β_{1-42})-induced neurotoxicity in rat hippocampal neurons were evaluated. *In vitro* studies revealed that these compounds demonstrated moderate to good multifunctional neuroprotective activity. Among the entire series, compounds **10e**, **10j**, **10n** and **10p** appeared to be the most active multifunctional neuroprotective agents. Studies indicate that compounds **10e**, **10f**, **10h**, **10i**, **10j**, **10n** and **10p** exhibit significant activities against OGD-induced neurotoxicity in rat cortical neurons, and **10e**, **10j**, **10n** and **10p** show prominent activities against H₂O₂- and A β_{1-42} -induced neurotoxicity in rat hippocampal neurons. Moreover, these derivatives did not exert conspicuous neurotoxicity in rat cortical neurons. Thus, the present study evidently shows that **10e**, **10j**, **10n** and **10p** are potent multifunctional neuroprotective agents, which may serve as promising lead candidates for anti-Alzheimer's disease drug development.

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1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is a multi-faceted neurodegenerative brain disorder featured by memory loss, progressive deficits in cognitive functions, linguistic decline, severe behavioral abnormalities and eventual death.¹ It was estimated by the World Alzheimer's Report that approximately 47 million people worldwide suffer from AD, which may double every 20 years.² Several characteristic pathological features, such as amyloid- β deposits (A β), tau protein aggregation, oxidative stress, decreased level of acetylcholine (ACh), neuro-inflammation and dyshomeostasis of biometals have been thought to play vital roles in the pathogenesis of AD. However, the etiology of AD is still not completely known.³ Although some anti-AD drugs, such as acetylcholinesterase inhibitors (AChEIs): donepezil, tacrine, rivastigmine, and galantamine and *N*-methyl-D-aspartate (NMDA) receptor antagonist, memantine have been approved for the treatment for AD, only the symptomatic relief of AD is provided rather than a cure. Therefore, there is an urgent

need for the development of novel anti-AD drugs that are safe and effective with minimal side effects.

Abundant evidence has demonstrated that A β , the major component of senile plaque, with a series of neurotoxicity, exerts direct or indirect injuries on the nervous system.⁴ Moreover, the excessive deposition and accumulation of A β in the brain initiate the generation of reactive oxygen species (ROS),⁵ eventually leading to oxidative damage, which may contribute to the progression of AD. Hydrogen peroxide (H₂O₂) produced during the redox process is the main form of ROS, which causes protein and lipid peroxidation, DNA damage and cell death.⁶ In addition, cerebral ischemia is characterized by an insufficient oxygen and glucose supply, which results in imbalanced energy metabolism and finally cell death, and is also believed to be a cause of AD.⁷ As an *in vitro* model of cerebral ischemia, oxygen–glucose deprivation (OGD) has now been extensively used. Based on these observations, the discovery of novel compounds with multifunction for protecting neurons from toxicity induced by OGD, H₂O₂ and A β may be an effective strategy for the treatment of AD.

Natural products offer great chemical diversity and provide rich sources of potential therapeutic agents (Fig. 1). Geniposide (**1**) is one of the main iridoid glycosides isolated from the gardenia fruit (*Gardenia jasminoides* Ellis, *Rubiaceae*), which is widely used as traditional Chinese medicine (TCM) due to its anti-inflammatory,⁸ anti-oxidation,⁹ neurotrophic and

^a Department of Medicinal Chemistry, Anhui University of Chinese Medicine, 103 Meishan Road, Hefei 230031, PR China. E-mail: yczhang2017@163.com

^b Anhui Province Key Laboratory of Chinese Medicinal Formula, Hefei, Anhui, 230012, China. E-mail: lijiaming2004@sina.com

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Fig. 1 Structures of geniposide, genipin and gardenamide A.

neuroprotective properties.¹⁰ Furthermore, geniposide improved cognition in an AD mouse model.¹¹ Interestingly, genipin (2), the aglycone of geniposide, is one of the main effective ingredients of the *G. jasminoides* fruit extract, particularly known for its neuritogenic and neuroprotective actions.¹² It protects neuronal cells against cytotoxicity induced by various neurotoxic agents including A β , 6-hydroxydopamine (6-OHDA), *tert*-butyl hydroperoxide (*t*BHP) and endoplasmic reticulum stress inducers.^{13–15} These injurious

stimuli represent etiologic agents of neurodegenerative diseases, such as AD and Parkinson's diseases (PD). Gardenamide A (GA, 3), the structural analogue of genipin, was first isolated from *Rothmannia urcelliformis*, which is a widespread small tree in the forests of East Africa,¹⁶ and 2-hydroxyethyl gardenamide A was also found in fruit of *G. jasminoides*.¹⁷ Previous research suggested that gardenamide A displays better neuroprotective effects to serum deprived- and 6-OHDA-induced impairments than genipin in PC12

Fig. 2 Designed strategy for the target compounds.

cells,¹⁸ and attenuate the accumulation of intracellular ROS and the loss of mitochondrial membrane potential. Gardenamide A was confirmed as an activator of both neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), and regulated the PI3K/AKT and ERK1/2 signaling pathway to reduce the level of apoptosis.¹⁹

Piperazine can be traced in a number of well-established commercially available drugs. The wide array of pharmacological activities exhibited by piperazine derivatives have made them indispensable linkers for the development of therapeutic agents for neurodegenerative diseases.²⁰ In our previous study, we designed a series of genipin derivatives as anti-AD agents using the scaffold of piperazine, among which the target compounds exhibited moderated inhibitory activity against AChE. Meanwhile, several compounds exhibited preferable neuroprotective efficacy than the positive reference donepezil.²¹ Thus, we adopted the piperazine scaffold in our designed molecular frame, which may provide better anti-AD efficacy and neuroprotective ability. In this study, we present the synthesis and pharmacological assessment of novel gardenamide A-based multifunctional compounds as anti-AD agents (Fig. 2). The pharmacological activity of these novel compounds against OGD-induced neurotoxicity in rat cortical neurons, and H₂O₂- and A β ₁₋₄₂-induced neurotoxicity in rat hippocampal neurons was evaluated. In addition, the neurotoxicity of the target compounds in rat cortical neurons was also assessed.

2. Results and discussion

2.1 Synthesis of compounds

The synthetic route for the substituted gardenamide A derivatives 10a–r is depicted in Scheme 1. The commercially available genipin (2) was transformed into the desired intermedi-

ate 4 via reaction with *tert*-butyldimethylsilyl chloride (TBSCl), and subsequent mild oxidation reaction by Dess–Martin periodinane (DMP) produced compound 5.¹⁸ The key intermediates 6a and b were obtained via lactamization and deprotection, which upon further reaction with methylsulfonyl chloride transformed into the methanesulfonyl esters 7a and b. Compounds 9a–i commenced with the substitution reaction of substituted benzyl chloride 8a–i and piperazine. The target compounds 10a–r were finally obtained by the substitution reaction of various substituted benzylpiperazines with the intermediates 7a and b under mild conditions and salification with concentrated hydrochloric acid in acetone.

2.2 Biological evaluation

2.2.1 Neuroprotection activity against OGD-induced neurotoxicity. Oxygen and glucose deprivation (OGD) is an acute model of lesion produced by free radical generation, followed by a re-oxygenation period.²² This model mimics what can occur in stroke or AD, in which the hippocampus is profoundly affected, and patients show the symptoms of short-term memory loss.²³ The results of all the target compounds against OGD-induced neurotoxicity and neuroprotective activity in rat cortical neurons were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay (Table 1). MTT reduction in basal conditions was considered as 100% cell viability without any treatment. Donepezil and gardenamide A were also tested as control. All the tested compounds exhibited significant protection effects. The cell viabilities ranged from 64.1% to 87.6% at 10 μ M. Interestingly, compounds 10e, 10f, 10h, 10i, 10j, 10n and 10p significantly increased the cell viability at a low concentration. The representative compounds 10e, 10j, 10n and 10p showed remarkable neuroprotective effects (cell

Scheme 1 Synthetic route for the target compounds 10a–r. Reagents and conditions: (i) TBSCl, imidazole, DMF, 0 °C; (ii) DMP, CH₂Cl₂, r. t.; (iii) (a) CH₃NH₂·EtOH or (CH₃)₂CHNH₂, pyridine, 65 °C; (b) TFA, THF, 65 °C; (iv) CH₃SO₂Cl, Et₃N, CH₂Cl₂, 0 °C; (v) CH₂Cl₂, 0 °C; and (vi) (a) substituted benzylpiperazine, K₂CO₃, DMF, r. t. and (b) CH₃COCH₃, HCl (Conc.).

Table 1 Neuroprotective effects of **10a–r** against OGD-induced neurotoxicity in rat cortical neurons^a

Comp.	R ₁	R ₂	OGD/R	Survival rate ± SD (%)		
				0.1 μM	1 μM	10 μM
10a	CH ₃	H	59.4 ± 1.5	58.6 ± 1.02 ^{##}	63.7 ± 1.5 ^{***##ΔΔ}	72.3 ± 0.7 ^{***##ΔΔ}
10b	CH ₃	4-CH ₃	59.6 ± 1.2	59.0 ± 2.16 ^{##}	66.9 ± 1.1 ^{***##}	72.6 ± 1.4 ^{***##ΔΔ}
10c	CH ₃	3-CH ₃	60.3 ± 1.4	60.8 ± 1.58 ^{##}	66.7 ± 1.5 ^{***##}	71.9 ± 2.3 ^{***##ΔΔ}
10d	CH ₃	3-OCH ₃	59.9 ± 1.5	59.5 ± 1.05 ^{##}	63.8 ± 0.6 ^{***##ΔΔ}	72.5 ± 1.5 ^{***##ΔΔ}
10e	CH ₃	4-F	59.6 ± 2.4	63.8 ± 0.67 ^{***ΔΔ}	71.3 ± 2.1 ^{**}	85.7 ± 1.0 ^{***ΔΔ}
10f	CH ₃	4-Cl	58.5 ± 1.1	62.7 ± 0.98 ^{***ΔΔ}	71.0 ± 0.7 ^{**}	80.1 ± 1.2 ^{***##}
10g	CH ₃	4-NO ₂	58.6 ± 1.3	60.0 ± 1.26 ^{##}	66.7 ± 1.2 ^{***##}	74.3 ± 1.0 ^{***##ΔΔ}
10h	CH ₃	4-CF ₃	60.3 ± 1.1	63.0 ± 1.19 ^{***ΔΔ}	72.2 ± 1.2 ^{**}	80.9 ± 1.2 ^{***##}
10i	CH ₃	3-CF ₃	59.7 ± 1.2	63.3 ± 0.61 ^{***ΔΔ}	72.5 ± 1.4 ^{***ΔΔ}	80.3 ± 0.5 ^{**}
10j	CH(CH ₃) ₂	H	59.4 ± 0.9	64.4 ± 0.98 ^{***ΔΔ}	71.8 ± 1.8 ^{**}	84.5 ± 1.6 ^{***ΔΔ}
10k	CH(CH ₃) ₂	4-CH ₃	60.5 ± 1.4	59.0 ± 1.87 ^{**}	73.1 ± 1.3 ^{***ΔΔ}	85.7 ± 1.8 ^{***ΔΔ}
10l	CH(CH ₃) ₂	3-CH ₃	59.3 ± 1.2	59.0 ± 1.37 ^{**}	71.3 ± 2.3 ^{**}	84.8 ± 2.2 ^{***ΔΔ}
10m	CH(CH ₃) ₂	3-OCH ₃	59.7 ± 2.0	60.6 ± 1.93 ^{**}	62.1 ± 1.8 ^{***ΔΔ}	64.0 ± 2.0 ^{***##ΔΔ}
10n	CH(CH ₃) ₂	4-F	59.5 ± 1.9	65.4 ± 1.68 ^{***ΔΔ}	74.4 ± 1.7 ^{***ΔΔ}	86.5 ± 1.1 ^{***ΔΔ}
10o	CH(CH ₃) ₂	4-Cl	59.5 ± 1.9	58.7 ± 2.01 ^{**}	64.3 ± 0.3 ^{***##ΔΔ}	71.9 ± 2.3 ^{***##ΔΔ}
10p	CH(CH ₃) ₂	4-NO ₂	58.9 ± 1.0	67.1 ± 1.62 ^{***ΔΔ}	74.3 ± 0.9 ^{***ΔΔ}	87.6 ± 1.5 ^{***ΔΔ}
10q	CH(CH ₃) ₂	4-CF ₃	61.2 ± 1.0	59.1 ± 1.6 ^{***##}	71.6 ± 2.2 ^{**}	85.7 ± 1.4 ^{***ΔΔ}
10r	CH(CH ₃) ₂	3-CF ₃	60.0 ± 1.5	58.7 ± 1.3 ^{##}	71.6 ± 1.2 ^{**}	85.5 ± 2.1 ^{***ΔΔ}
Donepezil			58.5 ± 0.6	65.5 ± 0.7	72.3 ± 0.5	85.9 ± 1.8
Gardenamide A			59.0 ± 0.9	59.0 ± 0.8	69.4 ± 0.6	79.3 ± 1.3

^a The cell viability in the control was taken as 100%. The positive control was donepezil and or gardenamide A. Compared with OGD/R group, “*” *P* < 0.05, “***” *P* < 0.01. Compared with donepezil group, “#” *P* < 0.05, “##” *P* < 0.01. Compared with GA, “Δ” *P* < 0.05, “ΔΔ” *P* < 0.01. All data were expressed as mean ± SD (*n* = 5).

viability: 85.7%, 84.5%, 86.5% and 87.6% at 10 μM, respectively) similar with the donepezil group (85.9% at 10 μM), which were more potent than the lead compound gardenamide A (79.3% at 10 μM). In general, the compounds with the 2-isopropyl on gardenamide A exhibited a higher cell survival ratio than the compounds with the 2-methyl group. It is a reasonable assumption that the compounds with 2-isopropyl exhibit improved lipophilicity, which make the crossing of the cell membrane easier. The benzyl group also had a significant influence on the cell viability. The compounds with electron-withdrawing groups such as **10e**, **10f**, **10h** and **10i** (cell viabilities: 85.7%, 80.1%, 80.9% and 80.3% at 10 μM, respectively) exhibited better cell viabilities than the compounds with electron-releasing groups such as **10b–d** and **10m** (cell viabilities: 72.6%, 71.9%, 72.5% and 64.1% at 10 μM, respectively). However, the substituent at the 4-position (**10b**, **10h**, **10k** and **10q**) or 3-position (**10c**,

10i, **10l** and **10r**) in the benzene ring may render the neuroprotection ineffective.

2.2.2 Neuroprotection activity against H₂O₂-induced neurotoxicity. AD is a deadly progressive neurodegenerative disorder, with ongoing neuron degeneration, which is the result of excessive ROS production.²⁴ Therefore, a neuroprotective strategy is considered as permanent therapy for AD besides other symptomatic relief strategies. To achieve this goal, the neuroprotective potential of the target compounds against H₂O₂-induced neurotoxicity in rat hippocampal neurons was assessed using the MTT colorimetric assay. The cells were treated with H₂O₂ (150 μM) for 48 h, which resulted in a marked decrease in cell viability (42.67%, *p* < 0.001) compared to the untreated control. The co-treatment of cells with compounds **10e**, **10j**, **10n** and **10p** ameliorated H₂O₂-induced neurotoxicity in a concentration-dependent manner (Table 2). Compounds **10e**, **10j**, **10n** and **10p** demonstrated significant

Table 2 Neuroprotection against H₂O₂-induced neurotoxicity in hippocampal neurons^a

Comp.	Survival rate (%) ± SD			
	H ₂ O ₂	0.1 μM	1 μM	10 μM
L-NAC	42.6 ± 0.8	62.6 ± 0.9 ^{**}	69.5 ± 1.1 ^{**}	79.1 ± 0.5 ^{**}
GA	42.3 ± 1.4	59.0 ± 1.1 ^{**}	65.8 ± 1.7 ^{**}	70.9 ± 1.3 ^{**}
10e	43.2 ± 0.7	62.5 ± 0.4 ^{***ΔΔ}	70.3 ± 0.7 ^{***ΔΔ}	78.5 ± 0.8 ^{***ΔΔ}
10j	42.0 ± 0.2	60.3 ± 1.1 ^{***##}	71.0 ± 0.7 ^{***ΔΔ}	76.2 ± 0.8 ^{***##ΔΔ}
10n	41.6 ± 0.5	60.4 ± 1.4 ^{***##}	67.3 ± 0.9 ^{***##}	72.4 ± 0.8 ^{***##}
10p	43.2 ± 1.0	57.6 ± 1.7 ^{***##}	73.8 ± 1.1 ^{***##ΔΔ}	82.1 ± 1.3 ^{***##ΔΔ}

^a All data were expressed as mean ± SD (*n* = 5). Compared with H₂O₂ group, “*” *P* < 0.05, “***” *P* < 0.01. Compared with L-NAC group, “#” *P* < 0.05, “##” *P* < 0.01. Compared with GA group “Δ” *P* < 0.05, “ΔΔ” *P* < 0.01.

Table 3 Neuroprotection against A β_{1-42} -induced neurotoxicity in hippocampal neurons^a

Comp.	Survival rate (%) $\pm$ SD			
	A β	0.1 μ M	1 μ M	10 μ M
Donepezil	43.5 $\pm$ 1.3	47.3 $\pm$ 1.2**	72.0 $\pm$ 1.1**	81.3 $\pm$ 1.2**
GA	42.2 $\pm$ 1.4	60.0 $\pm$ 1.0**	66.2 $\pm$ 1.2**	71.1 $\pm$ 1.3**
10e	43.0 $\pm$ 1.1	64.2 $\pm$ 0.3*** $\Delta\Delta$	71.4 $\pm$ 1.3*** $\Delta\Delta$	80.8 $\pm$ 1.1*** $\Delta\Delta$
10j	42.8 $\pm$ 1.3	61.9 $\pm$ 0.5***	70.3 $\pm$ 1.1*** $\Delta\Delta$	76.4 $\pm$ 1.0*** $\Delta\Delta$
10n	42.1 $\pm$ 0.5	61.2 $\pm$ 0.6***	68.0 $\pm$ 0.3***	74.0 $\pm$ 0.9*** $\Delta\Delta$
10p	43.1 $\pm$ 0.9	58.3 $\pm$ 0.9***	71.5 $\pm$ 0.9*** $\Delta\Delta$	84.1 $\pm$ 1.7*** $\Delta\Delta$

^a All data were expressed as mean $\pm$ SD ($n = 5$). Compared with A β_{1-42} group, “*” $P < 0.05$, “***” $P < 0.01$. Compared with L-NAC group, “#” $P < 0.05$, “###” $P < 0.01$. Compared with GA group, “ Δ ” $P < 0.05$, “ $\Delta\Delta$ ” $P < 0.01$.

neuroprotectivity at different concentrations and cell viabilities of 78.59%, 76.22%, 72.40% and 82.19%, respectively at 10 μ M with gardenamide A (70.97%) and L-NAC (79.17%) as internal standards. Both of them exhibited remarkably high cell viability at a lower tested concentration. These results exhibited that compounds **10e**, **10j**, **10n** and **10p** successfully reduced the H₂O₂-induced neurotoxicity in rat hippocampal neurons.

2.2.3 Neuroprotection activity against A β_{1-42} -induced neurotoxicity. To elucidate the extensive neuroprotective potential of compounds **10e**, **10j**, **10n** and **10p**, their protective effect against A β_{1-42} -induced neurotoxicity in rat hippocampal neurons was also tested. The results showed that the co-treatment of cells with both compounds for 48 h ameliorated A β_{1-42} -induced neurotoxicity in a concentration-dependent manner and exhibited neuroprotective effects at concentrations ranging from 0.1 to 10 μ M by CellTiter-Glo[®] luminescent cell viability assay (Table 3). Donepezil and gardenamide A were also tested as controls. The CellTiter-Glo[®] luminescent cell viability assay is dependent on the luciferase/luciferin reaction, which in the presence of Mg²⁺ and ATP produces oxyluciferin and energy in the form of light. ATP is a valid parameter of cell viability and overall metabolic health, which is synthesized in the mitochondrial inner membrane. Thus, the result of the CellTiter-Glo[®] assay may reflect the activity of both the cell and mitochondrion. The four compounds **10e**, **10j**, **10n** and **10p** exhibited 74.0–84.1% of cell viability at 10 μ M, which was higher compared with that of the positive control gardenamide A

(71.1% at 10 μ M). All of them still exhibited significantly active cell viability at lower tested concentrations. These results suggest that these novel compounds exerted significant neuroprotection against A β_{1-42} -induced neurotoxicity, which confirms their potential as multifunctional agents with a neuroprotective nature.

2.2.4 Cytotoxicity. The cytotoxicity of compounds **10e**, **10j**, **10n** and **10p** was examined by exposing rat cortical neurons to them with different concentrations ranging from 3.7 μ M to 100 μ M (Table 4). After exposing the cells to these compounds for 48 h, the cell viability was evaluated using the CellTiter-Glo[®] assay. The results showed a dose-dependent effect of the tested compounds on the viability of the rat cortical neurons at different concentrations. Fortunately, nearly all the selected compounds (**10e**, **10j**, **10n** and **10p**) did not produce a significant cytotoxic effect on the cells at the concentrations of 3.7 μ M and 11.1 μ M after 48 h treatment. Thus, the results indicate that these compounds did not exhibit significant cytotoxicity at the tested concentration.

3. Conclusions

Current research focuses on developing small molecules with *in vitro* neuroprotective properties rather than predefining molecular targets. Thus, in the present study, a series of derivatives possessing the gardenamide A structure were prepared and evaluated for their neuroprotective activities against OGD-, H₂O₂- and A β_{1-42} -induced neurotoxicity in rat cortical neurons or hippocampal neurons. Among these derivatives, compounds **10e**, **10j**, **10n** and **10p** exhibited moderate *in vitro* neuroprotective effects in all the tested models.

The preliminary SAR study indicated that: a. the compounds with the 2-isopropyl on gardenamide A exhibited a higher cell survival ratio than the compounds with the 2-methyl group and b. the electron-withdrawing substitutions at the benzene ring favored the neuroprotective activity.

The present results can provide useful information for the development of novel gardenamide A neuroprotective agents for the treatment of AD. Further studies related to optimizing the structure, clarifying the action mechanism and evaluating the octanol–water coefficient to predict the permeability of these compounds are in progress.

Table 4 Cytotoxicity of compounds **10e**, **10j**, **10n** and **10p** in rat cortical neurons^a

Comp.	Survival rate (%) $\pm$ SD			
	3.7 μ M	11.1 μ M	33.3 μ M	100 μ M
10e	98.4 $\pm$ 4.7	96.2 $\pm$ 8.3	85.1 $\pm$ 7.4**	78.5 $\pm$ 0.9**
10j	90.7 $\pm$ 2.4**	96.3 $\pm$ 3.2	91.7 $\pm$ 2.0**	82.5 $\pm$ 1.8**
10n	94.2 $\pm$ 2.8	98.4 $\pm$ 3.7	88.4 $\pm$ 2.8**	80.9 $\pm$ 2.1**
10p	97.2 $\pm$ 1.0	99.7 $\pm$ 2.9	88.7 $\pm$ 0.7**	78.5 $\pm$ 1.8**

^a All data were expressed as mean $\pm$ SD ($n = 3$). The survival rate of blank cell was 100 $\pm$ 2.8. “*” $P < 0.05$, “**” $P < 0.01$.

4. Experimental section

4.1 Experimental

Chemical synthesis. The detailed experimental procedures for all new compounds can be found in the ESI.†

4.2 Biological assays

Primary culture. All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, Revised 1996), and all efforts were made to minimize the number of animals used. Embryos were rapidly removed from the uteri of pregnant Sprague–Dawley rats at 17–18 days of gestation. The cortices were dissected, and the dura mater was removed in cold Hank's balanced salt solution (HBSS, Sigma-Aldrich, St. Louis, MO). Tissues were collected and washed in HBSS, and 0.05% trypsin (Gibco, Grand Island, NY, USA) was added for digestion at 37 °C for 15 min. The digestion was stopped by the addition of fetal bovine serum (FBS, Hyclone, Logan, UT, USA) to a final concentration of 10% (v/v). Cells were collected by centrifugation at 800 rpm for 10 min to remove the HBSS and were resuspended in neurobasal/B27 medium with 100 U per mL penicillin and 100 mg per mL streptomycin (Sigma-Aldrich). The cells were plated in 6-well chamber slides coated with 5 µg per mL poly-D-lysine at 37 °C in a humidified incubator with 5% CO₂ and 95% air. The cells were allowed to grow for 7 days before treatment.

Neuroprotection against OGD-induced neurotoxicity. Rat cortical neurons were seeded at a density of 4×10^4 cells per well in 48-well plates, and were pre-treated with various concentrations (0.1, 1.0 and 10 µM) of compound, donepezil, gardenamide A at 37 °C in 5% CO₂ atmosphere for 24 h. On the second day, the cell culture Dulbecco's modified Eagle's medium (DMEM) was removed and the cells were washed with glucose-free DMEM and placed in fresh glucose-free DMEM. The OGD group was changed to glucose-free DMEM and transferred to a hypoxia chamber (Thermo, Germany), containing a mixture of 95% N₂/5% CO₂ at 37 °C for 2 h. Then, for reoxygenation, serum was resupplied and glucose was added to a final concentration of 1 mg L⁻¹, followed by incubation for 24 h under normal conditions. For the measurement of cell viability, 20 µL per well thiazoyl blue tetrazolium bromide (MTT; 5 mg mL⁻¹) was added 4 h prior to the completion of incubation periods. Thereafter, the reaction mixture was carefully taken out and DMSO (150 µL) solution was added to each well to achieve solubilisation of the formazan crystals formed in the viable cells. Viable cells were quantified spectrophotometrically at 570 nm using a multimode microplate reader (Berthold, Germany). All assays were performed in quintuplicate, and the results are shown as a percentage of the control.

Neuroprotection against H₂O₂-induced neurotoxicity. Rat hippocampal neurons were cultured in 48-well chamber slides at a density of 4.0×10^4 cells per well. After 24 h of incubation, the medium was replaced with fresh DMEM consisting of 150 µM H₂O₂ and various concentrations (0.1,

1.0 and 10 µM) of the compounds, L-NAC or Gardenamide A at 37 °C for 24 h. Rat hippocampal neurons were also cultured without any treatment as the control group and cell viability was quantified using the standard MTT assay. All assays were performed in quintuplicate, and the results are shown as a percentage of the control.

Neuroprotection against Aβ₁₋₄₂-induced neurotoxicity. Rat hippocampal neurons were cultured in 48-well chamber slides at a density of 4.0×10^4 cells per well. After 24 h of incubation, the culture medium was replaced with fresh medium containing 2 µM Aβ₁₋₄₂ (Sigma-Aldrich) and incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. Then, the culture medium was changed to fresh medium containing 2 µM Aβ₁₋₄₂ and various concentrations (0.1, 1.0 and 10 µM) of the compounds, donepezil or gardenamide A at 37 °C in a 5% CO₂ atmosphere. After 48 h of treatment, the plate was equilibrated at room temperature for approximately 30 min. The volumes were adjusted to the same level, 100 µL of CellTiter-Glo® reagent (Promega, USA) was added, the plates were sealed and shaken for 20 min at speed 10, and then incubated for 10 min before luminescence was measured using a multimode microplate reader (Berthold, Germany).

Cytotoxicity assay. Rat cortical neurons were cultured in 48-well chamber slides at a density of 4.0×10^4 cells per well. The cytotoxicity was determined using the CellTiter-Glo® luminescent cell viability assay kit according to the manufacturer's instructions (Promega, USA). The cells were treated with various concentrations (3.7, 11.11, 33.33, and 100 µM) of compound at 37 °C under 5% CO₂ for 48 h, and then CellTiter-Glo® reagent was added to each well. The luminescent signals were read using a multimode microplate reader (Berthold, Germany).

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Anhui Medical University and Experiments were approved by the Animal Ethics Committee of Anhui University of Chinese Medicine.

Statistical analysis. Statistical comparisons were performed with one-way analysis of variance (ANOVA), followed by the Dunnett *post hoc* test using GraphPad Prism 7.01 (GraphPad Software, San Diego, CA, USA). Values of *P* < 0.05 were considered significant. The means of the data are presented together with the SE.

Author contributions

All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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