Med Chem Res
DOI 10.1007/s00044-017-2101-9

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Discovery of a novel multifunctional carbazole–aminoquinoline dimer for Alzheimer's disease: copper selective chelation, anti-amyloid aggregation, and neuroprotection

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Received: 23 August 2017 / Accepted: 19 October 2017

Abstract A novel multifunctional carbazole–aminoquinoline dimer **PZ001** was designed, synthesized, and evaluated. The results indicated that **PZ001** possessed selective copper chelation, and inhibited copper-induced $A\beta_{1-42}$ aggregation. Furthermore, **PZ001** exerted powerful neuroprotection against glutamate-induced HT22 cell death. These results suggest that **PZ001** may be a promising multifunctional anti-AD compound.

Keywords Multifunctional dimer · Copper selective chelation · Anti-amyloid aggregation · Neuroprotection · Alzheimer's disease

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that causes problems with memory, thinking, and behavior, and seriously leads to death. The World Alzheimer Report 2016 shows that there are about 47 million people worldwide that are living with AD in 2016. The number is predicted to increase to more than 131 million by 2050, as the aging of population (Alzheimer's disease international 2016). Research found that the pathogenesis of AD is associated with many pathways including the deficiency of cholinergic neurotransmitters, the formation of neurotoxic beta-amyloid (Aβ) peptide, tau protein hyperphosphorylation, metal ion disturbance, oxidative stress, and so on (Fang et al. 2013; Sreenivasachary et al. 2017; Huang and Mucke 2012; Horton et al. 2017; Villarroya et al. 2007). AD has been discovered more than 100 years ago, but only five drugs have been clinically used to treat AD (Rampa et al. 2012; Schneider et al. 2014). However, these drugs, acting on a single target, could only attenuate the symptom of AD instead of curing the disease.

Facing the multiple pathogenesis of AD, the classical therapeutic method aiming at one target may be inadaptable to this complex disease. Therefore, developing multifunctional molecules as multi-target direct ligands (MTDLs)

Published online: 31 October 2017

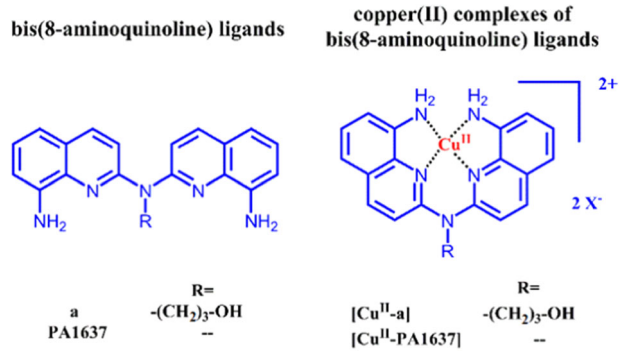


Fig. 1 General structure of bis(8-aminoquinoline) ligands a and PA1637, and their copper(II) complexes [CuII-a] and [CuII-PA1637], respectively

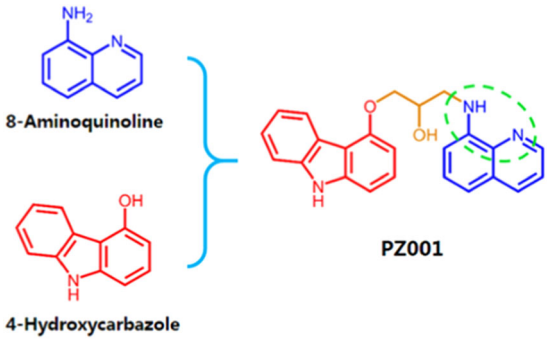


Fig. 2 Design of PZ001

has become a promising strategy for designing new anti-AD agents (Leon et al. 2013; Lu et al. 2012; Rosini 2014).

Senile plaques is one of the main pathological characteristics of AD. The amyloid proteins (Aβ) is the main component of senile plaques (Hardy and Selkoe 2002; Salahuddin et al. 2016). The post-mortem analysis of amyloid plaques indicate the levels of copper, iron, and zinc are about 5.7, 2.9, and 2.8 times than the levels in normal brains, respectively (Dong et al. 2003). Cu–amyloids induce oxidative damage, and then produce reactive oxygen species (ROS) resulting in neuron death (Robert et al. 2015). The mitochondrial dysfunction is also associated with Aβ–Cu(II) (Jiang et al. 2007). NMDA receptor activation, APP processing, and tau phosphorylation are also affected by the abnormal metal ions in AD patients (Ayton et al. 2013). Bis(8-aminoquinolines) ligan ds (Fig. 1), tetradentate copper chelators, generate the Cu(II)−bis(8-aminoquinoline) complex by competing with Cu–Aβ for Cu (Nguyen et al. 2015). They can also decrease oxidative damage induced by Cu–Aβ (Nguyen et al. 2014). PA1637 (Fig. 1) has high selectivity for Cu^{2+} chelation and incapacity to complex zinc ions. It could fully reverse the memory deficit in $A\beta_{1-42}$ injected mice (Ceccom et al. 2012). In consideration of the high activities of Bis(8-aminoquinolines) ligands, we took 8-aminoquinoline as one moiety for development of multifunctional anti-AD agents.

Oxidative stress is one of the most important nosogenetic factors in AD, and closely associated with other hypotheses (Bonda et al. 2010). ROS, the production of oxidative stress, causes oxidative damage leading to neuron damage and death. Oxidative damage plays an important part in the decrease of neurons. Therefore, the antioxidant could be a potential strategy to prevent and treat AD (Nunomura et al. 2006; Rosini et al. 2014). Carbazole, an important nitrogen aromatic heterocyclic compound, possesses a lot of biological activities associated with AD (Tang and Liu 2007). It has been reported that carbazole derivatives could scavenge ROS, protect neurons against oxidative damage (De Jesus-Cortes et al. 2012; Fang et al. 2016; MacMillan et al. 2011; Naidoo et al. 2014; Pieper et al. 2014, 2010; Tesla et al. 2012; Wang et al. 2014a, 2014b), inhibit the aggregation of Aβ (Zall et al. 2011; Saturnino et al. 2014), and inhibit cholinesterase (Thiratmatrakul et al. 2014; Tsutsumi et al. 2016). In our previous research, carbazole derivatives showed potent neuroprotective effects (Zhu et al. 2013). These specific functions of carbazole derivatives make them to become ideal anti-AD lead structures.

Herein, we combined 8-aminoquinoline as a copper selective chelate moiety, and 4-hydroxycarbazole as a neuroprotective moiety to develop potential multifunctional anti-AD agents. The compound (**PZ001**) was synthesized by connecting 8-aminoquinoline to 4-hydroxycarbazole via the 2-hydroxypropil chain (Fig. 2). Biological evaluation demonstrated that **PZ001** was a multifunctional anti-AD agent with selective copper chelation, Aβ aggregation inhibition, and also neuroprotection.

Material and methods

Chemistry

Reagents and solvents were purchased from commercial sources and were used without further purification unless stated. The progress of the reactions was monitored by thin-layer chromatography on a glass plate coated with silica gel with fluorescent indicator (GF254) and visualized with UV light. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance spectrometer 400 at 400 and 100 MHz, respectively. Chemical shifts are given in ppm (δ) referenced to $CDCl_3$ with 7.26 for ^{1}H and 77.10 for ^{13}C, and to d6-DMSO with 2.50 for ^{1}H and 4.90 for ^{13}C. In the case of multiplet, the signals are reported as intervals. Signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are expressed in hertz. High-resolution mass spectrometric data (HRMS) were collected on a Shimadzu LCMS–IT–TOF mass spectrometer. The purity of all final compounds was

determined by Agilent 1260 high performance liquid chromatography (HPLC) system using an Eclipse XDB-C_{18} column. In HPLC conditions, flow rate was set at 1 mL/min, and gradient elution was used 70% MeOH/H_2O with 0.1% TFA for 15 min.

Synthesis of 4-(oxiran-2-ylmethoxy)-9H-carbazole (1)

To a suspension of 4-hydroxy carbazole (1 g, 5.5 mmol) and K_2CO_3 (1.52 g, 11 mmol) in acetonitrile (20 mL), epichlorohydrin (5.0 mL, 6.4 mmol) was added. The reaction mixture was refluxed for 24 h. After completion of the reaction, as indicated by TLC, the acetonitrile in the reaction mixture was removed under reduced pressure. The obtained residue was dissolved in ethyl acetate (50 mL), organic phase was washed with water (3 × 20 mL), separated, dried with anhydrous Na_2SO_4, evaporated under reduced pressure, and purified by column chromatography over silica gel (1:1, dichloromethane/petroleum ether) to give **1** as a white solid (0.86 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (d, $J = 7.9$, 0.9 Hz, 1H), 8.10 (s, 1H), 7.46–7.39 (m, 2H), 7.35 (t, $J = 8.0$ Hz, 1H), 7.27 (dd, $J =$ 5.8, 2.2 Hz, 1H), 4.49 (dd, $J = 11.0$, 3.3 Hz, 1H), 4.30 (dd, $J = 11.0$, 5.4 Hz, 1H), 3.59 (dddd, $J = 5.6$, 4.1, 3.4, 2.6 Hz, 1H), 3.03 (dd, $J = 5.0$, 4.1 Hz, 1H), 2.92 (dd, $J = 5.0$, 2.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.02, 141.01, 138.76, 126.62, 125.14, 123.24, 122.55, 119.78, 112.92, 110.00, 104.09, 101.39, 68.84, 50.40, 44.92. MS ESI: 239.1.

Synthesis of 1-((9H-carbazol-4-yl)oxy)-3-(quinolin-8-ylamino)propan-2-ol (PZ001)

A solution of 4-(oxiran-2-ylmethoxy)-9H-carbazole (300 mg, 1.26 mmol) in 15 mL of MeOH was added to the 8-aminoquinoline (222 mg, 1.5 mmol). The mixture was heated at 50 °C for 16 h. The solution was evaporated in vacuo. The crude products was purified by column chromatography over silica gel (10:3, petroleum ether/ethyl acetate) to afford **PZ001** as a faint yellow solid (298 mg, 62%). ^{1}H NMR (400 MHz, DMSO) δ11.26 (s, 1H), 8.73 (d, $J = 3.7$ Hz, 1H), 8.28 (d, $J = 7.8$ Hz, 1H), 8.20 (d, $J = 8.1$ Hz, 1H), 7.49 (dd, $J = 8.2$, 4.2 Hz, 1H), 7.44 (d, $J = 8.1$ Hz, 1H), 7.30 (dq, $J = 16.0$, 8.0, 7.5 Hz, 3H), 7.12 (t, $J = 7.4$ Hz, 1H), 7.06 (dd, $J = 8.0$, 4.0 Hz, 2H), 6.76 (d, $J = 7.7$ Hz, 1H), 6.70 (d, $J = 7.9$ Hz, 1H), 5.56 (s, 1H), 4.43–4.34 (m, 1H), 4.26 (d, $J = 5.1$ Hz, 2H), 3.68 (d, $J = 10.2$ Hz, 1H), 3.48 (dd, $J = 12.8$, 7.0 Hz, 1H).^{13}C NMR (100 MHz, DMSO) δ155.31, 147.42, 145.11, 141.60, 139.40, 138.03, 136.45, 128.78, 128.22, 126.95, 125.03, 122.99, 122.20, 122.17, 119.06, 113.85, 112.05, 110.84, 104.90, 104.45, 100.95, 70.79, 68.05, 55.38, 46.57.HRMS calcd. for $C_{24}H_{21}N_3O_2$ $[M+H]^+$: 384.1713. Found: 384.1707. HPLC purity: 98.4%.

Biological assay

Metal-chelating ability assay

The metal chelation was monitored using a UV–vis spectrophotometer in anhydrous ethanol buffer by screening the absorption spectra ranging from 200 to 600 nm. To examine the metal selectivity of **PZ001** for Cu(II), **PZ001** (2 μL, 10 mM, stock solution) and $CuCl_2$ (2 or 4 μL, 10 mM, stock solution), $FeCl_3$, $ZnCl_2$ or $FeCl_3$ (2 μL, 10 mM, stock solution) were added to 1 mL anhydrous ethanol. Then, the mixture was allowed to stand at room temperature for 30 min. The absorption spectra of **PZ001** (20 μM, final concentration) alone or in the presence of $CuCl_2$ (20 or 40 μM, final concentration), $FeCl_3$, $ZnCl_2$, and $FeCl_3$ (20 μM, final concentration) in ethanol were recorded at room temperature.

Thioflavin T (ThT) fluorescence assay

The inhibition of self-mediated and copper-mediated $A\beta_{1-42}$ aggregation was determined by ThT fluorescence assay as previously described (Lu et al. 2013). $A\beta_{1-42}$ (Millipore; counterion, NaOH) was dissolved in ammonium hydroxide (1% v/v) to give a stock solution (2000 μM), which was aliquoted into small samples and stored at −80 °C.

For the inhibition of self-mediated $A\beta_{1-42}$ aggregation experiment, the Aβ stock solution was diluted with 50 mM phosphate buffer (pH 7.4) to 50 μM before use. A mixture of the peptide (10 μL, 25 μM, final concentration) with or without the tested compound (**PZ001**,10 μL, 20 μM, final concentration) was incubated at 37 °C for 48 h. Blanks using 50 mM phosphate buffer (pH 7.4) instead of Aβ was also carried out. The sample was diluted to a final volume of 200 μL with 50 mM glycine–NaOH buffer (pH 8.0) containing ThT (5 μM). Then the fluorescence intensities were recorded 5 min later (excitation, 450 nm; emission, 485 nm). The percent inhibition of aggregation was calculated by the expression $(1-IF_i/IF_c) \times 100$, in which IF_i and IF_c are the fluorescence intensities obtained for Aβ in the presence and absence of inhibitors after subtracting the background, respectively.

For the inhibition of copper-mediated $A\beta_{1-42}$ aggregation experiment, the Aβ stock solution was diluted in 20 μM HEPES (pH 6.6) with 150 μM NaCl. The mixture of the peptide (10 μL, 25 μM, final concentration) with or without copper (10 μL, 25 μM, final concentration), and the tested compound (**PZ001**, 10 μL, 50 μM, final concentration) was incubated at 37 °C for 24 h. Then 20 μL of the sample was diluted to a final volume of 200 μL with 50 mM

glycine–NaOH buffer (pH 8.0) containing ThT (5 μM). The detection method was the same as that of self-mediated $A\beta_{1-42}$ aggregation experiment.

Cell culture

HT22 cells were maintained in Dulbecco's modified Eagle media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and incubated at 37 °C under 5% CO_2. Test compounds were dissolved in DMSO and diluted in DMEM supplemented with 10% (v/v) FBS. The final concentration of DMSO in the medium was less than 0.01% (v/v), which showed no influence on cell growth.

Assessment of cell viability by MTT assay

The cell viability was determined by MTT assay as previously described (Wang et al. 2016). To study the cytotoxicity and the neuroprotective effects of **PZ001**, HT22 cells were cultured in 96-well plates and incubated overnight. Cells were pretreated with tested compounds or the vehicle DMSO for 30 min followed with/without 2 mM glutamate (Glu) for 24 h. Ten microliters of 5 mg/mL MTT was added to each well and cells were incubated for 2 h at 37 °C. To dissolve purple formazan crystal, 100 μL DMSO was carefully added to replace the medium. After vigorously shaking for 15 min at 37 °C, the absorbance at 570 nm was measured using a microculture plate reader.

Statistical analysis

All analyses were repeated at least in duplicate. Statistical analyses among groups were tested by one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

Measurement of intracellular ROS

HT22 cells were grown in Corning 96-well plates at a cell density of 4×10^3 cells/well. After overnight attachment, cells were pretreated with the test compounds or the vehicle control DMSO for 30 min and then incubated with/without 4 mM Glu for 12 h. Cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 10 μM non-fluorescent dye DHE in serum-free medium for 30 min at 37 °C in the dark. Cells were subsequently washed twice with PBS and photographed using a (200×) fluorescence microscope.

PAMPA-BBB assay

Prediction of the brain penetration of compounds was evaluated using a parallel artificial membrane permeation assay (PAMPA) and porcine brain lipid (Avanti Polar Lipids) in a similar manner as previously described (Chen et al. 2014).

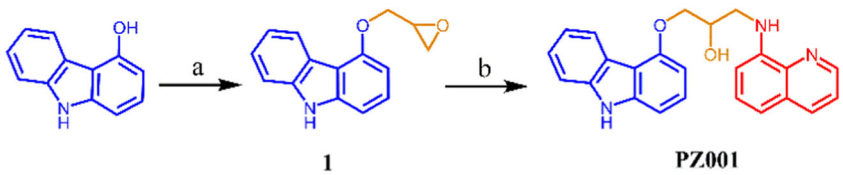
Results and discussion

Chemistry

The synthetic route for the novel compound **PZ001** is shown in Scheme 1. Commercially available 4-hydroxycarbazole was reacted with epichlorohydrin in the presence of K_2CO_3 to give 4-(oxiran-2-ylmethoxy)-9H-carbazole (**1**). Compound **1** was then made to react with 8-aminoquinoline at 50 °C to get the target compound **PZ001**.

Metal-chelating ability assay

In consideration of containing 8-aminoquinoline moiety, the metal-chelating ability of **PZ001** was investigated using UV–vis spectrometry. The results in Fig. 3a show that **PZ001** had the maximum absorption peaks at 285, 320, and 335 nm, and a shoulder peak. When $CuCl_2$ was added, the maximum absorption wavelength of **PZ001** shifted from 320 to 317 nm, and the shoulder peak disappeared. The results demonstrate the formation of an **PZ001**–Cu(II) complex. Interestingly, when $FeCl_2$, $FeCl_3$, or $ZnCl_2$ was mixed with **PZ001**, no changes in the maximum absorption peak wavelengths were observed, which suggested that **PZ001** could selectively chelate Cu(II). 8-aminoquinoline, a group of copper selective chelation, showed to selectively chelate Cu(II) in Fig. 3b. Clioquinol (CQ) is a unselective metal ion chelator. CQ could chelate all of the four metal ions in Fig. 3c. 4-hydroxycarbazole showed no metal ion chelation, which indicated copper selective chelation of **PZ001** comes from 8-aminoquinoline.



Scheme 1 Synthetic route of PZ001. Reagents and conditions: **a** epichlorohydrin, K_2CO_3, acetonitrile, reflux, 24 h; **b** 8-aminoquinoline, MeOH, 50 °C, 16 h

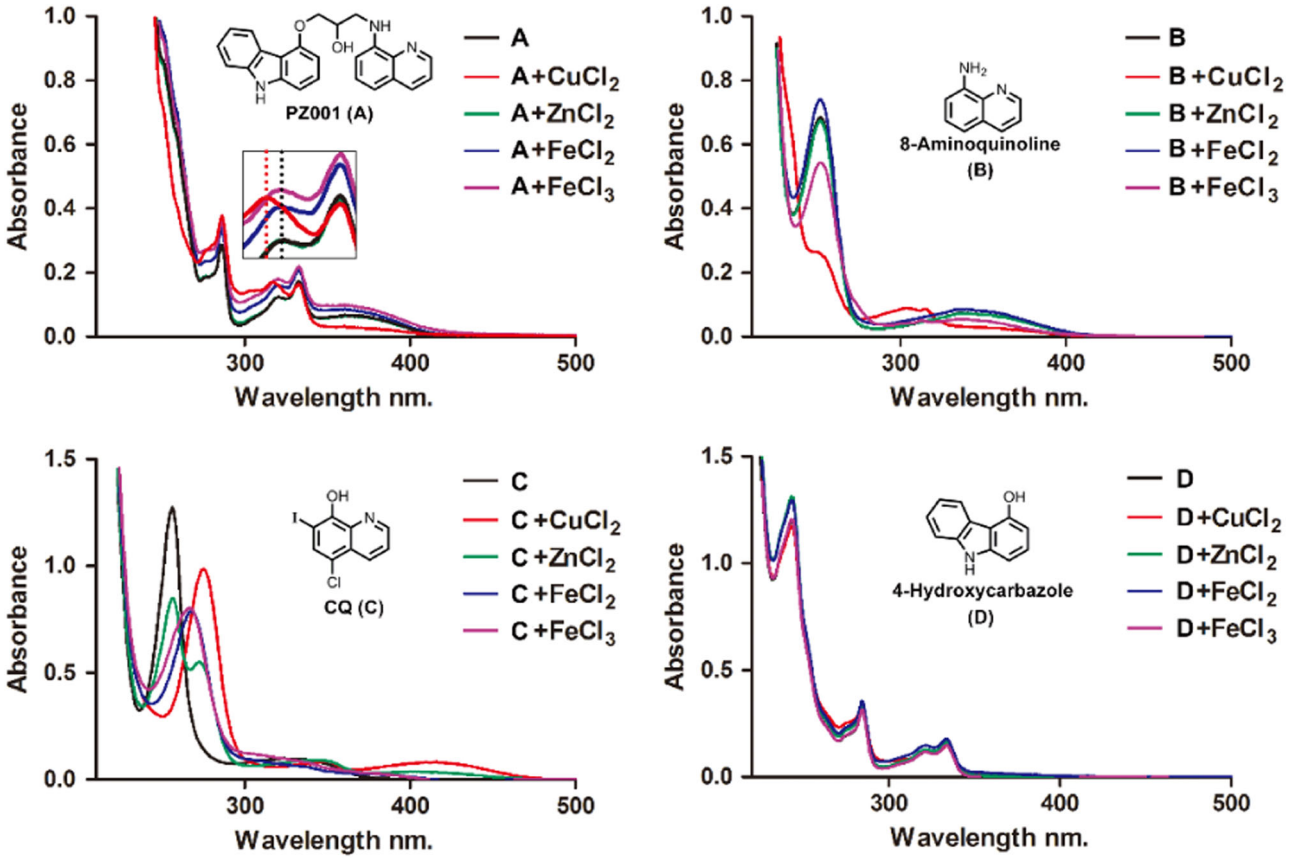


Fig. 3 Metal ion chelating activity assay. UV–vis spectrum of **PZ001**, 8-aminoquinoline, CQ, or 4-hydroxycarbazole (20 μM) alone and in the presence of $CuCl_2$ (20 μM), $FeCl_2$ (20 μM), $FeCl_3$ (20 μM), or $ZnCl_2$ (20 μM) in anhydrous ethanol buffer was measured at room temperature

Table 1 Inhibitory activities of PZ001 against $A\beta_{1-42}$ self/copper-induced aggregation

Compound	Inhibition of self-mediated $A\beta_{1-42}$ aggregation	Inhibition of Cu-mediated $A\beta_{1-42}$ aggregation
PZ001[a]	22.9	22.3
Congo Red[b]	70.6	–
Clioquinol[c]	–	12.3

[a] The final concentration of PZ001 was 20 μM

[b] The positive control was 5 μM congo red

[c] The positive control was 20 μM clioquinol

$A\beta_{1-42}$ anti-aggregating activity

The inhibitory activity of **PZ001** against self-mediated $A\beta_{1-42}$ aggregation was evaluated using a ThT fluorescence assay. The result showed that **PZ001** inhibited Aβ self-aggregation (22.9%) (Table 1). Furthermore, given that copper-mediated $A\beta_{1-42}$ aggregation plays an important role in AD pathogenesis and **PZ001** could chelate Cu(II), the ability of the inhibition of copper-mediated $A\beta_{1-42}$ aggregation was studied. CQ was used as the positive control compound. The fluorescence of Aβ treated with Cu(II) and the tested compound decreased dramatically (**PZ001**, 22.3% inhibition of copper-mediated $A\beta_{1-42}$ aggregation; CQ, 12.3% inhibition). These results indicated **PZ001** inhibited copper-mediated $A\beta_{1-42}$ aggregation.

Neuroprotection of PZ001 against oxidative stress

The neurotoxicity induced by Glu in HT22 cells, is considered to be an excellent model for studying the influence of oxidative stress in neurons. Our results (Fig. 4) showed that **PZ001** possessed good neuroprotection in Glu-induced cell death at 10 μM. 4-hydroxycarbazole also had significant neuroprotection at 10 μM, which suggestted neuroprotection of **PZ001** comes from 4-hydroxycarbazole.

Cell toxicity of PZ001

In order to evaluate the cytotoxicity, the HT22 cells was exposed to PZ001 at 50–200 μM for 24 h and then MTT assay was applied. Results (Fig. 5a) indicated that the OD decreased significantly at 50 μM. The decrease of OD may be related to cell death or the inhibition of cell proliferation lead by **PZ001**. In order to search for the reason, we

observed cells through a photomicroscope. In Fig. 5b, no cell death was found even under 50 μM of **PZ001**. Then the lactate dehydrogenase (LDH) release was examined, the results (Fig. 5c) showed the HT22 cells, treated with **PZ001** at 20–100 μM, had no obvious effect on LDH release. Therefore, **PZ001** did not cause the cell death. The decrease of OD may be connected with the inhibition of cell proliferation.

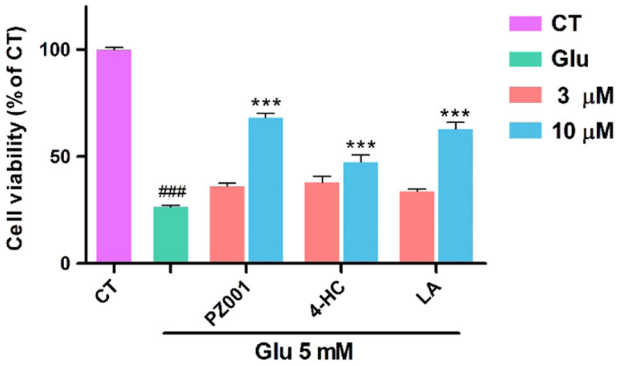


Fig. 4 The neuroprotective effects of PZ001, 4-hydroxycarbazole(4-HC), alpha lipoic acid (LA) on glutamate-induced cytotoxicity in HT22 cells. HT22 cells were pretreated with different compounds (3, 10 μM) for 0.5 h, followed by stimulation with glutamate (5 mM) for 24 h. Cell viability was determined by the MTT assay. Data are presented as means ± S.D. One-way ANOVA followed by Tukey's test. $^{###}P < 0.001$ vs. control group; $^{***}P < 0.001$ vs. glutamate-treated group

ROS scavenging assay

Carbazole derivatives exhibit antioxidant activity by directly scavenging ROS. Thus, we measured the free radical scavenging ability of **PZ001** by fluorescent probe DHE assay. The results showed that all of PZ001, 4-HC, and LA exhibited the ability of scavenging ROS at 10 μM (Fig. 6).

Prediction of BBB permeability

The penetration of blood–brain barrier (BBB) is a crucial detection index for central nervous system (CNS) drugs. To investigate whether **PZ001** could penetrate the BBB, we used a PAMPA for the BBB (PAMPA-BBB) (Di et al. 2003). The results (Table 2, S1) showed that **PZ001** was CNS+, which indicated **PZ001** could penetrate the BBB.

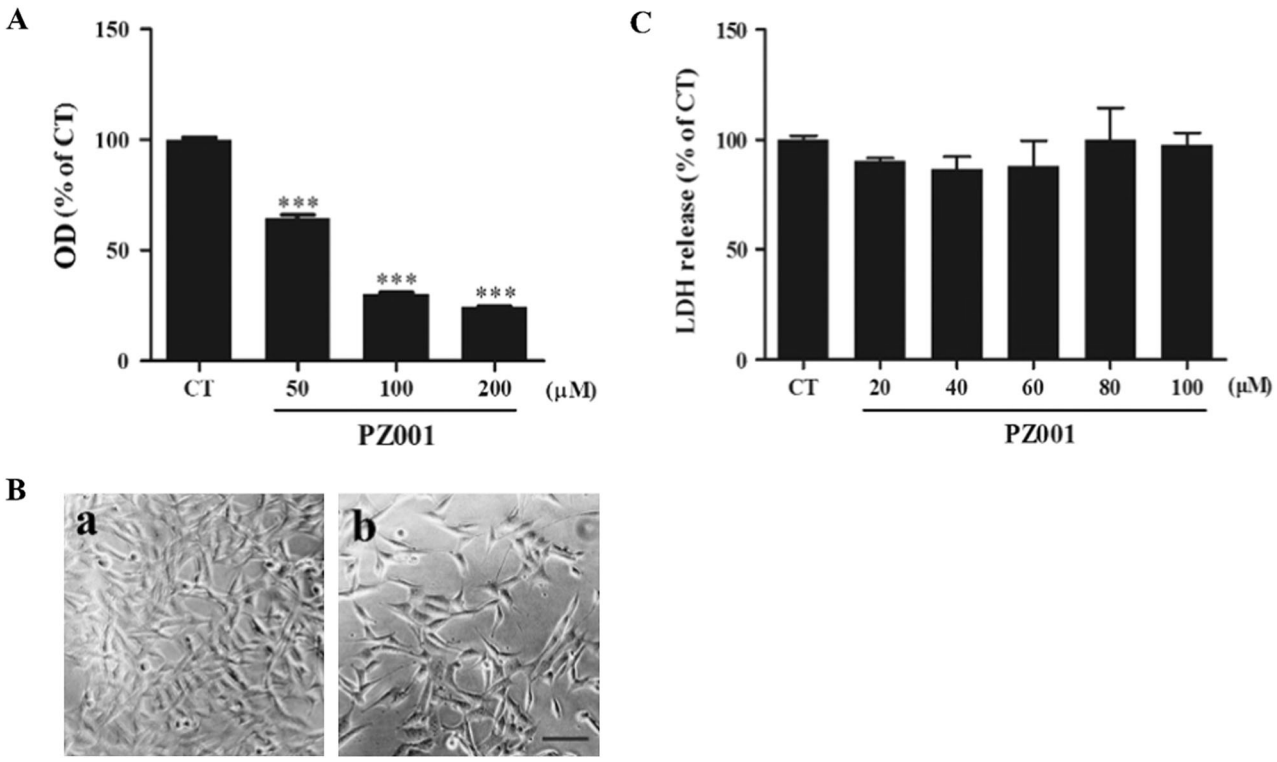


Fig. 5 Cell cytotoxicity of PZ001 in HT22 cells. **a** HT22 cells were treated with PZ001 (50–200 μM) for 24 h. OD was tested by MTT assay. **b** Cells were photographed using a photomicroscope (×200). (a) CT; (b) PZ001 (50 μM); scale bar, 50 μm. **c** Cells were treated with PZ001 (20–100 μM) for 24 h. LDH release was tested by LDH cytotoxicity assay kit. Data are presented as means ± S.D. One-way ANOVA followed by Tukey's test. $^{***}P < 0.001$ vs. control group

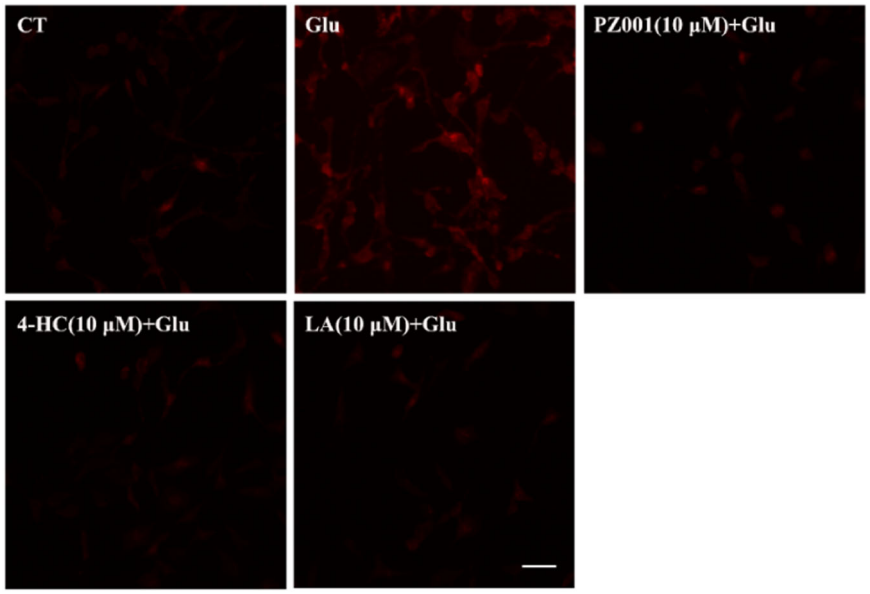


Fig. 6 Effects of PZ001, 4-hydroxycarbazole (4-HC) and alpha lipoic acid (LA) on intracellular ROS levels. Cells were treated with glutamate (4 mM) in the absence or presence of different compounds (10 μM), and 12 h later ROS generation inside the cells. DHE fluorescence was analyzed by visual observation of cell morphology through fluorescence microscopy (200×) equipped with a UV filter. Scale bar, 50 μm

Table 2 Permeability (Pe × 10^{-6} cm s^{-1}) in the PAMPA-BBB assay for **PZ001** and their predicted penetration into the CNS

Cmpd	P_e (×10^{-6} cm s^{-1})	Prediction
PZ001	16.3 ± 0.6	CNS+

Conclusion

In conclusion, basing on the copper selective chelation of 8-aminoquinoline and the neuroprotective effect of 4-hydroxycarbazole, we designed and synthesized a novel multifunctional compound **PZ001**, which contains two pharmacophores. **PZ001** exhibited good copper selective chelation and inhibition of copper-mediated $A\beta_{1-42}$ aggregation, also slight inhibition of self-mediated $A\beta_{1-42}$ aggregation. In addition, due to the effect of 4-hydroxycarbazole, **PZ001** showed significant neuroprotective effect against Glu-induced cell death in HT22 cells at 10 μM and low toxicity. In summary, these results suggest that compound **PZ001** will be a novel promising multifunctional anti-AD hit compound.

Acknowledgements We thank Prof. Bernard Meunier for fruitful discussions on copper chelators. This study was supported by Guangdong Provincial International Cooperation Project of Science & Technology (No. 2013B051000038), National Natural Science Foundation of China (Nos. 81673608, 31371070, and 81671264) and the Fundamental Research Funds for the Central Universities (No.15ykjc08b).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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