Neuroprotective effects of ZL006 in Aβ1–42-treated neuronal cells

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Graphical Abstract

ZL006 inhibits Aβ1–42-induced oxidative stress and neuronal injury by activating the Akt/Nrf2/HO-1 pathway

Abstract

Amyloid beta (Aβ)-induced neurotoxicity and oxidative stress plays an important role in the pathogenesis of Alzheimer’s disease (AD). ZL006 is shown to reduce over-produced nitric oxide and oxidative stress in ischemic stroke by interrupting the interaction of neuronal nitric oxide synthase and postsynaptic density protein 95. However, few studies are reported on the role of ZL006 in AD. To investigate whether ZL006 exerted neuroprotective effects in AD, we used Aβ1–42 to treat primary cortical neurons and N2a neuroblastoma cells as an in vitro model of AD. Cortical neurons were incubated with ZL006 or dimethyl sulfoxide for 2 hours and treated with Aβ1–42 or NH3•H2O for another 24 hours. The results of cell counting Kit-8 (CCK-8) assay and calcein-acetoxymethylester/propidium iodide staining showed that ZL006 pretreatment rescued the neuronal death induced by Aβ1–42. Fluorescence and western blot assay were used to detect oxidative stress and apoptosis-related proteins in each group of cells. Results showed that ZL006 pretreatment decreased neuronal apoptosis and oxidative stress induced by Aβ1–42. The results of CCK8 assay showed that inhibition of Akt or NF-E2-related factor 2 (Nrf2) in cortical neurons abolished the protective effects of ZL006. Moreover, similar results were also observed in N2a neuroblastoma cells. ZL006 inhibited N2a cell death and oxidative stress induced by Aβ1–42, while inhibition of Akt or Nrf2 abolished the protective effect of ZL006. These results demonstrated that ZL006 reduced Aβ1–42-induced neuronal damage and oxidative stress, and the mechanisms might be associated with the activation of Akt/Nrf2/heme oxygenase-1 signaling pathways.

Key Words: Akt; Alzheimer’s disease; amyloid-beta; apoptosis; heme oxygenase-1; neurotoxicity; Nrf2; oxidative stress; treatment; ZL006

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Introduction

More than 47 million people worldwide have Alzheimer’s disease (AD), which is a major cause of dementia in the elderly (Alzheimer’s Association, 2016). The neuropathological hallmarks of AD include extracellular plaques consisting of amyloid beta (Aβ) and intraneuronal neurofibrillary tangles (Selkoe and Hardy, 2016). The generation and accumulation of Aβ is shown to initiate the pathological cascade in AD, leading to the dysfunction and death of neuronal cells (Hardy and Higgins, 1992; Selkoe and Hardy, 2016). Furthermore, reducing Aβ levels alleviates Aβ-induced apoptosis and cognitive impairment (Aminyavari et al., 2018; Gan et al., 2018). The production of free radicals and oxidative stress is a key factor in the progression of AD (Querfurth and LaFerla,
2010). Aβ has been shown to induce the generation of H$_2$O$_2$ in vitro (Huang et al., 1999). Accumulated Aβ leads to dys- function of mitochondria and induces extensive production of reactive oxygen species (ROS), followed by lipid peroxidation, protein oxidation, and DNA/RNA oxidation (Yu et al., 2018). Furthermore, increased oxidative stress results in impaired degradation of proteins in AD (Haynes et al., 2004). We have shown that several herb extracts, including Hopeahainol A, Diammonium glycyrrhizinate and Orientin, exert anti-oxidative effects and protect against Aβ-induced neuro- nal death and cognitive dysfunction (Zhu et al., 2012, 2013; Yu et al., 2015). Although the use of antioxidant supplements as primary prevention of AD is controversial, several clinical trials have shown that antioxidant might be associated with the reduced risk and progression of AD (Morris et al., 2002a, b; Barnes and Yaffe, 2005; Turner et al., 2015; Kryscio et al., 2017).

Akt pathway plays an essential role in the regulation of cell apoptosis, and is downregulated in the brains of AD rats (Wang et al., 2016). Accumulating evidence suggests that activation of Akt pathway protects neuronal cells from Aβ-induced detrimental effects (Chong et al., 2007; Yi et al., 2018). The nuclear transcription factor NF-E2-related factor 2 (Nrf2) is a downstream target of Akt, and it regulates the redox status in the central nervous system (de Vries et al., 2008). Under oxidative stress, Nrf2 translocates into the nucleus and binds to heme oxygenase-1 HO-1 (Tan et al., 2013). The levels of nuclear Nrf2 are decreased in the hippocampal CA1 region of AD patients (Rojo et al., 2017). In addition, the levels of oxidative stress are increased in the hippocampus of Nrf2−/− APP mice (Rojo et al., 2017). Therefore, the Akt/Nrf2/HO-1 pathway is a potential target for the treatment of AD.

ZL006 is designed and synthesized to disrupt the interaction of neuronal nitric oxide synthase (nNOS) and postsynaptic density 95 (PSD95) and shows promising therapeutic effects in several neurological disorders (Zhou et al., 2010; Yi et al., 2018). To investigate the toxic dose of ZL006 to cells, we treated cells with ZL006 in a concentration gradient (NH$_4$H$_2$O group; DMSO group; ZL006-treated groups: 5, 10, 20, 50, 100, 150 μM). To select the effective dose of ZL006, we also set a concentration gradient (NH$_4$H$_2$O group; Aβ$_{1-42}$ group; DMSO group; ZL006-treated groups: 5, 10, 20, 50, 75, 100, 150 μM). Then the cells were divided into four groups: NH$_4$H$_2$O group (NH$_4$H$_2$O, < 1 ‰), Aβ$_{1-42}$ group (neurons: 2 μM Aβ$_{1-42}$; N2a neuroblastoma cells: 20 μM Aβ$_{1-42}$), DMSO group (neurons: 2 μM Aβ$_{1-42}$ + 1 % DMSO; N2a neuroblastoma cells: 20 μM Aβ$_{1-42}$ + 1 % DMSO), and ZL006-treated groups (neurons: 75 μM ZL006 + 2 μM Aβ$_{1-42}$; N2a neuroblastoma cells: 10 μM ZL006 + 20 μM Aβ$_{1-42}$). The primary cortical neurons and N2a neuroblastoma cells were incubated with ZL006 or DMSO for 2 hours and treated with Aβ$_{1-42}$ or NH$_4$H$_2$O for another 24 hours, and then the following experiments were performed.

To investigate the role of Akt/Nrf2 pathway, Akt inhibitor VIII group (neurons: 5 μM Akt inhibitor VIII + 75 μM ZL006 + 2 μM Aβ$_{1-42}$); N2a neuroblastoma cells: 5 μM Akt inhibitor VIII + 10 μM ZL006 + 20 μM Aβ$_{1-42}$) or ML385 group (neurons: 5 μM ML385 + 75 μM ZL006 + 2 μM Aβ$_{1-42}$; N2a neuroblastoma cells: 5 μM ML385 + 10 μM ZL006 + 20 μM Aβ$_{1-42}$) was included. The cells of these two groups were incubated with ZL006 and Akt inhibitor VIII or ML385 for 2 hours and treated with Aβ$_{1-42}$ or NH$_4$H$_2$O for another 24 hours. Then, CCK8 assay was performed.

CCK8 assay
Cell viability was determined using the CCK8 assay (Beyo- time Biotechnology) according to the manufacturer’s instructions. Briefly, primary cortical neurons at day-in-vitro (DIV) 11–13 or N2a neuroblastoma cells were incubated with 10 μL CCK8 for 2 hours at 37°C in a 96-well plate, and the absorbance was measured at 450 nm in a plate reader (Bio-Rad, Hercules, CA, USA). Cell viability was expressed as the percentage live cells over the control ones (NH$_4$H$_2$O group).

Cell apoptosis assay
The apoptotic rate of neurons or N2a neuroblastoma cells was determined by an Annexin V-FITC/propidium iodide
(PI) kit (Vazyme, Nanjing, China). Briefly, the neurons at DIV 11–13 were incubated with the binding buffer containing Annexin V-FITC for 5 minutes in the dark, and the fluorescence was detected using a fluorescence microscope (Olympus, Tokyo, Japan). The N2a neuroblastoma cells were collected and suspended in 0.5 mL binding buffer containing 5 μL Annexin V and 10 μL PI, and incubated for 5 minutes at 37°C in the dark. The apoptotic rates including early apoptotic (AV+/PI-) and late apoptotic (AV+/PI+) N2a neuroblastoma cells were analyzed using a flow cytometer (BD Biosciences, Carlsbad, CA, USA).

Western blot assay
Western blot assay was performed as described previously (Yu et al., 2017). Primary cortical neurons or N2a neuroblastoma cells were pretreated with ZL006 or DMSO vehicle for 2 hours and then treated with Aβ1–42 or NH4•H2O vehicle for 24 hours. Then total proteins were extracted using RIPA lysis buffer (Beyotime, Nanjing, China; P0013C) and 30 μg protein of each group was separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride membranes. These membranes were blocked with 5% non-fat milk for 1 hour and incubated overnight at 4°C with the following primary antibodies: rabbit anti-Bax monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Bcl-2 monoclonal antibody (1:1000; Cell Signaling Technology), rabbit anti-Phospho-Akt mononclonal antibody (Ser473) (1:1000; Cell Signaling Technology), rabbit anti-Akt monoclonal antibody (1:1000; Cell Signaling Technology), rabbit anti-Nrf2 monoclonal antibody (1:1000, Abcam, Cambridge, MA, USA), mouse anti-HO-1 polyclonal antibody (1:2000, Abcam), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (1:5000; Bioworld), mouse anti-microtubule-associated protein-2 (MAP2) monoclonal antibody (1:500, Abcam), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (1:5000; Bioworld, Louis Park, MN, USA) and rabbit anti-β-tubulin polyclonal antibody (1:2000, Bioworld). After washed three times with TBS/Tween 20, the membranes were incubated with corresponding secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG) at room temperature for 2 hours. The proteins were visualized with the ECL kit (Millipore, Billerica, MA, USA). The intensity of bands was quantified using ImageJ (https://imagej.nih.gov/ij/, NIH, Bethesda, USA). The relative protein expression was expressed as the band intensity of each group/ the band intensity of the loading control β-tubulin or GAPDH.

Measurement of mitochondrial membrane potential
Mitochondrial membrane potential (MMP) was measured using JC-1 fluorescence assay (Beyotime, Nanjing, China) as described previously (Zhu et al., 2012). Briefly, the neurons at DIV 11–13 were incubated with the JC-1 working solution for 20 minutes in the dark, and the fluorescence was detected using a fluorescence microscope (Olympus). The monomer form of JC-1 (green) represents decreased MMP and aggregate form (red) represents relatively intact MMP. The N2a neuroblastoma cells were collected and suspended in JC-1 staining buffer, and incubated for 20 minutes at 37°C in the dark. The MMP of N2a neuroblastoma cells was examined using a flow cytometer (BD Biosciences, San Diego, CA, USA).

ROS detection
The intracellular ROS of primary cortical neurons was detected using a ROS assay kit (Genmed, Shanghai, China). Briefly, the neurons at DIV 11–13 were incubated with the staining solution (mixture of reagent B and reagent C) for 30 minutes in the dark and washed with Reagent D. Then the fluorescence was detected using a fluorescence microscope (Olympus). The intracellular ROS of N2a neuroblastoma cells was detected using a ROS assay kit (Jiancheng Bioengineering, Nanjing, China). N2a neuroblastoma cells were incubated with 10 μM 2,7-dichloro-dihydrofluorescein diacetate at 37°C for 30 minutes in the dark, and subjected to the measurement of 2,7-dichloro-dihydrofluorescein diacetate fluorescence by a fluorescence microplate reader (Hitachi, Tokyo, Japan).

Calcein acetoxy-methyl-ester/propidium iodide AM/PI staining assay
The viability of neurons was also determined by a calcein-acetoxy-methyl-ester/propidium iodide (AM/PI) Double Stain Kit (Invitrogen, Carlsbad, CA, USA). Cortical neurons at DIV 11–13 were incubated with the calcein-AM and PI buffer at 37°C for 15 minutes in the dark, and the viable cells (green fluorescence) and dead cells (red fluorescence) were observed by an inverted fluorescence microscope (Olympus) and the ratio of viable cells (green fluorescence) to total cells (green fluorescence and red fluorescence) was counted.

Immunostaining
The cells were fixed in 4% paraformaldehyde for 20 minutes, washed with PBS-T for 30 minutes, and blocked with 2% bovine serum albumin for 2 hours. Then the cells were incubated with anti-cleaved caspase-3 polyclonal antibody (1:200; Cell Signaling Technology) and mouse anti-microtubule-associated protein-2 (MAP2) monoclonal antibody (1:500, Abcam) at 4°C overnight, and washed with PBS-T for 30 minutes, then incubated with Alexa Fluor Plus 488, goat anti-mouse IgG (H+L) secondary antibody and Alexa Fluor Plus 594, goat anti-mouse IgG (H+L) secondary antibody (1:500; Invitrogen, Carlsbad, CA, USA) for 60 minutes at room temperature. DAPI reagent (1:1000; Bioworld, Louis Park, MN, USA) was used for the nucleus staining. Images were taken using a fluorescence microscope (Olympus, Japan) and the ratio of cleaved-caspase3 positive cells (red fluorescence) to MAP2 positive cells (green fluorescence) was counted.

Nitrite analysis
The concentration of nitric oxide was measured by a nitric oxide detection kit (Beyotime, Nanjing, China). The supernatant of primary cortical neurons was collected and added with Griess reagent, and the absorbance was obtained at 540 nm with sodium nitrite as a standard curve.
Statistical analysis
Results were expressed as the mean ± SEM and analyzed by SPSS 16.0 statistical analytical software (SPSS, Chicago, IL, USA). Statistical analysis among groups was performed using one-way analysis of variance followed by Bonferroni’s post hoc test and P < 0.05 was considered statistically significant.

Results
ZL006 protects against Aβ1–42-induced primary cortical neuronal cell death
To determine the potential neurotoxicity of ZL006, different concentrations of ZL006 were added to the media of neurons. As shown in Figure 1B, ZL006 moderately decreased the viability of primary cortical neuron at the concentration of 150 μM, and it did not show any cytotoxicity at lower concentrations. ZL006 (50–100 μM) significantly increased the cell viability in Aβ1–42-treated neurons (P < 0.05; Figure 1C). In addition, Aβ1–42 treatment induced oxidative neuronal cell death as shown by PI staining, and ZL006 pretreatment partially rescued the neurotoxicity (P < 0.01; Figure 1D and E).

ZL006 ameliorates Aβ1–42-induced apoptosis in primary neurons
To access whether ZL006 protected against Aβ1–42-induced apoptosis, Annexin V staining was performed. As shown in Figure 2A and B, Annexin-V-FITC+ neurons were significantly increased after Aβ1–42 treatment, while pretreatment of ZL006 significantly decreased the Annexin-V-FITC+ neurons (P < 0.01). In addition, the level of cleaved caspase-3 was significantly reduced in ZL006-pretreated neurons (P < 0.05; Figure 2C and D). ZL006 decreased the level of Bax (an apoptotic activator) and increased the level of Bcl-2 (an anti-apoptotic protein) in Aβ1–42-treated neurons (Figure 2E and F). These data demonstrated that ZL006 attenuated Aβ1–42-induced apoptosis in primary neurons.

ZL006 attenuates Aβ1–42-induced oxidative stress in primary cortical neurons
To evaluate the potential anti-oxidative effects of ZL006, the levels of MMP and intracellular ROS were examined in Aβ1–42-treated primary cortical neurons. As shown in Figure 3A and B, Aβ1–42 significantly decreased the MMP in primary cortical neurons, while ZL006 pretreatment partially reversed the MMP loss (P < 0.01). Intracellular ROS was increased after Aβ1–42 treatment, and ZL006 pretreatment significantly inhibited the ROS generation in neurons (P < 0.01; Figure 3C and D). In addition, Aβ1–42 treatment induced a significant increase of NO release in the supernatant (P < 0.05). However, the level of NO was decreased by ZL006 pretreatment (P < 0.05; Figure 3E). These results suggested that ZL006 decreased the oxidative stress in Aβ1–42-treated neurons.

ZL006 protects against Aβ1–42-induced neurotoxicity partially by Akt/Nrf2/HO-1 pathway in primary cortical neurons
Given that Akt/Nrf2/HO-1 pathways played an important role in the pathogenesis of AD, we examined whether ZL006 could modulate the Akt/Nrf2/HO-1 pathway. As shown in Figure 4A and B, the level of p-Akt was significantly decreased after Aβ1–42 treatment (P < 0.01), while ZL006 pretreatment induced the phosphorylation of Akt (P < 0.01). In addition, ZL006 pretreatment increased the levels of Nrf2 and HO-1 in Aβ1–42-treated primary neurons (P < 0.05; Figure 4A and C). Furthermore, Akt inhibitor VIII (Figure 4D) or ML385 (Figure 4E) pretreatment reversed the beneficial effects of ZL006 in Aβ1–42-treated neurons, suggesting that ZL006 protects primary cortical neurons partially by activating the Akt/Nrf2/HO-1 pathway.

ZL006 inhibits Aβ1–42-induced neurotoxicity and oxidative stress by Akt/Nrf2/HO-1 pathway in N2a cells
To further confirm the neuroprotective effects of ZL006 against Aβ1, N2a neuroblastoma cells were used. Similarly, Aβ1–42 treatment resulted in cell viability reduction in N2a neuroblastoma cells, and ZL006 partially rescued the detrimental effects (Figure 5A). In addition, ZL006 significantly reduced the apoptotic rate in Aβ1–42-treated N2a neuroblastoma cells (Figure 5B and C). The MMP loss (Figure 5E) resulted in cell viability reduction in N2a neuroblastoma cells. As expected, ZL006 induced the phosphorylation of Akt, and upregulated the expressions of Nrf2 and HO-1 in Aβ1–42-treated N2a neuroblastoma cells. ZL006 pretreatment reversed the ROS production (Figure 5F). Next we examined the effects of ZL006 on Akt/Nrf2/HO-1 pathway in Aβ1–42-treated N2a neuroblastoma cells. As expected, ZL006-induced oxidative stress by Akt/Nrf2/HO-1 pathway counteracted the protective effects of ZL006 (Figure 5J). These results showed that ZL006 protected N2a neuroblastoma cells against Aβ1–42-induced neurotoxicity and oxidative stress, and activated the Akt/Nrf2/HO-1 pathway, which suggested that ZL006 might be an alternative compound for AD treatment.

Discussion
Oxidative stress plays an important role in the progression of AD, making it a potential target for the treatment (Li et al., 2018a; Onyango, 2018; Weinstein, 2018). In this study, we found that Aβ1–42 reduced neuronal viability and promoted neuronal apoptosis and oxidative stress in primary cortical neurons and N2a neuroblastoma cells. However, ZL006 treatment decreased neuronal apoptosis and oxidative stress induced by Aβ1–42. In addition, ZL006 protected against Aβ1–42-induced neurotoxicity partially by the activation of the Akt/Nrf2/HO-1 pathway.

The neuroprotective effects of ZL006 have been extensively studied in ischemic stroke, traumatic brain injury, Parkinson’s disease and hemorrhage-induced thalamic pain (Zhou et al., 2010; Hu et al., 2014; Cai et al., 2018; Qu et al., 2020). ZL006 is designed to selectively interrupt the interaction between PSD95 and nNOS, which plays a critical role in synaptic transmission and neuronal functions. ZL006 treatment ame-
Neuroprotective effects of ZL006 in Aβ1–42-treated neuronal cells. Tao WY, Yu LJ, Jiang S, Cao X, Chen J, Bao XY, Li F, Xu Y, Zhu XL (2020). Neural Regen Res 15(12):2296-2305. doi:10.4103/1673-5374.285006

Figure 1 ZL006 protects against Aβ1–42-induced neuronal cell death.
(A) The chemical structure of ZL006. (B) Primary cortical neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours, then treated with Aβ1–42 (2 μM) or NH4·H2O vehicle (< 1‰) for 24 hours, and cell viability was accessed. n = 6 cells per group. (C) Primary cortical neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (2 μM) or NH4·H2O vehicle (< 1‰) for 24 hours, and cell viability was accessed. n = 6 cells per group. (D) Primary cortical neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (2 μM) or NH4·H2O vehicle (< 1‰) for 24 hours, and caspase-3 activity was determined. n = 6 cells per group. (E) Primary cortical neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (2 μM) or NH4·H2O vehicle (< 1‰) for 24 hours, and Annexin V-FITC staining analysis was performed. Original magnification, 20×, scale bar: 50 μm. (F) Quantitative analysis of the PI+ cells. n = 6 cells per group. Data were expressed as mean ± SEM (one-way analysis of variance followed by Bonferroni’s post hoc test). *P < 0.05, **P < 0.01, vs. NH4·H2O vehicle group; #P < 0.05, ##P < 0.01, vs. Aβ1–42 + DMSO group. Aβ: Amyloid-beta; DAPI: 4′,6-diamidino-2-phenylindole; DIV: day-in-vitro; DMSO: dimethyl sulfoxide; FITC: fluoresceine isothiocyanate; MAP2: microtubule-associated protein 2.

Figure 2 ZL006 ameliorates Aβ1–42-induced apoptosis in primary neurons.
Primary neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (2 μM) or NH4·H2O vehicle (< 1‰) for 24 hours, and Annexin V-FITC staining analysis was performed. Original magnification, 20×, scale bar: 50 μm. (B) Quantitative analysis of green fluorescence intensity. n = 3 cells per group. (C) The level of cleaved caspase-3 and MAP2 of primary cortical neurons at DIV 11–13 was determined by immunostaining. Original magnification, 20×, scale bar: 50 μm. (D) Quantitative analysis of the level of cleaved caspase-3. n = 4 cells per group. (E) The protein expressions of Bax and Bcl-2 in primary neurons at DIV 11–13 were examined by western blot assay. n = 4 cells per group. Data were expressed as mean ± SEM (one-way analysis of variance followed by Bonferroni’s post hoc test). *P < 0.05, **P < 0.01, vs. NH4·H2O vehicle group; #P < 0.05, ##P < 0.01, vs. Aβ1–42 + DMSO group. Aβ: Amyloid-beta; AM: acetoxymethylester; PI: propidium iodide; DIV: day-in-vitro; DMSO: dimethyl sulfoxide; FITC: fluoresceine isothiocyanate; MAP2: microtubule-associated protein 2.

ZL006 alleviates ischemic injury in oxygen-glucose deprivation (OGD)-treated primary cortical neurons and in middle cerebral artery-occluded mice and rats (Zhou et al., 2010). In addition, ZL006 treatment promotes the migration and differentiation of transplanted neural stem cells in ischemic stroke models by upregulating the activity of cAMP responsive element binding protein (Wang et al., 2017). ZL006 treatment attenuates apoptosis and neurological deficits in the controlled cortical impact mouse model, indicating that ZL006 might be used to treat traumatic brain injury (Qu et al., 2020). Hu et al. (2014) reported that ZL006 reduced neuronal apoptosis and oxidative stress by upregulating sirtuin 3 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP+) -treated neurons. Moreover, systemic or intra-amygdala treatment of ZL006 alleviated conditioned fear, while it did not affect object recognition memory and spatial memory, suggesting that ZL006 is a potential drug for the treatment of fear-related disorders (Li et al., 2018b; Song et al., 2019). In this study, we found...
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Figure 3 ZL006 attenuates Aβ1–42-induced oxidative stress in primary cortical neurons.

(A) Cortical neuronal cells at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (2 μM) or NH₃•H₂O vehicle (< 1‰) for 24 hours, and MMP was measured using JC-1 fluorescence assay (JC-1 fluorescent probe; green: JC-1 monomers; red: JC-1 aggregates). Original magnification, 40×, scale bar: 20 μm. (B) Quantitative analysis of green/red fluorescence ratio. n = 3 cells per group. (C) The level of intracellular ROS in neurons at DIV 11–13 was measured by fluorescence assay. Original magnification, 20×, scale bars: 50 μm. (D) Quantitative analysis of ROS. n = 4 cells per group. (E) The NO release in the supernatants of neurons at DIV 11–13 was examined by Griess reaction. n = 3–4 cells per group. Data were expressed as mean ± SEM (one-way analysis of variance followed by Bonferroni’s post hoc test). *P < 0.05, **P < 0.01, vs. NH₃•H₂O group; #P < 0.05, vs. Aβ1–42 + DMSO group. Aβ: Amyloid-beta; DIV: day-in-vitro; DMSO: dimethyl sulfoxide; JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine; NO: nitric oxide; ROS: reactive oxygen species.

Figure 4 ZL006 protects against Aβ1–42-induced neurotoxicity partially by the Akt/Nrf2/HO-1 pathway.

(A) Primary neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) for 24 hours, and the levels of p-Akt/Akt, Nrf2 and HO-1 were determined by western blot assay. (B, C) Quantitative analysis of the relative levels of p-Akt/Akt. n = 4 cells per group. (D) Primary neurons at DIV 10–12 were pretreated with ZL006 (75 μM) or DMSO vehicle (< 1‰) with or without Akt inhibitor VIII (5 μM) for 24 hours and then treated with Aβ1–42 (2 μM) or NH₃•H₂O vehicle (< 1‰) for 24 hours, and cell viability was determined by CCK8 assay. n = 12 cells per group (analysis of variance with Bonferroni’s post hoc test). *P < 0.05, **P < 0.01, vs. NH₃•H₂O group; #P < 0.05, vs. Aβ1–42 + DMSO group; Aβ: Amyloid-beta; DMSO: dimethyl sulfoxide; Nrf2: NF-E2-related factor 2; HO-1: heme oxygenase-1.

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that ZL006 ameliorated Aβ1–42-induced oxidative stress and neuronal apoptosis. Interestingly, ZL006 did not affect spatial memory, which was compromised in the early stage of AD, in normal conditions (Zhou et al., 2010; Li et al., 2018b). Whether ZL006 treatment improves spatial memory in AD models needs further investigations.

Neuronal apoptosis plays a critical role in the development and homeostasis of the central nervous system, and Aβ induced neuronal apoptosis contributes to the pathogenesis of AD (Obulesu and Lakshmi, 2014). Although the underlying mechanisms are not fully defined, it is generally considered that Aβ directly or indirectly targets mitochondria, and induces the depolarization of MMP, which results in the release of cytochrome c and activation of caspase-9 and caspase-3. Aβ treatment also triggers the upregulation of pro-apoptotic protein including Bax and downregulation of anti-apoptotic protein including Bcl-2. Humanin is able to bind to Bax and prevents the translocation of Bax to the mitochondria to inhibit cellular apoptosis. In addition, humanin and its derivatives protect against learning and memory deficits in Aβ-induced AD models (Li et al., 2013; Chai et al., 2014; Romeo et al., 2017). A point mutation of Beclin 1 (F121A) disrupts the interaction of Beclin 1 and Bcl-2, and attenuates cognitive impairment in AD mice (Rocchi et al., 2017). Several chemicals, including Sulforaphane, Tanshinone IIA, and Dalesconol B, have been reported to decrease Aβ-induced neuronal apoptosis and memory decline in vitro and in vivo (Zhu et al., 2014; Li et al., 2016; Hou et al., 2018). Here, we showed that ZL006 mitigated Aβ1–42-induced neuronal apoptosis and promoted the survival of neurons, which might contribute to the neuroprotective effects of ZL006.

It is well known that increased Aβ induces extensive oxidative stress, which in turn contributes to the accumulation of amyloid plaques through the amyloidogenic pathway (Gu et al., 2013; Zuo et al., 2015). The generation of ROS and oxidant molecules and enzymes is imbalanced in AD, which leads to the aggravation of oxidative stress (Zuo et al., 2015). In addition, Aβ-induced oxidative stress contributes to the pathology of tau phosphorylation by activating the JNK/p38 mitogen-activated protein kinase pathway, suggesting the association between oxidative stress and tau hyperphosphorylation (Giraldo et al., 2014). Moreover, inhibition of oxidative stress seems to be an attractive strategy for AD treatment (Cheignon et al., 2018; Wojsiat et al., 2018). Resveratrol upregulates the activity of sirtuin 1 and attenuates spatial memory impairment and synaptic dysfunctions in Aβ1–42-treated rats (Gomes et al., 2018). Our previous results also demonstrate that Hopeahainol A and Diammonium glycyrrhizinate decrease the oxidative response and improve spatial memory in AD mice (Zhu et al., 2012, 2013). Here, we identified that ZL006 pretreatment could significantly inhibit the oxidative stress in Aβ1–42-treated neurons, which was consistent with previous studies showing the antioxidant effects of ZL006 (Hu et al., 2014; Liu et al., 2017).

Increasing evidence shows that activation of the Akt pathway is a potential therapeutic strategy for AD treatment (Heras-Sandoval et al., 2014; Kitagishi et al., 2014; Rai et al., 2019). The level of Akt phosphorylation and the activity of Akt1 are decreased in the brain samples of AD patients (Lee et al., 2009). Constitutive activation of Akt protects hippocampal neurons against mutant presenilin-1-induced cell death (Weihl et al., 1999). Our previous data show that Dalesconol B attenuates memory deficits and Aβ1–42-induced neuronal apoptosis by activating the Akt pathway (Zhu et al., 2014). However, the effects of Aβ on the Akt pathway are still controversial. Akt activity and phosphorylation are decreased in human neuroblastoma SH-SY5Y cells expressing Aβ, while the PI3K activity is not affected (Lee et al., 2009). Acute Aβ1–42 exposure induces phosphorylation of Akt in primary neurons, which is dependent of the activation of both NMDA receptor and 7 nicotinic receptor. The level of Akt phosphorylation is compromised in the hippocampus of 13-month-old AD transgenic (TAS10) mice (Abbott et al., 2008). We have demonstrated that Aβ1–42 treatment inhibits the Akt activity and phosphorylation in SH-SY5Y cells and in Aβ1–42-treated mice (Zhu et al., 2014). In this study, the phosphorylation of Akt was inhibited in Aβ1–42-treated primary cortical neurons and N2a neuroblastoma cells, which might be due to the diversity of Aβ species.

Akt modulates the pathogenesis of AD by regulating downstream kinases including GSK3β, which has been extensively studied as a tau kinase (Hernandez et al., 2013; Bhat et al., 2018). Nrf2/HO-1 axis is a downstream target of the Akt pathway (Martin et al., 2004; Dai et al., 2007; Rong Bhat et al., 2018). Nrf2 is decreased in the brains of AD mice, and activation of Nrf2 pathway or Nrf2 overexpression protects neurons against Aβ-induced neurotoxicity (Kanninen et al., 2008). Nrf2 deficiency exacerbates Aβ or Tau-associated neuropathology and spatial memory deficits in APP or Tau transgenic mice (Rojo et al., 2017). Furthermore, HO-1 is localized with neurofibrillary tangles and senile plaque in the brains of AD patients, and is associated with the neurofibrillary pathology of AD (Smith et al., 1994). The plasma level of HO-1 is significantly decreased in AD patients (Schipper et al., 2000; Ishizuka et al., 2002), indicating that HO-1 might be a biomarker and therapeutic target for AD. Sulforaphane activates the Nrf2/HO-1 pathway to exert its anti-inflammatory effect against Aβ (An et al., 2016). Orientin promotes Nrf2 translocation from cytoplasm to nucleus and attenuates spatial memory deficits in Aβ1–42-induced AD mice (Yu et al., 2015). In this study, it was shown that ZL006 pretreatment activated the Akt/Nrf2/HO-1 pathway in Aβ1–42-treated neuronal cells, which contributed to the neuroprotective effects of ZL006.

In conclusion, the current study demonstrated that ZL006 could protect neurons against Aβ1–42-induced neurotoxicity and inhibit oxidative stress. In addition, ZL006 activated the Akt/Nrf2/HO-1 pathway in Aβ1–42-treated neurons, which might be associated with its neuroprotective effects. Thus, these results suggest that ZL006 might be a potential compound for AD treatment. Since ZL006 is able to cross the blood-brain barrier quickly without major side effects (Zhou et al., 2010), further studies are needed to investigate the neuroprotective effects of ZL006 and the potential role of the Akt/Nrf2/HO-1 pathway in AD models.
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Figure 5 ZL006 inhibits Aβ1–42-induced neurotoxicity and oxidative stress by Akt/Nrf2/HO-1 pathway in N2a neuroblastoma cells.

(A) N2a neuroblastoma cells were pretreated with ZL006 (10 μM) or DMSO vehicle (< 1‰) for 2 hours and then treated with Aβ1–42 (20 μM) or NH4+H2O vehicle (< 1‰) for 24 hours, the cell viability was determined using the CCK8 assay. n = 8–12 cells per group.

(B) The apoptotic rate of N2a neuroblastoma cells (early apoptotic (AV+/PI–) and late apoptotic (AV+/PI+)) was accessed using AnnexinV/PI flow cytometry. (C) Quantitative analysis of the apoptotic rate. The apoptotic cells included the AnnexinV-FITC+/PI− and AnnexinV-FITC+/PI+ cells. n = 4 cells per group. (D) The MMP of N2a neuroblastoma cells was examined by JC-1 flow cytometry. (E) Quantitative analysis of JC-1 assay. n = 3 cells per group. (F) The level of ROS in N2a neuroblastoma cells was measured by flow cytometry, n = 4 cells per group. (G) The protein levels of p-Akt/Akt, Nrf2 and HO-1 in N2a neuroblastoma cells were determined by western blot assay. (H, I) Quantitative analysis of the relative protein levels of p-Akt/Akt, n = 3 cells per group. (J) N2a cells were pretreated with ZL006 (10 μM) with or without Akt inhibitor VIII (5 μM) or ML385 (5 μM) for 2 hours and then treated with Aβ1–42 (20 μM) for 24 hours, and cell viability was determined by the CCK8 assay. n = 10–12 cells per group. Data were expressed as the mean ± SEM (one-way analysis of variance followed by Bonferroni’s post hoc test). *P < 0.05, **P < 0.01, vs. NH4+H2O group; #P < 0.05, ##P < 0.01, vs. Aβ1–42 + DMSO group; &&P < 0.01, vs. Aβ1–42 + ZL006 group. Aβ: Amyloid-beta; DMSO: dimethyl sulfoxide; JC-1: 5,5′,6,6′-tetraethyl-imidacarbocyanine; Nrf2: NF-E2-related factor 2; HO-1: heme oxygenase-1.

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