Regular Article

7-(4-Hydroxyphenyl)-1-phenyl-4E-hepten-3-one, a Diarylheptanoid from Alpinia officinarum, Protects Neurons against Amyloid-β Induced Toxicity

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Alzheimer’s disease (AD) is one of the major causative agents of Alzheimer’s disease (AD), the most common neurodegenerative disorder characterized by progressive cognitive impairment. While effective drugs for AD are currently limited, identifying anti-Aβ compounds from natural products has been shown as a promising strategy which may lead to breakthroughs for new drug candidate discovery. We have previously reported that 7-(4-hydroxyphenyl)-1-phenyl-4E-hepten-3-one (AO-1), a diarylheptanoid extracted from the plant Alpinia officinarum, has strong effects on neuronal differentiation and neurite outgrowth in vitro and in vivo. The present study further uncovers that AO-1 exerts neuroprotective effects against the neurotoxicity caused by Aβ. Under the damage of Aβ oligomers, the major pathological forms of Aβ, dendrites of neurons become atrophic and simplified, but such impairments were substantially alleviated by AO-1 treatment. Moreover, AO-1 reduced apoptotic levels and oxidative stress triggered by Aβ. Further analysis showed that the anti-caspase and dendrite protective effects of AO-1 were dependent on activation of phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathways. These findings collectively identify AO-1 as a beneficial compound to ameliorate the deleterious effects of Aβ on dendrite integrity and cell survival, and may provide new insights on drug discovery of AD.

Key words Alzheimer’s disease (AD); amyloid-β (Aβ); dendrite; mammalian target of rapamycin (mTOR); neuroprotection; phosphatidylinositol 3-kinase (PI3K)

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease in the elderly which is characterized by progressive cognitive decline and movement dysfunction. Being a disease with extremely complex etiology, the accumulation of amyloid-β (Aβ) peptides is believed as a main pathogenic event that directly causes neurodegeneration.1–3 Aβ peptides, which are normally constituted by 40–42 amino acids, are released by the aberrant cleavage of amyloid precursor protein (APP) and deposit at synapses. Aβ peptides are the major component of the senile plaques observed in AD brain. Moreover, soluble oligomers formed by aggregation of monomeric Aβ42 (1–42 a.a) peptides are the most pathogenic forms during early progression of AD. The damaging effects of Aβ42 includes synapse loss, neurite dystrophy and dendritic simplification.4,5) Thus, one of the current efforts for drug development against AD is to generate platforms that aim at antagonizing the devastating effects of Aβ oligomers. Such approaches have been proven promising for therapeutic design and drug discovery of AD.6–8)

A variety of compounds derived from natural sources have been identified effective for counteracting Aβ-induced neurotoxicity.9,10) Such anti-Aβ activities could be achieved through several ways. For instance, Aβ production or aggregation is prevented, or Aβ clearance is accelerated. Moreover, Aβ-induced signaling pathways could be blocked or down-regulated. A great number of natural compounds have shown powerful anti-Aβ effects through multiple actions mentioned above.11–15) Notably, curcumin and its related compounds, a group of natural diarylheptanoids found in the spice turmeric of the ginger family (Zingiberales), have long gained attention for their abilities of Aβ aggregation/signaling antagonism and memory enhancement.16–20) Resveratrol, a natural polyphenolic compound found in many plants, is demonstrated to have multi-neuroprotective effects against Aβ toxicity.21,22) Both compounds have been extensively studied in animals and have shown great potentials as therapeutic agents for AD.

We previously reported that two naturally occurring curcumin-like diarylheptanoids, 7-(4-hydroxyphenyl)-1-phenyl-4E-hepten-3-one (AO-1; Fig. 1) and 7-(4-hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one (AO-2), promotes differentiation and neurite outgrowth in both Neuro-2a cells and cultured hippocampal neurons through activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways, and AO-1 accelerates differentiation of newborn neurons in vivo.23) Both diarylheptanoids were isolated from the rhizomes of Alpinia officinarum, a Zingiberales plant long used as a traditional Chinese medicine and a popular spice. Given the beneficial effects of AO-1 on neuronal differentiation in vitro and in vivo, this study aims at further interrogating whether this compound protects Aβ-injured neurons to retain their viability and differentiation abilities.

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RESULTS

AO-1 Alleviated Dendritic Impairments Induced by Aβ42  The toxic effects of Aβ oligomers during the early progression of AD include pathological changes of neuronal morphologies, such as synapse loss, neurite dystrophy and dendritic simplification.24,25) Given that AO-1 shows neurite outgrowth promoting effects, it is tempting to hypothesize whether they have beneficial effects for neurite integrity after Aβ injuries. We observed that Aβ42 oligomers induced severe dendritic simplification, as Sholl analysis showed that the dendritic intersections at 20–160 µm from the cell bodies were remarkably fewer in Aβ42-treated neurons than those in control neurons (Figs. 2A, B). Notably, although normal dendritic complexity levels were not fully restored, pretreatment of AO-1 (0.5 µM) largely increased dendritic intersections at 40–150 µm from the cell bodies when compared to those of Aβ42-treated neurons (Figs. 2A, B). Moreover, the total dendritic branching numbers of AO-1 pretreated neurons were relatively retained normal (Fig. 2C). These findings together suggest that AO-1 exerts ameliorative effects toward Aβ42 toxicity on dendrite integrity.

AO-1 Protects Neurons against Aβ42-Induced Apoptosis and Oxidative Stress  To examine whether AO-1 promotes neuronal survival after Aβ42 damage, we used terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, which labels apoptotic cells by recognizing DNA fragmentation resulted from apoptotic signaling cascades. Exposure to Aβ42 oligomers for 48 h led to elevated levels of apoptosis of hippocampal neurons, indicated by significantly more Aβ42-treated neurons (ca. 16%) labeled by TUNEL than control neurons (ca. 10%) (Figs. 3A, B). However, pretreatment of AO-1 (0.5 µM) totally blocked Aβ42-induced neuronal apoptosis, resulting in only ca. 5.5% of apoptotic neurons (Figs. 3A, B).

Mitochondria dysfunction and oxidative stress are main causes of neuronal damage induced by Aβ. To further investigate the possible mechanism of AO-1 against Aβ42 toxicity, we examined levels of released reactive oxygen species (ROS), which is a direct indicator of oxidative stress. Aβ42 treated neurons showed remarkably increased ROS level compared to control, whereas pretreatment of AO-1 at 0.5 µM totally reversed the elevated ROS level (Fig. 4). Interestingly, curcumin, a known ROS scavenger who shares similar structure

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Fig. 1. The Chemical Structure, Chemical Formula, and Molecular Weight of AO-1

Fig. 2. AO-1 Ameliorates Aβ42-Induced Dendritic Simplification of Hippocampal Neurons  
(A) Cultured hippocampal neurons (15 DIV) which were sparsely labeled with EGFP were pre-treated with AO-1 (0.5 µM) at 15 DIV for 2h, followed by oligomerized Aβ42 (1 µM) treatment for 24h. Scale bar, 20 µm. (B) Numbers of dendritic intersections at 0–200 µm from the cell bodies were accessed by Sholl analysis. (C) Total dendritic branch number was quantified. ***p<0.001, Aβ42 vs. Control; ###p<0.001, Aβ42+AO-1 vs. Aβ42. From 30 to 40 neurons from three independent experiments in each group were measured. Error bars depict the mean±S.E.M.
with AO-1, was only effective to inhibit Aβ-induced ROS release at a higher concentration (10 μM). Together, both the apoptosis analysis and ROS assay showed that AO-1 exhibits strong protective effects against Aβ42-induced lesion in neurons.

**AO-1 Inhibits Caspase-3 Dependent Apoptosis Induced by Aβ42, Which Is through Regulating PI3K-Mammalian Target of Rapamycin (mTOR) Signaling Pathway**

To understand how AO-1 acts on neuroprotection, we performed mechanistic studies. Caspase-3, a major member of caspase family, is activated by numerous cell death signal pathways. The levels of its cleaved fragment, cleaved-caspase-3, represent apoptosis degrees. Cultured hippocampal neurons exposed to Aβ42 oligomers showed an increased level of cleaved-caspase-3, which was partially reversed by addition of AO-1 (Figs. 5A, C). Thus, the decreased cell apoptosis induced by AO-1 may take place through downregulation of caspase-3 cleavage. Our previous study suggested that AO-1-promoted neurite growth is dependent on activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-ERK and PI3K-Akt signaling pathways. We then asked whether AO-1 regulates the same pathways during neuroprotection. Interestingly, blockade of MEK activity by U0126 did not inhibit AO-1’s effect on downregulating caspase-3 cleavage (Figs. 5B, D), suggesting MEK-ERK may not be involved. However, either blockade of PI3K by LY294002 or its substrate mTOR by Torin1 led to significant increase of cleaved-caspase-3 even in the presence of AO-1 (Figs. 5B, D). Therefore, the effect of AO-1 on neuroprotection against Aβ42 is probably through activation of PI3K-mTOR pathway.

**AO-1 Mediated Anti-Aβ Effect on Dendrite Protection Is Dependent on PI3K-mTOR Signaling Pathway**

We further asked whether PI3K-mTOR signaling pathway is important for AO-1 to protect dendrite integrity upon Aβ insult. LY294002 and Torin1 were pretreated to block PI3K and mTOR activities, respectively, followed by application of AO-1 and Aβ42. Indeed, treatment of either inhibitor totally blocked the effect of AO-1 on dendrite protection, resulting in similar dendrite dystrophy when compared to Aβ42 treatment alone (Figs. 6A, B). Thus, AO-1 mediated dendrite protection against Aβ42 is through PI3K-mTOR signaling pathway.

**DISCUSSION**

The current study identifies that AO-1, a natural diarylheptanoid derived from the rhizomes of *Alpinia officinarum*, exerts protective effects in neurons against Aβ-induced apoptosis and dendrite atrophy. Moreover, we showed that AO-1 is capable of attenuating the activated caspase pathway triggered by Aβ42, which is dependent on PI3K-mTOR activities but not MEK activity. Given that we previously reported that...
AO-1 promotes neuronal differentiation and neurite growth, this study further reveals that this compound not only has beneficial effects for normal neuronal development, but also protects neurons from insults commonly occurred during AD pathogenesis.

PI3K/mTOR pathway is crucial for almost all cellular events including cell growth, survival, metabolism and adaptation to stresses. The activities of PI3K/mTOR must be precisely regulated in a balanced state to maintain healthy cells. Normal activation of PI3K/mTOR pathway is important for protection and regeneration of neurons in different injury and degeneration models.31–33) Interestingly, mTOR is often found hyperactivated in AD, which could lead to aberrant autophagy and continuously elevated global protein synthesis that overloads the endoplasmic reticulum. Consistently, inhibitors of mTOR have been shown in animal models to ameliorate some pathological features such as cognitive functions in short term paradigms.34) However, sustained downregulation of mTOR also causes insufficient protein synthesis and other deficits, which eventually leads to synaptic failure and cognitive dysfunction. In fact, due to the signaling complexity and versatile roles of mTOR, conflict observations have been made regarding the changes of mTOR levels in different in vivo and in vitro AD models.35) Therefore, data obtained from manipulation of PI3K/mTOR activities require careful analysis and interpretation in the therapeutic design of AD. For example, a recent study identifies a small molecule which prevents neurodegeneration through only partial restoration of one of the mTOR downstream signaling pathways.36) The current study shows that PI3K/mTOR activation contributes to AO-1 induced inhibition of caspase-3 cleavage. Since a number of signaling events can be triggered by PI3K/mTOR, such as transcription/translation and cytoskeletal rearrangement, it still remains to be fully elucidated what events are specifically responsible for the neuroprotective effects of AO-1. More importantly, in vivo studies are needed to determine whether AO-1 is indeed beneficial for mitigating AD pathology. AO-1 has been proven to be able of crossing the blood brain barrier and promote differentiation of newborn neurons in the dentate gyrus.23) Further studies using AD animal models will be carried out to test the effects of AO-1 administration. Moreover, curcumin and its derivatives, a class of well-known anti-Aβ compounds that are structurally close to AO-1, directly bind to Aβ and inhibits its oligomerization.37–39) It will thus be interesting to verify whether the protective effect of AO-1 is through direct binding to Aβ.

MATERIALS AND METHODS

Chemicals and Reagents The rhizomes of Alpinia officinarum...
ficinarum HANCE were collected in October of 2010 from Xuwen County, Guangdong Province of P. R. China, and were authenticated by Prof. Guang-Xiong Zhou at the Institute of Traditional Chinese Medicine & Natural Products, Jinan University (voucher specimen No. 101029). The extraction method of AO-1 (purity more than 98%) was described previously.\(^{23}\) AO-1 was dissolved in dimethyl sulfoxide (DMSO) in a concentration of 10 mM as stock solution. From 0.1 to 0.5 \(\mu\)M of AO-1 was used to study the protective effects of the compound, and DMSO with equal volume of the paired AO-1 treatment was added as the vehicle control in each experiment. When two concentrations of AO-1 were tested in one experiment, the volume of DMSO vehicle control was equal to the volume of AO-1 treated in higher concentration.

The primary antibody against caspase-3 was purchased from Cell Signaling Technology, MA, U.S.A.; \(\alpha\)-tubulin was from Sigma-Aldrich, MO, U.S.A.; MAP2 was from Millipore, Germany, Pharmacological inhibitor LY294002 was from Millipore; U0126 was from Cell Signaling Technology; Torin1 was from Tocris, U.K.; Curcumin was from Sigma-Aldrich.

**A\(\beta\) Oligomerization** Human A\(\beta\)42 (1–42 a.a.) peptides (purchased from rPeptide, GA, U.S.A.) were oligomerized at 37°C for 7 d in a 5% CO\(_2\)-supplemented atmosphere.\(^{30}\) Soluble oligomerized A\(\beta\)42 peptides were added to neurons (equivalent to 1 \(\mu\)M peptides).

**Cell Culture** Primary cortical or hippocampal neurons were prepared from Sprague Dawley (SD) rat embryos at day 18 of pregnancy and plated on poly-d-lysine (1 mg/mL; Sigma-Aldrich) coated 18 mm coverslips or poly-l-lysine (0.1 mg/mL; Sigma-Aldrich) coated plates as previously described.\(^{41,42}\) The neurons were maintained in Neurobasal medium (Life Technologies, CA, U.S.A.) supplemented with 2% B27 (Life Technologies), 1 mM l-glutamine (Life Technologies) and 1% penicillin/streptomycin. Cortical or hippocampal neurons were plated at the density of 1×10⁵/coverslip for TUNEL assay and dendrite morphology analysis, and 6×10⁵/35 mm dish for Western blot analysis.

All experimental procedures involving the use of animals were approved by the Ethics Committee on Animal Experiments at Jinan University, China, and were strictly performed according to the guidelines of the Care and Use of Laboratory Animals. All efforts were made to minimize the suffering and the number of animals used.

**Dendrite Morphology Analysis** AO-1 (0.5 \(\mu\)M) was added to hippocampal neurons (13–15 d in vitro (DIV)) for 1 h, followed by A\(\beta\)42 treatment for 24 h to allow induction of dendrite atrophy and dendritic branching simplification. For inhibitor treatment, neurons were pre-treated with PI3K in-
hibitor (LY294002, 10 μM) or mTOR inhibitor (Torin1, 50 nm) for 30 min, followed by AO-1 treatment for 1 h. Aβ42 was then added to the neurons for 24 h. Dendrites were visualized by enhanced green fluorescent protein (EGFP) labeling using calcium phosphate transfection method,43) or by immunostaining for MAP2, a dendrite marker. Dendritic morphology and total branching number (the sum of the number of primary dendrites and the number of all branch points) were examined by Sholl analysis using ImageJ software.43)

**TUNEL Assay** Aβ42 was added to hippocampal neurons (7 DIV) for 48 h with or without pre-treated AO-1 for 1 h. The apoptotic cells were determined by TUNEL assay using in situ Cell Death Detection kit, TMR red (Roche, Switzerland), and were photographed using Zeiss Axiom Imager A2 microscope (Carl Zeiss AG). Apoptosis rate was analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.).

**Quantification of ROS Generation** ROS were measured with 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Santa Cruz, TX, U.S.A.). Aβ42 was added to cortical neurons (7 DIV) for 24 h after AO-1 or curcumin was pretreated for 1 h. Neurons were then washed with Dulbecco’s phosphate-buffered saline (DPBS) and incubated with 10 μM DCFH-DA in dark for 30 min at 37°C incubator with 5% CO2. The fluorescence intensity of the converted DCF represents the levels of ROS, which was measured using a DTX880 multimode detector (Beckman Coulter, Brea, CA, U.S.A.). The excitation and emission wavelengths were set as 488 and 528, respectively. The results were showed as percentages of control group.

**Western Blot Analysis** Cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (60 mM Tris–HCl, pH 7.4, 1% SDS, 25% glycerol, 12.5% β-mercaptoethanol), and equal amounts of protein from each sample were separated by 10% SDS-PAGE. Proteins were transferred onto polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked with 5% fat-free powdered milk in Tris buffered saline with Tween-20 (TBST) for 1 h followed by an overnight incubation with anti-caspase-3 antibody (1:1000) or anti-α-tubulin antibody (1:3000) dissolved in TBST with 1% bovine serum albumin at 4°C. After washed 3 times, 10 min/time in TBST, the membranes were subsequently incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by detection using enhanced chemiluminescence (GE Healthcare, U.K.). Band intensities were quantified using Quantity One-4.6.2 software.

**Statistical Analysis** At least three independent experiments were performed in each method. Data were expressed as the mean±standard error of the mean (S.E.M.). Statistically significance was determined by one-way ANOVA. The level of significance was set at p<0.05.

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**Conflict of Interest** The authors declare no conflict of interest.

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