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Research Paper

# A natural diarylheptanoid protects cortical neurons against oxygen–glucose deprivation-induced autophagy and apoptosis

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**Keywords**
apoptosis; autophagy; ischaemic; neuroprotection; oxygen–glucose deprivation/reperfusion

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## Abstract

**Objectives** This study aims to investigate the neuroprotective effects of curcumin analogues, 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (AO-2) on oxygen–glucose deprivation and re-oxygenation (OGD/R) induced injury in cortical neurons, which is a widely accepted in-vitro model for ischaemic reperfusion.

**Methods** In this study, AO-2 was added to cortical neurons for 2 h as pretreatment, and then cortical neurons were subjected to OGD/R in the presence of AO-2 for 4 h. Cell viability was tested by 2′, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay and apoptosis by flow cytometry and Live & Dead cell assay. Western blot analysis detected the change in AKT/mTOR (mammalian target of rapamycin) signalling pathway.

**Key findings** Treatment of AO-2 increased cell survival of OGD/R-treated cortical neurons. Transient AKT/mTOR inhibition, induction of the autophagy marker LC3-II (microtubule-associated protein 1A/1B-light chain 3 phosphatidylethanolamine conjugate), and cleavage of the apoptosis marker Caspase-3 were observed at different stages of OGD/R, and AO-2 reversed all three events. Importantly, treatment of the mTOR inhibitor rapamycin blocked the neuroprotective effects of AO-2 on reducing LC3-II and cleaved Caspase-3 expression and cancelled AO-2-mediated neuronal survival.

**Conclusions** These results demonstrate that AO-2 increases resistance of cortical neurons to OGD/R by decreasing autophagy and cell apoptosis, which involves an mTOR-dependent mechanism.

## Introduction

Ischaemic stroke is a major global cause of death and permanent disability. After therapy in patients with ischaemic stroke, cerebral ischaemic reperfusion (I/R) injury often happens,[1,2] which leads to irreversible neuronal damage in ischaemic core region.[3] Most cells undergo apoptosis or necrosis in the ischaemic penumbra region. Investigation showed that autophagy plays an essential role in the pathological processes of ischaemic.[4,5] Autophagy, through an autophagosomal-lysosomal pathway, plays a key role in degrading undesired proteins and organelles and attribute to providing energy and cellular material recycling.[4,6] As autophagy can lead to cell death and survival, its role in ischaemia still remains controversial. Nonetheless, it is more reported that inhibiting autophagy protects cortical neuron apoptosis in animal models of I/R injury,[7–13] although some studies think that the activation of autophagy is neuroprotective.[14–16] Moreover, it has been shown that activation of mTOR signalling inhibits autophagy, which protects neuronal apoptosis.[17] Neuroprotective effect of this signalling pathway has been widely explored in ischaemic stroke.[7–9] These investigations demonstrate that inhibition of autophagy and apoptosis may become a potential therapeutic strategy for stroke.

Natural compounds have been widely used as neuroprotective agents in treating neuronal injuries in vitro and in vivo. For instance, curcumin has been demonstrated to have a variety of pharmacological capabilities, including anti-inflammation, anti-oxidation stress and anti-apoptotic effects.[18–20] Importantly, curcumin protects oxygen–glucose deprivation and re-oxygenation (OGD/R)-induced


 

neuronal injury in vitro and in ischaemic brain injury.[21–23] However, native curcumin easily degrades and has low oral bioavailability.[24] 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (AO-2) (Figure 1a), a curcumin-like diarylheptanoid from *Alpinia officinarum*, has been shown to decrease amyloid beta (Aβ)-induced cytotoxicity through PI3K (phosphoinositide 3-kinase)/AKT/mTOR (mammalian target of rapamycin) signalling pathway.[25] AO-2 also promotes hippocampal neuronal differentiation and neurite growth.[26] Moreover, 7-(4-hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (AO-1), which has a very similar structure to AO-2 but is less abundant in *A. officinarum*,[27] promotes neurogenesis of mouse hippocampal dentate gyrus after intraperitoneal administration, which indicates AO-2 has brain blood barrier permeability. However, whether AO-2 has protective effects on OGD/R injury is still unknown. Therefore, it would be of interest to investigate whether AO-2 has a protective role against OGD/R.

In the present study, we investigated the neuroprotective effects of AO-2 in OGD/R-treated primary cortical neurons. Further, we verified whether AO-2 exerts protective effect through inhibiting autophagy and apoptosis. Cell viability and apoptosis were measured to assess the effect of AO-2 against OGD/R. The changes in AKT/mTOR signal pathway, LC3-I/II (microtubule-associated protein 1A/1B-light chain 3 cytosolic form/phosphatidylethanolamine conjugate) and C-Casp-3 (cleaved caspase-3) were evaluated in OGD/R model, and whether AO-2 reverses these molecular changes under OGD/R was examined. The mTOR inhibitor rapamycin was used to verify whether the

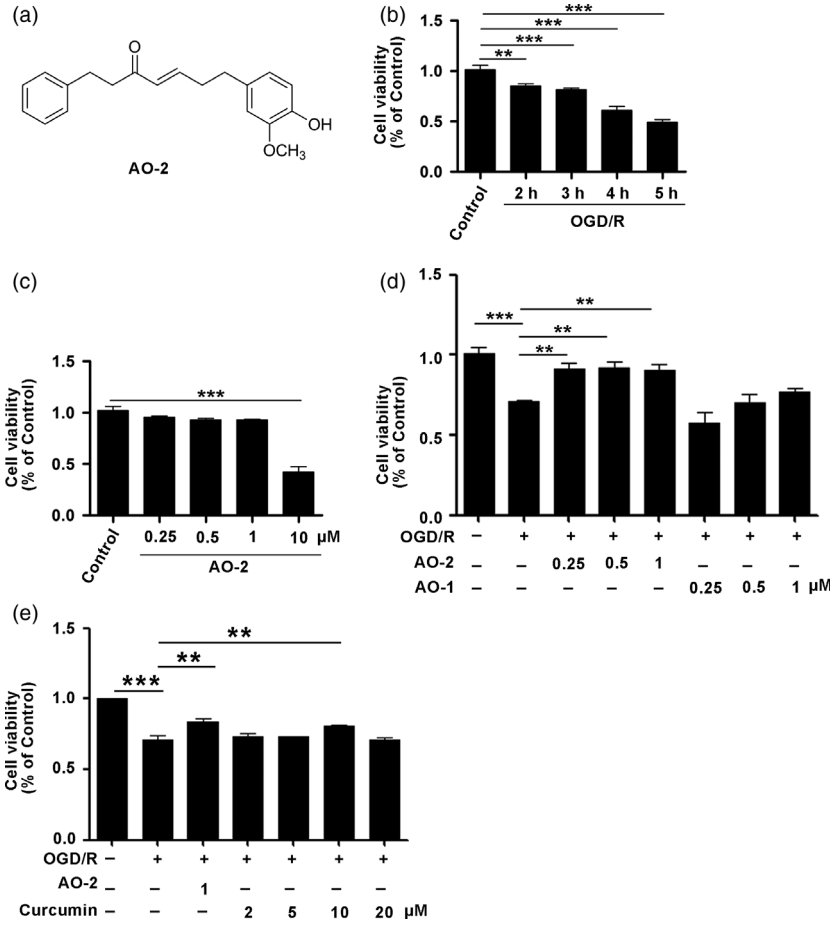


**Figure 1** 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one (AO-2) protects cortical neurons against oxygen–glucose deprivation/reoxygenation (OGD/R)-induced injury. (a) The chemical structure of AO-2 is shown. (b) Cell viabilities after different OGD time in cortical neurons. $^{**}P < 0.01$, $^{***}P < 0.001$. (c) Cortical neurons were treated with different concentrations of AO-2, cell viabilities were determined at 24 h. $^{***}P < 0.001$. (d) AO-2 prevented OGD/R-induced cell damage. Different concentrations of AO-2 and AO-1 were added in cortical neurons 2 h before OGD and continuously present during OGD, and then re-oxygenation was introduced for 24 h. Cell viability was determined. $^{***}P < 0.001$, $^{**}P < 0.01$. (e) Neuro-2a cells were pretreated with AO-2 (1 μM) and different concentrations of curcumin for 2 h and were challenged with OGD for 4 h followed by 24 h reperfusion. Cell viability was determined. $^{***}P < 0.001$, $^{**}P < 0.01$. Data are presented as mean ± standard error of the mean of three independent experiments.

neuroprotective effect of AO-2 was dependent on mTOR signal pathway. These findings showed that AO-2 alleviates ischemic brain injury by mTOR-dependent mechanism and inhibition of autophagy, which may provide a novel neuroprotective strategy against cerebral ischaemia.

## Materials and Methods

### Ethics statement

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.

### Chemicals, reagents and materials

AO-2, as previously reported, was extracted from the rhizomes of *A. officinarum* Hance and identified as 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one by Nuclear magnetic resonance spectroscopy methods, and the purity was more than 98% analysed by high-performance liquid chromatography.[25,26,28] The stock solution of AO-2 was prepared in dimethyl sulfoxide (DMSO) and kept at −80 °C. The final concentration of AO-2 was applied to cultured cortical neurons, and DMSO was added as the vehicle control.

Neurobasal culture medium, DMEM/F12 culture medium, B27 and D-Hank's solution were purchased from Gibco (Life Technologies, Carlsbad, CA, USA); Poly-L-lysine was from Sigma-Aldrich (St. Louis, MO, USA); Rabbit monoclonal anti-LC3, anti-C-Casp-3, anti-AKT, anti-p-AKT, anti-mTOR and anti-p-mTOR were from Cell Signaling Technology (Danvers, MA, USA); 2′, 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) and DMSO were from Sigma-Aldrich.

### Primary culture of rat cortical neurons

Cortical neurons were collected from E18 Sprague-Dawley rat embryos.[26] Briefly, cortical neurons were dissociated by incubation for 15 min at 37 °C in 5 ml of 0.125% trypsin. Dissociated neurons were fed with Neurobasal medium (Life Technologies) supplemented with 2% B27 (Life Technologies). Neuronal cells were seeded on 96-well plates ($1 \times 10^5$ per well) for MTT assay and 35-mm dishes ($8 \times 10^5$ per well) for Western blot analysis. The plates and dishes were precoated with Poly-L-lysine (0.1 mg/ml, Sigma-Aldrich) for 24 h at 37 °C. The medium was half-changed every 3 days. The neurons were cultured for 7 days in vitro (DIV) before experiments.

### OGD/R and drug exposure

The compounds were added to cortical neurons 2 h before OGD as pretreatment. Then the culture media were removed carefully without disturbing cells, the glucose-free DMEM (Life Technologies) was added in an amount equal to culture volume. If required, AO-2 was added into glucose-free DMEM. And then, the neurons were placed into a hypoxia environmental condition in a Modular Incubator Chamber (MIC-101; Billups-Rothenberg Inc, San Diego, CA, USA) with a saturated atmosphere of 95% N2 and 5% CO2 for 4 h. After the episode of OGD, the neurons were replaced with normal culture medium and returned to the normoxia environment in the incubator. Cell viability, cell apoptosis and cell death were measured at 24 h after OGD, and different signalling proteins were examined at different time after OGD. In each experiment, cultures exposed to OGD were compared with normoxic controls supplied with normal growth media in standard incubation conditions.

### Analysis of neuronal cell viability

Cell viability was tested by MTT assay. Briefly, MTT was added to each well for 4 h, and then the formazan was dissolved in DMSO. The OD (optical density) values were detected at 595 nm in a multimode detector (Beckman Coulter, S.Kraemer Boulevard Brea, CA, USA). Cell viability was determined by the following equation: cell viability (%) = absorbance value of sample/absorbance value of control × 100%.

### Detection of apoptosis by flow cytometry

The neuroprotective effect of AO-2 against OGD/R-induced cell apoptosis was detected using the Annexin V-FITC/PI (propidium iodide) Apoptosis Detection Kit (Biouniquer Tech, Nanjing, China) according to the manufacturer's instructions.[25] Briefly, cortical neurons were harvested by centrifugation, washed twice with cold PBS (phosphate-buffered saline) and resuspended in 1 × Annexin-binding buffer. Subsequently, Annexin V-FITC and PI were added to cortical neurons and incubated for 15 min in the dark at room temperature. The apoptosis rate was analysed by flow cytometry.

### Live & Dead cell assay

Cortical neurons were plated at a density of $6 \times 10^4$/coverslip. Cell death was examined using Live and Dead Cell Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, 1000 × buffer was diluted to 5 × buffer using PBS. Cells were washed twice with PBS at room temperature. The 5 × buffer was added to cortical

  

neurons and incubated for 15 min in the dark. The live cells and dead cells were stained separately and were imaged by Zeiss Axio Imager A2 microscope (Carl Zeiss AG, Jena, Germany).

### Western blot analysis

Cell medium was removed, and cells were washed with cold PBS for twice. Then cells were lysed with RIPA supplemented with protease inhibitors (Bimake, Houston, TX, USA) for 30 min on ice. Samples were collected and centrifuged at 11 900 *g* for 15 min at 4 °C. The supernatant was transferred to a new tube. Protein concentrations were quantified using Pierce BSA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) and were adjusted across equal different samples. The proteins were separated using 8%–10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gel, transferred to PVDF (polyvinylidene fluoride) membranes, which were then incubated with antibody against LC3 (1 : 1000), p-AKT (1 : 1000), AKT (1 : 1000), p-mTOR (1 : 1000), mTOR (1 : 1000), or C-Casp-3 (1 : 1000) at 4 °C overnight. Peroxidase-conjugated secondary antibodies (antimouse and antirabbit antibodies, Cell Signaling Technology) were added to recognize the above primary antibodies. The protein bands were visualized with ECL (enhanced chemiluminescence) Plus and Amersham Imager 600 (GE Healthcare, Chicago, IL, USA) and were quantified with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

Data processing and analysis were performed using GraphPad Prism 5 software (La Jolla, CA, USA). Standard errors of the mean values presented in the figures were calculated from three independent experiments. Differences between groups were assessed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test.

## Results

### AO-2 protects cortical neuronal viability after OGD/R

Cortical neurons (7 DIV) were subjected to OGD/R for 2–5 h, followed by 24 h of re-oxygenation. Cell damage became more severe as the time of OGD increased, that is, cell viability decreased by 10–50% when the time of OGD increased from 2 to 5 h (Figure 1b), suggesting that OGD/R significantly induced cortical neurons damage. In the following studies, we chose 4 h of OGD, which induced ~35% damage, to examine the neuroprotective effect of AO-2. To screen the nontoxic dosage of AO-2, different concentrations of the compound (0.25–10 μM) were added in cortical neurons for 24 h, and cell viability was measured (Figure 1c). This result shows that AO-2 had no cytotoxicity at concentrations up to 1 μM (Figure 1c). To test whether AO-2 protects cortical neuron injuries induced by OGD/R, AO-2 (0.25, 0.5 or 1 μM) was pretreated for 2 h before OGD. Notably, the cell viability of cortical neurons after OGD/R was reversed from ~65% to ~90% of control neurons. (Figure 1d). By contrast, AO-1 at similar concentrations showed no neuroprotective activity (Figure 1d). We also compared the activity between AO-2 and curcumin. Curcumin only showed protective effects when its concentration reached to 10 μM, which was 10–40 times higher than the effective doses of AO-2. The results suggest that treatment of AO-2 substantially protects neurons from OGD/R-induced damage.

### AO-2 inhibits OGD/R-induced neuronal apoptosis

To examine whether increased cell viability by AO-2 reflects reduced neuronal apoptosis, we tested the effects of AO-2 (0.25, 0.5 and 1 μM) against OGD/R in Neuro-2a cells using the Annexin V-FITC/PI Apoptosis Detection Kit by flow cytometry (Figure 2a). Notably, the robust induction of apoptosis by OGD/R was substantially inhibited by AO-2 at all three doses (Figure 2b). Consistently, 1 μM AO-2 remarkably decreased the number of apoptotic cortical neurons induced by OGD/R (Figure 2c and 2d). Furthermore, we used Live and Dead Cell Assay Kit to label individual live and dead cells using different fluorescent dyes. Consistent with the result of flow cytometry, the percentage of dead cells was significantly decreased by AO-2 treatment (Figure 2e and 2f). These findings suggest that AO-2 protects cortical neurons against OGD/R-induced cell apoptosis.

### OGD/R-induced autophagic activity and apoptosis at different stages

It has been suggested that autophagy is closely linked with the detrimental effects of OGD/R. High level of autophagy may generate autophagic cell death or apoptosis at severe stress.[4] Upon autophagy, LC3 is processed from an 18-kDa form (LC3-I) to a 16-kDa form (LC3-II), which is recruited to autophagosomes and acts as an indicator of autophagosome formation.[5] The AKT/mTOR pathway is the most extensively studied signalling pathways which inhibit autophagy. We explored the activation of AKT/mTOR signalling and LC3-II expression at different stages of OGD/R. Interestingly, AKT/mTOR signalling was inhibited during OGD, but was rapidly recovered to normal levels upon re-oxygenation (Figure 3a–3c). On the other hand, LC3-II form was detected after OGD and was continuously present during reperfusion, suggesting that

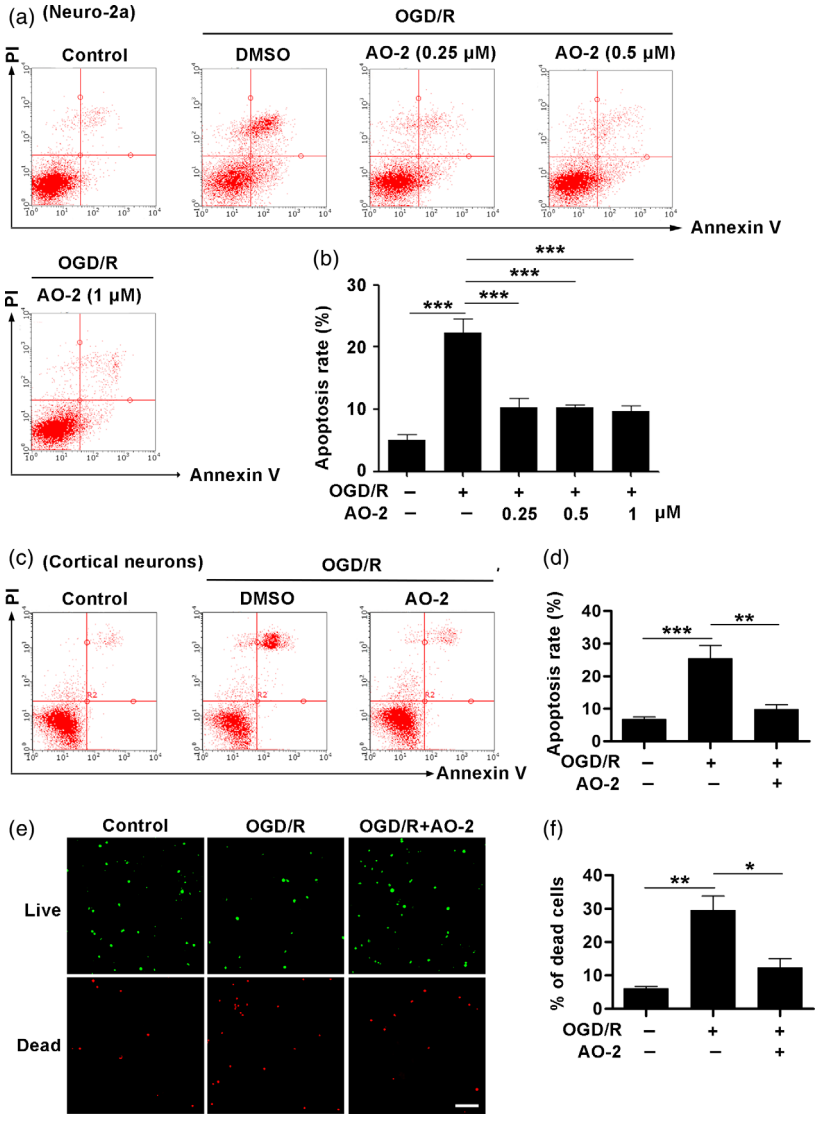


**Figure 2** 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one (AO-2) prevents oxygen-glucose deprivation/re-oxygenation (OGD/R)-induced neuronal apoptosis. Neuro-2a cells were pretreated with different concentrations of AO-2 for 2 h followed by 4 h OGD and 24 h re-oxygenation. (a) Apoptotic cells were detected using Annexin V/PI staining by flow cytometry. (b) Cell apoptosis is presented as Annexin V+/PI- and Annexin V+/PI+ percentage. $^{**}P < 0.01$, $^{***}P < 0.001$. (c) Cortical neurons were pretreated with 1 μM AO-2 for 2 h followed by 4 h OGD and re-oxygenation for 24 h. Apoptotic cells were detected using Annexin V/PI staining by flow cytometry. (d) Cell apoptosis is presented as Annexin V+/PI- and Annexin V+/PI+ percentage. $^{**}P < 0.01$, $^{***}P < 0.001$. (e) The live cells and dead cells were detected using live and dead cell assay kit and imaged. Scale bar, 50 μm. (f) The percentage of dead cells was counted. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are presented as mean ± standard error of the mean of three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

autophagy was activated during the whole OGD/R course (Figure 3a and 3d). To understand when apoptosis happens during OGD/R, we examined the cysteine protease Caspase-3, the cleaved form of which precipitates in apoptotic cell death. Notably, Caspase-3 cleavage was not activated after OGD and at early re-oxygenation phase, but was gradually increased at late re-oxygenation phase, suggesting that apoptosis only happens at the late phase of OGD/R (Figure 3a and 3e). Together, these results revealed that transient AKT/mTOR inhibition, autophagy and apoptosis occur in a defined sequence during OGD/R.

## AO-2 protects cortical neurons from OGD/R-induced autophagic apoptosis

In the previous study, we reported that AO-2 protects PC12 cells and neurons against Aβ1-42 induced cell apoptosis by activating AKT/mTOR pathway.[25] Here, we

  

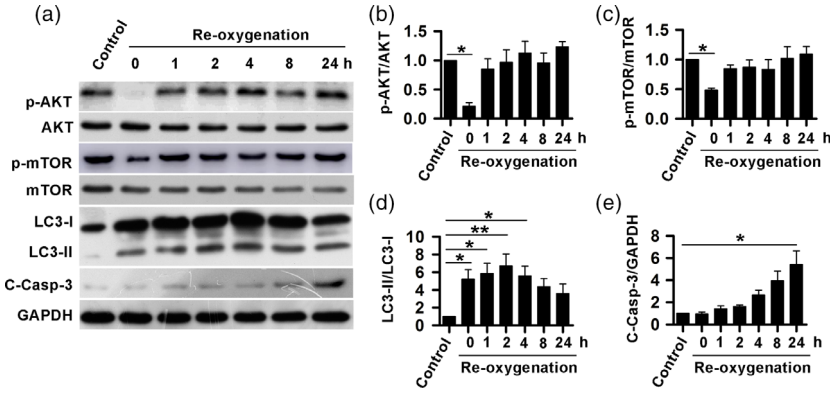


**Figure 3** oxygen-glucose deprivation/re-oxygenation (OGD/R) leads to AKT/mTOR (mammalian target of rapamycin) inhibition, autophagy and apoptosis at different stages. (a) Cortical neurons were subjected to OGD for 4 h by re-oxygenation for different times (0, 1, 2, 4, 8, 24 h). Cell lysates at different time points were collected for Western blot analysis of p-AKT, AKT, p-mTOR, mTOR, LC3-I/II (microtubule-associated protein 1A/1B-light chain 3 I/II) and C-Casp-3 (cleaved Caspase-3). Data were presented as p-AKT (b), p-mTOR (c), LC3-I/II (d) and C-Casp-3 (e) levels normalized to Control. $*P < 0.05$, $**P < 0.01$. Data are presented as mean ± standard error of the mean from at least three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

examined the effects of AO-2 on AKT/mTOR pathway, autophagy and apoptosis at different stages of OGD/R (Figure 4a). Indeed, OGD-induced inhibition of AKT/mTOR was completely cancelled by AO-2 (Figure 4b–4d). Moreover, AO-2 remarkably reversed the increase in LC3-II and C-Casp-3 at 2 and 24 h of re-oxygenation (Figure 4b, 4e, and 4f), respectively, suggesting that AO-2 inhibits OGD/R-mediated autophagy and apoptosis.

### AO-2 suppresses OGD/R-induced autophagy and apoptosis in mTOR-dependent manner

To determine whether mTOR activity is required for the neuroprotective effects of AO-2, we treated the neurons with rapamycin (1 μM), an mTOR inhibitor, 1 h before OGD. Although rapamycin itself did not further exacerbate LC3-II and C-Casp-3 expressions, it fully blocked AO-2's effect on the inhibition of the two proteins (Figure 4g–4i). This suggests that the inhibition of autophagy and apoptosis by AO-2 is through activation of mTOR signalling pathway. Consistently, the protection on cell viability by AO-2 was blocked by rapamycin (Figure 4j). To identify the role of autophagy in OGD/R-induced neuronal damage, 3-Methyladenine (3-MA), an inhibitor of autophagy, was add to cortical neurons as pretreatment. Similar to the effect of AO-2, 3-MA decreased C-Casp-3 expression and increased cell viability (Figure 4k–4n), suggesting inhibiting autophagy at early stage of OGD protects neurons from damaging effects by OGD/R. Thus, these findings suggest that the neuroprotective effect of AO-2 in OGD/R-treated cortical neurons is dependent on the activation of mTOR signalling pathway and inhibition of autophagy.

## Discussion

Ischaemic stroke is associated with high rate of disability and death. Studies have reported that neuronal damage is usually observed in ischaemic stroke, including apoptosis and necrosis.[2,27] In this study, we showed that AO-2, a natural diarylheptanoid, reduces OGD/R-induced autophagy and apoptosis in cortical neurons through an mTOR-dependent mechanism.

In ischaemic stroke and reperfusion, neurons face severe damages that are difficult to repair. According to the degree of ischaemia, autophagy may play different roles.[4] High level of autophagy may create cell damage and finally result in autophagic cell death or apoptosis, whereas low levels of autophagy are protective effects.[11,29] In certain kinds of ischaemia such as traumatic brain injury, autophagy mediates neuroprotective effects.[4] In the opposite, autophagy is mostly found to contribute the devastating effects, probably because the damage induces excessive autophagy that leads to cell death.[11] Some compounds have been shown to protect neuronal apoptosis by suppressing excessive autophagy after transient global cerebral ischaemia in rats.[1,12] Consistently, our study shows that activation of mTOR signalling pathway by AO-2 inhibits autophagy, which decreases cell apoptosis.[10,11,30] These studies demonstrated that I/R injury can be rescued by inhibiting autophagy.

In this study, we showed that the early molecular and cellular changes include inhibition of AKT/mTOR and induction of autophagy. It is worth to note that AKT/mTOR pathway is only inhibited by OGD but not reperfusion, and autophagy induced by OGD is not further exacerbated during reperfusion. This may suggest that autophagy is mainly stimulated by OGD but not reperfusion. By

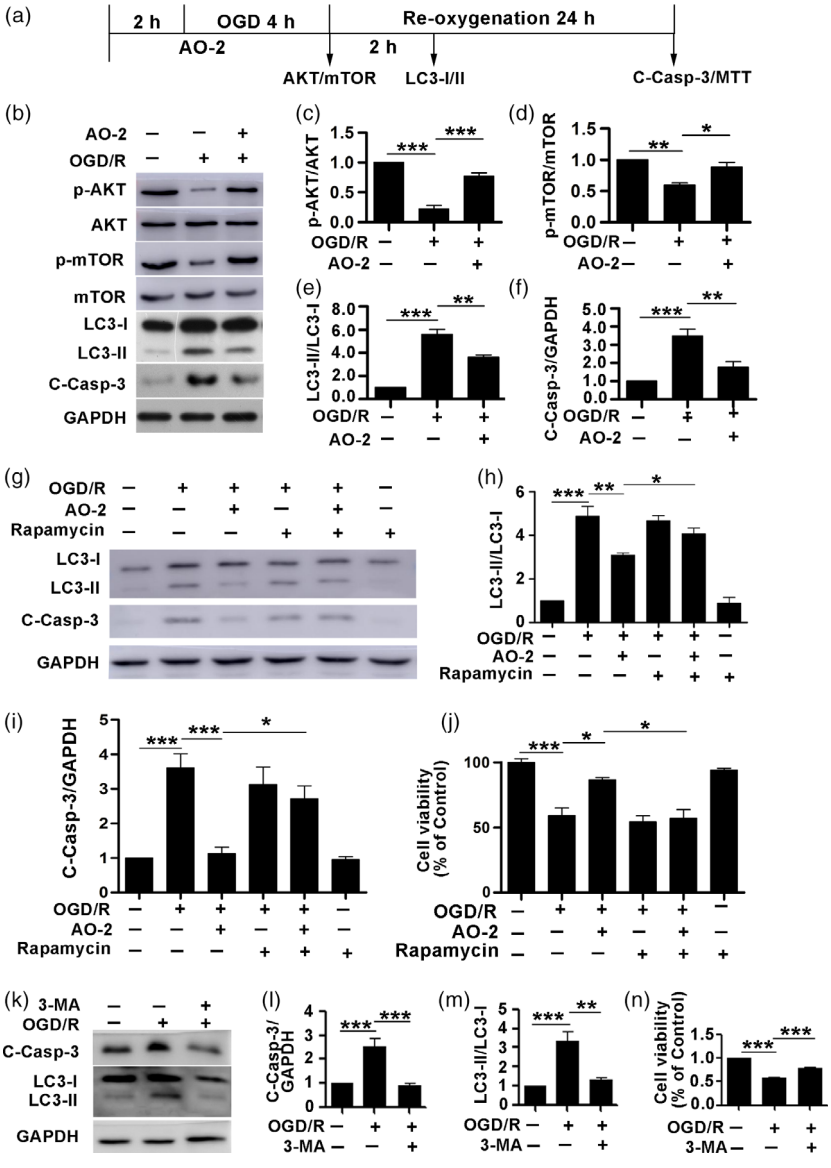


**Figure 4** 7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one (AO-2) suppresses oxygen-glucose deprivation/re-oxygenation (OGD/R)-induced autophagy and apoptosis in mTOR (mammalian target of rapamycin)-dependent manner. (a) The experimental plan. (b) The levels of p-AKT and p-mTOR were recovered by AO-2 treatment after OGD/R. The expression levels of LC3-I/II (microtubule-associated protein 1A/1B-light chain 3 I/II) and C-Casp-3 (cleave-caspase-3) were inhibited at 2 h and 24 h re-oxygenation, respectively, by AO-2. Relative band densities of p-AKT (c), p-mTOR (d), LC3-II (e), C-Casp-3 (f), normalized to total AKT, total mTOR, LC3-I and GAPDH, respectively, were measured with Quantity One and expressed as fold changes of control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (g) Cortical neurons were treated with or without rapamycin (1 μM) at the last 1 h before OGD/R and continuously present under OGD-inducing conditions for 4 h. The protein levels of LC3-II and C-Casp-3 were measured by Western blot analysis. (h) and (i) Data were presented as C-Casp-3 and LC3-II normalized levels. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. (j) MTT assay showed that treatment of rapamycin blocked neuroprotective effect of AO-2 on cell viability. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. (k) Cortical neurons were treated with or without 3-Methyladenine (3-MA, 10 mM) at the last 1 h before OGD/R and continuously present during OGD for 4 h. The protein levels of C-Casp-3 (l) and LC3-II (m) were measured by Western blot analysis. Data were presented as C-Casp-3 and LC3-II normalized levels. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. (n) MTT assay showed that treatment of 3-MA exerted neuroprotective effect on cell viability as AO-2 did. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Data are presented as mean ± standard error of the mean from at least three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

contrast, apoptosis only occurs at the late phase of reperfusion. Thus, intervention at the early phase, in particular by re-activating AKT/mTOR or inhibiting autophagy during OGD, may be critical to reduce the subsequent, relatively irreversible apoptosis. Consistent with this notion, we showed that treatment of AO-2 only at the OGD phase fully

restores AKT/mTOR and autophagy levels, which is enough to prevent apoptosis. The PI3K/AKT/mTOR signalling pathway is the most studied pathway that negatively regulates autophagy.[31,32] We have shown that the mTOR inhibitor rapamycin blocked the neuroprotective effect of AO-2, suggesting an mTOR-dependent mechanism. However, how AO-2 regulates the mTOR signalling pathway to achieve neuroprotective effects remains unclear, which awaits further investigation.

We have previously shown that 7-(4-hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (AO-1) promotes neurogenesis of mouse hippocampal dentate gyrus after intraperitoneal administration.[25,28] This suggests that AO-1 could penetrate brain blood barrier (BBB). As AO-2 has a very similar structure to AO-1 (only lack a methoxy group), we propose that AO-2 also has BBB permeability. Further studies would be required to access the in-vivo effects of AO-2 using ischaemic stroke animal models and examine whether neuronal autophagy and apoptosis were inhibited by AO-2 through mTOR. Furthermore, neuroinflammation is another detrimental effect induced by I/R that causes neuronal injuries. We have shown that AO-2 reduces the levels of phosphor-p38 MAPK, a signalling molecule that leads to inflammatory responses.[25] Indeed, accumulating evidence indicates diarylheptanoids has anti-inflammatory roles.[13,33,34] It would be thus of interest to examine whether AO-2 also inhibits OGD-induced neuroinflammation in glial cells such as microglia.

## Conclusions

In summary, we demonstrated that AO-2 increased resistance of cortical neurons to OGD/R by decreasing autophagy and cell apoptosis. Notably, this effect was blocked by the mTOR inhibitor rapamycin, which indicated that the neuroprotection was involved an mTOR-dependent mechanism.

## Declarations

### Conflict of interest

The authors declare no conflict of interest.

### Funding

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### Authors' contributions

Lei Shi, Ying Wang and Xiaoqi Zhang conceived and designed the experiments; Qinghua Zhang and Qiaoyun Shi performed the experiments; Qinghua Zhang and Qiaoyun Shi analysed the data; Yinghui Peng contributed reagents/materials/analysis tools; Qinghua Zhang, Qiaoyun Shi, Lei Shi wrote the paper.

## References

1. Feng D *et al.* Pre-ischemia melatonin treatment alleviated acute neuronal injury after ischemic stroke by inhibiting endoplasmic reticulum stress-dependent autophagy via PERK and IRE1 signalings. *J Pineal Res* 2017; 62: e12395.
2. Strong K *et al.* Preventing stroke: saving lives around the world. *Lancet Neurol* 2007; 6: 182–187.
3. Bi M *et al.* Tau exacerbates excitotoxic brain damage in an animal model of stroke. *Nat Commun* 2017; 8: 473.
4. Galluzzi L *et al.* Autophagy in acute brain injury. *Nat Rev Neurosci* 2016; 17: 467–484.
5. Zhang X *et al.* Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy* 2013; 9: 1321–1333.
6. Feng Y *et al.* The machinery of macroautophagy. *Cell Res* 2014; 24: 24–41.
7. Carloni S *et al.* Activation of autophagy and Akt/CREB signaling play an equivalent role in the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia. *Autophagy* 2010; 6: 366–377.
8. Hou S *et al.* Neuroprotective effect of salvianolic acids against cerebral ischemia/reperfusion injury. *Int J Mol Sci* 2016; 17: 1190.
9. Chen GZ *et al.* Remote ischemic post-conditioning protects the brain from focal ischemia/reperfusion injury by inhibiting autophagy through the mTOR/p70S6K pathway. *Neurol Res* 2018; 40: 182–188.
10. Lu Q *et al.* Autophagy in neonatal hypoxia ischemic brain is associated

with oxidative stress. *Redox Biol* 2015; 6: 516–523.
11. Shi R *et al.* Excessive autophagy contributes to neuron death in cerebral ischemia. *CNS Neurosci Ther* 2012; 18: 250–260.
12. Sun D *et al.* BFGF plays a neuroprotective role by suppressing excessive autophagy and apoptosis after transient global cerebral ischemia in rats. *Cell Death Dis* 2018; 9: 172.
13. Thampithak A *et al.* Transcriptional regulation of iNOS and COX-2 by a novel compound from *Curcuma comosa* in lipopolysaccharide-induced microglial activation. *Neurosci Lett* 2009; 462: 171–175.
14. Fletcher L *et al.* Rapamycin treatment improves neuron viability in an in vitro model of stroke. *PLoS One* 2013; 8: e68281.
15. Wang P *et al.* Induction of autophagy contributes to the neuroprotection of nicotinamide phosphoribosyltransferase in cerebral ischemia. *Autophagy* 2012; 8: 77–87.
16. Yan HJ *et al.* Histamine H3 receptors aggravate cerebral ischaemic injury by histamine-independent mechanisms. *Nat Commun* 2014; 5: 3334.
17. Gao X *et al.* The protective effect of alpha-lipoic acid against brain ischemia and reperfusion injury via mTOR signaling pathway in rats. *Neurosci Lett* 2018; 671: 108–113.
18. Rane JS *et al.* Curcumin inhibits tau aggregation and disintegrates preformed tau filaments in vitro. *J Alzheimers Dis* 2017; 60: 999–1014.
19. Dhouib IB *et al.* Neuroprotective effects of curcumin against acetamiprid-induced neurotoxicity and oxidative stress in the developing male rat cerebellum: biochemical, histological, and behavioral changes. *Environ Sci Pollut Res Int* 2017; 24: 27515–27524.
20. Wynn JK *et al.* The effects of curcumin on brain-derived neurotrophic factor and cognition in schizophrenia: a randomized controlled study. *Schizophr Res* 2018; 195: 572–573.
21. Dong HJ *et al.* Curcumin attenuates ischemia-like injury induced IL-1 beta elevation in brain microvascular endothelial cells via inhibiting MAPK pathways and nuclear factor-kappa B activation. *Neurol Sci* 2014; 35: 1387–1392.
22. Liu ZJ *et al.* Curcumin protects neurons against oxygen-glucose deprivation/reoxygenation-induced injury through activation of peroxisome proliferator-activated receptor-c function. *J Neurosci Res* 2014; 92: 1549–1559.
23. Wu JX *et al.* Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. *PLoS One* 2013; 8: e59843.
24. Tsuda T. Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. *Food Funct* 2018; 9: 705–714.
25. Xiao HL *et al.* 7-(4-Hydroxy-3-methoxypheny1)-1-pheny1-4E-hepten-3-one alleviates A beta 1-42 induced cytotoxicity through PI3K-mTOR pathways. *Biochem Biophys Res Commun* 2017; 484: 365–371.
26. Tang G *et al.* A natural diarylheptanoid promotes neuronal differentiation via activating ERK and PI3K-Akt dependent pathways. *Neuroscience* 2015; 303: 389–401.
27. Liu Z *et al.* Determination of diarylheptanoids from *Alpinia officinarum* (Lesser Galangal) by HPLC with photodiode array and electrochemical detection. *Phytochem Anal* 2005; 16: 252–256.
28. Zheng KL *et al.* Activation of Akt by SC79 protects myocardiocytes from oxygen and glucose deprivation (OGD)/re-oxygenation. *Oncotarget* 2017; 8: 14978–14987.
29. Gabryel B *et al.* Neuronal autophagy in cerebral ischemia – a potential target for neuroprotective strategies? *Pharmacol Rep* 2012; 64: 1–15.
30. Qin AP *et al.* Autophagy was activated in injured astrocytes and mildly decreased cell survival following glucose and oxygen deprivation and focal cerebral ischemia. *Autophagy* 2010; 6: 738–753.
31. Zhu YC *et al.* Tanshinone IIA protects hippocampal neuronal cells from reactive oxygen species through changes in autophagy and activation of phosphatidylinositol 3-kinase, protein kinas B, and mechanistic target of rapamycin pathways. *Curr Neurovasc Res* 2017; 14: 132–140.
32. Liu Y *et al.* Lupeol induces apoptosis and cell cycle arrest of human osteosarcoma cells through PI3K/AKT/mTOR pathway. *Technol Cancer Res Treat* 2016; 15: 16–24.
33. Huang X *et al.* 7-(4-Hydroxyphenyl)-1-phenyl-4E-hepten-3-one, a diarylheptanoid from *Alpinia officinarum*, protects neurons against amyloid-beta induced toxicity. *Biol Pharm Bull* 2016; 39: 1961–1967.
34. Lee CJ *et al.* Oregonin inhibits lipopolysaccharide-induced iNOS gene transcription and upregulates HO-1 expression in macrophages and microglia. *Br J Pharmacol* 2005; 146: 378–388.