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Activation of anti-oxidant of curcumin pyrazole derivatives through preservation of mitochondria function and Nrf2 signaling pathway

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ABSTRACT

Oxidative stress is an important cause of neurodegenerative diseases. Antioxidant is an potential important method to treat such diseases. The aim of this study is to discover new and effective antioxidants and their mechanism. The neuroprotective effect of six curcumin pyrozole compounds were first evaluated on sodium nitroprusside (SNP) - induced PC12 cell injury by testing cell viability and LDH release. The results showed that four compounds (C1-C4) have more significant protective effects compared to curcumin and edaravone. Furthermore, compounds C1-C4 can attenuate the intracellular ROS, and compound C3 is the most effective one which can preservate the mitochondria function by inhibiting the mitochondrial membrane potential loss and enhance nuclear translocation of Nrf2 in PC12 cell. These results indicated that C3 may be a potential candidate drug for treating neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are diseases associated with injury of neurons in the brain. As aging progresses, the prevalence of neurodegenerative diseases has steadily increased (Kinlay, 2011). There is no ideal drug for neurodegenerative disorders, and it's urgent to find an effective remedy (Firuzi et al., 2011; Yang et al., 2017). Accumulating evidence indicates that oxidative stress plays a significant role during the occurrence and development of NDs. Excessive reactive oxygen species (ROS) can cause irreversible damage in brain via attacking DNA, RNA and protein (Cheignon et al., 2017). It has been reported that usage of antioxidant is an promising strategy for neurodegenerative treatment (Dumont and Beal, 2011; Zaidun et al., 2018).

In the central nervous system (CNS), mitochondria is the energy-generating center and provides energy necessary for neuronal survival and normal physiological processes (Covill-Cooke et al., 2018). The brain is more susceptible to injury during mitochondrial dysfunction (Citi et al., 2018) which is associated with excessive ROS production. Such behavior is the basis of several NDs (Pérez et al., 2018). In recent years, mitochondria has been used as target for neurodegenerative drugs (Covill-Cooke et al., 2018).

Nuclear factor E2-related factor 2 (Nrf2), a transcription factor, is closely related to the regulation of antioxidant protein expression (Lee et al., 2018). If antioxidant system is unbalanced, antioxidant enzymes such as superoxidedismutase (SOD) and glutathione (GSH), will be up-regulated to remove excess free radicals. Transferring Nrf2 from the cytoplasm to the nucleus is a prerequisite for inducing expression of antioxidant genes (Hou et al., 2018). Recent studies proposed that Nrf2 has gained increasing interest in the treatment of neurodegenerative disorders (Dinkova-Kostova et al., 2018).

Curcuma longa L. is a traditional Chinese medicine that has a therapeutic effect on stroke. Curcumin is Curcuma longa L's main active component and is used to study neuroprotective effect in recent years (Taylor et al., 2011). However, the structural instability and low bioavailability of the compound are two defects that hinder its use in clinic (Yallapu et al., 2015; Bonferoni et al., 2017). It's been reported that the instability of the β-keto structural fragment (shown in Fig. 1) in the curcumin structure is the cause of its low bioavailability and rapid metabolism in vivo (Liang et al., 2009). Curcumin pyrazole derivatives have been documented as having multiple pharmacological functions, such as antioxidation, anticancer and inhibiting gluconeogenesis. In this study, we synthesized six curcumin pyrazole derivatives and evaluated their neuroprotective effect, mechanism and signal pathway.

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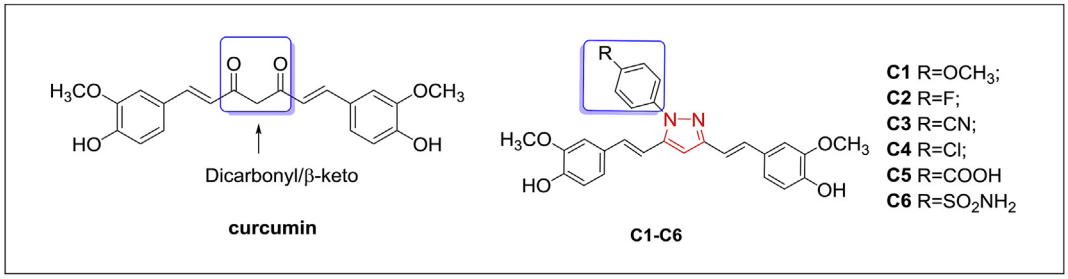

Fig. 1. Curcumin and curcumin pyrazole derivatives.

2. Experimental

2.1. Materials

Curcumin was purchased from Beijing Hengye Zhongyuan Chemical Co., Ltd (Beijing, China). Sodium nitroprusside (SNP) was purchased from Sigma, and edaravone from Aladdin (Shanghai China). PC12 cells were generously provided by Researcher, J. K. Chen. Nrf2 primary antibody was from Abcom. Lactate dehydrogenase (LDH) assay kit, Methythiazolydiphenyl-tetrazolium bromide (MTT), Hoechst, Rhodamin, LDH, ROS, JC-1 and NO assay kit were purchased from Beyotime. Other Culture medium were purchased from Hyclone and Gibco (USA). All other chemicals are from commercial sources.

2.2. Synthesis

Compounds C1-C6 were prepared according to the previous procedure (Sherin and Rajasekharan, 2015). Curcumin, phenylhydrazine hydrochloride, anhydrous methanol (20 ml), and water (20 ml) were added to a 250 ml three-necked flask. Reaction was stirred over night at 80 °C. The reaction process were monitor by TLC until the reaction was completed and stopped the reaction. After evaporating off the solvent, the reaction solution was diluted with water and then extracted with ethyl acetate (100 ml × 3). The organic phase was dried over anhydrous magnesium sulfate, and the solvent was evaporated under reduced pressure to obtain the aim compounds.

2.3. Cell cultures

PC12 cells were cultured in DMEM medium containing 10% FBS, 5% HS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2. When the cell coverage rate was about 90%, the cells were digested and passaged by 0.25% trypsin −0.02% EDTA.

2.4. Cell viability

PC12 cells were cultured in 96-well plates for 24 h. The cells were then pre-treated with compounds for 24 h, and then incubated with 300 μM SNP for another 24 h. Cells without the compound treatment were set as the control or SNP group. 10 μl of 5 mg/ml MTT was added to previous treated cells for 3 h at 37 °C and 5% CO_2. Discarded the supernatant, 100 μl of DMSO was added to 96-well pates and the solutions were mixed thoroughly. The absorbance was measured at 570 nm. The experiments were repeated at least 3 times. All % cell viability were normalized to the control group.

2.5. Measurement of lactate dehydrogenase (LDH) release

PC12 cells were cultured in 96-well plates for 24 h. The cells were then pre-treated with compounds for 24 h, and then incubated with 300 μM SNP for another 24 h. Cells without the compound treatment were set as the control or SNP group. The LDH release amount of treated-cells in 96-well pates was determined by LDH assay kit according to the instructions. All % LDH release values were normalized to the SNP group.

2.6. Observation of PC12 cell morphologic changes

Differentiated PC12 cells were cultured in DMEM containing 10% FBS, 5% HS for 24 h. The cells were then pre-treated with C1-C4 for 24 h respectively and then incubated with 300 μM SNP for another 24 h. Cells without the compound treatment were set as the control or SNP group. Then the cells were co-incubated with Rhodamin (5 μM) and Hoechst 33258 (10 μg/ml) for 30 min in cell incubator. The medium was removed, and washed with DMEM 2 times.

2.7. Determination of ROS level

Differentiated PC12 cells were cultured in DMEM containing 10% FBS, 5% HS for 24 h. The cells were then pre-treated with C1-C4 for 24 h respectively and then incubated with 300 μM SNP for another 9 h. Cells without the compound treatment were set as the control or SNP group. Then 10 μM DCFH-DA was co-incubated for 30 min. The medium was removed, and washed with DMEM 2 times.

2.8. Change in mitochondrial membrane potential

Differentiated PC12 cells were cultured in DMEM containing 10% FBS, 5% HS for 24 h. The cells were then pre-treated with C1-C4 for 24 h respectively and then incubated with 300 μM SNP for another 9 h. Cells without the compound treatment were set as the control or SNP group. Then JC-1 was co-incubated for 20 min. The medium was removed, and washed with JC-1 buffer 2 times.

2.9. SOD, GSH-px, and GSH assay

PC12 cells were cultured in 6-well plates for 24 h. The cells were then pre-treated with C3 (5, 2.5 1.25 μM) for 24 h, and then incubated with 300 μM SNP for another 24 h. The cells were lysed on ice, then the lysates were centrifuged at 12,000 g for 5–10 min. The supernatant was collected to measure the levels of SOD, GSH-px (Beyotime, China) and GSH (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions and quantified protein concentration at the same time.

2.10. Western blot analysis

PC12 cells were seeded in six-well plates and treated with different concentrations of C3 (1.25, 2.5, 5 μM) for 12 h or 24 h. Cells lysate, nuclear and cytoplasmic protein extraction kit (containing 1 mM PMSF) were used to lyse cells to prepare whole cell extracts, nuclear and cytoplasmic proteins, respectively. Protein was quantified using BCA protein quantification kit, and then separated and transferred by gel electrophoresis as previous methods. The membrane was blocked with 5% skim milk for 2 h at room temperature and then incubated overnight at 4 °C with Nrf2 primary antibody (1:1000). Thereafter, they were further incubated with the corresponding secondary antibody (1:5000).

The membrane was incubated with the chemilumines cent kit for 2 min and the signal was obtained by a developing device. Next, used Image J. to quantify the bands.

2.11. Inhibitor treatment

PC12 cells were incubated with C3 or LY294002 (PI3K/AKT pathway inhibitor) or co-incubated with LY294002 and C3 for 12 h. The level of Nrf2 was determined by Western blotting. And incubated with C3 or LY294002 or co-incubated with LY294002 and C3 for 1 h, drug-treated cells were further treated with or without 300 μM SNP for 24 h. Then the cell viability of PC12 cells was determined by MTT assay.

3. Results and discussion

3.1. Primary screening of protective effect of curcumin pyrazole derivatives

NO-mediated oxidative stress has been considered as an important pathology in many neurodegenerative diseases (Castillo et al., 2000; Calcerrada et al., 2011). NO excessive release will directly lead to neurotoxicity, participating in a series of neuropathological processes. Sodium nitroprusside (SNP), is widely used as a NO donor in experimental studies. NO release is caused by reduction of $Fe(CN)_5NO_2$ anions in SNP (Wang et al., 2015). Rat pheochromocytoma PC12 cells are commonly used in the study of neurodegenerative diseases (Zheng et al., 2016). In this study, the SNP-induced cell death model was first established through optimizing concentration to PC12. Different concentration of SNP from 100 to 800 μM were incubated with cells for 24 h. As shown in Fig. 2A and 300 μM SNP causing 60% cell death will be used for the subsequent experiments.

As shown in Fig. 2B and C, compounds with different substituents behave differently in this assay. The protective effect of 5 μM C1-C6 on SNP-induced cell death was examined by MTT assay as the preliminary screening. Edaravone and curcumin were used as positive control agents (Edaravone is known as a free radical scavenger (Lee and Xiang, 2018; Satoh et al., 2002) and used to treat stroke in Japan). The result showed that C1-C4 can effectively protect cell from death, and have better activity than both curcumin and edaravone. Among these heterocycle derivatives, compounds containing substituent of methoxy (C1), fluorine (C2), cyano (C3), and chlorine (C4) showed more significant effects. In contrast, C5 and C6 showed lower protective effect.

3.2. Cytotoxicity experiment of curcumin pyrazole derivatives

The cytotoxicity experiment then showed that (Fig. 3A and Fig. 3B), 10 μM of C5 and C6 were able to reduce the survival of PC12 cell. These two compounds didn't show protection in the preliminary experiment. Such result may be related to the toxicity.

3.3. C1-C4 improved PC12 cells survival against SNP-induced apoptosis

We further evaluated the protective effect of C1-C4 at different concentrations. As shown in Fig. 4A, these four compounds all showed a dose-dependent protective effect. Cell vitality was significantly improved at 5 μM. Compartively, the cell survival rate of SNP group decreased significantly and the low concentration (1.25 μM) had no significant protection. The potency of 5 μM C1-C4 was similar to the performance of 100 μM edaravone. If cells were pretreated with 5 μM of C1-C4, the cell viability increased to 94.50 ± 3.77, 90.37 ± 5.26, 93.75 ± 1.14, 90.53 ± 4.37 (SNP group 58.41 ± 1.75). The results showed that pretreatment with C1-C4 significantly prevented the PC12 cell against SNP-induced apoptosis. There was no obvious difference between the four compounds.

3.4. C1-C4 decreased intracellular lactate dehydrogenase (LDH)

It was reported that destruction of the cell membrane structure releases lactate dehydrogenase (LDH), an indicator of cell death (Perez et al., 2010), from the cytoplasm into the culture fluid. The release rate of LDH was examined to determine the level of cell injury. Fig. 4B presents when pretreated with 5 μM of C1-C4, the LDH release rate of cells were distinctly reduced by 32.54 ± 0.80, 31.83 ± 1.54, 51.67 ± 6.44, 41.36 ± 4.60 in contrast to SNP group. The potency of 5 μM C1-C4 was similar to the performance of 100 μM edaravone, which is consistent with MTT assay. From this experiment, C3 was more active.

3.5. C1-C4 blocked SNP-induced PC12 cell morphological changes

In order to examine the cell morphology change more intuitively, Hoestch and Rhodamin were performed. The result in Fig. 5 showed that the cell number, cell density, and morphology were clear in control group. In contrast, the number of damaged group was less than control group. Degeneration of cytoplasm (atrophy and round shape) were obsd, besides nucleus disappeared or were densely stained. Although the drug groups had some degree of damage, the number of cells were significantly higher compared to injury group and the morphology was more clearer. This further proved that these compounds can antagonize SNP-induced PC12 cells injury. The cell number in the C3 group was the highest and the morphology was also significantly improved. This result was consistent with the LDH assay.

3.6. C1-C4 attenuated oxidative damage induced by SNP

Evidence showed that overproduction of reactive oxygen species is one of the major causes of neurodegenerative diseases. Increased ROS levels would lead to neuronal cell death through lipid peroxidation and DNA degradation (Gill et al., 2017). DCFH-DA was used to determine ROS levels. DCFH-DA can be hydrolyzed into DCFH by intracellular esterase. DCFH does not penetrate the cell membrane. Active oxygen can oxidize non-fluorescent DCFH to green-fluorescence DCF and the green fluorescence intensity is proportional to the level of reactive oxygen species. As shown in Fig. 6, after 9 h of incubation with SNP, green-fluorescence-enhanced cells were clearly observed in the SNP group compared to control cell. C1-C4 at 5 μM significantly reduced the fluorescence intensity. C3 had the significant effect among C1-C4. This was consistent with the result of the LDH assay.

3.7. Protection of mitochondrial membrane potential of C1-C4

Several aspects of neuronal function are particularly dependent on cellular energy donated from mitochondria. Excess ROS can destroy the mitochondrial membrane potential and cause neurodegenetive diseases. Based on that research, more and more neuroprotective agents target at mitochondrial. We used the JC-1 to detect SNP-induced mitochondrial damage in PC12 cell. In Fig. 7, the red fluorescence of the normal group was more noticeable than the green fluorescence, indicating that the cell mitochondria was in a healthy state. After treatment with SNP for 9 h, green-fluorescence enhanced cells were observed in the SNP-treated cell, suggesting that the mitochondrial membrane potential (MMP) were destroyed. Pretreated with 5 μM C1-C4, the red fluorescence intensity of cells significantly increased, suggesting MMP had been significantly restored. C1-C4 may enhanced its neuroprotection through MMP recovery. Among them, C3 was the most obvious, which is consistent with results above.

3.8. Involvement of Nrf2 for the protection of C3

Transferring of Nrf2 from the cytoplasm to the nucleus is a prerequisite for inducing expression of antioxidant genes. We next

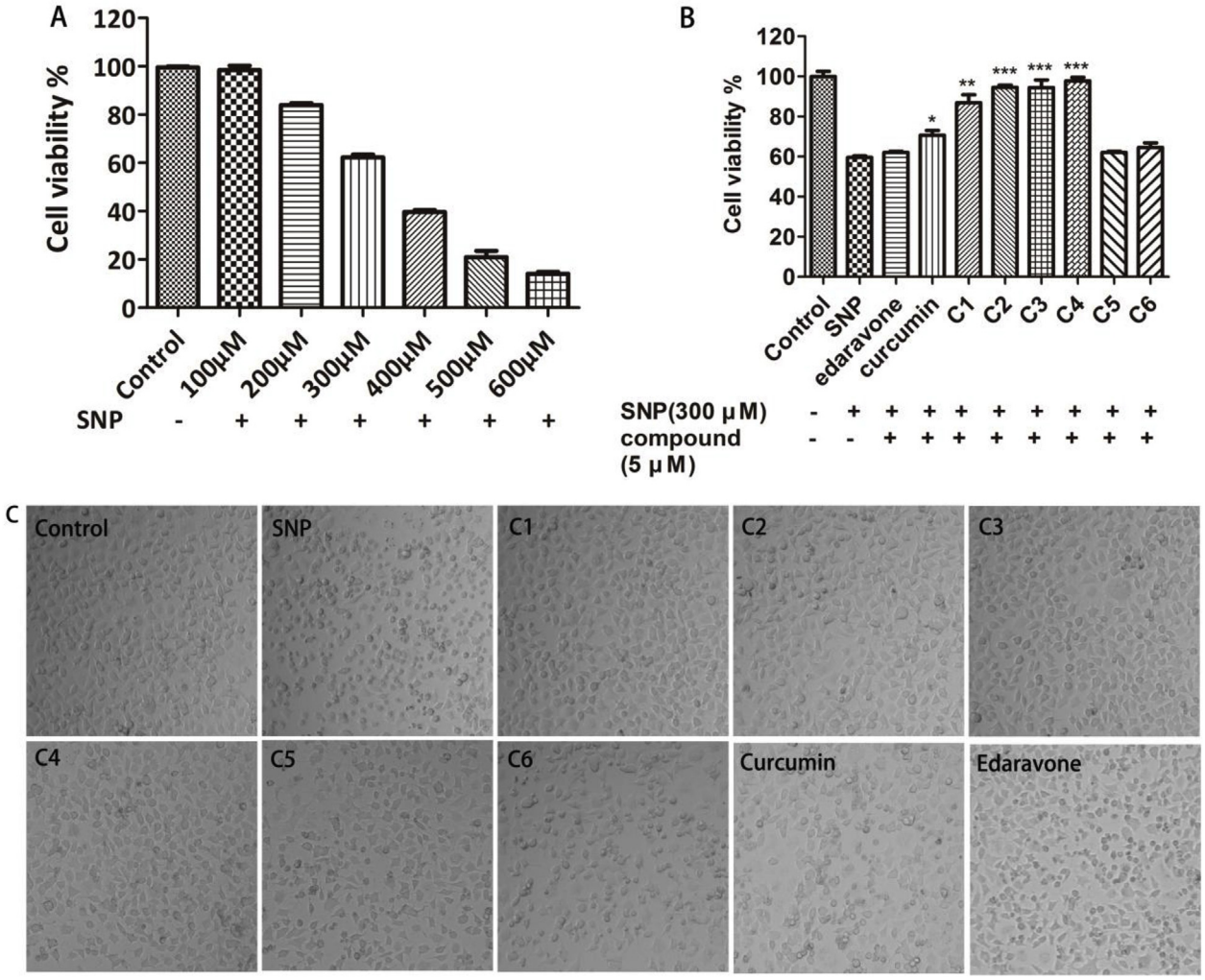

Fig. 2. PC12 cell viability (A) PC12 cells were treated with different doses of SNP followed by incubation for 24 h. (B) PC12 cells were pretreated with 5 μM edaravone, curcumin and its derivations for 24 h followed by treatment with 300 μM SNP for another 24 h. (C) Cell morphology changes in (B). (Data are expressed as the mean ± SD of three replicates ***p < 0.001,**p < 0.01 and *p < 0.5 compared to SNP group).

explored the induction of Nrf2 nuclear translocation of C3. After treatment with C3 for 12 h and 24 h, we obtained the total cell extracts, nuclear proteins, and proteoproteins, respectively. The results showed that the level of nuclear Nrf2 protein was increased while the cytosolic Nrf2 level decreased, and the change of total Nrf2 was not obvious between 12 h and 24 h of C3 treatment (Fig. 8A and Fig. 8E). The quantitative results were shown in Fig. 8B–D and Fig. 8F–H. These results indicate that C3 promotes the translocation of Nrf2 to the nucleus.

To confirm whether Nrf2 is involved in the neuroprotective effects of C3, PI3K inhibitor LY294002 was used to down-regulate Nrf2 expression. As shown in Fig. 9A, LY294002 decreased the expression of total Nrf2. While, western blotting showed that the down-regulation of LY294002 on Nrf2 expression were reversed by C3. MTT analysis showed that 5 μM C3 showed approximately 25% neuroprotective activity compared to the SNP group, which is similar to the results shown in Fig. 2B. However, the addition of LY294002 resulted in a decrease in

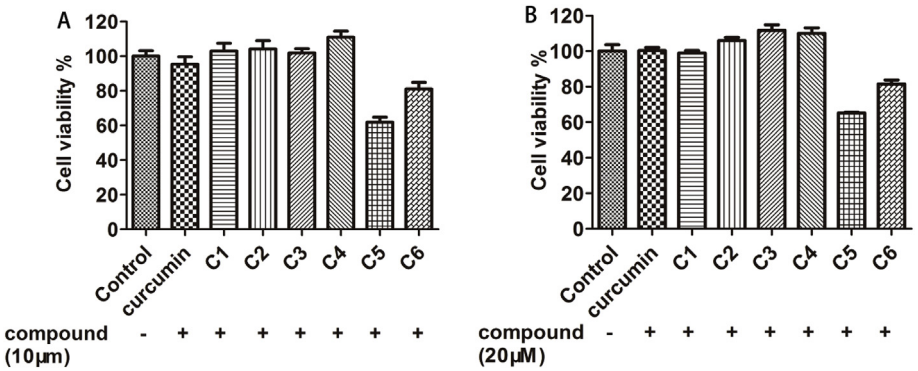

Fig. 3. The cytotoxicity of curcumin and its derivatives. (A) PC12 cells were co-incubated with 10 μM compounds for 24 h. (B) PC12 cells were co-incubated with 20 μM compounds for 24 h.

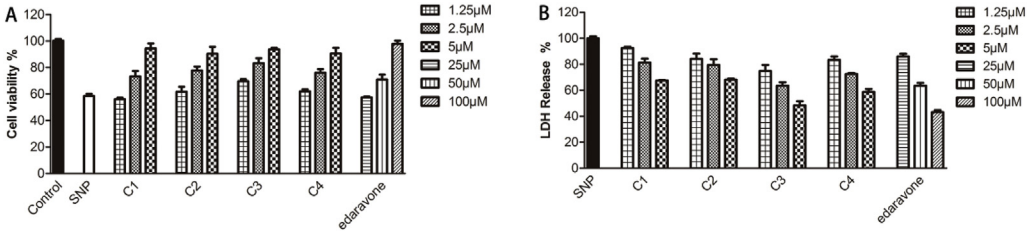

Fig. 4. Effect of C1-C4 and edaravone at three doses on SNP-induced PC12 injury. PC12 cells were pretreated with C1-C4 and edaravone for 24 h followed by treatment with 300 μM SNP for another 24 h and measured by MTT(A) and LDH (B) assay (Data are expressed as the mean ± SD of three independent assays).

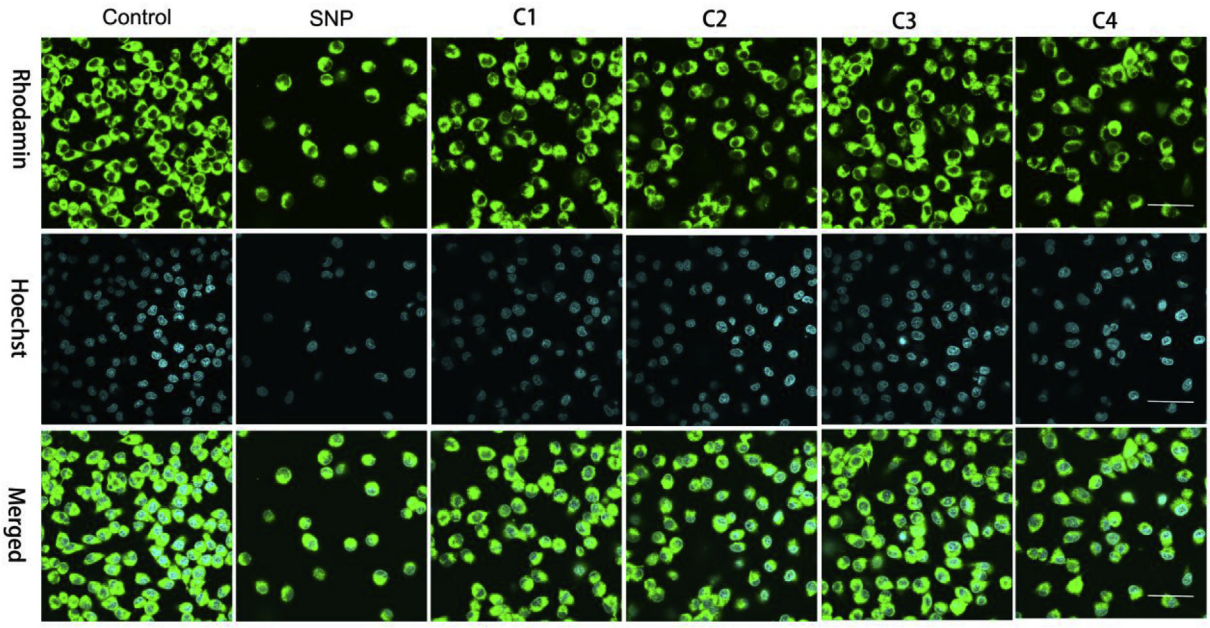

Fig. 5. Confocal images of SNP-induced PC12 cells by 5 μM C1-C4 (60X). Cell were pre-treated with 5 μM C1-C4 or 10% FBS, 5% HS DMEM for 24 h and then incubated with or without 300 μM SNP for another 24 h at 37 °C. 10 μg/ml Hoechst and 5 μM Rhodamin were co-incubated for 30 min, then detected by Laser confocal. Rhodamin stained cells showing cytoplasm location. Hoechst stained cells showing nucleus location. Scale bar = 50 μm.

the neuroprotective capacity of C3 for SNP-induced cell death compared to co-treatment with the C3 and SNP groups. These results indicate that the Nrf2 pathway is at least partially involved in the neuroprotective effects of C3 on PC12 cells.

Nrf2 is involved in the regulation of antioxidant enzymes and non-enzymatic antioxidants (Liu et al., 2018; Sha et al., 2015). Antioxidant defense system is essential for scavenging free radicals, and protecting protein and DNA from damage. As the main intracellular nonenzymatic antioxidant, GSH was measured. In addition, GSH-px and SOD (enzymatic antioxidants) were also determined. Results form Table 1 show that the content of GSH-px, SOD and GSH were reduced with the SNP treatment. However, C3 can improve that concentrations to some extent. The above results indicated that C3 possessed anti-oxidant activities in PC12 cells exposed to SNP possibly by regulating Nrf2.

4. Discussion

Neuroprotection is an important strategy for the treatment of NDs, and the use of neuroprotective agents is a potential approach (Enogieru et al., 2018). The pathogenesis of NDs is a complex process, and exact biomarkers and mechanisms are not fully understood (Ning et al., 2014; Wang et al., 2017). Clinical drugs that are currently widely used are very limited (Butterfield and Boyd-Kimball, 2018; Fachel et al., 2019) and have some adverse effects (Enogieru et al., 2018). For example, memantine hydrochloride, a glutamate receptor antagonist for moderate to severe AD, potentially increase the risk of adverse effects, including dizziness, headache, vomiting or agitation (Francois et al., 2018; Deardorff and Grossberg, 2016). Donepezil hydrochloride is a palliative medication used to improve the cognitive and behavioral symptoms of AD patients, with common side effects such as loss of appetite, gastrointestinal upset, diarrhea, or vomiting (Ruela et al., 2015). Rasagiline, a novel MAO-B inhibitor for treatment of idiopathic early PD, may also cause headache, dizziness or orthostatic hypotension (Solís-García del Pozo et al., 2013; Poewe et al., 2015). In addition, these drugs are now mainly focused on the symptoms and not the ideal drugs. (Enogieru et al., 2018).

Previous studies have reported the neuroprotective effects of curcumin (Maiti and Dunbar, 2018), but its clinical application is limited due to its low in vivo bioavailability (Sun et al., 2017; Bisceglia et al., 2018). Carbonyl is believed to be the key to reducing the bioavailability of curcumin (Liang et al., 2009; Zhao et al., 2013), so subsequent studies have focused on the structure modification including hydroxy etherification and modification of the β-diketone structure. Studies on the neuroprotective effects of curcumin and its derivatives have been reported, and its mechanism of action includes scavenging free radicals, anti-oxidation (Marta et al., 2018), and anti-inflammatory (Sorrenti et al., 2018), yet research on signaling pathways has rarely been reported.

Evidence shows that mitochondrial damage and oxidative stress are the early pathogenesis of various NDs (Elfawy and Das, 2019). In this work, The neuroprotective effects of compounds were explored from these two perspectives. We synthesized six curcumin pyrozole

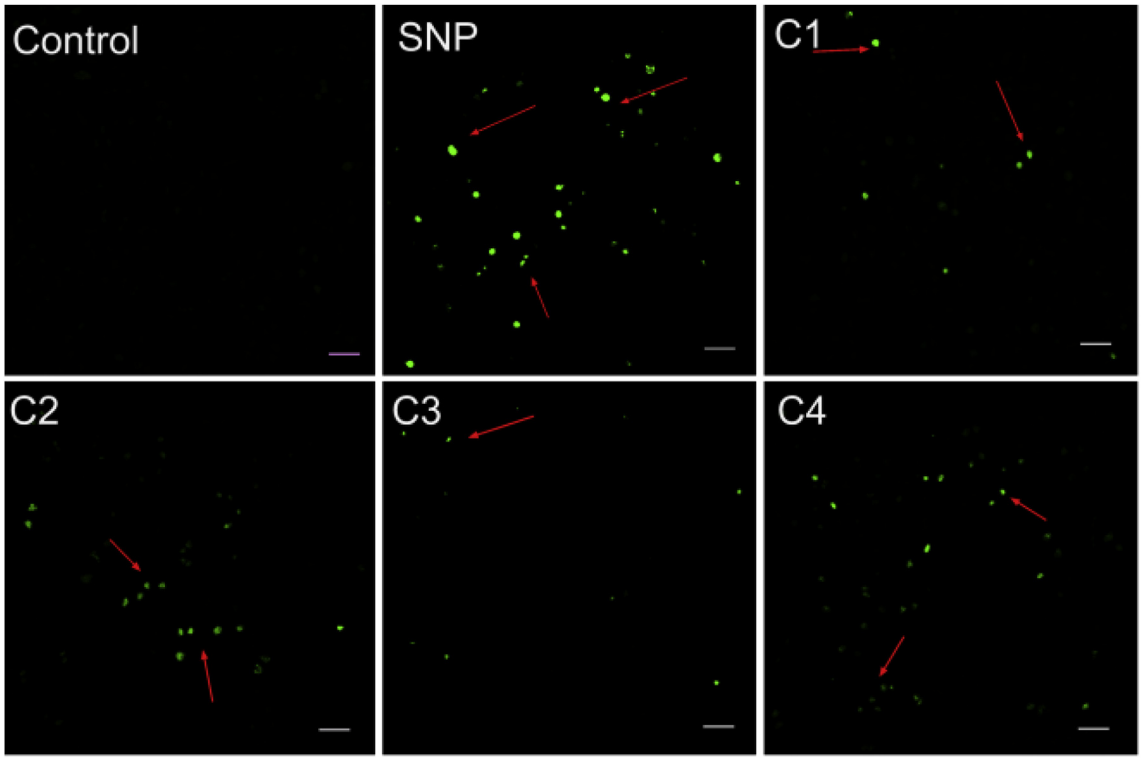

Fig. 6. Changes in ROS levels (20X). (A) C1-C4 inhibit SNP-induced increase in ROS levels. Pretreated with 5 μM C1-C4 for 24 h, SNP (300 μM) was added for another 9 h. Then 10 μM DCFH-DA was incubated for 30 min. The excitation and emission wavelength was 488 nm and 525 nm respectively. Scale Bar = 50 μm.

compounds and evaluated their neuroprotection on sodium nitroprusside (SNP) - induced PC12 cell injury by testing cell viability and LDH release, and investigated the pathway of action by preserving mitochondria function and Nrf2 signal in PC12 cell.

Oxidative stress has been a crucial pathophysiological factor resulting in nerve cell death in neurodegenerative diseases and free radicals are recognized as the main attackers (Losada-Barreiro and Bravo-Díaz, 2017). Free radicals include reactive oxygen (ROS) and nitrogen

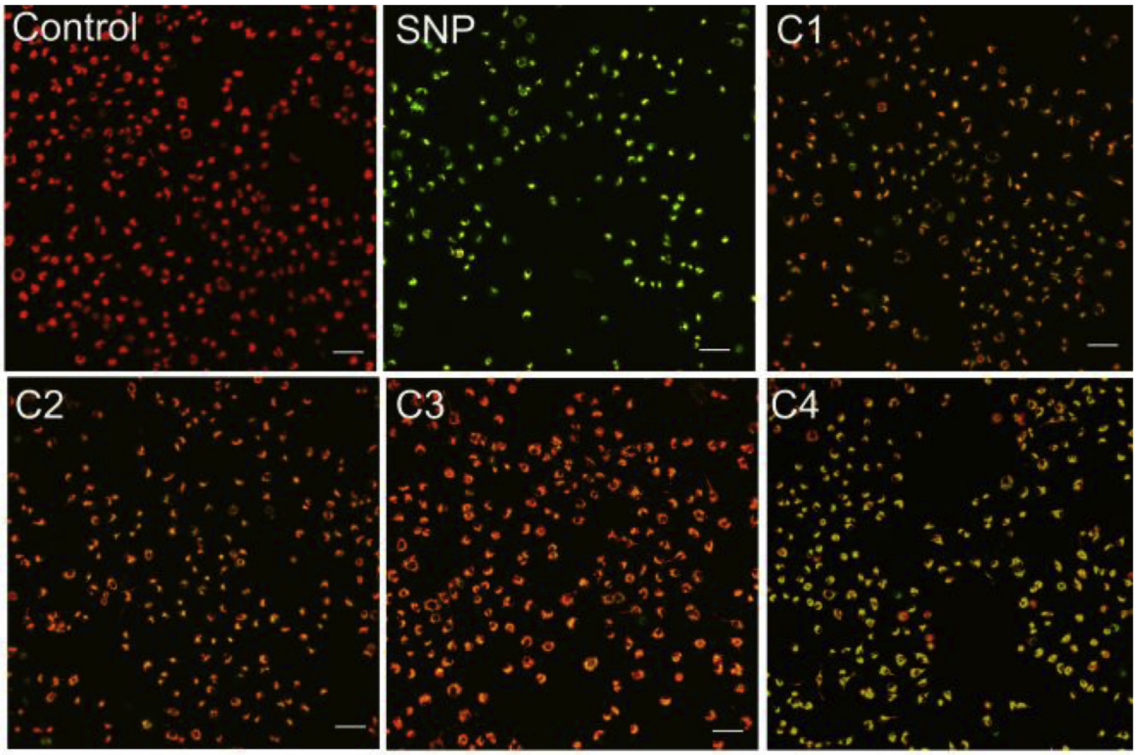

Fig. 7. C1-C4 restored SNP-induced Mitochondrial Membrane Potential in PC12 Cells (20X). After pretreated with 5 μM C1-C4 for 24 h, SNP (300 μM) was added for another 9 h. Mitochondrial membrane potential was determinated using JC-1 dye. Scale Bar = 50 μm.

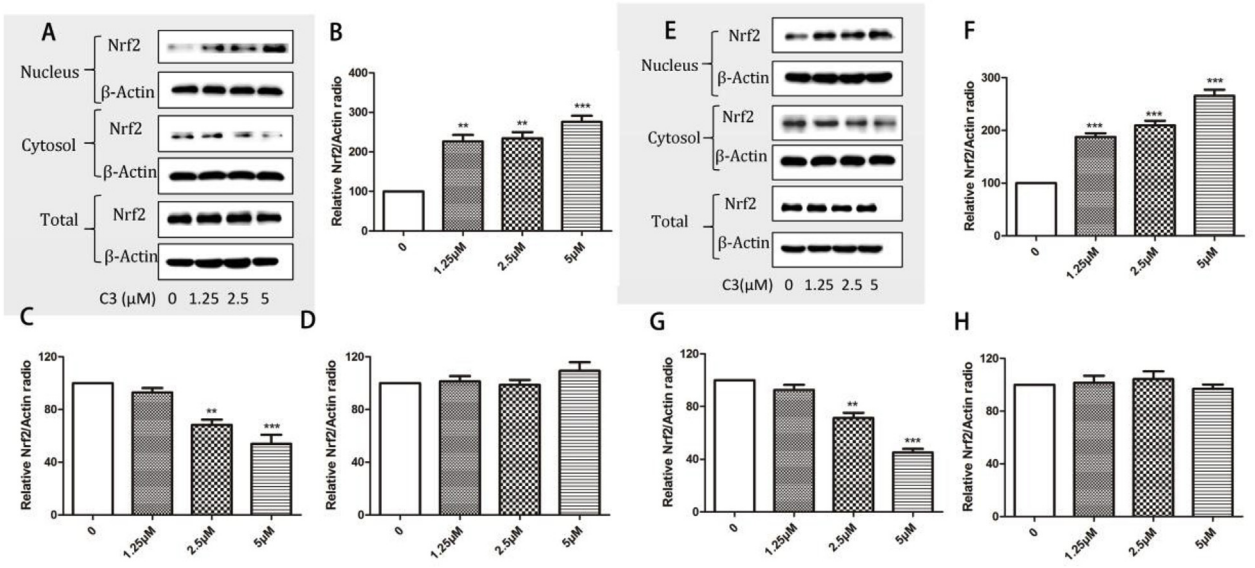

Fig. 8. C3 promoted Nrf2 nuclear translocation. PC12 cells were treated with C3 (5, 2.5, 1.25 μM) for 12 or 24 h. Nuclear Nrf2, cytosolic Nrf2, and total Nrf2 were analyzed by Western blots. (A) PC12 cells were treated with C3 for 12 h. (E) PC12 cells were treated with C3 for 24 h. Quantification of the blots is shown in panels B–D and F-H. Results from three independent experiments are expressed as means ± SD. ***p < 0.001,**p < 0.01 and *p < 0.5 vs the control group.

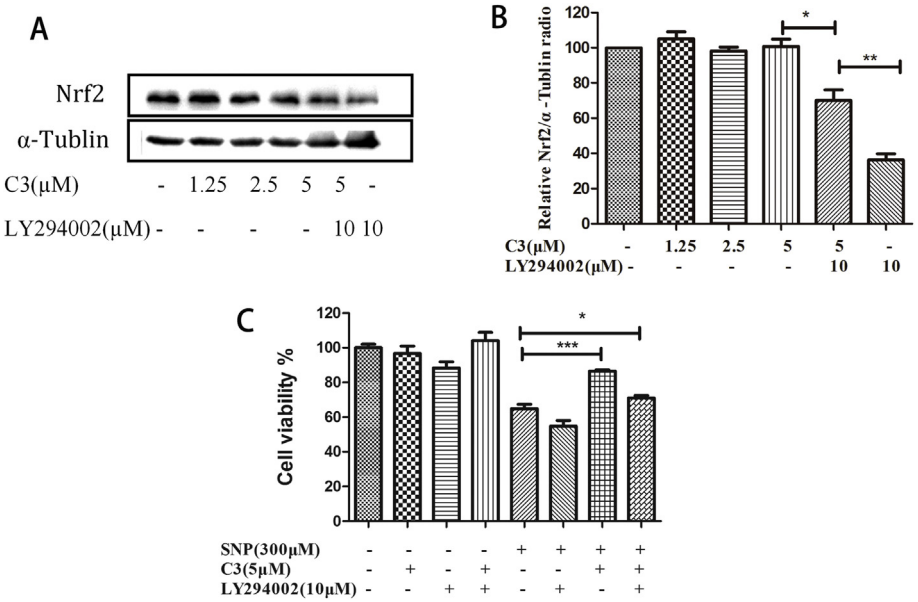

Fig. 9. Nrf2 is involved in the cytoprotection of C3. (A) Validation of Nrf2 levels of LY294002 in PC12 cells. (B) Quantification of the blot in A. (C) PI3K inhibitor LY294002 attenuated C3 neuroprotection in PC12 cells.

Table 1
Effect of C3 on GSH, GSH-px and SOD on SNP-treated PC12 cells (Mean ± S.D.). Cells were pre-treated with C3 (5, 2.5 1.25 μM) for 24 h, and then incubated with 300 μM SNP for another μ24 h. The cells were lysed on ice and the supernatant was collected to measure the levels of SOD, GSH-px and GSH according to the instructions and quantified protein concentration at the same time. Results from three independent experiments are expressed as means ± SD. **p < 0.01 and *p < 0.5 vs the control group;$^{\#\#}$p < 0.01 and $^{\#}$p < 0.5 vs the SNP group.

	GSH(nM/mg)	GSH-px (mU/mg)	SOD (U/mg)
Control	88.82 ± 0.70	50.46 ± 0.68	34.47 ± 1.45
SNP (300 μM)	49.22 ± 1.18**	38.15 ± 1.80**	19.58 ± 1.15*
SNP + C3 (1.25 μM)	54.02 ± 3.16	38.97 ± 2.57	20.37 ± 0.51
SNP + C3 (2.5 μM)	61.86 ± 4.62	41.70 ± 3.72	28.14 ± 2.97
SNP + C3 (5 μM)	67.04 ± 1.86$^{\#}$	48.21 ± 0.76$^{\#\#}$	28.23 ± 1.24$^{\#}$

(RNS) species (Li et al., 2015; Satta et al., 2017). Nitric oxide (NO), a RNS, has been considered as an important neuromodulator and involved in many pathological processes (Li et al., 2013). NO is associated with brain damage and early neurological deterioration, including acute ischemic stroke or other neurologically related diseases (Zheng et al., 2016; Chung, 2010). Excessive free radicals can cause damage to protein and DNA, which ultimately leads to cell death. Therefore, searching for antioxdant candidates is becoming a promising approach to recover neurodegenerative disorder (Panahi et al., 2017; Velusamy et al., 2017). In this study, after SNP treatment, ROS production was augmented and more PC12 cells were dying. However, MTT and LDH assays showed C1-C4 can reversed the effects of SNP on PC12 in a concentration manner. 5 μM of C1-C4 treatment improved cell viability by about 30% which are better than curcumin, and could decrease ROS intensity distinctly.

Mitochondria plays a key role in cell life cycle by providing sufficient energy for cellular activity (Chien et al., 2018). It's an electron transport center that is susceptible to oxidative damage which can cause mitochondrial dysfunction. Mitochondrial dysfunction also contributes to NDs pathology (Chan et al., 2016). Mitochondrial ROS intensifys lipid peroxidation, DNA and protein damage in the substantia nigra cells, finally resulting in loss of DA neurons in PD (Kim et al., 2016). MMP directly reflects the condition of mitochondria (Lv et al., 2017). Our result showed ROS-mediated damage during SNP treatment is able to impair MPP, which could be repaired by C1-C4 pretreatment. Results indicates that curcumin pyrozole compounds protect damaged neuron by scavenging free radicals and stabilizing mitochondrial membranes by targeting mitochondria. The result provides a new proof for the novel neuroprotective agents that exert neuroprotective effects by targeting mitochondria.

Nrf2 is a transcription factor and is taken for regulating the expression of antioxidant genes. When cells were activated by oxidative stress, Nrf2 would translocate to the nucleus. Nrf2 is involved in the protection of neurons, which is detected abnormal or insufficient in NDs. Activation of Nrf2 has been an interest in protecting against neurodegenerative diseases. Endogenous antioxidants such as GSH-px, SOD (enzymatic defense) and GSH (non-enzymatic defense) activities can be boosted by activating Nrf2 signaling pathway (Liu et al., 2018; Sha et al., 2015). SOD which is an important antioxidant enzyme in living organisms, is a naturally occurring superoxide radical scavenger. GSH-px enable to eliminate peroxides in cells and plays a key role in protecting cells from free radical attack. GSH can bind to peroxides and free radicals to protect thiols and thiolase from damage. These enzymes and non-enzymatic antioxidant defense systems play key roles in the development of oxidative stress. In our results, C3 significantly increased the levels of Nrf2 in nucleus at 12 h and 24 h and it can also reverse the effect by PI3K inhibitor LY294002 to some extent. The concentrations of GSH, GSH-px and SOD were remarkably decreased by the treatment of SNP, but C3 can increase them. These results suggested that curcumin pyrozole compound could protect damaged neuron via the Nrf2 pathway. It also provides evidence that novel neuroprotective agents activate Nrf2 pathway and enhance the antioxidant defense system to provide neuroprotection.

5. Conclusions

In this work, we evaluated the neuroprotective effects of six curcumin pyrazole derivatives on their antioxidant effect in SNP-induced PC12 neuron. Results showed that 5 μM C1-C4 can antagonize SNP-mediated PC12 cell death and effectively reduce reactive oxygen species (ROS) levels. Additionally C1-C4 are more effective compared to curcumin and edaravone, and C3 is the most active compound which may be related to electron-withdrawing substituent. Our results also revealed that the protective effect of C3 is related to the protection of mitochondria and Nrf2 signaling pathway. In conclusion, C3 may be a potential candidate compound for neuroprotectant in the future.

Conflicts of interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.01.026.

References

Bisceglia, F., Seghetti, F., Serra, M., Zusso, M., Gervasoni, S., Verga, L., Vistoli, G., Lanni, C., Catanzaro, M., Lorenzi, E.D., Belluti, F., 2018. Prenylated curcumin analogues as multipotent tools to tackle alzheimer's disease. ACS Chem. Neurosci. https://doi.org/10.1021/acschemneuro.8b00463.

Bonferoni, M.C., Rossi, S., Sandri, G., Ferrari, F., 2017. Nanoparticle formulations to enhance tumor targeting of poorly soluble polyphenols with potential anticancer properties. Semin Cancer Biol 10, 205–214.

Butterfield, D.A., Boyd-Kimball, D., 2018. Redox proteomics and amyloid β-peptide: insights into alzheimer disease. J. Neurochem. https://doi.org/10.1111/jnc.14589.

Calcerrada, P., Peluffo, G., Radi, R., 2011. Nitric oxide-derived oxidants with a focus on peroxynitrite: molecular targets, cellular responses and therapeutic implications. Curr. Pharmaceut. Des. 17, 3905–3932.

Castillo, J., Rama, R., Dávalos, A., 2000. Nitric oxide-related brain damage in acute ischemic stroke. Stroke 31, 852–857.

Chan, F., Lax, N.Z., Davies, C.H., Turnbull, D.M., Cunningham, M.O., 2016. Neuronal oscillations: a physiological correlate for targeting mitochondrial dysfunction in neurodegenerative diseases? Neuropharmacology 102, 48–58.

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., Collin, F., 2017. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. Redox Biol 14, 450–464.

Chien, L., Liang, M.Z., Chang, C.Y., Wang, C., Chen, L.Y., 2018. Mitochondrial therapy promotes regeneration of injured hippocampal neurons. BBA-MOL. Basis. Dis. 1864, 3001–3012.

Chung, K.K.K., 2010. Modulation of pro-survival proteins by S-nitrosylation: implications for neurodegeneration. Apoptosis 15, 1364–1370.

Citi, V., Calderone, V., Martelli, A., Breschi, M.C., Testai, L., 2018. Pathophysiological role of mitochondrial potassium channels and their modulation by drugs. Curr. Med. Chem. 25, 2661–2674.

Covill-Cooke, C., Howden, J.H., Birsa, N., Kittler, J.T., 2018. Ubiquitination at the mitochondria in neuronal health and disease. Neurochem. Int. 117, 55–64.

Deardorff, W.J., Grossberg, G.T., 2016. A fixed-dose combination of memantine extended-release and donepezil in the treatment of moderate-to-severe Alzheimer's disease. Drug Des. Dev. Ther. 10, 3267–3279.

Dinkova-Kostova, A.T., Kostov, R.V., Kazantsev, A.G., 2018. The role of Nrf2 signaling in counteracting neurodegenerative diseases. FEBS J. 285, 3576–3590.

Dumont, M., Beal, M.F., 2011. Neuroprotective strategies involving ROS in Alzheimer disease. Free Radic. Biol. Med. 51, 1014–1026.

Elfawy, H.A., Das, B., 2019. Crosstalk between mitochondrial dysfunction, oxidative stress, and age related neurodegenerative disease: etiologies and therapeutic strategies. Life Sci. 218, 165–184.

Enogieru, A.B., Haylett, W., Hiss, D.C., Bardien, S., Ekpo, O.E., 2018. Rutin as a potent antioxidant: implications for neurodegenerative disorders. Oxid. Med. Cell. Longev. 1–17.

Fachel, F.N.S., Schuh, R.S., Veras, K.S., Bassani, V.L., Koester, L.S., Henriques, A.T., Braganhol, E., Teixeira, H.F., 2019. An overview of the neuroprotective potential of rosmarinic acid and its association with nanotechnology-based delivery systems : a novel approach to treating neurodegenerative disorders. Neurochem. Int. 122, 47–58.

Firuzi, O., Miri, R., Tavakkoli, M., Saso, L., 2011. Antioxidant therapy: current status and future prospects. Curr. Med. Chem. 18, 3871–3888.

Francois, M., Sicsica, J., Fleury, N.P., 2018. Drugs for dementia and excess of hospitalization: a longitudinal French study. J. Alzheimers. Dis. 61, 1627–1637.

Gill, I., Kaur, S., Kaur, N., Dhiman, M., Mantha, A.K., 2017. Phytochemical ginkgolide B attenuates amyloid-β1-42 induced oxidative damage and altered cellular responses in human neuroblastoma SH-SY5Y cells. J. Alzheimers Dis. 60, S25–S40.

Hou, Y., Peng, S., Li, X., Yao, J., Xu, J., Fang, J., 2018. Honokiol alleviates oxidative stress-induced neurotoxicity via activation of Nrf2. ACS Chem. Neurosci. 9, 3108–3116.

Kim, H., Kim, S.H., Cha, H., Kim, S.R., Lee, J.H., Park, J.W., 2016. IDH2 deficiency promotes mitochondrial dysfunction and dopaminergic neurotoxicity: implications for Parkinson's disease. Free Radic. Res. 50, 853–860.

Kinlay, S., 2011. Changes in stroke epidemiology, prevention, and treatment. Circulation 124, 494–496.

Lee, J., Song, K., Huh, E., Oh, M.S., Kim, Y.S., 2018. Neuroprotection against 6-OHDA toxicity in PC12 cells and mice through the Nrf2 pathway by a sesquiterpenoid from Tussilago farfara. Redox. Biol. 18, 6–15.

Lee, X.R., Xiang, G.L., 2018. Effects of edaravone, the free radical scavenger, on outcomes in acute cerebral infarction patients treated with ultra-early thrombolysis of recombinant tissue plasminogen activator. Clin. Neurol. Neurosurg. 167, 157–161.

Li, J.W., Ning, N., Ma, Y.Z., Zhang, R., Tan, F., Chen, N.H., 2013. Claulansine F suppresses apoptosis induced by sodium nitroprusside in PC12 cells. Free Radic. Res. 47, 488–497.

Li, S., Tan, H.Y., Wang, N., Zhang, Z.J., Lao, L.X., Wong, C.W., Feng, Y.B., 2015. The role of oxidative stress and antioxidants in liver diseases. Int. J. Mol. Sci. 16, 26087–26124.

Liang, G., Yang, S., Zhou, H., Shao, L., Huang, K., Xiao, J., Huang, Z., Li, X., 2009. Synthesis, crystal structure and anti-inflammatory properties of curcumin analogues. Eur. J. Med. Chem. 44, 915–919.

Liu, Y., Li, A., Feng, X.L., Jiang, X.Y., Sun, X., Huang, W.Z., Zhu, X.S., Zhao, Z.X., 2018. L-Menthol alleviates cigarette smoke extract induced lung injury in rats by inhibiting oxidative stress and inflammation: via nuclear factor kappa B, p38 MAPK and Nrf2 signalling pathways. RSC Adv. 8, 9353–9363.

Losada-Barreiro, S., Bravo-Díaz, C., 2017. Free radicals and polyphenols: the redox chemistry of neurodegenerative diseases. Eur. J. Med. Chem. 133, 379–402.
Lv, C., Yuan, X., Zeng, H.W., Liu, R.H., Zhang, W.D., 2017. Protective effect of cinnamaldehyde against glutamate-induced oxidative stress and apoptosis in PC12 cells. Eur. J. Pharmacol. 815, 487–494.
Maiti, P., Dunbar, G.L., 2018. Use of curcumin, a natural polyphenol for targeting molecular pathways in treating age-related neurodegenerative diseases. Int. J. Mol. Sci. 19, 1637.
Marta, R., Giacomo, R., Stefania, C., Stefania, M., Arianna, S., Alessio, M., Daniela, R., Laura, C., Milena, S., Mayra, P., Daniela, C., Letizia, V., Dirk, S., Bernhard, W., Klaus, R.L., Guido, C., Vittorio, P., Ernst, U., Simona, C., 2018. Identification of dual Sigma1 receptor modulators/acetylcholinesterase inhibitors with antioxidant and neurotrophic properties, as neuroprotective agents. Eur. J. Med. Chem. 158, 353–370.
Ning, X.L., Guo, Y., Wang, X.W., Ma, X.Y., Tian, C., Shi, X.Q., Zhu, R.Z., Cheng, C., Du, Y.S., Ma, Z.Z., Zhang, Z.L., Liu, J.Y., 2014. Design, synthesis, and biological evaluation of (E)-3,4-Dihydroxystyryl aralkyl sulfones and sulfoxides as novel multifunctional neuroprotective agents. J. Med. Chem. 57, 4302–4312.
Panahi, Y., Rajaee, S.M., Johnston, T.P., Sahebkar, A., 2017. Neuroprotective effects of antioxidants in the management of neurodegenerative disorders: a literature review. J. Cell. Biochem. 1–7.
Perez, J., Hill, B.G., Benavides, G.A., Dranka, B.P., Darley-Usmar, V.M., 2010. Role of cellular bioenergetics in smooth muscle cell proliferation induced by platelet-derived growth factor. Biochem. J. 428, 255–267.
Pérez, M.J., Ponce, D.P., Aranguiz, A., Behrens, M.I., Quintanilla, R.A., 2018. Mitochondrial permeability transition pore contributes to mitochondrial dysfunction in fibroblasts of patients with sporadic Alzheimer's disease. Redox Biol 10, 290–300.
Poewe, W., Seppi, K., Fitzer-Attas, C.J., Wenning, G.K., Gilman, S., Low, P.A., Giladi, N., Barone, P., Sampaio, C., Eyal, E., Rascol, O., 2015. Efficacy of rasagiline in patients with the parkinsonian variant of multiple system atrophy: a randomised, placebo-controlled trial. Lancet Neurol. 14, 145–152.
Ruela, A.L.M., Carvalho, F.C., Pereira, G.R., 2015. Exploring the phase behavior of monoolein/oleic acid/water systems for enhanced donezepil administration for alzheimer disease treatment. J. Pharm. Sci-US 105, 71–77.
Satoh, K., Ikeda, Y., Shioda, S., Tobe, T., Yoshikawa, T., 2002. Edarabone scavenges nitric oxide. Redox Rep. 7, 219–222.
Satta, S., Wilkinson, F.L., Yvonne, A.M., White, S.J., Mahmoud, A.M., 2017. The role of Nrf2 in cardiovascular function and disease. Oxid. Med. Cell. Longev. 9237263.
Sha, L.K., Sha, W., Kuchler, L., Daiber, A., Giegerich, A.K., Weigert, A., Knape, T., Snodgrass, R., Schröder, K., Brandes, R.P., Brüne, B., von, K.A., 2015. Loss of Nrf2 in bone marrow-derived macrophages impairs antigen-driven CD8(+) T cell function by limiting GSH and Cys availability. Free Radic. Biol. Med. 83, 77–88.
Sherin, D.R., Rajasekharan, K.N., 2015. Mechanochemical synthesis and antioxidant activity of curcumin-templated azoles. Arch. Pharm. 348, 908–914.
Solís-García del Pozo, J., Mínguez-Mínguez, S., De Groot, P.W.J., Jordan, J., 2013. Rasagiline meta-analysis: a spotlight on clinical safety and adverse events when treating Parkinson's disease. Expert Opin. Drug Saf. 12, 479–486.
Sorrenti, V., Contarini, G., Sut, S., Dall'Acqua, S., Confortin, F., Pagetta, A., Giusti, P., Zusso, M., 2018. Curcumin prevents acute neuroinflammation and long-term memory impairment induced by systemic lipopolysaccharide in mice. Front. Pharmacol. 9, 183.
Sun, D., Zhou, J.K., Zhao, L.S., Zheng, Z.Y., Li, J., Pu, W.C., Liu, S.Y., Liu, X.S., Liu, S.J., Zheng, Y., Zhao, Y., Peng, Y., 2017. Novel curcumin liposome modified with hyaluronan targeting CD44 plays an anti-leukemic role in acute myeloid leukemia in vitro and in vivo. ACS Appl. Mater. Interfaces 9, 16857–16868.
Taylor, M., Moore, S., Mourtas, S., Niarakis, A., Re, F., Zona, C., La Ferla, B., Nicotra, F., Masserini, M., Antimisiaris, S.G., Gregori, M., Allsop, D., 2011. Effect of curcumin-associated and lipid ligand-functionalized nanoliposomes on aggregation of the Alzheimer's Aβ peptide. Nanomedicine 7, 541–550.
Velusamy, T., Panneerselvam, A.S., Purushottam, M., Anusuyadevi, M., Pal, P.K., Jain, S., Essa, M.M., Guillemin, G.J., Kandasamy, M., 2017. Protective effect of antioxidants on neuronal dysfunction and plasticity in Huntington's disease. Oxid. Med. Cell Longev. 1–15.
Wang, L.X., Yi, F., Fu, L.N., Yang, J.P., Wang, S., Wang, Z.X., Suzuki, K., Sun, L., Xu, X.L., Yu, Y., Qiao, J., Belmonte, J.C.I., Yang, Z., Yuan, Y., Qu, J., Liu, G.H., 2017. CRISPR/Cas9-mediated targeted gene correction in amyotrophic lateral sclerosis patient iPSCs. Protein Cell 8, 365–378.
Wang, R., Sun, Y., Huang, H., Wang, L., Chen, J., Shen, W., 2015. Rutin, a natural flavonoid protects PC12 cells against sodium nitroprusside-induced neurotoxicity through activating PI3K/Akt/mTOR and ERK1/2 pathway. Neurochem. Res. 40, 1945–1953.
Yallapu, M.M., Nagesh, P.K.B., Jaggi, M., Chauhan, S.C., 2015. Therapeutic applications of curcumin nanoformulations. AAPS J. 17, 1341–1356.
Yang, B., Li, W., Satani, N., Nghiem, D.M., Xi, X.P., Aronowski, J., Savitz, S.I., 2017. Transl. Stroke Res 130, 1–11.
Zaidun, N.H., Thent, Z.C., Latiff, A.A., 2018. Combating oxidative stress disorders with citrus flavonoid: Naringenin. Life Sci. 208, 111–122.
Zhao, C., Liu, Z., Liang, G., 2013. Promising curcumin-based drug design: mono-carbonyl analogues of curcumin (MACs). Curr. Pharmaceut. Des. 19, 2114–2135.
Zheng, W., Chong, C.M., Wang, H., Zhou, X., Zhang, L., Wang, R., Meng, Q., Lazarovici, P., Fang, J., 2016. Artemisinin conferred ERK mediated neuroprotection to PC12 cells and cortical neurons exposed to sodium nitroprusside-induced oxidative insult. Free Radic. Biol. Med. 97, 158–167.