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Oridonin, A natural diterpenoid, protected NGF-differentiated PC12 cells against MPP^+- and kainic acid-induced injury

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ABSTRACT

Oridonin (ORI) is a natural diterpenoid presented in some medicinal plants. The effects of pre-treatments from ORI against MPP^+- or kainic acid (KA)-induced damage in nerve growth factor (NGF)-differentiated PC12 cells were investigated. Results showed that pre-treatments of ORI at 0.25–2 μM enhanced the viability and plasma membrane integrity of NGF-differentiated PC12 cells. MPP^+ or KA exposure down-regulated Bcl-2 mRNA expression, up-regulated Bax mRNA expression, increased caspase-3 activity and decreased Na^+-K^+ ATPase activity. ORI pre-treatments at test concentrations reversed these changes. ORI pre-treatments decreased reactive oxygen species production, raised glutathione level, and increased glutathione peroxidase, glutathione reductase and catalase activities in MPP^+ or KA treated cells. ORI pre-treatments lowered tumor necrosis factor-alpha, interleukin (IL)-1beta, IL-6 and prostaglandin E_2 levels in MPP^+ or KA treated cells. ORI also diminished MPP^+ or KA induced increase in nuclear factor-κB binding activity. MPP^+ exposure suppressed tyrosine hydroxylase (TH) mRNA expression and decreased dopamine content. KA exposure reduced glutamine synthetase (GS) mRNA expression, raised glutamate level and lowered glutamine level. ORI pre-treatments at 0.5–2 μM up-regulated mRNA expression of TH and GS, restored DA and glutamine content. These findings suggested that oridonin was a potent neuro-protective agent against Parkinson's disease and seizure.

1. Introduction

Oridonin (ORI) is a diterpenoid presented in *Rabdosia rubescens* and *Rabdosia excisa*, and these two herbs are commonly used in traditional Chinese medicine to treat inflammatory disorders (Wu et al., 2012). It has been documented that ORI exhibited *in vitro* and *in vivo* inhibitory effects against lung cancer, liver cancer and breast cancer through limiting nuclear factor (NF) κB transcription, angiogenesis and epithelial-mesenchymal transition (Wang et al., 2014; Li et al., 2018). So far, the neuro-protective effects of ORI have attracted attention. Zhang et al. (2013) reported that ORI could alleviate inflammatory reactions in microglial cells, decrease β-amyloid deposition and suppress microglial activation in brain of transgenic mice. The study of Wang et al. (2016) revealed that ORI rescued Aβ1-42 induced synaptic loss and improved mitochondrial activity in synaptosomes of mice with Alzheimer's disease. Those previous literatures suggest that ORI is able to protect brain and neuronal system. However, less attention was paid to the potent of ORI to attenuate Parkinson's disease (PD) and/or seizure.

PD is a neurodegenerative disease due to the loss of nigrostriatal dopamine (DA) neurons and the accumulation of α-synuclein in astrocytes, which further cause neurotransmitters deficiency and even neurotoxicity at striatum (Devi et al., 2008; Chen et al., 2015). Both

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Abbreviations

COX	cyclooxygenase
DA	dopamine
DCFH-DA	dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPX	glutathione peroxidase
GR	glutathione reductase
GS	glutamine synthetase
GSH	glutathione
IL	interleukin
KA	kainic acid
LDH	lactate dehydrogenase
MFI	mean fluorescence intensity
MMP	mitochondrial membrane potential
MPP^+	1-methyl-4-phenylpyridinium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF	nuclear factor
NGF	nerve growth factor
OD	optical density
ORI	oridonin
PD	Parkinson's disease
PGE	prostaglandin E
RFU	relative fluorescence unit
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
TH	tyrosine hydroxylase
TNF	tumor necrosis factor

inflammatory and oxidative responses are involved in pathological development of PD (Hald and Lotharius, 2005; Yadav et al., 2014). High levels of inflammatory cytokines such as interleukin (IL)-1beta, IL-6 and tumor necrosis factor (TNF)-alpha have been found in nigrostriatal DA regions and lumbar cerebrospinal fluid of PD patients (Nagatsu et al., 2000). In addition, tyrosine hydroxylase (TH) is the rate-limiting enzyme for the conversion of tyrosine to DA and other catecholamines. The reduced TH activity or expression resulted in DA loss and favored the progression of PD (Bademci et al., 2012). 1-Methyl-4-phenylpyridinium (MPP^+) is the metabolite of an environmental toxin, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine. MPP^+ limits mitochondrial complex I, leads to ATP depletion, induces oxidative injury and cell death (Smeyne and Jackson-Lewis, 2005). Thus, MPP^+ has been widely used as a neurotoxin to induce PD symptoms in cells or rodents' models (Liu et al., 2015).

Seizure is a neurological disorder due to the over-reactivity of nerve cells in brain, especially in the area of hippocampus. Both inflammatory and oxidative stresses also play crucial roles in the etiopathogenesis of seizure (Vezzani et al., 2011; Huang et al., 2012). The massive release of inflammatory cytokines and reactive oxygen species (ROS) impairs nerve cell integrity, and initiates nerve cell apoptosis (Han et al., 2012). In addition, glutamate excitotoxicity due to the increased extracellular glutamate level in brain hyper-excitable areas promotes neuronal excitability and enhances seizure induction (Takanashi et al., 2015). Glutamine synthetase (GS) is in charge of glutamate clearance through converting glutamate to glutamine (Eid et al., 2013). It is reported that kainic acid (KA) could disturb nerve impulse transmission, depolarize neuronal membranes, cause focal hippocampal lesion and evoke seizure-like neuronal excitability (Malva et al., 2003). Thus, KA treated neuronal cells or rodents have been often used as seizure research models (Han et al., 2012; Huang et al., 2012).

In our present cell line study, the effects of pre-treatments from ORI at different concentrations against MPP^+- or KA- induced damage in nerve growth factor (NGF)-differentiated PC12 cells were investigated. The effects of ORI upon cell survival, plasma membrane integrity, DA and glutamine levels, and mRNA expression of TH and GS were examined. In addition, the influence of ORI upon the change in Na^+-K^+ ATPase activity, an indicator of mitochondrial malfunction and even motor neuron death (Ellis et al., 2003), was evaluated. These results could enhance our understanding regarding the possibility of using ORI as an anti-PD or anti-seizure agent.

2. Materials and methods

2.1. Materials

ORI (98%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). MPP^+, KA (99.5%) and NGF (99%) were purchased from Wako Chemical Co. (Tokyo, Japan). Antibiotics, medium and plates used for cell culture were obtained from Difco Laboratory (Detroit, MI, USA).

2.2. Cell culture and treatments

PC12 cells were regularly cultured with Dulbecco's modified Eagle's medium (DMEM) under 95% air and 5% CO_2 at 37 °C. PC12 cells were first treated with NGF at 50 ng/ml, and followed by a 5-day incubation at 37 °C for cell differentiation. Differentiated cells were washed twice with serum-free DMEM, and cultivated in 96 well plates. Cell numbers were adjusted to 10^5/ml by phosphate-buffered saline (PBS, pH 7.2). ORI was suspended in 1% carboxymethylcellulose at a concentration of 2 mg/ml, and further diluted by DMEM. MPP^+ or KA was directly dissolved in PBS. Differentiated cells were treated with ORI at 0.25, 0.5, 1, 2, 4, 8, 16 or 32 μM for 48 h at 37 °C. As shown in Fig. 1, ORI treatments at 8–32 μM caused cell death and ORI treatment at 4 μM had

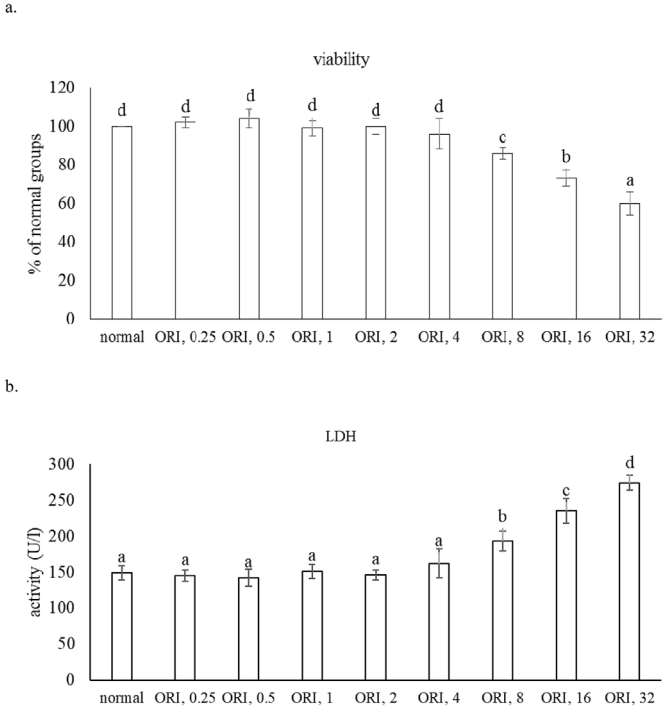

Fig. 1. Effects of oridonin (ORI) alone upon cell viability (a) and lactate dehydrogenase (LDH) activity (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0 (normal groups), 0.25, 0.5, 1, 2, 4, 8, 16 or 32 μM. Data are mean ± SD (n = 8). $^{a\text{-}d}$Values among bars without a common letter differ, $P < 0.05$.

greater standard deviation (SD) values in both cell viability and plasma membrane damage. Thus, the used concentrations of ORI for this study were 0.25–2 μM. Subsequently, ORI-treated cells were exposed to 100 μM MPP^+ or 150 μM kA for 24 h according to the methods of Hosseinzadeh et al. (2017) and Hou (2011). Cells without ORI, MPP^+ or KA were normal groups. Cells without ORI, but with MPP^+ or KA were control groups.

2.3. Assays of viability and plasma membrane damage

Cells were scraped and washed twice with PBS. The harvested cells were centrifuged at 1000 × *g* for 10 min. Cell suspension was collected and further mixed with 0.25 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After this mixture was incubated at 37 °C for 3 h, MTT formazan product was quantified by using a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) to measure the absorbance at 570 nm. Cell viability was shown in a percentage of normal groups. Plasma membrane damage was measured by detecting lactate dehydrogenase (LDH) activity (U/l). After centrifugation, cell supernatant at 50 μl was used for LDH activity determination by an assay kit (Sigma Chem. Co., St. Louis, MO, USA).

2.4. Measurement of DNA fragmentation and mitochondrial membrane potential (MMP)

Cells (10^5/ml) were added into 1 ml lysis buffer (5 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 20 mM EDTA), and incubated for 30 min at 25 °C. After centrifugation at 250 × *g* for 10 min, 20 μl of cell supernatant was mixed with 80 μl of immunoreagents (anti-histone biotin and anti-DNA peroxidase) and followed by incubating for 2 h at 25 °C. Reactive substrate (3-ethylbenzthiazoline-6-sulphonic acid) was added, and further incubated for 15 min at 25 °C. The absorbance values at both 405 nm and 490 nm were recorded by a microplate reader. The value, (absorbance of sample)/(absorbance of normal groups), was defined as an enrichment factor to indicate DNA fragmentation. Rh123, a fluorescent dye, at 100 μg/l was mixed with cell suspension, and incubated for 30 min at 37 °C. The value of mean fluorescence intensity (MFI), an indicator of MMP, was determined by a flow cytometry (FC500 Model, Beckman Coulter, Fullerton, CA, USA) after washed twice with PBS.

2.5. Determination of caspase-3 and Na^+-K^+ ATPase activities

Lysed cells were centrifuged at 10,000 × *g* for 15 min at 4 °C. The collected supernatant was mixed with a solution containing dithiothreitol and *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide. After incubating for 1 h at 37 °C, caspase-3 activity was measured by a fluorescent kit (Upstate, Lake Placid, NY, USA) via using a fluorophotometer. Data were shown as percentages of the normal groups. Na^+-K^+ ATPase activity was monitored according to the method of Torlinska and Grochowalska (2004), in which the amount of released inorganic phosphate from ATP was measured. Cells at 10^5/ml were centrifugated at 5,000 × *g* for 10 min at 4 °C to obtain pellet. Then, the pellet was fractionated to collect mitochondrial fraction via a mitochondria isolation kit (Pierce, Rockford, IL). Subsequently, cellular mitochondria were mixed with a solution containing 30 mM Tris-HCl buffer (pH 7.4), 100 mM NaCl and 20 mM KCl. ATP was added to initiate the assay process, and 15% trichloroacetic acid was added to terminate this assay after incubating for 15 min at 37 °C. The absorbance at 640 nm was read by a fluorophotometer. Data were shown as percentages of the normal groups.

2.6. Evaluation of oxidative and inflammatory parameters

Glutathione (GSH) content was analyzed by a kit (EMD Biosciences Inc., San Diego, CA, USA). ROS level was detected by 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Cell homogenate at 100 μl was mixed with 2 mg/ml DCFH-DA and further incubated for 30 min at 37 °C. Fluorescence values at 525 nm and 488 nm were monitored by a Molecular Devices fluorescence microplate reader (Sunnyvale, CA, USA). Result was expressed as a relative fluorescence unit (RFU) per mg protein. The activities of glutathione peroxidase (GPX), glutathione reductase (GR) and catalase were measured by assay kits obtained from OxisResearch Co. (Portland, OR, USA). TNF-alpha, IL-1beta, IL-6 and prostaglandin E $(PGE)_2$ levels were quantified by assay kits (Cayman Chem. Co., Ann Arbor, MI, USA). Cyclooxygenase (COX)-2 activity was determined by an ELISA kit (Cayman Chem. Co., Ann Arbor, MI, USA). Protein concentration of cell homogenate was determined by a kit (Pierce, Rockford, IL, USA).

2.7. Analyses for NF-κB binding activity

Cellular nuclear protein was extracted and NF-κB p50/65 binding activity was measured by a kit purchased from Chemicon Int. Co. (Temecula, CA, USA). Ten μg protein extract was mixed with 3, 3′, 5, 5′-tetramethylbenzidine, a NF-κB p50/p65 antibody. After incubating for 1 h at 25 °C, horseradish peroxidase-conjugated antibody was added. Sample was incubated for another 1 h, the absorbance at 450 nm was recorded by a microplate reader. Result was shown as an optical density (OD) per mg protein.

2.8. Determination of mRNA expression

mRNA expression was determined by real-time polymerase chain reaction (RT-PCR). Total RNA was extracted and isolated from cells, and its concentration was assayed by monitoring the absorbance at 260 nm cDNA was synthesized by 5 μg RNA through a cDNA synthesis kit (Legene Biosciences, San Diego, CA, USA). Subsequently, cDNA was used for RT-PCR process. The amplification conditions of cDNA were as follow: activation at 95 °C for 10 min, denaturation at 95 °C for 15s, annealing and elongation at 56 °C for 1 min. Forty cycles were performed for Bcl-2, Bax, TH and GS, and 32 cycles were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The generated fluorescence was quantified by a SYBR Green I Masterkit (Sigma Chem. Co., St. Louis, MO, USA) and RT sequence detection system, ABI 7500 PCR instrument (Foster City, CA, USA). mRNA level was expressed as percentage of normal groups. Both sense and antisense primers of target oligonucleotides included Bcl-2, forward, 5′-GTG GAT GAC TGA GTA CCT GAA C-3′, reverse, 5′-GAG ACA GCC AGG AGA AAT CAA-3'; Bax, forward, 5′-GCT GAT GGC AAC TTC AAC TG-3′, reverse, 5′-ATC AGC TCG GGC ACT TTA G-3'; TH, forward, 5′-GAT TGC TAC CTG GAA GGA GGT-3′, reverse, 5′-AGT CCA ATG TCC TGG GAG AAC-3'; GS, forward, 5′-CCA CTG TCC CTG GGC TTA GTT TA-3′, reverse, 5′-AGT GAC ATG CTA GTC CCA CCA A-3'; GAPDH, forward, 5′-AGA GGC AGG GAT GTT CTG-3′, reverse, 5′-GAC TCA TGA CCA CAG TCC ATG C-3'.

2.9. Measurement of DA content

DA content in normal cells and MPP^+ treated cells was assayed. Cells, 10^5/ml, were treated with 1 ml 40 mM KCl for 6 min at 37 °C to release cellular DA into culture medium. Then, media was collected and centrifuged for 1 min at 10,000 × *g*. Supernatants were collected and further filtered through a 0.22 μm filter. DA content in the supernatant was determined by a HPLC method (Richardson et al., 2006). HPLC (Agilent 1200 Series Capillary) system was equipped with a photodiode electrode array detector. Absorbance at 202 nm and 280 nm were read. The detection limit and the quantification limit were 0.25 and 10 nmol/mg protein, respectively. DA content in sample was quantified by comparison with a standard curve. The linear concentration range was 0–12.5 nmol/mg protein.

2.10. Measurements of glutamate and glutamine levels

Glutamate and glutamine levels in normal cells and KA treated cells were measured. Cell homogenate was mixed with buffer of sodium citrate or lithium citrate, respectively, for 1 h at 25 °C. After centrifugation, the released glutamate and glutamine in supernatants were determined by a Hitachi amino acid analyzer (L-8800, Tokyo, Japan). Glutamate and glutamine levels in sample were quantified by external standard curves. The detection limit and the quantification limit for glutamate were 50 and 2000 ng/mg protein, respectively. The linear concentration range for glutamate was 0–2000 ng/mg protein. For glutamine, the detection limit and the quantification limit, respectively, were 50 and 1250 ng/mg protein. The linear concentration range was 0–1000 ng/mg protein.

2.11. Statistical analyses

Each treatment had 8 different preparations (n = 8). Data were shown as mean ± SD. One-way analysis of variance (ANOVA) were applied for statistical analyses. Post-hoc comparison was processed by Dunnett's *t*-test. Difference among means was defined as significant if P value was lower than 0.05.

3. Results

3.1. ORI at high concentrations impaired cell survival

The effects of ORI treatments alone upon cell viability and plasma membrane damage determined by LDH activity are presented in Fig. 1. After 48 h treatment, ORI at 0.25–4 μM did not affect the viability and plasma membrane stability of NGF-differentiated PC12 cells ($P > 0.05$). However, ORI treatments for 48 h at 8–32 μM dose-dependently decreased cell viability and increased LDH activity ($P < 0.05$).

3.2. ORI enhanced cell survival

As shown in Fig. 2, pre-treatments of ORI for 48 h at 0.25–2 μM protected NGF-differentiated PC12 cells and their plasma membranes against subsequently MPP^+ or KA caused death and plasma membrane damage ($P < 0.05$). MPP^+ or KA exposure for 24 h down-regulated Bcl-2 mRNA expression and up-regulated Bax mRNA expression (Fig. 3, $P < 0.05$). ORI pre-treatments for 48 h increased Bcl-2 mRNA expression and decreased Bax mRNA expression ($P < 0.05$). ORI pre-treatments at 2 μM did not further raise Bcl-2 mRNA expression than 1 μM in MPP^+ and KA exposed cells ($P > 0.05$). As shown in Table 1, ORI pre-treatments at 0.25–2 μM for 48 h alleviated MPP^+ or KA induced increase in DNA fragmentation and decrease in MMP ($P < 0.05$). ORI pre-treatments at 2 μM failed to further reduce DNA fragmentation and raise MMP in MPP^+ or KA treated cells when compared with 1 μM pre-treatments ($P > 0.05$). MPP^+ or KA exposure for 24 h increased caspase-3 activity and decreased Na^+-K^+ ATPase activity (Fig. 4, $P < 0.05$). ORI pre-treatments for 48 h at 0.25–2 μM reduced caspase-3 activity and at 0.5–2 μM elevated Na^+-K^+ ATPase activity ($P < 0.05$).

3.3. ORI alleviated oxidative and inflammatory stress

As presented in Table 2, ORI pre-treatments for 48 h at 0.25–2 μM concentration-dependently lowered ROS production and increased catalase activity in MPP^+ or KA exposed cells ($P < 0.05$). ORI pre-treatments at 0.25–2 μM, without concentration-dependent effects, also raised GSH level, GPX and GR activities in MPP^+ or KA treated cells ($P < 0.05$). ORI pre-treatments for 48 h at 0.25–2 μM concentration-dependently lowered TNF-alpha, IL-1beta, IL-6 and PGE_2 levels in MPP^+ or KA treated cells (Table 3, $P < 0.05$). ORI pre-treatments at 0.25–2 μM, without concentration-dependent effects, also declined COX-2 activity in MPP^+ or KA treated cells ($P < 0.05$). ORI pre-treatments for 48 h at 0.25–2 μM diminished MPP^+ or KA induced increase in NF-κB binding activity (Fig. 5, $P < 0.05$), in which concentration-dependent effect was shown in reducing KA-induced elevation of NF-κB binding activity ($P < 0.05$).

3.4. ORI mediated TH, DA, GS, glutamate and glutamine

MPP^+ exposure for 24 h suppressed TH mRNA expression (Fig. 6a, $P < 0.05$), and decreased DA content (6b, $P < 0.05$). ORI pre-treatments for 48 h at 0.5–2 μM up-regulated TH mRNA expression and restored DA content ($P < 0.05$). As shown in Fig. 7, KA exposure for 24 h reduced GS mRNA expression (7a, $P < 0.05$), raised glutamate level and lowered glutamine level (7b, $P < 0.05$). ORI pre-treatments for 48 h at 0.5–2 μM increased GS mRNA expression, decreased glutamate level and restored glutamine content ($P < 0.05$).

4. Discussion

The protective effects of ORI, a diterpenoid, for brain and neuronal system against β-amyloid deposition, microglial inflammation and synaptic dysfunction have been reported (Zhang et al., 2013; Wang et al., 2016). Our present study extended the neuronal protective possibilities of ORI toward PD and seizure. We found that ORI pre-treatments at 0.25–2 μM markedly decreased caspase-3 activity, stabilized Na^+-K^+ ATPase activity, attenuated oxidative and inflammatory stress, and enhanced cell survival in MPP^+ and KA treated NGF-differentiated PC12 cells. Furthermore, ORI at these concentrations substantially restored mRNA expression of TH and GS, increased DA and glutamine content. These novel findings suggested that ORI could execute its actions in molecular levels to protect neuronal cells against PD and

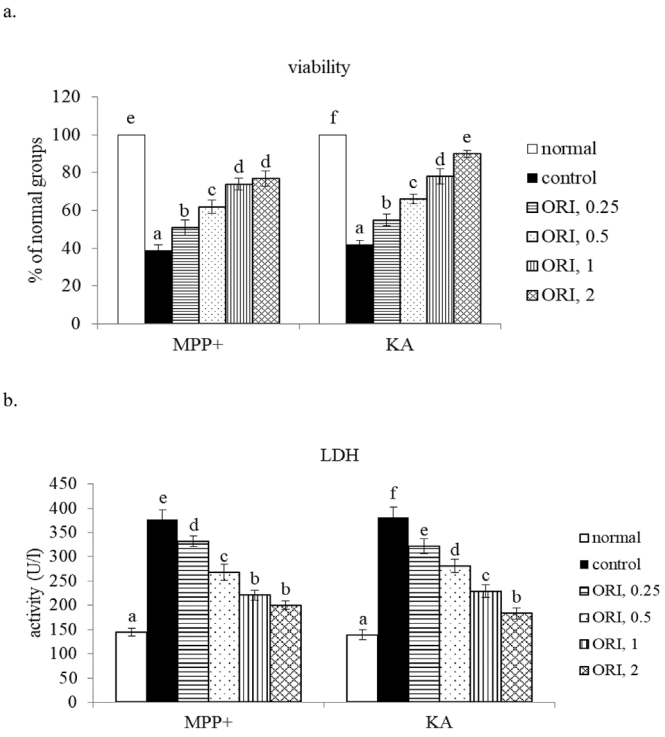

Fig. 2. Effects of oridonin (ORI) upon cell viability (a) and lactate dehydrogenase (LDH) activity (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using 1-methyl-4-phenylpyridinium (MPP^+) or kainic acid (KA) to induce injury. Normal group had no ORI, MPP^+ or KA. Control group had no ORI, but with MPP^+ or KA. Data are mean ± SD (n = 8). $^{a\text{-}f}$Values among bars without a common letter differ, $P < 0.05$.

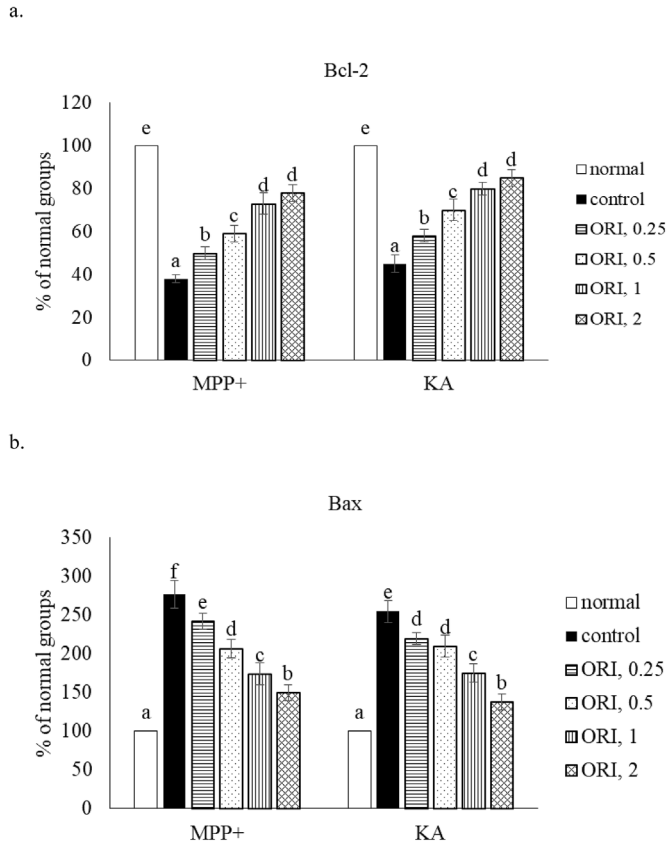

Fig. 3. Effects of oridonin (ORI) upon mRNA expression of Bcl-2 (a) and Bax (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using 1-methyl-4-phenylpyridinium (MPP^+) or kainic acid (KA) to induce injury. Normal group had no ORI, MPP^+ or KA. Control group had no ORI, but with MPP^+ or KA. Data are mean ± SD (n = 8). $^{a\text{-}f}$Values among bars without a common letter differ, $P < 0.05$.

Table 1
Effects of ORI upon DNA fragmentation and MMP. NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM and followed by using MPP^+ or KA to induce injury. Normal group had no ORI, MPP^+ or KA. Data are mean ± SD (n = 8). $^{a\text{-}e}$Values in a column without a common letter differ, $P < 0.05$.

	DNA fragmentation Folds of normal groups	MMP % of normal groups
Normal	1.00^{a}	100^{e}
MPP+	2.75 ± 0.19^{e}	32 ± 2^{a}
ORI, 0.25	2.31 ± 0.12^{d}	47 ± 5^{b}
ORI, 0.5	1.90 ± 0.15^{c}	62 ± 3^{c}
ORI, 1	1.49 ± 0.13^{b}	78 ± 5^{d}
ORI, 2	1.38 ± 0.1^{b}	83 ± 6^{d}
Normal	1.00^{a}	100^{e}
KA	2.48 ± 0.16^{e}	40 ± 3^{a}
ORI, 0.25	2.13 ± 0.09^{d}	53 ± 5^{b}
ORI, 0.5	1.72 ± 0.11^{c}	67 ± 4^{c}
ORI, 1	1.51 ± 0.07^{b}	81 ± 3^{d}
ORI, 2	1.43 ± 0.14^{b}	85 ± 4^{d}

seizure. Further animal studies are definitely required to examine the penetrative ability of this diterpenoid through blood brain barrier, and verify its effects, action modes and safety for PD and seizure improvement. In addition, our data revealed that ORI treatments alone at higher concentrations, 8–32 μM, caused the loss of NGF-differentiated PC12 cells. It seems that ORI at high dosages might be a cytotoxic substance. Thus, the used dosages of ORI for further applications must be carefully considered.

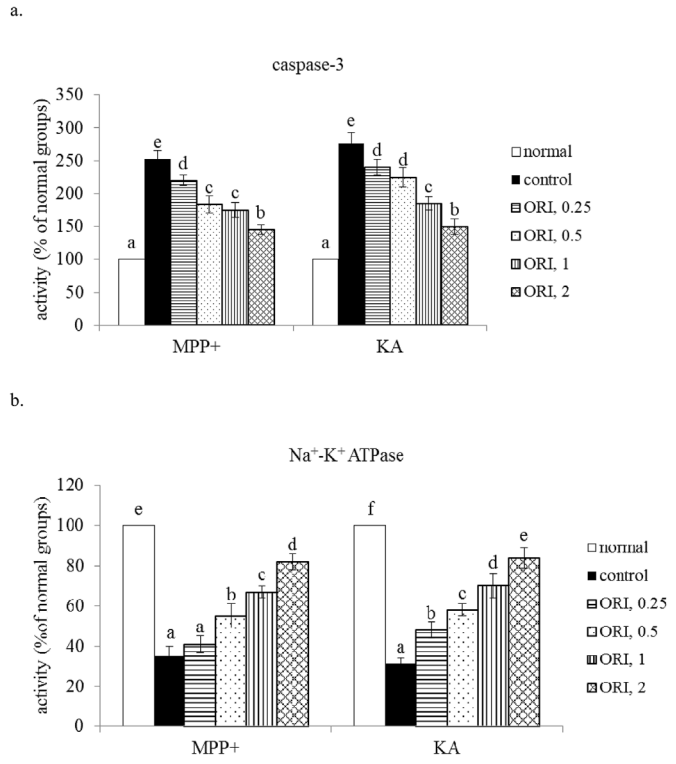

Fig. 4. Effects of oridonin (ORI) upon the activity of caspase-3 (a) and Na^+-K^+ ATPase (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using 1-methyl-4-phenylpyridinium (MPP^+) or kainic acid (KA) to induce injury. Normal group had no ORI, MPP^+ or KA. Control group had no ORI, but with MPP^+ or KA. Data are mean ± SD (n = 8). $^{a\text{-}f}$Values among bars without a common letter differ, $P < 0.05$.

MPP^+ or KA down-regulated mRNA expression of Bcl-2, an anti-apoptotic molecule; stimulated mRNA expression of Bax, an apoptotic molecule; as well as raised the activity of caspase-3, a mitochondrial apoptotic executor (Liu et al., 2010; Yalcin et al., 2010). These events resulted in cell death. However, ORI pre-treatments at 0.25–2 μM effectively reversed these changes, and increased cell viability. Na^+-K^+ ATPase, a transmembrane protein, is in charge of ion exchange between intracellular Na^+ and extracellular K^+. It is reported that decreased activity of this enzyme impaired neuronal ion homeostasis and caused cell death (Mark et al., 1995). In our present study, MPP^+ or KA disturbed plasma membrane and reduced mitochondrial membrane potential, both events subsequently lowered Na^+-K^+ ATPase activity, promoted LDH activity and DNA degradation. However, ORI at test concentrations protected DNA, stabilized mitochondrial membrane potential and plasma membrane integrity, as well as maintained Na^+-K^+ ATPase activity, which eventually benefited cell survival. These results indicated that ORI was able to penetrate into NGF-differentiated PC12 cells, and mediated several crucial apoptotic and anti-apoptotic factors.

MPP^+ or KA caused oxidative and inflammatory injury in brain or neuronal cells (Hald and Lotharius, 2005; Huang et al., 2012). Our results agreed those previous studies, which were evidenced by greater production of ROS, TNF-alpha, IL-1beta, IL-6 and PGE_2, higher COX-2 activity, lower GSH content, and reduced activity of GPX, GR and catalase. However, ORI at test concentrations efficiently and dose-dependently suppressed MPP^+ or KA induced oxidative and inflammatory response through decreasing COX-2 activity, restoring GPX, GR and catalase activities, and retaining GSH content. Since ORI was able to modulate GPX, GR, catalase and COX-2 activities, it was reasonable to observe lower formation of ROS, PGE_2, TNF-alpha, IL-1beta and IL-6. It is reported that the activation of NF-κB pathway in brain or neuronal

Table 2
Effects of ORI upon level or activity of ROS, GSH, GPX, GR and catalase. NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM and followed by using MPP^+ or KA to induce injury. Normal group had no ORI, MPP^+ or KA. Data are mean ± SD (n = 8). [a-f]Values in a column without a common letter differ, $P < 0.05$.

	ROS RFU/mg protein	GSH ng/mg protein	GPX U/mg protein	GR U/mg protein	catalase U/mg protein
Normal	0.09 ± 0.03[a]	91 ± 3[e]	70.3 ± 2.9[e]	65.4 ± 1.7[e]	67.6 ± 2.2[f]
MPP^+	2.55 ± 0.13[f]	30 ± 3[a]	33.6 ± 1.8[a]	30.2 ± 1.1[a]	29.9 ± 0.8[a]
ORI, 0.25	2.21 ± 0.17[e]	39 ± 2[b]	39.3 ± 2.1[b]	37.9 ± 2.0[b]	36.2 ± 1.0[b]
ORI, 0.5	1.68 ± 0.09[d]	52 ± 5[c]	47.6 ± 1.5[c]	45.5 ± 1.3[c]	44.1 ± 1.7[c]
ORI, 1	1.14 ± 0.12[c]	68 ± 4[d]	56.2 ± 1.0[d]	54.2 ± 1.4[d]	51.0 ± 1.2[d]
ORI, 2	0.72 ± 0.1[b]	75 ± 5[d]	59.6 ± 1.9[d]	57.0 ± 1.5[d]	58.3 ± 1.6[e]
Normal	0.12 ± 0.05[a]	93 ± 4[e]	68.1 ± 1.8[e]	66.7 ± 2.0[e]	68.0 ± 1.8[f]
KA	2.34 ± 0.16[f]	34 ± 2[a]	35.2 ± 0.9[a]	31.6 ± 1.3[a]	32.3 ± 1.0[a]
ORI, 0.25	1.91 ± 0.09[e]	47 ± 4[b]	41.5 ± 1.2[b]	38.5 ± 1.1[b]	39.7 ± 0.7[b]
ORI, 0.5	1.57 ± 0.11[d]	64 ± 5[c]	49.3 ± 1.0[c]	48.4 ± 0.7[c]	46.5 ± 1.4[c]
ORI, 1	1.23 ± 0.14[c]	78 ± 6[d]	58.7 ± 1.3[d]	56.9 ± 1.2[d]	53.8 ± 1.3[d]
ORI, 2	0.86 ± 0.13[b]	81 ± 3[d]	61.0 ± 2.1[d]	59.2 ± 1.7[d]	60.6 ± 1.2[e]

Table 3
Effects of ORI upon TNF-alpha, IL-1beta, IL-6 and PGE_2 levels, and COX-2 activity. NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM and followed by using MPP^+ or KA to induce injury. Normal group had no ORI, MPP^+ or KA. Data are mean ± SD (n = 8). [a-f]Values in a column without a common letter differ, $P < 0.05$.

	TNF-alpha pg/mg protein	IL-1beta pg/mg protein	IL-6 pg/mg protein	PGE_2 pg/mg protein	COX-2 U/mg protein
Normal	8 ± 2[a]	9 ± 3[a]	10 ± 4[a]	120 ± 8[a]	0.11 ± 0.03[a]
MPP^+	145 ± 7[f]	132 ± 8[f]	107 ± 9[f]	383 ± 16[f]	1.72 ± 0.17[e]
ORI, 0.25	124 ± 5[e]	108 ± 4[e]	81 ± 6[e]	331 ± 10[e]	1.36 ± 0.1[d]
ORI, 0.5	98 ± 6[d]	89 ± 7[d]	69 ± 4[d]	290 ± 13[d]	1.04 ± 0.12[c]
ORI, 1	73 ± 4[c]	64 ± 5[c]	52 ± 3[c]	251 ± 9[c]	0.67 ± 0.08[b]
ORI, 2	50 ± 3[b]	43 ± 2[b]	37 ± 5[b]	207 ± 15[b]	0.58 ± 0.06[b]
Normal	11 ± 5[a]	7 ± 1[a]	9 ± 3[a]	123 ± 11[a]	0.08 ± 0.04[a]
KA	137 ± 8[f]	112 ± 6[f]	120 ± 9[f]	360 ± 18[f]	1.55 ± 0.13[e]
ORI, 0.25	115 ± 5[e]	93 ± 5[e]	100 ± 6[e]	319 ± 13[e]	1.2 ± 0.15[d]
ORI, 0.5	94 ± 3[d]	74 ± 4[d]	82 ± 3[d]	272 ± 9[d]	0.94 ± 0.09[c]
ORI, 1	70 ± 4[c]	58 ± 3[c]	65 ± 2[c]	225 ± 12[c]	0.61 ± 0.14[b]
ORI, 2	48 ± 6[b]	40 ± 2[b]	45 ± 5[b]	196 ± 10[b]	0.54 ± 0.1[b]

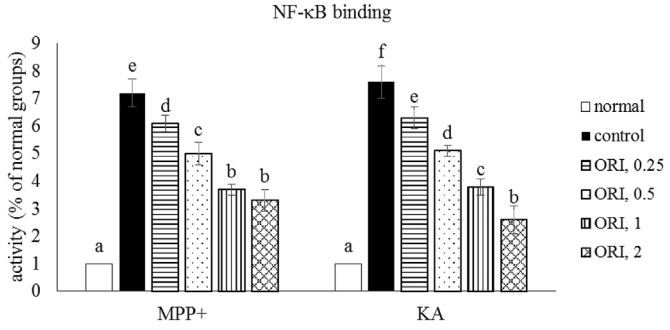

Fig. 5. Effects of oridonin (ORI) upon NF-κB binding activity. NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using 1-methyl-4-phenylpyridinium (MPP^+) or kainic acid (KA) to induce injury. Normal group had no ORI, MPP^+ or KA. Control group had no ORI, but with MPP^+ or KA. Data are mean ± SD (n = 8). [a-f]Values among bars without a common letter differ, $P < 0.05$.

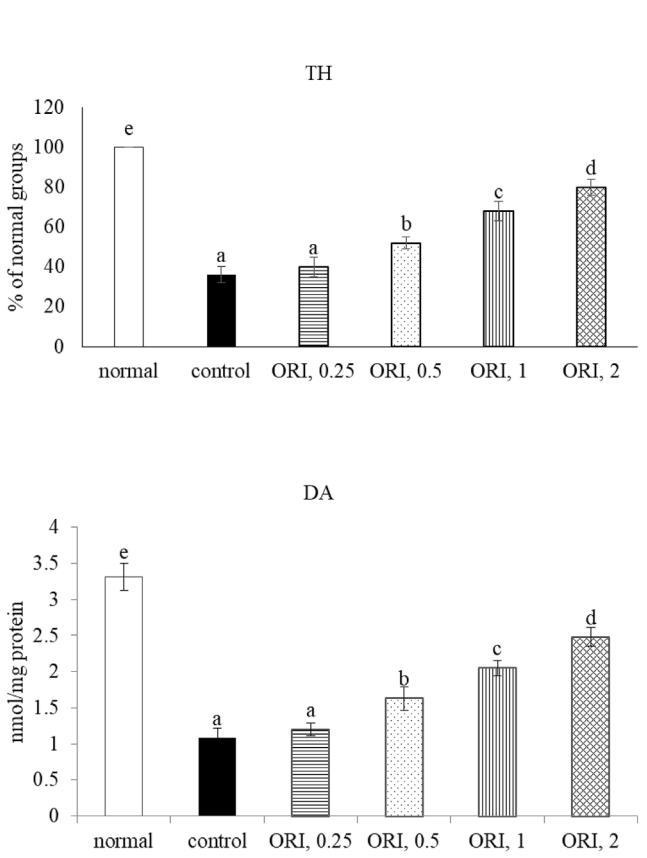

Fig. 6. Effects of oridonin (ORI) upon mRNA expression of tyrosine hydroxylase (TH, a) and dopamine (DA) content (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using 1-methyl-4-phenylpyridinium (MPP^+) to induce injury. Normal group had no ORI or MPP^+. Control group had no ORI, but with MPP^+. Data are mean ± SD (n = 8). [a-e]Values among bars without a common letter differ, $P < 0.05$.

cells increased the down-stream generation of oxidants and inflammatory cytokines, which promoted the progression of PD or seizure (Mattson and Meffert, 2006). In our present study, MPP^+ or KA exposure stimulated NF-κB binding activity, which contributed to facilitate oxidative and inflammatory reactions in NGF-differentiated PC12 cells. We found ORI pre-treatments markedly limited NF-κB binding activity, which in turn decreased the production of ROS and inflammatory cytokines. These results revealed that ORI might ameliorate PD and seizure associated oxidative and inflammatory stress via inhibiting NF-κB activation.

TH is a key enzyme responsible for the biosynthesis of DA and other catecholamines such as noradrenaline and adrenaline (Laverty, 1978). The study of Nagatsu and Sawada (2007) found that both activity and mRNA level of TH were decreased in substantia nigra and striatum of brain tissues from PD patients. Thus, restoring TH expression or activity has been considered as a therapeutic target for PD because DA and

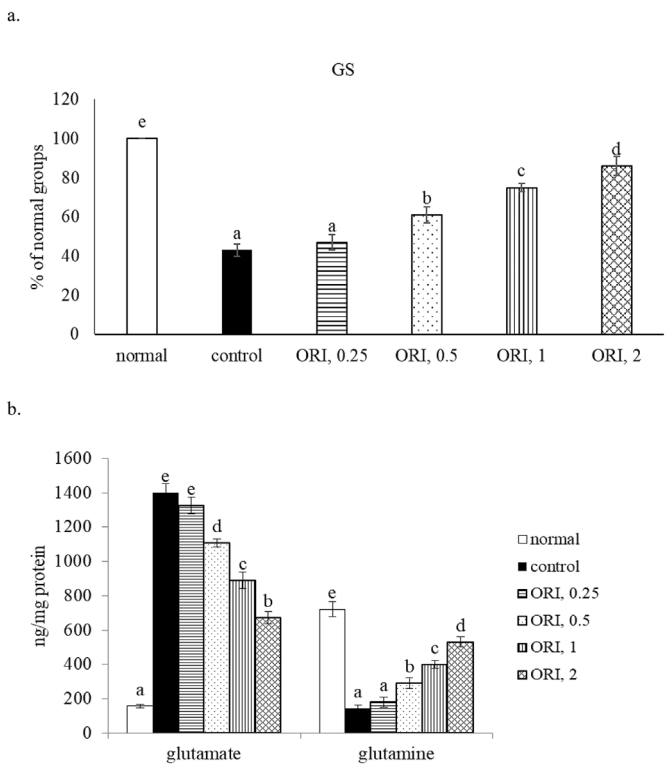

Fig. 7. Effects of oridonin (ORI) upon mRNA expression of glutamine synthetase (GS, a) and glutamine and glutamate levels (b). NGF-differentiated PC12 cells were pre-treated with ORI at 0.25, 0.5, 1 or 2 μM, and followed by using kainic acid (KA) to induce injury. Normal group had no ORI or KA. Control group had no ORI, but with KA. Data are mean ± SD (n = 8). [a-e]Values among bars without a common letter differ, $P < 0.05$.

other catecholamines are crucial neurotransmitters in the central nervous system (Feve, 2012). In addition, it is reported that TH itself is a major target of oxidative or nitrosative injury in PD's pathogenesis (Khan et al., 2012). Our data revealed that ORI pre-treatments at 0.25–2 μM diminished MPP^+ induced oxidative stress, and at 0.5–2 μM increased TH mRNA expression and DA content in NGF-differentiated PC12 cells. Obviously, ORI could benefit DA biosynthesis via alleviating oxidative stress and maintaining TH mRNA expression. These results suggested that ORI could be considered as a potent anti-PD agent because it was able to restore TH expression and increase DA level.

Glutamate accumulation in neuronal system evokes excitation, which plays an important role in the pathological development of seizure (During and Spencer, 1993). GS could convert glutamate to glutamine, and attenuate glutamate excitotoxicity (van Gassen et al., 2009). It is reported that lower GS mRNA expression due to KA exposure in rats weakened this conversion, led to glutamate accumulation, induced synaptic excitation and caused seizure attack (Kanamori, 2017). We found that ORI pre-treatments at 0.5–2 μM markedly upregulated GS mRNA expression under KA exposure condition. The lower glutamate level and greater glutamine level in ORI pre-treated NGF-differentiated PC12 cells agreed that the GS regulated conversion had been improved. These results revealed that ORI was a potent anti-seizure agent because it could benefit GS mRNA expression and diminish glutamate excitotoxicity. It is reported that massive glutamates interfere cellular uptake for cysteine and decrease GSH biosynthesis due to the deficiency of its precursor, cysteine (Lewerenz et al., 2006). Since ORI pre-treatments lowered glutamate accumulation, the greater GSH production under KA exposure condition could be partially explained.

In conclusion, oridonin is a diterpenoid naturally occurred in some medicinal plants. Our present study indicated that ROI could provide multiple protective activities in PD and seizure cell models. The natural property of ORI and our observations suggest that this agent at appropriate dosages could be applied for the prevention and/or amelioration of PD and seizure.

Conflicts of interest

All authors stated that no Conflict of Interest.

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Transparency document

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References

Bademci, G., Vance, J.M., Wang, L., 2012. Tyrosine hydroxylase gene: another piece of the genetic puzzle of Parkinson's disease. CNS Neurol. Disord. - Drug Targets 11, 469–481.
Chen, Y.M., Liu, S.P., Lin, H.L., Chan, M.C., Chen, Y.C., Huang, Y.L., Tsai, M.C., Fu, R.H., 2015. Irisflorentin improves α-synuclein accumulation and attenuates 6-OHDA-induced dopaminergic neuron degeneration, implication for Parkinson's disease therapy. Biomedicine 5, 4.
Devi, L., Raghavendran, V., Prabhu, B.M., Avadhani, N.G., Anandatheerthavarada, H.K., 2008. Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. J. Biol. Chem. 283, 9089–9100.
During, M.J., Spencer, D.D., 1993. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. Lancet (London, England) 341, 1607–1610.
Eid, T., Tu, N., Lee, T.S., Lai, J.C., 2013. Regulation of astrocyte glutamine synthetase in epilepsy. Neurochem. Int. 63, 670–681.
Ellis, D.Z., Rabe, J., Sweadner, K.J., 2003. Global loss of Na, K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis. J. Neurosci. 23, 43–51.
Feve, A.P., 2012. Current status of tyrosine hydroxylase in management of Parkinson's disease. CNS Neurol. Disord. - Drug Targets 11, 450–455.
Hald, A., Lotharius, J., 2005. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? Exp. Neurol. 193, 279–290.
Han, J.Y., Ahn, S.Y., Kim, C.S., Yoo, S.K., Kim, S.K., Kim, H.C., Hong, J.T., Oh, K.W., 2012. Protection of apigenin against kainate-induced excitotoxicity by anti-oxidative effects. Biol. Pharm. Bull. 35, 1440–1446.
Hosseinzadeh, L., Monaghash, H., Ahmadi, F., Ghiasvand, N., Shokoohinia, Y., 2017. Bioassay-guided isolation of neuroprotective fatty acids from Nigella sativa against 1-methyl-4-phenylpyridinium-induced neurotoxicity. Pharmacogn. Mag. 13, 627–633.
Hou, C.W., 2011. Pu-Erh tea and GABA attenuates oxidative stress in kainic acid-induced status epilepticus. J. Biomed. Sci. 18, 75.
Huang, H.L., Lin, C.C., Jeng, K.C., Yao, P.W., Chuang, L.T., Kuo, S.L., Hou, C.W., 2012. Fresh green tea and gallic acid ameliorate oxidative stress in kainic acid-induced status epilepticus. J. Agric. Food Chem. 60, 2328–2336.
Kanamori, K., 2017. Faster flux of neurotransmitter glutamate during seizure - evidence from 13C-enrichment of extracellular glutamate in kainate rat model. PLoS One 12, e0174845.
Khan, M.S., Tabrez, S., Priyadarshini, M., Priyamvada, S., Khan, M.M., 2012. Targeting Parkinson's - tyrosine hydroxylase and oxidative stress as points of interventions. CNS Neurol. Disord. - Drug Targets 11, 369–380.
Laverty, R., 1978. Catecholamines: role in health and disease. Drugs 16, 418–440.
Lewerenz, J., Klein, M., Methner, A., 2006. Cooperative action of glutamate transporters and cystine/glutamate antiporter system Xc- protects from oxidative glutamate toxicity. J. Neurochem. 98, 916–925.
Li, C., Wang, Q., Shen, S., Wei, X., Li, G., 2018. Oridonin inhibits VEGF-A-associated angiogenesis and epithelial-mesenchymal transition of breast cancer in vitro and in vivo. Oncol. Lett. 16, 2289–2298.
Liu, W., Kong, S., Xie, Q., Su, J., Li, W., Guo, H., Li, S., Feng, X., Su, Z., Xu, Y., Lai, X., 2015. Protective effects of apigenin against 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells. Int. J. Mol. Med. 35, 739–746.
Liu, W.B., Zhou, J., Qu, Y., Li, X., Lu, C.T., Xie, K.L., Sun, X.L., Fei, Z., 2010. Neuroprotective effect of osthole on MPP + -induced cytotoxicity in PC12 cells via inhibition of mitochondrial dysfunction and ROS production. Neurochem. Int. 57, 206–215.
Malva, J.O., Silva, A.P., Cunha, R.A., 2003. Presynaptic modulation controlling neuronal excitability and epileptogenesis: role of kainate, adenosine and neuropeptide Y receptors. Neurochem. Res. 28, 1501–1515.
Mark, R.J., Hensley, K., Butterfield, D.A., Mattson, M.P., 1995. Amyloid beta-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca2 + homeostasis and cell death. J. Neurosci. 15, 6239–6249.
Mattson, M.P., Meffert, M.K., 2006. Roles for NF-kappaB in nerve cell survival, plasticity,

and disease. Cell Death Differ. 13, 852–860.
Nagatsu, T., Mogi, M., Ichinose, H., Togari, A., 2000. Cytokines in Parkinson's disease. J. Neural Transm. Suppl. 58, 143–151.
Nagatsu, T., Sawada, M., 2007. Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects. J. Neural Transm. Suppl. 72, 113–120.
Richardson, J.R., Caudle, W.M., Wang, M., Dean, E.D., Pennell, K.D., Miller, G.W., 2006. Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. FASEB J. 20, 1695–1697.
Smeyne, R.J., Jackson-Lewis, V., 2005. The MPTP model of Parkinson's disease. Brain Res. Mol. Brain Res. 134, 57–66.
Takanashi, J., Mizuguchi, M., Terai, M., Barkovich, A.J., 2015. Disrupted glutamate-glutamine cycle in acute encephalopathy with biphasic seizures and late reduced diffusion. Neuroradiology 57, 1163–1168.
Torlinska, T., Grochowalska, A., 2004. Age-related changes of Na^+, K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in rat brain synaptosomes. J. Physiol. Pharmacol. 55, 457–465.
van Gassen, K.L., van der Hel, W.S., Hakvoort, T.B., Lamers, W.H., de Graan, P.N., 2009. Haploinsufficiency of glutamine synthetase increases susceptibility to experimental febrile seizures. Genes Brain Behav. 8, 290–295.
Vezzani, A., French, J., Bartfai, T., Baram, T.Z., 2011. The role of inflammation in epilepsy. Nat. Rev. Neurol. 7, 31–40.
Wang, Y.Y., Lv, Y.F., Lu, L., Cai, L., 2014. Oridonin inhibits mTOR signaling and the growth of lung cancer tumors. Anti Canccer Drugs 25, 1192–1200.
Wang, S., Yu, L., Yang, H., Li, C., Hui, Z., Xu, Y., Zhu, X., 2016. Oridonin attenuates synaptic loss and cognitive deficits in an Aβ1-42-induced mouse model of Alzheimer's disease. PLoS One 11, e0151397.
Wu, Y.X., Zhang, W., Li, J.C., Liu, N., 2012. Chemical constituents of flowers and fruits of Rabdosia excisa. Chin. J. Nat. Med. 10, 43–47.
Yadav, A., Agarwal, S., Tiwari, S.K., Chaturvedi, R.K., 2014. Mitochondria: prospective targets for neuroprotection in Parkinson's disease. Curr. Pharm. Des. 20, 5558–5573.
Yalcin, A., Armagan, G., Turunc, E., Konyalioglu, S., Kanit, L., 2010. Potential neuroprotective effect of gamma-glutamylcysteine ethyl ester on rat brain against kainic acid-induced excitotoxicity. Free Radic. Res. 44, 513–521.
Zhang, Z.Y., Daniels, R., Schluesener, H.J., 2013. Oridonin ameliorates neuropathological changes and behavioural deficits in a mouse model of cerebral amyloidosis. J. Cell Mol. Med. 17, 1566–1576.