Multifunction of Chrysin in Parkinson’s Model: Anti-Neuronal Apoptosis, Neuroprotection via Activation of MEF2D, and Inhibition of Monoamine Oxidase-B

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Supporting Information

ABSTRACT: Chrysin, a flavonoid compound existing in several plants, is applied as a dietary supplement because of its beneficial effects on general human health and alleviation of neurological disorders. However, mechanisms underlying neuroprotection of chrysin has not been fully elucidated, and the effects of chrysin on the Parkinson’s disease (PD) model in vivo have not been investigated. It is here shown that chrysin protects primary granular neurons against 1-methyl-4-phenylpyridinium ion insult via antiapoptosis by reversing the dysregulated expression of Bcl-2, Bax, and caspase 3. The mechanisms also involved activating transcriptional factor myocyte enhancer factor 2D (MEF2D) via regulation of AKT-GSK3β signaling. In this in vivo model of PD, chrysin rescued the dopaminergic neurons loss and alleviated the decrease in dopamine level induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. Moreover, chrysin markedly inhibited monoamine oxidase-B activity in vitro and in vivo. In conclusion, chrysin exerted beneficial effects to PD, possibly through multitarget mechanisms including antineuronal apoptosis, activation of the AKT-GSK3β/MEF2D pathway, and inhibition of the MAO-B activity.

KEYWORDS: chrysin, Parkinson’s disease, neuroprotection, MEF2D, MAO-B

INTRODUCTION

Parkinson’s disease (PD) ranks second for the most common neurodegenerative disorder in humans and is featured as the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). To date, it is still incurable. Currently used medications such as precursor of dopamine (levodopa) and monoamine oxidase-B (MAO-B) inhibitors (selegiline and rasagiline) have modest effects on symptoms but no obvious disease-modifying potential because their primarily target is not the alleviation of DA neuron loss. The ideal PD therapy would to treat both the symptoms and root causes, concurrently relieving the PD-associated symptoms and arresting or delaying the relentless progression of SNpc DA neuron loss.

Despite a number of candidate disease-modifying agents that have shown promising therapeutic effects in preclinical trials, no licensed PD neuroprotective drugs are currently available. In recent years, emphasis has been placed on identifying and characterizing potentially active plant-derived pharmaceutical ingredients to meet this unmet demand. Indeed, natural products remain an enormous untapped resource for the continued discovery and development of therapeutic agents for a wide range of disease conditions. Flavonoids have attracted considerable attention from researchers because they exert beneficial effects desired for human health through multiple-target mechanisms of action.

Chrysin is a bioflavonoid compound mainly isolated from honey. Propolis, several plant species, fruits, passion flowers, and even mushrooms are also rich of chrysin. Increasing lines of evidence indicate that chrysin possesses beneficial effects to neurological diseases. It was found to attenuate neurological deficit scores and infarct volumes through antineuroinflammation in a mouse model of stroke, protect against age-related memory decline through its strong antioxidative effects and through modulation of brain-derived neurotropic factor production, improve both motor and sensory functions via modulation of neuronal apoptosis and endogenous biomarkers, and so attenuate neurological deficits resulted from spinal cord injury. In addition, chrysin was found to protect 1-methyl-4-phenylpyridinium ion (MPP+) -induced mesencephalic cultures injury, which suggests that it would be beneficial for PD treatment. Our recent work examined the synergistic neuroprotective effects of chrysin and...
protocatechuic acid, two polyphenols coexisting in the fruits of *Alpinia oxyphylla*, with chrysin potentiating the protective effect of protocatechuic acid against 6-hydroxydopamine-induced PC12 cell damage and DA neurons loss in a zebrafish model. However, the therapeutic effect of chrys in 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP)-induced PD mouse model has not been investigated, and the mechanisms underlying neuroprotective effect exerted by chrysin has not been fully elucidated.

Cerebellar granule neurons (CGNs) are the most abundant and homogeneous neuronal types in the mammalian central neural system, which would undergo apoptosis when exposure to certain neurotoxins (e.g., 6-OHDA, MPP⁺, glutamate, and β-amyloid). Because of the abundance and homogeneity of CGNs and the easy and reliable reduction of apoptosis, cultured CGNs have been demonstrated to be a useful in vitro model system for the evaluation of candidate neuroprotectants.

The aim of the present study was to evaluate the protective effects of chrysin against MPP⁺-induced injury of primary CGNs in vitro and to assess the therapeutic effect of chrysin against MPTP-induced DA neuron loss in mice. The mechanisms underlying its neuroprotection were also investigated.

### MATERIALS AND METHODS

**Reagents.** Chrysin (purity ≥98) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All cell culture media and supplements were purchased from Gibco Invitrogen (Carlsbad, CA). All primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA) except the monoclonal antibody against tyrosine hydroxylase (TH) purchase from Millipore (Billerica, MA). Wortmannin and LY-294002 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), Halt phosphatase inhibitor cocktail, BCA protein assay kits, and ECL advanced Western blotting detection kit were purchased from Pierce Biotecnology (Rockford, IL). An MAO-Glo assay kit and recombinant human MAO-B (rhMAO-B) enzyme were purchased from Promega (Madison, WI). All other reagents were from Sigma-Aldrich (St. Louis, MO) except where stated otherwise.

**Primary Cerebellar Granule Neuron Culture.** Primary cerebellar granule neurons (CGNs) were isolated from the cerebellum of 8 day old Sprague–Dawley rats (Laboratory Animal Center of Guangdong Province, Guangzhou, China), as described in a previous publication. Experiments were done at 8 days in vitro (DIV) because CGNs acquired several features of mature neurons after 7 DIV culture. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jinan University.

**Drug Treatment.** Chrysin was dissolved in DMSO (50 mM stock) and then diluted in culture medium to the indicated final concentrations to pretreat cells for 2 h. The DMSO (final concentration 0.1%) was added as vehicle control, which was demonstrated to be nontoxic to cells. To evaluate the neuroprotection of chrysin, we added 150 μM MPP⁺ for another 36 h of incubation.

**Measurement of Cell Viability.** CGNs were seeded in 96 well plates at a density of 1.5 × 10⁴ per well in a total volume of 100 μL. A total of 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 mg/mL) in PBS was added for 4 h of incubation at 37 °C after drug treatment. The medium was then replaced with 100 μL of DMSO to dissolve the formazan. The absorbance was measured at 570 nm wavelength on a microplate reader (BioTek Synergy HT, Winooski, VT). Cell viability of treatment group was expressed as a percentage of the control cells.

**Fluorescein Diacetate–Propidium Iodide Double Staining Assay.** CGNs were cultivated in 6 well plates at a density of 2 × 10⁶ per well in 1.5 mL. After drug treatment, neurons were washed twice with ice-cold PBS. Following incubation with fluorescein diacetate (FDA) (10 μg/mL) and propidium iodide (PI) (5 μg/mL) for 15 min, the neurons were observed and photographed by a fluorescence microscope (BX51, Olympus Corp., Japan).

**MEF2 Luciferase Reporter Assay.** PC12 cells were transfected with a MEF2/pGreenFire1 reporter lentivector (System Biosciences, Mountain View, CA) as described previously. After transfection for 48 h, cells were incubated with serial concentrations of chrysin for 24 h, and then cellular extracts were assessed by luciferase assay system (Promega, Fitchburg, WI).

**MEF2D siRNA Transfection.** At 8 DIV of CGN in a 96 well plate, neurons were transfected with MEF2D siRNA or scrambled RNA according to the instructions of manufacturer (Santa Cruz Biotechnology). Drug treatment was performed after 48 h of transfection, and cell viability was determined by the MTT assay.

**Western Blotting.** Primary CGNs were cultured in six well plate at a density of 2 × 10⁶ per well in 1.5 mL. At 8 DIV, cells were pretreated with chrysin for 2 h before incubation with 150 μM MPP⁺ for another 12 h. After being washed with ice-cold PBS, cells were homogenized with RIPA lysis buffer containing 1 mM PMSF and 1% Hal Phosphatase Inhibitor Cocktail on ice. Lysates were then centrifuged at 12500g at 4 °C for 20 min. The concentration of protein in the supernatant was determined using the BCA protein assay kit (Pierce). Protein samples (30 μg) were separated by SDS-PAGE and transferred to PVDF membranes. After incubation with the appropriate primary antibodies (1:1000) and peroxidase-conjugated secondary antibodies (1:2500), the blots of protein of interest were visualized using an ECL advanced Western blotting detection kit. Quantitative analysis of protein bands were performed with a Molecular Imager ChemiDoc XRS equipped with a QuantityOne software (Bio-Rad, Hercules, CA).

**Animals and Treatment.** A total of 48 male C57BL/6 mice (28 ± 2 g body weight, 8–9 weeks of age) were purchased from the Laboratory Animal Center of Guangdong Province and were fed, six animals per cage, under a 12 h light/dark cycle with ad libitum access to food and water. Mice were divided into four groups (n = 12 per group) for this study. MPTP (30 mg/kg/day) was injected ip once a day for 5 consecutive days to induce subacute experimental Parkinsonism, as described previously except that the control group mice were given an equal volume of saline. On the eighth day, either chrysin (10 or 100 mg/kg/day) or vehicle (10% DMSO) was administered ip once a day for 7 days. It has been reported that DMSO is well-tolerated at 10 mL/kg for 3 days in mice (ip), and 5 mL/kg is safe in a week study. The experimental protocols were
approved by the IACUC of Jinan University (approval no SCXK2013-0034).

**Tissue Processing and Immunohistochemistry.** A total of 24 h after the last dose of drug was administered, six mice were randomly chosen from each treatment group and sacrificed. Brain-tissue processing and immunohistochemistry were performed as described previously.30,31 Briefly, mouse brains were fixed with 4% PFA after transcardiac perfusion, paraffin-embedded, and cut to make 10 μM sections for TH immunohistochemistry. The TH-positive cells were counted by observers blind to the experimental protocol on a stereomicroscope (BX51, Olympus Corp., Tokyo, Japan) using the optical fractionator method, as described previously.32,33

**Determination of Levels of Dopamine and Its Metabolites, 3,4-Dihydroxyphenylacetic Acid and Homovanillic Acid Using Electrochemical HPLC.** The striatal tissue from the remaining six mice in each group were homogenized in 0.1 M perchloric acid (HClO₄) containing 0.01% EDTA, and the homogenate was centrifuged at 10000g (4 °C) for 10 min to precipitate proteins. After filtration with 0.22 μM filter membrane, the supernatants was analyzed using a high-pressure liquid chromatography (HPLC) system coupled to a 2465 electrochemical detector (Waters) as described previously.34 Concentrations of DA and its metabolites were expressed as ng per mg of tissue weight.

**MAO-B Inhibition Activity.** The MAO-B inhibition activity of chrysin was determined according to the instructions of MAO-Glo assay kit. Briefly, chrysin at serial concentrations were incubated in 96 well opaque white plates with MAO substrate and rhMAO-B (final concentration: 0.25 mg protein/mL). Selegiline, a known MAO-B inhibitor, served as a positive control. Samples were incubated for 1 h at room temperature. Reactions were terminated by addition of luciferin detection reagent, and samples were incubated for an additional 20 min to allow the development of luciferase- and esterase-dependent luminescence. Luminescence was determined with a luminometer and was corrected for background using no-MAO-B controls. Results are presented as percent of vehicle (total MAO-B activity).

For in vivo MAO-B inhibition activity, a single dose of chrysin (10 or 100 mg/kg), selegiline (10 mg/kg), or vehicle (saline containing 10% DMSO) were given to male C57BL/6 mice ip. A total of 24 mice were used (six mice per group). Mice were killed 24 h after drug administration, and the tissue homogenates of cerebellum, liver, and intestine were prepared as reported by Tipton et al.35 MAO-B activity of homogenates was determined as aforementioned except no rhMAO-B or

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**Figure 1.** Prevention of MPP⁺-induced neuronal damage to CGNs. (A) Chrysin increases the cell viability of MPP⁺-treated CGNs. CGNs were treated with chrysin at the indicated concentrations or with vehicle for 2 h and then exposed to 150 μM MPP⁺ for 36 h. Cell viability was measured using an MTT assay. Data from three independent experiments were expressed as mean ± SEM; ###, P < 0.001 versus control; *, P < 0.05; and **, P < 0.01 versus vehicle treatment, respectively. (B) Chrysin blocks MPP⁺-induced CGNs death. CGNs were preincubated with 25 μM chrysin or vehicle and then exposed to 150 μM MPP⁺. At 36 h after the MPP⁺ challenge, viable and dead CGNs were assayed with FDA–PI double staining. Scale bar: 20 μM.
Inhibitory drugs were added. The percentage of MAO-B inhibition was calculated as follows: \( \frac{(A_{\text{vehicle}} - A_{\text{drug}})}{A_{\text{vehicle}}} \times 100\% \), where \( A_{\text{vehicle}} \) and \( A_{\text{drug}} \) mean MAO-B activity of vehicle-treated sample and drug-treated sample, respectively.

**Molecular Docking.** Molecular docking analyses were performed by SYBYL software (Tripos Inc., St. Louis, MO).\(^{36}\) Three-dimensional (3D) crystal structure of MAO-B in complex with safinamide, a selective inhibitor, was retrieved from the protein data bank (PDB code: 2VSZ).\(^{37}\) The 3D structure of MAO-B in complex with chrysin was constructed by SYBYL standard geometric parameters and then was optimized using the Powell method. Docking analysis was accomplished with the Surflex-Dock program, which uses an empirically derived scoring function based on the binding affinities of protein–ligand complexes and has been proven to be efficient in treating various receptors.\(^{38}\) The active site of MAO-B was defined relative to the coordinates of safinamide. The rotatable bonds of the ligands were defined, and the receptor was kept rigid during the simulations. Surflex-Dock scores were used to estimate the binding affinity, which were expressed in \(-\log_{10} (K_d)\) units.

**Data and Statistical Analysis.** Statistical analysis was undertaken using Prism 6.0 software (GraphPad Software). All data are here expressed as mean ± SEM, and multiple comparisons were assessed using one-way ANOVA followed by the Tukey’s post hoc test. The level of significance was set to \( P < 0.05 \).

# RESULTS

**Chrysin Prevention of Primary CGNs from MPP⁺-Induced Neuronal Damage In Vitro.** In the pilot study, MPP⁺ decreased the cell viability of CGNs in a concentration- and time-dependent manner (data not shown). MPP⁺ at 150 \( \mu \text{M} \) for 36 h treatment caused a decrease of 50.58 ± 7.26% in CGN viability; thus, this treatment condition was used in the following study except where otherwise stated. Pretreatment of chrysin, at the concentration range 6−25 \( \mu \text{M} \), significantly and concentration-dependently increased the cell viability of CGNs insulted by MPP⁺ (Figure 1A). To further verify the protective effects of chrysin against neurotoxicity induced by MPP⁺, we examined the neurons by FDA–PI double staining. Chrysin visibly increased the number of viable neurons (FDA positive) and decreased the number of dead neurons (PI positive) (Figure 1B).

It has been shown that chrysin can prevent tunicamycin-induced SH-SY5Y cell death via inhibition of caspase 3 activity and mitochondrial apoptotic pathways,\(^{39} \) which were also involved in MPP⁺-induced damage of mesencephalic DA neurons.\(^{40} \) Thus, proteins associated with apoptosis, including Bcl-2, Bax, cleaved caspase 3 (c-caspase 3), and caspase 3, were examined using Western blotting. Pretreatment of chrysin attenuated the MPP⁺-induced down-regulation of antiapoptotic protein Bcl-2, the up-regulation of pro-apoptotic Bax proteins, and c-caspase in a concentration-dependent manner (Figure 2A). The increased ratio of Bax/Bcl-2 and c-caspase/caspase was also alleviated by pretreatment of chrysin. This was even completely reversed by 25 \( \mu \text{M} \) chrysin (Figure 2B). The antiapoptotic effect of chrysin was also verified on a zebrafish model in vivo. Chrysin at 6−12 \( \mu \text{M} \) significantly inhibited l-hydroxyglutaric-acid-induced neuronal apoptosis in a concentration-dependent manner (Supplementary Figure 1).

**Contribution of Reversal of MEF2D Dysregulation to Protection of Chrysin against MPP⁺ Neurotoxicity.** Increasing amounts of evidence indicate that MEF2, MEF2D in particular, plays a critical role in neuronal survival of DA neurons\(^{38−40} \) and in MPP⁺ causing damage to DA neurons via inhibition of MEF2D transcriptional activity.\(^{41} \) For this reason, the possibility that chrysin might activate MEF2D against MPP⁺ insult was here examined. In consistent with previous finding,\(^{42} \) the expression of MEF2D protein dramatically decreased by MPP⁺ treatment (Figure 3A,B). Pretreatment of chrysin greatly reversed the inhibition of MEF2D expression caused by MPP⁺, and the expression level of the 25 \( \mu \text{M} \) chrysin-treated group was even higher than that of normal control cells (Figure 3A,B). The effect of chrysin on MEF2D transcriptional activity was tested further using a MEF2-dependent luciferase reporter assay. Chrysin visibly increased the MEF2 transcriptional activity in a concentration-dependent manner (Figure 3C). More importantly, the genetic silence of MEF2D by siRNA completely abolished the effect of chrysin on protecting CGNs from MPP⁺-induced neurotoxicity (Figure 3D), suggesting that the neuroprotection of chrysin was found to be dependent primarily on MEF2D.

**Chrysin Activation of MEF2D through Regulation of the Akt-GSK3β Pathway.** Because the MEF2 activity has been demonstrated to be regulated by the Akt-GSK3β pathway,\(^{39,41} \) the possibility that chrysin might enhance MEF2 activity through the regulation of Akt-GSK3β pathway was here evaluated. Chrysin significantly reversed the reduction of phosphorylation of Akt at Ser473 and phosphorylation of GSK3β at Ser9 in a concentration-dependent manner (Figure 4A,B). Further analysis indicated that LY294002, a specific inhibitor of PI3K, significantly abolished the MEF2D tran-
Figure 3. Involvement of MEF2D activation in the protection of chrysain against MPP⁺ neurotoxicity. (A,B) Chrysin reverses the down-regulation of MEF2D by MPP⁺ treatment in CGNs. CGNs were pretreated with chrysin or vehicle control for 2 h and then treated with 150 μM MPP⁺ for 36 h. The total proteins were extracted for Western blot analysis using specific antibodies. Representative blots are shown in (A), and densitometric analysis from three independent experiments is shown in (B). (C) Chrysin stimulates MEF2 transcriptional activation using a MEF2 transcriptional reporter assay. PC12 cells were transfected with MEF2 luciferase reporter construct, treated for 2 h with chrysin. The luciferase reporter activity was measured 24 h after treatment. UC, untransfected control; TC, transfected control without chrysin treatment. (D) Down-regulation of MEF2D by MEF2D siRNA abolishes the neuroprotection of chrysin. CGNs were transfected with MEF2D siRNA or scrambled RNA. A total of 48 h after transfection, CGNs were pretreated with 25 μM chrysin for 2 h or left untreated, exposed to MPP⁺, and then subjected to MTT assay for the measurement of cell viability. Data from three independent experiments are here expressed as mean ± SEM. In (A) and (D), #, P < 0.05 and ##, P < 0.01 vs control; and *, P < 0.05 and **, P < 0.01 vs vehicle treatment. In (C), *, P < 0.05; **, P < 0.01; and ***, P < 0.001 vs transfected control (TC) without chrysin treatment.

Figure 4. Chrysin potentiation of Akt against MPP⁺ to down-regulate MEF2D negative regulator GSK3β in CGNs. (A,B) Chrysin reverses the MPP⁺-induced decrease of phospho-Ser473 Akt (p-Akt) and phospho-Ser9 GSK3β (p-GSK3β). CGNs were pretreated with chrysin for 2 h and then exposed to MPP⁺, and cell lysates were immunoblotted with the indicated antibodies. Representative blots and densitometric analysis are shown in (A) and (B). Data from three independent experiments are expressed as mean ± SEM; #, P < 0.01 vs control; **, P < 0.01; and ***, P < 0.01 vs vehicle group. (C) Effects of Akt pathway inhibitor LY294002 and GSK3β inhibitor on MEF2D transcriptional activity. **, P < 0.01 and ***, P < 0.001 vs transfected control (TC) without drug treatment; and $**, P < 0.01 vs chrysin group. (D) Effects of Akt pathway inhibitors LY294002 and wortmannin (Wort), and GSK3β inhibitor on MPP⁺-induced neurotoxicity in CGNs. CGNs were pretreated with LY294002 (1 μM), wortmannin (2 μM), or LiCl (10 μM) for 2 h, incubated with or without chrysin for 2 h, and finally exposed to MPP⁺. Cell viability was examined using an MTT assay. Data from three independent experiments are here expressed as mean ± SEM $**, P < 0.01 vs untreated control; $***, P < 0.01 vs MPP⁺ group; $**, P < 0.01 vs chrysin plus MPP⁺ group.
scriptional activation exerted by chrysin, and GSK3β inhibitor LiCl treatment alone notably activated MEF2D transcriptional activity (Figure 4C). Consistent with this, LY294002 and another PI3K inhibitor, wortmannin, significantly abolished the neuroprotection exerted by chrysin, while LiCl treatment alone substantially protected cells (Figure 4D). It suggests that the Akt-GSK3β/MEF2D pathway was involved in the neuroprotective effect of chrysin against MPP+-induced neurotoxicity in CGNs.

Chrysin Rescue of SNpc DA Neurons from MPTP-Induced Death and Attenuated Decrease of Dopamine and Its Metabolites Level in Mice. The effect of chrysin on the loss of SNpc DA neurons was further tested in MPTP-induced C57BL/6 mice, which is a widely accepted in vivo model of PD. MPTP resulted in a significant loss of TH-positive DA neurons in the SNpc. Mice treated with chrysin at 100 mg/kg post-MPTP injection retained much more DA neurons than that of vehicle treatment (Figure 5A,B). Consistent with the change in SNpc DA neurons, levels of the striatal dopamine and its metabolites DOPAC and HVA all decreased dramatically by MPTP injection, and chrysin treatment attenuated the decrease in the striatal contents of dopamine, DOPAC and HVA (Figure 5C). These results suggest that chrysin protected SNpc DA neurons from MPTP-induced toxicity in vivo effectively.

Chrysin Inhibition of MAO-B Activity in Vitro and in Vitro. It has been reported that several compounds derived from Ginkgo biloba, including chrysin, have in vitro MAO-inhibiting properties. The MAO-B inhibition of chrysin was investigated both in vitro and in vivo and by computational simulation. Chrysin inhibited MAO-B activity in a concentration-dependent manner with an IC₅₀ of 12.3 μM in vitro (Figure 6A). Similar to a previous report, the positive control selegiline significantly inhibited MAO-B with an IC₅₀ of 0.08 μM. The ip injection of chrysin notably inhibited MAO-B activity in the mice brain and intestine but not liver (Figure 6B). Selegiline potently inhibited MAO-B in all three tissues. Molecular docking analysis revealed that chrysin occupied the MAO-B active site (PDB code: 2V5Z), which consists of a substrate cavity in front of the flavin and an entrance cavity close to the loop from the Phe99 to Tyr112, with a Surflex-Dock score of 6.28 (Figure 6C). As a reference, a Surflex-Dock of 5.17 was obtained for safinamide, a compound known to bind to MAO-B (Figure 6C). Chrysin might form two hydrogen bonds with side chains of Pro102 and Try326, respectively. The dihydroxy-chromen moiety of chrysin was located near the entrance cavity, and the benzene ring of chrysin was predicted to occupy the substrate cavity.

DISCUSSION

The protective effect of chrysin against neurotoxin insult to neurons has been investigated previously. TUNEL and TH immunohistochemistry were used to demonstrate that chrysin at 40 μM could protect mesencephalic cultures from injury by MPP⁺. In SH-SYSY cells, chrysin at 4–20 μM prevented neuronal cell death via inhibition of caspase-3 activity and the mitochondrial apoptosis pathway in a concentration-dependent
MPP⁺-induced CGNs insult was used to investigate the neuroprotective effect of chrysin. Our results are consistent with aforementioned findings; at similar concentration ranges, 3–25 μM chrysin protected CGNs from MPP⁺ insult in a concentration-dependent manner, possibly through antineuronal apoptosis effect, as evidenced by the reversal of the altered expression ratio of pro-apoptotic marker Bax to the antiapoptotic marker Bcl-2 and the activation of caspase-3. The antiapoptotic effect of chrysin was confirmed in LGA-induced neuronal apoptosis in zebrafish in vivo.

The molecular mechanisms underlying DA neuron death still remain elusive. It is well-accepted to be multifactorial, and oxidative stress plays a pivotal role in the pathogenesis of this disease. Oxidative stress can be generated from multiple sources, including dopamine metabolism, mitochondrial dysfunction, neuroinflammation, iron homeostasis, and aging. Because all of the aforementioned processes nearly regulate the survival factor MEF2D, targeting MEF2D is an attractive therapeutic strategy. It has been reported that the PD-related neurotoxin MPP⁺ can damage DA neurons via the inhibition of MEF2D transcriptional activity. Enhancing MEF2D by recombinant viral approach in vivo was found to protect SNpc DA neurons from PD-associated neurotoxicity. However, this recombinant viral approach obviously limits its practical applications. The present study demonstrated that the natural flavonoid chrysin is a potent agent of MEF2D activator with the capability of protecting DA neuronal cells from MPP⁺-induced damage. Although it is possible that there are other chrysin effectors, MEF2D is believed to be the major downstream mediator of its neuroprotective effect in this model system because chrysin was found to significantly up-regulate MEF2D protein expression and increase MEF2 transcriptional activity in a concentration-dependent manner. In addition, knockdown of MEF2D was found to completely block the effect of chrysin with respect to protecting cells from toxic stress. Chrysin may provide an effective and practical approach to modulation of MEF2 activity in neurons therapeutically in vivo.

Several studies suggest that MEF2D is tightly regulated in neurons by several signaling pathways including Akt-GSK3β, which directly phosphorylates MEF2D to regulate its activity. The PI3K/Akt pathway has been shown to participate in the death and survival of DA neurons in PD model. Inhibition of GSK3β prevents SH-EP1 cells from MPP⁺-induced death in vitro and DA neurons from MPTP toxicity in mice. The current MPP⁺ model reveals clearly that chrysin modulates MEF2 through multiple mechanisms to synergistically increase neuroprotective activity, including the down-regulation of MEF2 inhibitor GSK3β via activation of Akt and elevation of MEF2D expression via yet-unknown signaling pathways.

Figure 6. Inhibition of MAO-B activity of chrysin. (A) Chrysin inhibited MAO-B activity in vitro. (B) Chrysin inhibited MAO-B activity in vivo. (C) Molecular docking simulation of interaction between chrysin with MAO-B. Left panel: low-energy conformation of chrysin bound to the pocket of human MAO-B generated by molecular-docking software (SYBYL, Tripos Inc., St. Louis, MO). The human MAO-B was displayed in ribbon form. Chrysin was depicted as a ball-and-stick model showing carbon (white), oxygen (red), and hydrogen (blue). The binding surface of MAO-B was represented as a green translucent surface, and the flavin cofactor was depicted as cyan. Right panel: overlay of chrysin with a reported MAO-B inhibitor safinamide (purple).
mechanisms. It is notable that chrysin at the concentration of 6 μM increases GSK3β phosphorylation significantly but mildly increases Akt phosphorylation without statistical significance. Although AKT inhibits GSK3β by phosphorylating Ser9, GSK3β is also modulated by the Wnt signaling cascade. It has been reported that methylated chrysin induces suppression of the canonical Wnt signaling pathway with a simultaneous elevation in the expression of phospho-GSK3β/Ser9 in the early hepatocarcinogenesis rat model. Therefore, the effect of chrysin on the Wnt signaling cascade in our present model shall warrant further in-depth investigation.

Our recent publication has demonstrated that the oral extract of Fructus A. oxyphylla, which contains chrysin, protects PC12 cells from 6-hydroxydopamine-induced damage, as least in part, via the activation of the P13K/Akt pathway and the combination of chrysin and protocatechuic acid can simultaneously activate nuclear factor-erythroid 2-related factor 2 (Nrf2) and inhibit nuclear factor-κB (NF-κB) pathways in the context of 6-hydroxydopamine-induced neurotoxicity, exhibiting its enhanced neuroprotective effects through antioxidant and anti-inflammation. Specifically, chrysin at the concentration of 6 μM increases cell viability significantly; it, however, mildly increases AKT phosphorylation but without statistical significance. The discrepancy implies that other than AKT signaling, mechanisms including the activation of Nrf2 and the inhibition of NF-κB may also contribute to the neuroprotection of chrysin on MPP+ neurotoxicity. This indicates that chrysin modulates multiple targets to protect neurons from MPP+ insult. This one-molecule-multitarget agent could be more effective in treating PD, which is multifactorial in nature.

MAO-B mainly metabolizes dopamine. PD is associated with elevated levels of MAO-B in the brain. MAO-B inhibitors block the dopamine metabolism in the brain, making more dopamine available and providing modest benefit for the motor features of PD. The neuroprotective effect of MAO-B inhibitors has remained controversial. Numerous studies have reported that rasagiline can prevent cell death induced by various stimuli. However, that is independent of its MAO-B inhibition. Chrysin notably inhibited the MAO-B activity in vitro and in vivo. Computational simulation postulates that chrysin may act as a reversible MAO-B inhibitor because no potential covalent bonds between MAO-B and chrysin can be formed. Importantly, the IC₅₀ of in vitro MAO-B inhibition is about 12.5 μM, which is also the effective concentration to provide neuroprotection in vitro. If such a concentration can be achieved in vivo, chrysin may provide symptom-relieving benefit and disease-modifying potential concurrently for PD therapy.

The beneficial effect of chrysin is dependent on its bioavailability and achievable drug concentration in vivo. It has been reported that the oral bioavailability of chrysin is only 0.03−0.02%, and the maximum serum concentration for flavonoid aglycones is estimated to 1 μM in general. In the present study, it has been demonstrated that in vivo neuroprotection and MAO-B inhibition by ip injection of chrysin. However, the preferred method of PD drug administration is the oral route. Further development of new oral formulations to improve the bioavailability of chrysin for the treatment of PD is urgently needed.

Current PD therapies mainly offer symptomatic relief with a marginal effect on the disease modification because no neuroprotective therapy is available for use in clinical settings. The development of multifunctional agents targeting both sympatric relief and neuroprotection is the central challenge of further PD therapy. The present study showed that the natural flavonoid chrysin protected CGNs from MPP+−induced neurotoxicity in vitro, rescued SNpc DA neurons from MPTP-induced death, and attenuated depletion of dopamine and its metabolites in mice, possibly through multifunctional mechanisms of action including antineuronal apoptosis, neuro-protection via activating the AKT/GSK3β/MEF2D pathway, and inhibition of MAO-B activity. Chrysin may provide symptom-relieving benefits and a disease-modifying potential concurrently for PD therapy.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website via DOI: 10.1021/acs.jafc.6b01707.

A description of LGA-induced neuronal apoptosis. A figure showing chrysin-inhibited neuronal apoptosis in zebrafish larvae induced by LGA. (PDF)

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### Funding

This study was supported by grants from by the Natural National Science Foundation of China (NSFC 81303251), the Natural Science Foundation of Guangdong Province (2015A030313317), and the Science and Technology Program of Guangzhou (20141400097) and partially by grants from the Science and Technology Development Fund (FDCT) of Macao SAR (ref no. 134/2014/A3), the Research Committee of University of Macau (MYRG139(Y1-L4)-ICMS12-LMY and MYRG2015-00214-ICMS-QRCM), and the Overseas and Hong Kong, Macau Young Scholars Collaborative Research Fund by the Natural National Science Foundation of China (81328025).

Notes: The authors declare no competing financial interest.

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DOI: 10.1021/acs.jafc.6b01707

*J. Agric. Food Chem.* 2016, 64, 3224–3233


