Identification of Licopyranocoumarin and Glycyrurol from Herbal Medicines as Neuroprotective Compounds for Parkinson’s Disease

Takahiro Fujimaki1, Shinji Saiki2, Etsu Tashiro1, Daisuke Yamada2, Mitsuhiro Kitagawa1, Nobutaka Hattori2, Masaya Imoto1*

1 Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama, Japan, 2 Department of Neurology, Juntendo University School of Medicine, Bunkyo, Tokyo

Abstract

In the course of screening for the anti-Parkinsonian drugs from a library of traditional herbal medicines, we found that the extracts of choi-joki-to and daio-kanzo-to protected cells from MPP⁺⁻ induced cell death. Because choi-joki-to and daio-kanzo-to commonly contain the genus Glycyrrhiza, we isolated licopyranocoumarin (LPC) and glycyrurol (GCR) as potent neuroprotective principals from Glycyrrhiza. LPC and GCR markedly blocked MPP⁺⁻ induced neuronal PC12D cell death and disappearance of mitochondrial membrane potential, which were mediated by JNK. LPC and GCR inhibited MPP⁺⁻ induced JNK activation through the suppression of reactive oxygen species (ROS) generation, thereby inhibiting MPP⁺⁻ induced neuronal PC12D cell death. These results indicated that LPC and GCR derived from choi-joki-to and daio-kanzo-to would be promising drug leads for PD treatment in the future.

Introduction

Parkinson’s disease (PD) is a common neurodegenerative disease characterized by progressive dopaminergic neuronal cell death in the substantia nigra pars compacta of the midbrain. The main symptoms of PD are movement disorders such as tremors, bradykinesia/akinesia, rigidity, postural instability, and gait abnormalities. Although deep-brain stimulation and oral administration of L-dopa, dopamine agonists and amantadine hydrochloride have been well established as symptomatic treatments, there are no therapies to completely cure patients with the disorder [1]. Mitochondrial dysfunction, especially dysfunction of the mitochondrial electron transport chain mainly relying on complex I activity, has been implicated in the disease’s pathogenesis. In addition to defects of complex I in postmortem brains, skeletal muscle and platelets of patients with PD [2,3,4,5,6], cybrid cells containing mtDNA derived from PD platelets have indicated complex I defects [7,8,9]. Because various rodent models treated with mitochondrial toxins such as rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺) show motor deficits associated with selective loss of dopaminergic neurons, they have been widely used as acquired PD models [10,11,12,13,14,15]. Selegiline, a medication widely used at present, has the capacity to protect dopamine neurons by inhibiting MAO-B oxidation for conversion of MPTP into MPP⁺ and blocking the formation of free radicals derived from the oxidative metabolism of dopamine [16,17]. Also, MPP⁺ models offer unexploited therapeutic potential for some atypical antipsychotics (olanzapine, aripiprazole, and ziprasidone) and the anticonvulsant zonisamide in PD, and new mechanisms of neuroprotective effects of FLZ (which activates HSP27/HSP70) and paenomillin (which modulates autophagy) have led to treatments for PD [18,19,20,21].

Herbal medicines are employed to treat PD in ancient medical systems in Asian countries such as India, China, Japan, and Korea based on anecdotal and experience-based theories [22]. The traditional herbal medicines yi-gan san and modified yeolddahanso-tang have neuroprotective effects and can rescue dopaminergic neurons from MPP⁺/MPTP toxicity using both in vitro and in vivo methods [23,24]. Several compounds derived from herbal medicines also exert anti-Parkinsonian activities. For instance, ginsenoside Rh1 isolated from Panax ginseng C. A. Meyer, 5-O-demethylswertipunicoside isolated from S. punica, and salidroside isolated from Rhodiola rosea L., have been reported to attenuate MPP⁺ induced neurotoxicity in PC12 cells in vitro [25,26,27]. However, clinical evidence for the efficacy and safety of these herbal medicines for PD is
Therefore, in this study, we screened a library containing 128 traditional herbal medicines, which have been used clinically for at least 10 years in Japan, focusing on their neuroprotective effects using PD-like cellular models of cell death by mitochondrial toxins, and found the anti-Parkinsonian herbal medicines *choi-joki-to* and *daio-kanzo-to*. Moreover, we identified licopyranocoumarin and glycyrrul from the genus *Glycyrrhiza* as common components contained in these two herbal medicines, and found they exerted neuroprotective effects against MPP⁺-induced toxicity.

**Results**

**Identification of *choi-joki-to* and *daio-kanzo-to* as potent neuroprotective herbal medicines using in vitro PD-like model screening**

Rotenone, a direct inhibitor of mitochondria complex I, is usually employed to mimic Parkinsonism in vitro and in vivo [29]. Treatment of NGF-differentiated PC12D cells [30] with 0.3 μM rotenone for 48 h caused marked cell death as evaluated by the trypan blue dye exclusion assay. Using this PD-like model, we screened a library containing 128 traditional herbal medicines, which have been used clinically in Japan, focusing on preventive effects against rotenone-induced cell death of NGF-differentiated PC12D cells.

As a result, several ethyl acetate (EtOAc) extracts of herbal medicines showed suppressive effects against rotenone-induced cell death generally, but two traditional herbal medicines, *choi-joki-to* and *daio-kanzo-to* exerted significant neuroprotective effects against rotenone-induced neurotoxicity (Figure 1A). Furthermore, the EtOAc extracts of *choi-joki-to* or *daio-kanzo-to* also conferred dose-dependent protection from neuronal cell death induced by MPP⁺, another well-known PD-like cellular model (Figure 1B).
Licopyranocoumarin and glycyrurol isolated from Glycyrrhiza as potent neuroprotective compounds

Next, we attempted to identify the major components responsible for neuroprotective effects contained in choi-joki-to and daio-kanzo-to. First, we noted that both choi-joki-to and daio-kanzo-to commonly contain rhubarb and Glycyrrhiza species, at the ratio of 2:1 (Table 1). Therefore, we examined whether this 2:1 ratio of rhubarb to Glycyrrhiza is important for neuroprotective effects against MPP+-induced toxicity. As shown in Figure 2, rhubarb and Glycyrrhiza contained in choi-joki-to and daio-kanzo-to at 2:1 is not a special ratio necessary for neuroprotective effects, but rather increased Glycyrrhiza content potentiated the neuroprotective activity against MPP+-induced cell death. Thus, we attempted to isolate the active principle responsible for neuroprotective effects from EtOAc extract of Glycyrrhiza by monitoring the inhibitory activity of MPP+-induced NGF-differentiated PC12D cell death using a trypan blue dye exclusion assay. As a result, we isolated 10.8 mg of licopyranocoumarin (LPC) and 4.0 mg of glycyrurol (GCR) from 50 g of Glycyrrhiza powder as potent neuroprotective compounds (Figure 3A, B). Both LPC and GCR markedly blocked MPP+-induced cell death in a dose-dependent manner with IC50 values of 0.9 μM and 1.2 μM, respectively (Figure 3C). Furthermore, both LPC and GCR did not show cytotoxic effects against other toxins, such as taxol and cisplatin (CDDP) even at 3 μM concentration, which significantly suppressed MPP+-induced cell death in PC12D cells. Therefore, cytotoxic ability of LPC and GCR may specific for mitochondrial toxins (Figure 3D). To further verify the inhibitory effect of LPC and GCR on MPP+-induced cell death, PC12D cells were labeled with PI and histogram analysis-related nuclear DNA contents were ascertained by flow cytometry. By the treatment of PC12D cells with 0.3 mM of MPP+ and NGF-differentiated PC12D cells with DNA content below G1 phase levels (defined as hypodiploid sub-G1 peak) were distinguishable in the population as compared with control levels (49.63±6.41% versus 7.23±1.04% of cells in sub-G1, respectively) (Figure 4A,B). LPC or GCR alone did not show any effects on the overall population of cells. However, they decreased the percentage of MPP+-induced cell death by 11.2–29.0% and 11.4–28.0% (values are the mean of average of three data), respectively (Figure 4A,B), confirming that LPC and GCR inhibited MPP+-induced cell death.

Licopyranocoumarin and glycyrurol attenuate the MPP+-induced decrease in mitochondrial membrane potential

MPP+ is a well-known inhibitor of mitochondria complex I and induces mitochondrial dysfunction. Because LPC or GCR suppressed MPP+-induced cell death, we next surveyed the effect of LPC and GCR on MPP+-mediated loss of mitochondrial membrane potential (ΔΨmit) using JC-1 dyes. As shown in Figure 5, by the treatment of PC12D cells with 0.3 mM of MPP+ for 48 h, ΔΨmit was decreased to 45–50% as estimated from decrease of JC-1 aggregate fluorescence. LPC or GCR alone did not affect ΔΨmit. Compared with the group treated with MPP+ alone, fluorescent intensities increased in a dose-dependent manner following addition of LPC and GCR individually, indicating that LPC and GCR each inhibited MPP+-induced decrease of ΔΨmit.

Licopyranocoumarin and glycyrurol counteract MPP+-induced ROS production

MPP+ has been extensively reported to evoke generation of reactive oxygen species (ROS). Figure 6 showed cytofluorometric histograms of NGF-differentiated PC12D cells after 12 h of treatment with 0.3 mM MPP+ upon staining with CMH2DCFDA. ROS levels were significantly increased from 100±7.8% (control level) to 247±14.9% (p<0.001). However, the generation of intracellular ROS was reduced to 164±15.7% (p<0.01) and 133±13.0% (p<0.01) by the addition of 3 μM LPC and 5 μM GCR, respectively.

Antioxidant activities of licopyranocoumarin and glycyrurol in vitro

Because treatment of PC12D cells with LPC and GCR each effectively reduced MPP+-induced ROS generation, the free radical scavenging activities of these two compounds were
examined. When the antioxidant activity of LPC and GCR were evaluated by β-carotene bleaching assay, LPC and GCR inhibited less than 10% of the carotene bleaching even at the final concentration of 30 μM (Figure 7A). The DPPH free radical scavenging potentials of LPC and GCR at 30 μM each showed little to no scavenging activity (Figure 7B). These results indicated that LPC and GCR did not possess antioxidant activity in vitro.

Licopyranocoumarin and glycyrurol attenuate JNK activity induced by MPP⁺

It is well-established that JNK plays a central role in the mediation of MPP⁺-induced neurotoxicity [31,32,33,34]. Particularly, MPP⁺-induced ROS generation is reported to be closely associated with JNK activation [35]. Thus, we investigated whether the ability of LPC or GCR to reduce MPP⁺-induced cell death involves the alteration of JNK signaling in MPP⁺-induced neurotoxicity. As shown in Figure 8A, phosphorylated JNK levels were increased after exposure to MPP⁺ for 36 h, and treatment with LPC or GCR significantly reduced the expression levels of the phosphorylated protein. In addition, a JNK inhibitor, SP600125, led to attenuation of the MPP⁺-induced neuronal cell death and decreased ΔΨᵅ [Figure 8B, C]. These results suggest that MPP⁺-induced lowering of ΔΨᵅ, which leads to neuronal cell death, were mediated by JNK, and neuroprotective activity of LPC and GCR against MPP⁺-induced neuronal cell death might be due to downregulation of ROS generation, resulting in the inhibition of JNK activation.

Discussion

Both choi-joki-to and daio-kanzo-to are traditional herbal medicines available in Japan (called kanpo in Japan in particular) that are usually used for laxative products. In the laboratory, choi-joki-to exhibited oxygen radical scavenging capacity [36] and inhibited the progression of atheroma in a KHC rabbit model [37]. On the other hand, daio-kanzo-to has provided inhibition of amylase
activity in mouse plasma and gastrointestinal tube [38], inhibition of cholera toxin [39], and inhibitory effects on drug oxidations [40]. In this study, we have demonstrated that choi-joki-to and daio-kanzo-to had neuroprotective effects against MPP⁺- and rotenone-induced toxicity in NGF-differentiated neuronal PC12D cells. Furthermore, we identified that Glycyrrhiza, commonly contained in these two herbal medicines, possessed potent neuroprotective activity against MPP⁺-induced toxicity. The correlation coefficient between neuroprotective effects of traditional herbal medicines and their contents of Glycyrrhiza. The correlation coefficient between neuroprotective effects of traditional herbal medicines and contents of Glycyrrhiza in each herbal medicine was calculated at 0.20 (Figure S1), indicating a very weak relationship. This weak relationship might be explained by our finding that higher concentration of Glycyrrhiza (300 µg/ml) showed cytotoxic effect in PC12D cells (Figure S2). Another possible explanation is that other constituent of traditional herbal medicines, such as rbhurab, also exerted neuroprotective effects in PC12D cells (Figure 2). Major components of Glycyrrhiza are triterpenoid saponins, and glycyrrhizin and its metabolite. These compounds show several potential health effects including anti-inflammatory, anti-viral, hepatoprotective, anti-cancer and immunomodulatory effects [41]. Therefore, at first we predicted that glycyrrhizin might be an active principle contained in Glycyrrhiza that suppressed MPP⁺- and rotenone-induced toxicity, but glycyrrhizin did not show such activities. Instead, we isolated the coumarin derivatives, licopyranocoumarin (LPC) and glycyrurol (GCR), as the most potent neuroprotective compounds in Glycyrrhiza. LPC isolated from Glycyrrhiza sp. has been reported to show several bioactivities, including anti-HIV effects and inhibition of CYP3A4 and the aryl hydrocarbon receptor antagonist [42,43,44]. On the other hand, GCR, which was very recently isolated from Glycyrrhiza uralensis, shows antithrombotic effects [45]. However, so far the neuroprotective effects of these two compounds have not yet been reported. This study has indeed revealed, for the first time, the potent neuroprotective activity of LPC and GCR in a PD-like cellular model system. LPC and GCR also inhibited rotenone-induced cell death in HeLa cells; however, the effects in HeLa cells were quite weak when compared to that seen in PC12D cells (Figure S3). Therefore, LPC and GCR seem to prefer to exert cytoprotection in neuronal cells. Oxidative stress associated with a general dysfunction of mitochondrial homeostasis is a leading hypothesis as a potential mechanism for dopaminergic neuronal degeneration in PD [46]. Postmortem analyses of the substantia nigra from PD patients confirm several oxidative stress-related alterations [47,48,49], and several toxins (rotenone, paraquat, and MPP⁺) used to produce PD-animal models directly or indirectly inhibit mitochondrial function, induce the production of ROS, and promote oxidative damage. Therefore, antioxidant ingredi- ents are considered to be promising approach to prevent the disease progression. For example, α-tocopherol, coenzyme Q₁₀ and catechols have been reported to exert neuroprotective effects by attenuating rotenone-induced oxidative stress on rotenone models in vitro [50,51,52]. Likewise, we found that LPC and GCR attenuated the MPP⁺-induced increase in intracellular ROS generation (Figure 6A), indicating that inhibition of MPP⁺- mediated ROS generation is closely related to the neuroprotective effects of LPC and GCR. Several lines of evidence have suggested that ROS generation induces the activation of JNK signaling, and JNK represents one of the major signaling pathways implicated in PD pathogenesis. JNK activity is increased in MPTP animal models [53,54,55,56], MPP⁺-treated cell culture models [53,54], and rotenone neurotoxicity [57,58]. Moreover, ROS-mediated activation of JNK almost inevitably leads to cell death. Indeed, we also confirmed that a JNK inhibitor, SP600125, suppressed MPP⁺- induced cell death (Figure 8B), and MPP⁺-induced activation of JNK and cell death were found to be inhibited by LPC and GCR under conditions where LPC or GCR inhibited the MPP⁺-mediated ROS generation (Figure 8A). Although the potential mechanisms by which JNK participates in MPP⁺-induced cell death remains to be fully determined, activation of JNK has been reported to mediate cell death by participating in the induction of mitochondrial permeability transition (mPT) and decrease of ΔΨₘ in subsets of cell types [59,60]. Because in our assay system SP600125 inhibited both cell death and the decrease in ΔΨₘ induced by MPP⁺ (Figure 8B and C), we consider the inhibition of the decrease in MPP⁺-induced ΔΨₘ caused by LPC and GCR (Figure 5) to be due to the inhibition of ROS-mediated JNK activation.

Several neuroprotective compounds have significant antioxidant and free radical-scavenging activities. LPC and GCR are members of the coumarin compound family. There have been several reports on the antioxidant activities of coumarins [61,62,63], and LPC and GCR each inhibited MPP⁺-induced ROS generation. Nevertheless, neither LPC nor GCR possessed ROS scavenging activity in vitro. Increased amount of ROS can be generated by an imbalance of antioxidant enzymes and activation of the oxidase.

Table 1. Crude drugs constituents of “choi-joki-to” and “daio-kanzo-to”.

| choi-joki-to | daio-kanzo-to |
|-------------|--------------|
| Scientific names | Contents (g) | Scientific names | Contents (g) |
| rhubarb | 2 | rhubarb | 4 |
| glycyrrhiza | 1 | glycyrrhiza | 2 |
| Salt cake | 0.5 | |

doi:10.1371/journal.pone.0100395.t001

Figure 4. Licopyranocoumarin and glycyrurol attenuated MPP⁺-induced apoptosis. (A) NGF-differentiated PC12D cells were treated with various concentrations of licopyranocoumarin or glycyrurol in the presence of 0.3 mM MPP⁺ for 48 h. Collected cells were stained with PI and analyzed by flow cytometry. (B) The sub G₁ ratio was analyzed. Values are the means of three independent experiments; bars, s.d. *p<0.01 compared with MPP⁺ group cells.
Identification of Neuroprotective Compounds for PD

A

| licopyranocoumarin (μM) | control | MPP⁺ 0.3 mM |
|-------------------------|---------|-------------|
|                        | 17.9%   | 50.9%       |
|                        | 18.4%   | 49.4%       |
|                        | 18.7%   | 36.0%       |
|                        | 22.9%   | 23.4%       |
|                        | 16.3%   | 45.2%       |
|                        | 15.9%   | 42.0%       |
|                        | 17.8%   | 30.6%       |
|                        | 17.4%   | 24.2%       |

JC-1 aggregate

B

- Cells with reduced mitochondrial membrane potential (%)
- w/o MPP⁺
  - 0 μM: 20 ± 2
  - 0.3 μM: 22 ± 2
  - 1 μM: 24 ± 2
  - 3 μM: 26 ± 2

- MPP⁺ 0.3 mM
  - 0 μM: 20 ± 2
  - 0.3 μM: 22 ± 2
  - 1 μM: 24 ± 2
  - 3 μM: 26 ± 2

- Cells with reduced mitochondrial membrane potential (%)
- w/o MPP⁺
  - 0 μM: 10 ± 2
  - 0.3 μM: 12 ± 2
  - 1 μM: 14 ± 2
  - 3 μM: 16 ± 2

- MPP⁺ 0.3 mM
  - 0 μM: 10 ± 2
  - 0.3 μM: 12 ± 2
  - 1 μM: 14 ± 2
  - 3 μM: 16 ± 2
system. Membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is known to be a neurotoxin-related oxidase enzyme system [64,65], and enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin reductase (TPx) and catalase [66]. Therefore, it is likely that LPC and GCR might induce the imbalance by inhibiting oxidase activity directly or neurotoxin-induced activation of oxidase system. Furthermore, we can’t exclude the possibility that LPC and GCR could induce the expression or activation of antioxidant enzymes.

In summary, we identified choi-joki-to and daio-kanzo-to as neuroprotective herbal medicines, and both LPC and GCR were identified as neuroprotective substances from Glycyrrhiza contained in choi-joki-to and daio-kanzo-to. LPC or GCR exert their neuroprotective effects by inhibiting MPP⁺-induced ROS production and thus limiting JNK activation, and causing a subsequent decrease in ΔΨ mitochondrial potential. Our proposed mechanism is illustrated in Figure 9. Further studies are required to elucidate the molecular mechanisms for the suppression of ROS generation by LPC and GCR in PC12D cells. Our findings enliven the prospect of using LPC, GCR, choi-joki-to and daio-kanzo-to as effective and safe natural therapeutic agents in PD; in vivo trials in MPTP animal models are needed.

Figure 5. Licopyranocoumarin and glycyrurol protected cells against MPP⁺-induced disappearance of mitochondrial membrane potential. (A) NGF-differentiated PC12D cells were pre-incubated for 1 h with 3 μM licopyranocoumarin (LPC) or 3 μM glycyrurol (GCR), then treated with 0.3 mM MPP⁺ for 48 h. Collected cells were stained with JC-1 and analyzed by flow cytometry. (B) The ratio of cells exhibiting disappearance of mitochondrial membrane potential was analyzed. Values are the means of three independent experiments; bars, s.d. #p<0.01 compared with control cells.

doi:10.1371/journal.pone.0100395.g005

Figure 6. Licopyranocoumarin and glycyrurol decreased MPP⁺-induced intracellular ROS generation. (A) NGF-differentiated PC12D cells were pre-incubated for 1 h with 3 μM licopyranocoumarin (LPC) or 3 μM glycyrurol (GCR), then treated with 0.3 mM MPP⁺ for 12 h. Then, the samples were loaded with 2.5 μM CM-H₂DCFDA and the fluorescence intensities were measured by flow cytometry. (B) The ratio of cells exhibiting ROS production was analyzed. Values are the means of four independent experiments; bars, s.d. **p<0.01 compared with control cells.

doi:10.1371/journal.pone.0100395.g006
Identification of Neuroprotective Compounds for PD

Materials and Methods

Reagents

MPP⁺, Rotenone, linoleic acid, 2,2-Diphenyl-1-pycrylhydrazyl (DPPH), SP600125 and mouse monoclonal anti-β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Taxol, cisplatin, JC-1 and pyridinium iodide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nerve growth factors, CM-H2DCFDA, and mouse monoclonal anti-β-actin antibodies were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG used as a secondary antibodies were purchased from GE Healthcare (Little Chalfont, UK).

Cell cultures

PC12D was identified a new subline of PC12 pheochromocy-toma cells (PC12D cells) in which neurites are extended within 24 h in response to cAMP-enhancing reagents as well as in response to nerve growth factor (NGF) [30]. PC12D cells were cultured in Dulbecco’s modified Eagle medium supplemented with 5% (v/v) inactivated fetal bovine serum, 10% (v/v) inactivated horse serum, 100 U/mL penicillin G, 0.6 mg/mL L-glutamine, and 0.1 mg/mL kanamycin at 37°C with 5% CO₂. PC12D cells were differentiated by 100 ng/mL NGF treatment for 48 h.

Cell viability assays

For the trypan blue dye exclusion assay, differentiated PC12D cells were cultured in 48-well dishes. Drug-treated or untreated cells were stained with trypan blue (Sigma Chemical Co.), and the ratio of viable cells was determined using a hemocytometer. Cell viability (%) means the ratio of the number of trypan blue-impermeable cells to total cell count. IC₅₀ values were calculated by linear regression analysis from the inhibition of MPP⁺-induced cell death at different concentrations of the drug.

Cell cycle analysis

To examine apoptosis, differentiated PC12D cells were harvested after drug treatment. The cells were washed with PBS and fixed with 70% ethanol at 4°C for more than 1 h. The cells were then stained with propidium iodide (PI) solution according to a previously reported protocol [67]. The labeled nuclei were subjected to flow cytometry (FCM, Beckman-Coulter, Miami, FL).

Measurements of mitochondrial membrane potential

Changes in mitochondrial membrane potentials were assessed by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetracylbenzimidazoleylcarboxaninioide) (Wako) was used according to the manufacturer’s protocol. Briefly, treated cells were collected by pipetting and removing medium. Next, the cells were incubated in medium containing 2.5 μg/mL JC-1 for 20 min at 37°C. Cells were then washed with PBS. JC-1 fluorescence was measured by a flow cytometer.

Measurement of intracellular ROS

Intracellular ROS production was measured using CM-H₂DCFDA. The cells were plated at a density of 12×10⁴ cells per 12-well dish. The cells were treated with MPP⁺ and test compounds for 12 h, and then trypsinized and collected. After the cells were washed with PBS, incubated with 2.5 μM CM-H₂DCFDA in HBSS at 37°C for 30 min, and then washed again with PBS three times. The relative levels of fluorescence were quantified by using a flow cytometer.

β-carotene bleaching assay

This assay was carried out according to the β-carotene bleaching method [68]. A mixture of β-carotene and linoleic acid was prepared by adding a mixture of 0.3 mg of β-carotene in 3 mL chloroform, 40 mg linoleic acid and 400 mg Tween 20. Chloroform was removed and 100 mL of distilled water was added to form an emulsion with continuous shaking. Aliquots (0.1 mL) of the β-carotene/linoleic acid emulsion were mixed with 1 μL of sample solution and incubated in a water bath at 30°C. The oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60-min period. Control samples contained 1 μL of methanol. Antioxidant activity is expressed as percent inhibition relative to control after 60 min incubation using the following equation:

\[
AA(\%) = 100 \times (DR_c - DR_x)/DR_c,
\]
where AA = antioxidant activity; DRc = degradation rate of the control = \[\ln\left(\frac{a}{b}\right)/60\]; DRs = degradation rate in presence of the sample = \[\ln\left(\frac{a}{b}\right)/60\];

a = absorbance at time 0;
b = absorbance at 60 min.

DPPH radical scavenging assay

The DPPH radical scavenging effect of test compounds was determined according to the previously described method [68]. The reaction mixtures contained 100 μL ethanol, 125 μM DPPH, and test compounds. After 2 min of incubation at room temperature, the absorbance was recorded at 517 nm.

Extraction and isolation of licopyranocoumarin and glycyrurol from *Glycyrrhiza*

Compounds were extracted from dried and pulverized *Glycyrrhiza* (50 g) with 90% EtOH, then filtrated and concentrated in vacuo. This suspension was adjusted to pH 7.0, followed by extraction with EtOAc (5 L) twice; the organic layer was concentrated to yield residue (3.76 g). The EtOAc extract was fractionated by centrifugal partition chromatography (CPC) with CHCl₃:MeOH:H₂O (5:6:4). The obtained crude active extract

Figure 8. Licopyranocoumarin and glycyrurol attenuated MPP⁺-induced JNK activation. (A) NGF-differentiated PC12D cells were treated with various concentrations of licopyranocoumarin (LPC) or glycyrurol (GCR) and 0.3 mM MPP⁺ for 36 h, and JNK and phosphor-JNK level were detected by Western blot. NGF-differentiated PC12D cells were treated with SP600125 and 0.3 mM MPP⁺ for 48 h. Thereafter (B) cell viability was measured by trypan blue dye exclusion assay and (C) mitochondrial membrane potentials were assessed by JC-1 assay. Values of (B) are the means of three independent experiments; **p<0.01 compared with MPP⁺ group cells.

doi:10.1371/journal.pone.0100395.g008

Figure 9. Suggested model for neuroprotection of licopyranocoumarin and glycyrurol against MPP⁺-induced toxicity in PC12D cells. Both licopyranocoumarin and glycyrurol exert neuroprotective effects against MPP⁺-induced toxicity via suppression of ROS generation and of JNK activation.

doi:10.1371/journal.pone.0100395.g009
was applied on Sephadex LH20 column chromatography (Sephadex LH-20, 70 μM; GE Healthcare, NJ, USA), and eluted with MeOH. The active fraction (250.6 mg) was further purified by preparative octadecyl silyl (ODS) HPLC (YMC-Pack ODS-AQ, YMC Co. Ltd., Japan) with 40% aqueous CH3CN to give pure lipopyranocoumarin (10.8 mg) and glycyrroul (4 mg), respectively.

Western blotting
Cells were lysed in RIPA buffer (50 mM HEPES (pH 7.2), 1.5% Triton X-100 (Wako), 1% sodium deoxycholate (Wako), 0.1% SDS, 0.5 M NaCl (Wako), 5 mM EDTA, 50 mM NaF (Sigma), 0.1 mM sodium vanadate (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF) with sonication. The lysates were centrifuged at 13,000 rpm for 15 min to yield the soluble cell lysates. For immunoblotting, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a polyvinylidene fluoride membrane (Millipore) by electroblotting and probed with appropriate antibodies. Immune complexes were detected with an Immobilon Western kit (Millipore), and luminescence was detected with a LAS-1000 mini (Fujifilm Co., Tokyo, Japan).

Statistical analysis
All statistical analyses in bar plots were performed with a two-tailed paired Student’s t-test.

References

1. Dawson TM, Dawson VL (2002) Neuroprotective and neuroreconstructive strategies for Parkinson’s disease. Nature neuroscience 5 Suppl: 1030–1036.
2. Binder LA, Birch-Cooch M, Cardidge NE, Parker WD Jr, Turnbull DM (1989) Mitochondrial function in Parkinson’s disease. Lancet 2: 49.
3. Parker WD Jr, Parks JK, Sverdlow RH (2008) Complex I deficiency in Parkinson’s disease frontal cortex. Brain research 1109: 213–216.
4. Parker WD Jr, Sverdlow RH (1996) Mitochondrial dysfunction in idiopathic Parkinson disease. American journal of human genetics 62: 758–762.
5. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, et al. (1990) Mitochondrial complex I deficiency in Parkinson’s disease. Journal of neurochemistry 54: 823–827.
6. Sniegrodzki R, Parks J, Parker WD Jr (2004) High frequency of mitochondrial complex I mutations in Parkinson's disease and aging. Neurobiology of aging 25: 1273–1281.
7. Esteves AR, Domingues AF, Ferreira IL, Januario C, Swerdlow RH, et al. (2008) Characterization of cybrid cell lines containing mtDNA from Huntington’s disease patients. Biochemical and biophysical research communications 361: 701–704.
8. Trimmer PA, Berland MK, Keeney PM, Bennett JP Jr, Parker WD Jr (2004) Parkinson’s disease transgenic mitochondrial cybrids generate Lewy inclusion bodies. Journal of neurochemistry 88: 800–812.
9. Hekila RE, Hess A, Duvinov RC (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 224: 1451–1453.
10. Eberhardt O, Schulz JB (2003) Apoptotic mechanisms and antiapoptotic therapy in the MPTP model of Parkinson’s disease. Toxicology letters 139: 135–151.
11. Ribeiro RS, Chincu GC, Markey SP, Ebert MH, Jacobson DM, et al. (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proceedings of the National Academy of Sciences of the United States of America 80: 4546–4550.
12. Davis GC, Williams AC, Markey SP, Ebert MH, Caine ED, et al. (1979) Chronic Parkinsonism secondary to intravenous injection of meperidine. Brain research 15. Martinez TN, Greenamyre JT (2012) Toxin models of mitochondrial disease. Nature neuroscience 3: 1301–1306.
13. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, et al. (2000) Chronic systemic pesticide exposure produces features of Parkinson’s disease. Nature neuroscience 3: 1301–1306.
14. Martinez TN, Greenamyre JT (2012) Toxin models of mitochondrial dysfunction in Parkinson’s disease. Antioxidants & redox signaling 16: 920–934.
15. Cao BY, Yang YP, Luo WF, Mao CJ, Han R, et al. (2010) Paeoniflorin, a potent neuroprotective and neurorestorative reagent, effectively showed cytoprotective effects in neuronal cells. Neuropharmacology 59: 15. Martinez TN, Greenamyre JT (2012) Toxin models of mitochondrial disease. Nature neuroscience 3: 1301–1306.
16. Dawson TM, Dawson VL (2002) Neuroprotective and neurorestorative strategies for Parkinson’s disease. Nature neuroscience 5 Suppl: 1030–1036.
17. Cohen G, Pasik P, Cohen B, Leist A, Mytilineou C, et al. (1984) Paragonime and dengue prevent the neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) in monkeys. European journal of pharmacology 106: 209–210.
18. El-O Australian compound, protects PC12 cells from MPP+-induced oxidative stress and apoptosis in PC12 cells. Brain research 1383: 99–107.
19. Park SW, Lee CH, Lee JG, Kim IW, Shin BS, et al. (2011) Protective effects of atypical antipsychotic drugs against MPP+-induced oxidative stress in PC12 cells. Neuroscience research 69: 283–290.
20. Yurekli VA, Gurler S, Naziroglu M, Ugu AE, Koyuncuoglu HR (2013) Zonisamide ameliorates MPP+-induced oxidative toxicity through modulation of Ca2+ signaling and caspase-3 activity in neuronal PC12 cells. Cellular and molecular neurobiology 33: 205–212. 21. Maniv YB, Sanchez-Ramos JR (1999) Traditional and complementary therapies in Parkinson’s disease. Advances in nutrition 80: 565–574.
22. Bar N, Ahn T, Chung S, Oh MS, Ko H, et al. (2011) The neuroprotective effect of modified Yoolahamsan-tang via autophagy enhancement in models of Parkinson’s disease. Journal of ethnopharmacology 134: 313–322.
23. A62.2. Doo AR, Kim SN, Park JY, Cho KH, Hong J, et al. (2010) Neuroprotective effects of an herbal medicine, Yi-Gan San on MPP+/-MPTP-induced cytotoxicity in vitro and in vivo. Journal of ethnopharmacology 131: 433–442.
24. Hashimoto R, Yu J, Koizumi H, Osuji Y, Okabe T (2012) Ginsenoside Rb1 prevents MPP+-induced apoptosis in PC12 Cells by Stimulating Estrogen Receptors with Consequent Activation of ERK1/2, Akt and Inhibition of SAPK/JNK, p38 MAPK. Evidence-based complementary and alternative medicine: eCAM 2012: 697171.
25. Li X, Ye X, Sun X, Liang Q, Tao L, et al. (2011) Salidroside protects against MPP+-induced apoptosis in PC12 cells by inhibiting the NO pathway. Brain research 1302: 9–18.
26. Zhou J, Sun Y, Zhao X, Deng Z, Pu X (2013) 3-O-demethylcytoranine inhibits MPP+-induced oxidative stress and apoptosis in PC12 cells. Brain research 1508: 53–62.
27. Chung V, Liu L, Bian Z, Zhao Z, Leek Feng W, et al. (2006) Efficacy and safety of herbal medicines for idiopathic Parkinson’s disease: a systematic review. Movement disorders: official journal of the Movement Disorder Society 21: 1709–1715.
28. Beal MF (2001) Experimental models of Parkinson’s disease. Nature reviews Neuroscie 2: 325–334.
29. Kato C, Sembra K, Kataiwa S, Yamazaki Y, Sano M (1987) Neurite growth from a new subline of PC12 pheochromocytoma cells: cyclic AMP mimics the action of nerve growth factor. Journal of neuroscience research 17: 36–44.
31. Yao S, Li Y, Kong L (2006) Preparative isolation and purification of chemical constituents from the root of Polygonum multiflorum by high-speed counter-current chromatography. Journal of chromatography A 1115: 64–71.
32. Meilke K, Herdegen T (2000) JNK and p38 stress kinases—degenerative effectors of signal-transduction-cascades in the nervous system. Progress in neurobiology 61: 43–60.
33. Tatton WG, Chalmers-Redman R, Brown D, Tatton N (2003) Apoptosis in Parkinson's disease: signals for neuronal degradation. Annals of neurology 53 Suppl 3: 561–70, discussion 570–62.
34. Voss T, Ravina B (2008) Neuroprotection in Parkinson's disease: myth or reality? Current neurology and neuroscience reports 8: 304–309.
35. Kim SY, Kim MY, Mo JS, Park JW, Park HS (2007) SAG protects human neuroblastoma SH-SY5Y cells against 1-methyl-4-phenylpyridinium ion (MPP+)–induced cytotoxicity via the downregulation of ROS generation and JNK signaling. Neuroscience letters 413: 132–136.
36. Nishimura K, Owasa T, Watamabe K (2011) Evaluation of oxygen radical absorbance capacity in kampo medicine: Evidence-based complementary and alternative medicine: eCAM 2011: 812163.
37. Iizuka A, Iijima OT, Kondo K, Matsumoto A, Itakura H, et al. (1999) Antioxidative effects of Choi-oki-to and its ability to inhibit the progression of athreosis in KHC rabbits. Journal of atherosclerosis and thrombosis 6: 49–54.
38. Kobayashi K, Funayama N, Suzuki R, Yoshizaki F (2002) Survey of the influence of Chinese medicinal prescriptions on amylase activity in mouse plasma and gastrointestinal tube. Biological & pharmaceutical bulletin 25: 1108–1111.
39. Oh H, Matsuura D, Miyake M, Ueno M, Takai I, et al. (2002) Identification in traditional herbal medications and confirmation by synthesis of factors that inhibit cholera toxin-induced fluid accumulation. Proceedings of the National Academy of Sciences of the United States of America 99: 3042–3046.
40. Hasegawa A, Kawaguchi Y, Nakasa H, Nakamura H, Ohmori S, et al. (2002) Effects of Kampo extracts on drug metabolism in rat liver microsomes: Rhei Rhiosma extract and Glycyrrhizae Radix extract inhibit drug oxidation. Japanese journal of pharmacology 89: 164–170.
41. Asl MN, Hosseinzadeh H (2008) Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. Phytotherapy research: PTR 22: 709–724.
42. Hatano Y, Yasuhara T, Fukuda T, Noro T, Okuda T (1989) Photoin constituents of licorice. II. Structures of licoppyronomucarin, licoracoumolin and gisaldavone, and inhibitory effects of licorice phonics on xanthine oxidase. Chemical & pharmaceutical bulletin 37: 3005–3009.
43. Tsukamoto S, Aburatani M, Yoshida T, Yamashita Y, El-Beih AA, et al. (2005) CYTP3A4 inhibitors isolated from Licorice. Biological & pharmaceutical bulletin 28: 2000–2002.
44. Kasi A, Hiranatsu N, Hayakawa K, Yao J, Kitamura M (2000) Blockade of the dioxin pathway by herbal medicine Formula Bupleuri Minor: identification of active entities for suppression of AhR activation. Biological & pharmaceutical bulletin 31: 838–846.
45. Tao WW, Duan JA, Yang NY, Tang YP, Liu MZ, et al. (2012) Antiinflammatory phenolic compounds from Glycyrrhiza uralensis. Fitoterapia 83: 422–425.
46. Seaton TA, Cooper JM, Schapira AH (1997) Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. Brain research 777: 110–118.
47. Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, et al. (1997) Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. Journal of neurochemistry 69: 1196–1203.
48. Dexter DT, Carter CJ, Wells FR, Jovoy-Agid F, Agid Y, et al. (1989) Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. Journal of neurochemistry 52: 381–389.
49. Floor E, Wenzel MG (1998) Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. Journal of neurochemistry 70: 268–275.
50. Song JX, Sre SC, Ng TB, Lee CK, Leung GP, et al. (2012) Anti-Parkinsonian drug discovery from herbal medicines: what have we got from neurotoxic models? Journal of ethnomedical 130: 608–711.
51. Testa CM, Sherer TB, Greenamyre JT (2005) Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. Brain research Molecular brain research 134: 109–118.
52. Yang L, Calingasan NY, Wille EJ, Cormier K, Smith K, et al. (2009) Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases. Journal of neurochemistry 106: 1427–1439.
53. Saporito MS, Bosen EM, Miller MS, Carwell S (1999) CEP-1347/KT-7515, an inhibitor of c-Jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons In vivo. The Journal of pharmacology and experimental therapeutics 281: 421–427.
54. Xia XG, Harding T, Wellner M, Bieneman A, Uney JB, et al. (2001) Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 98: 10433–10438.
55. Hunot S, Vila M, Teismann P, Davis RJ, Hirsch EG, et al. (2004) JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 101: 665–670.
56. Park SW, Kim SH, Park KH, Kim SD, Kim JY, et al. (2004) Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease. Neuroscience letters 363: 243–246.
57. Newhouse K, Hsuan SL, Chang SH, Cai B, Wang Y, et al. (2004) Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. Toxicological sciences: an official journal of the Society of Toxicology 79: 137–146.
58. Klintworth G, Newhouse K, Li T, Choi WS, Fajig R, et al. (2007) Activation of c-Jun N-terminal protein kinase is a common mechanism underlying paraquat and rotenone-induced dopaminergic cell apoptosis. Toxicological sciences: an official journal of the Society of Toxicology 97: 149–162.
59. Hanawa N, Shinobara M, Saberl B, G organization WA, Han D, et al. (2006) Role of JNK translocation to mitochondria leading to induction of mitochondrial bioenergetics in acetaminophen-induced liver injury. The Journal of biological chemistry 283: 13565–13574.
60. Lin X, Wang YJ, Li Q, Hou YY, Hong MH, et al. (2009) Chronic high-dose morphine treatment promotes SH-SY5Y cell apoptosis via c-Jun N-terminal kinase-mediated activation of mitochondria-dependent pathway. The FEBS journal 276: 2022–2036.
61. Ng TB, Liu F, Wang ZT (2000) Antioxidative activity of natural products from plants. Life sciences 66: 709–723.
62. Fernandez-Puntero B, Barroso I, Iglesias I, Benedi J, Villar A (2001) Antioxidant activity of Fraxetin: in vivo and ex vivo parameters in normal situation versus induced stress. Biological & pharmaceutical bulletin 24: 777–784.
63. Vladimirov IU A, Parfenov EA, Epanchintseva OM, Smirnov LD (1991) [The antiradical activity of coumarin reductones]. Bulllettan' eksperimental'noi i klinichesko i biologii i meditsiny 112: 472–475.
64. Infanger DW, Sharma RV, Davison RL (2006) NADPH oxidases of the brain: distribution, regulation, and function. Antioxidants & redox signaling 10: 1503–1506.
65. Savada M, Imamura K, Nagatsu T (2006) Role of cytokines in inflammatory process in Parkinson's disease. Journal of neural transmission Supplement: 373–381.
66. Jin H, Kalnasasamy A, Ghosh A, Anantharam V, Kalyanaraman B, et al. (2013) Mitochondria-targeted antioxidants for treatment of Parkinson's Disease: Preclinical and clinical outcomes. Biochimica et biophysica acta.
67. Kavatsani M, Ushi M, Simu S, Osada H, Imuto M (2003) Transmembrane domain of Bel-2 is required for inhibition of ceramide synthesis, but not cytochrome c release in the pathway of inostamycin-induced apoptosis. Experimental cell research 296: 57–66.
68. Kumazawa S, Taniguchi M, Suzuki Y, Shimura M, Kwon MS, et al. (2002) Antioxidant activity of polyphenols in carob pods. Journal of agricultural and food chemistry 50: 373–377.