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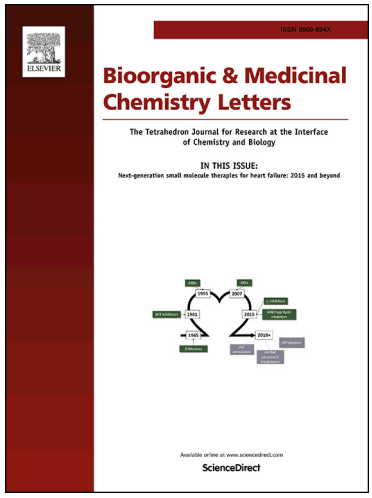

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Synthesis and biological evaluation of clovamide analogues with catechol functionality as potent Parkinson's disease agents in vitro and in vivo

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Abstract

- In this study, seven clovamide analogues (**1-7**) were designed and synthesized, and the neuroprotection of **1-7** as well as **8-15** (prepared in our previous work) against H_2O_2-induced oxidative stress was evaluated in SH-SY5Y cells. Results showed that **1-7** with catechol groups exhibited better neuroprotective effects than **8-15**, and their EC_{50} values ranged from 4.26 to 23.83 μM, especially **1**, indicating that the moiety of catechol governed the activities of these compounds. Furthermore, oral administration of **1** (10 or 20 mg/kg) was demonstrated to possess anti-PD effect through alleviating apoptosis and oxidative stress in vitro and in vivo, and to up-regulate the expression of heme oxygenase-1 (HO-1) via PI3K/AKT/mTOR pathway. Finally, the pharmacokinetic (PK) assessment of **1** was determined in rats. These findings suggested that **1** might be an effective candidate for PD therapy.

Keywords: Clovamide analogue; Catechol functionality; Oxidative stress; Heme oxygenase-1; Parkinson's disease; Pharmacokinetic assessment

Parkinson's disease (PD), the second most common age related neurodegenerative disease only to Alzheimer's disease, is affecting 1% of the population above the age of 65, and the risk of PD is

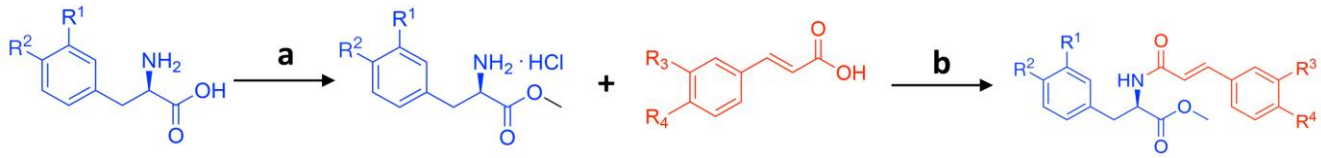
continually increased in the elderly **[1]**. Although the exact etiology of PD is still unknown, the pathogenesis is believed to be related with the progressive loss of dopamine-producing neurons in the substantia nigra (SNpc), destroying the balance between excitatory neurotransmitter (acctyl choline, ACh) and inhibitory neurotransmitter (dopamine, DA) **[2]**. Clinically, the symptoms of PD included motor dysfunctions (resting tremor, postural instability, and muscle rigidity) and non-motor-related symptoms (autonomic dysfunction, cognitive deficits, and sleep disorders) **[3]**. Since 1960's, replacement or supplement of DA has always been the main therapeutic for PD. The representative drugs are catechol-*O*-methyltransferase (COMT) inhibitors, monoamine oxidase type B (MAO-B) inhibitors, and levodopa (*L*-DOPA) **[4]**. Although these drugs can greatly alleviate the symptoms of PD, they cannot halt or reverse the disease progression induced by neuronal damage, and long-term use of levodopa even accompanied by motor complications **[5]**. Therefore, it is necessary to find out new therapeutic strategies that can protect neurons in SNpc to hamper PD progression.

Oxidative stress, as the major inducer of nigral loss in both sporadic and genetic forms of PD, represents the physiological response linked an imbalance between antioxidant defense systems and reactive oxygen production, and plays a central role in the progression of the disorder **[6]**. Heme oxygenase-1 (HO-1), also known as heat-shock protein 32 or inducible HO, is the rate-limiting enzyme in heme catabolism with strong antioxidant activities. Emerging evidences support the role for heme oxygenase-1 (HO-1) as important components of the cellular antioxidant and cytoprotective defense **[7, 8]**. Recently, the anti-oxidant agents for PD treatment have received much attention, and several anti-oxidants, such as Puerarin **[9]** and Desipramine **[10]**, were found to provide PD treatment involved in up-regulating the expression of HO-1, suggesting that HO-1 might represent a promising and novel therapeutic strategy in treating Parkinsonism.

Clovamide, an amide analogue of rosmarinic acid, was first reported in red clover **[11].** Evidences demonstrated that the protective effects of clovamides reducing oxidative stress-induced damage to blood platelets, plasma and cardio myoblasts have been reported **[12, 13]**. However, there are no significant data currently available on the neuroprotection against oxidative stress in vitro and in vivo. In addition, catechol group was deemed as an important antioxidant pharmacophore in various antioxidants, such as catecholamines and flavonoids **[14]**, and clovamide analogues with catechol group displayed significant effects of scavenging free radical **[15]**. Therefore, synthesizing and evaluating the neuroprotection of clovamide analogues containing catechol functionality are necessary in developing the neurodegenerative disease's therapeutic. In order to evaluate whether the catechol group plays an important role on the neuroprotective effects of clovamide analogues against

neuro-oxidative stress, seven representative clovamide analogues (**1-7**) with different position and quantity of catechol groups were designed and synthesized, and the neuroprotective effects of **1-7** as well as **8-15** without catechol functionality (prepared in our previous work **[16]**) against H_2O_2-induced oxidative stress was evaluated in SH-SY5Y cells

Among them, **1-7** provide neuroprotection with EC_{50} values ranging from 4.26 μM to 23.83 μM, while compounds **8-15** showed poor effects (**Table 1**), suggesting that catechol group might be the important pharmacophore for their neuroprotective effects in this model, and their protective effects are independent of the quantity of phenolic hydroxyl groups. Furthermore, the optimal one, N-[3', 4'-Dihydroxy-(E)-cinnamoyl]-L-phenylalanine methyl ester (**1**), was demonstrated to exhibit significant neuroprotection involved in attenuating apoptosis and up-regulating the expression of HO-1 via PI3K/AKT/mTOR pathway. In vivo, as far as we are aware**,** the anti-PD effects of **1** through apoptosis and oxidant pathways was firstly revealed in this study. Additionally, ADME-related descriptors were calculated to predict the pharmacokinetic properties of the compounds, and the pharmacokinetic (PK) assessment of **1** was determined in rats.



Scheme1. (a) $SOCl_2$, MeOH, 0 °C→ R.T., 12 h; (b) HBTU, DIPEA, CH_3CN, R.T., 2 h.

Table.1

Chemical structures and neuroprotective effects of compounds **1-15**.

Compound	R^1	R^2	R^3	R^4	EC_{50}, (μM)[a]
1	H	H	OH	OH	4.26 ± 1.37
2	H	OH	OH	OH	11.26 ± 1.02
3	OH	OH	H	H	14.33 ± 1.77
4	OH	OH	OH	OH	15.58 ± 0.74
5	OH	OH	OH	H	17.48 ± 1.53
6	OH	OH	H	OH	19.07 ± 0.48
7	OH	OH	OCH_3	OH	23.83 ± 2.16
8	H	OH	H	OH	N.D.[b]

9	H	OH	H	H	N.D.
10	H	OH	OH	H	N.D.
11	H	OH	OCH_3	H	N.D.
12	H	H	H	OH	N.D.
13	H	H	H	H	N.D.
14	H	H	OH	H	N.D.
15	H	H	OCH_3	OH	N.D.
NAC[c]					1.68 ± 0.41 (mM)
Caffeic acid[d]					33.82 ± 3.22

Note: [a]EC_{50}, 50% concentration of maximal effect (means ± S.E.M. of three experiments); [b] N.D. means the EC_{50} was not detected because of the poor activity observe in the primary screening at 100 μM; [c] NAC (N-Acetyl-L-cysteine) as the positive control (mM); [d] Caffeic acid: synthetic substrate of the compounds.

Initially, in order to confirm the optimal concentration of H_2O_2, cells were incubated with various concentrations of H_2O_2 (100–1000 μM) for 24 h. As shown in **Figure 1A**, 600 μM H_2O_2 could decrease the cell viability to 52.66 ± 2.34 % of control. Therefore, 600 μM H_2O_2 was chosen for the subsequent experiments. Among the analogues, seven compounds (**1-7**) exhibited potent protection rates (>50%) at 100 μM, while others (**8-15**) showed poor protection rates (＜50%) at 100 μM (**Figure 1B**). The EC_{50} values of these compounds **1-7** were 4.26 ± 1.37, 11.26 ± 1.02, 14.33 ± 1.77, 15.58 ± 0.74, 17.48 ± 1.53, 19.07 ± 0.48, 23.83 ± 2.16 μM, respectively. Above all, **1** exhibited the most remarkable protective effect (EC_{50} = 4.26 ± 1.37 μM) (**Table 1**), and was considered as a potential lead in further evaluation. Treatment alone with **1** at different concentrations (1-100 μM) for 24 h didn't affect the cell viability (**Figure 1C**). The effect of **1** against H_2O_2-induced cell death was shown in **Figure 1D**, pretreatment with **1** at 1 and 10 μM could improve cell viability to 68.31 ± 2.09% and 88.99 ± 1.19% of control, respectively. The neurotoxicity of H_2O_2 and the neuroprotective effect of **1** were further evaluated by lactate dehydrogenase (LDH) assay. As shown in **Figure 1E**, the leakage of LDH in H_2O_2 group (339.93 ± 3.66 U/L) was much more than control group (154.87 ± 1.41 U/L). In contrast, pretreatment with **1** (1, 10 μM) reduced the LDH release in a dose-dependent manner (269.12 ± 2.47 and 180.66 ± 2.23 U/L, respectively). Moreover, **1** alone treatment also has no influence on cell viability and LDH release. The neuroprotection of **1** was further confirmed by observing the cell morphological under the inverted microscope (**Figure 1F**).

The above results confirmed that **1** at 1 and 10 μM could provide neuroprotection without cytotoxicity.

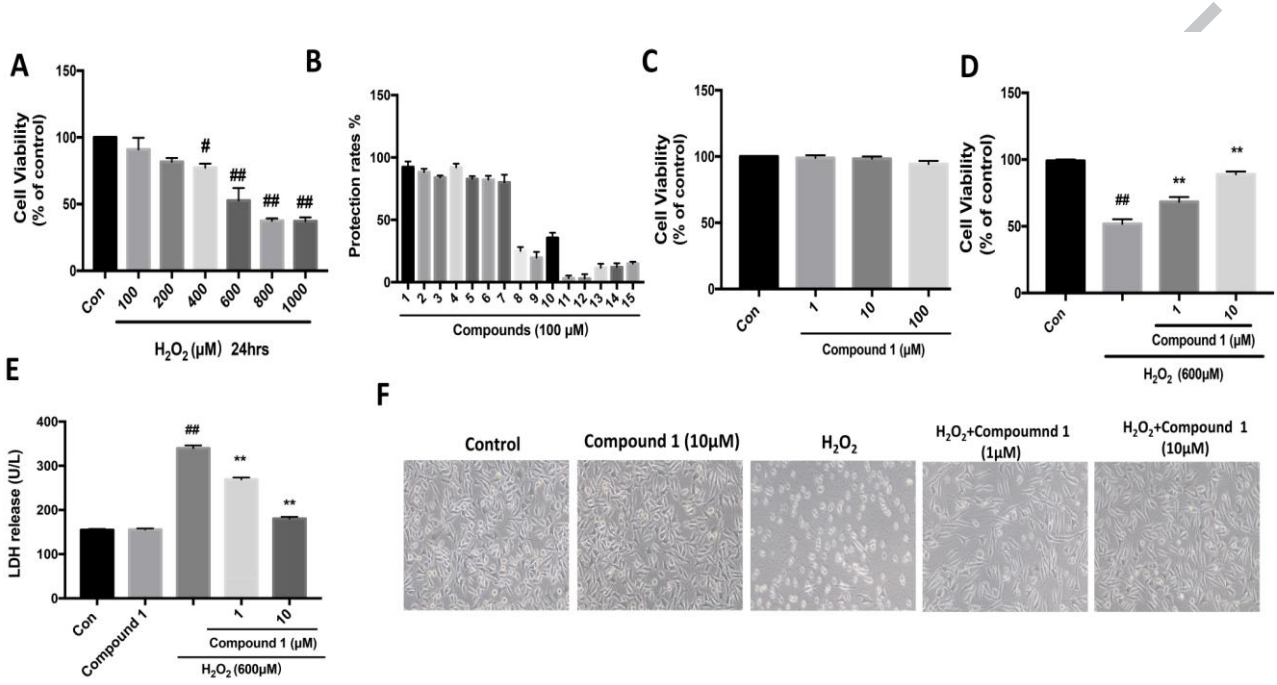


Figure 1. Neuroprotection of **1** in SH-SY5Y cells. (A) Cytotoxicity of different concentrations of H_2O_2 in SH-SY5Y cells. (B) Dose-dependent neuroprotection of pretreatment with **1** against H_2O_2-induced cytotoxicity in SH-SY5Y cells. (C) Protective effect of the plasma membrane damage was analyzed by LDH release. (D) Treated with **1** alone at 1, 10, 100 μM had no effect on cell viability. (E) The observation of the cell morphological under the inverted microscope followed by above treatments. All the groups were treated with less than 0.1% DMSO. The data were represented as mean ± S.E.M. of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control and $^{**}p < 0.01$ compared with H_2O_2 treatment group.

As is known to all, reactive oxygen species (ROS), as the mitochondrial by-products in aerobic respiration process, regulate many metabolic reactions **[17]**. However, the excessively increased of ROS generation can induce oxidative stress and result in cell death via apoptosis in a wide variety of cell types **[18]**. To confirm whether or not the protective effect of **1** via anti-oxidant**,** the level of intracellular ROS was determined by dihydroethidium (DHE) fluorescence dye. Results showed that the cells treated with H_2O_2 could increase the accumulation of intracellular ROS, which was about 4.0-fold relative to that of control cells. While pretreatment with **1** (1,10 μM) could significantly decrease the ROS production in a dose-dependent manner, suggesting the anti-oxidative effects of it

via inducting ROS production (**Figures 2A-C**).

Mitochondria is a major site of ROS generation, the destruction of it integrity could cause the increasing consumption of ATP and accumulating of intracellular ROS production **[19]**. Previous study has confirmed that oxidative stress is involved in mitochondrial dysfunctions, as contributing to open the mitochondrial permeability transition pore, resulting in depolarization of the mitochondrial membrane potential (MMP) **[20]**. In this study, therefore, the effect of **1** reversed the loss of MMP induced by H_2O_2 was detected using Rho 123 assay. **Figures 2D-F** showed that **1** at 1 and 10 μM could respectively increase the MMP to 79.43 ± 2.51% and 98.37 ± 1.87% compared to H_2O_2 group (26.31 ± 3.32%) of control. These results suggested that **1** could prevent the loss of MMP to provide neuroprotection, and we hypothesized that the ability of **1** to inhibit ROS generation may attribute to the preservation of MMP loss. Moreover, under physiological conditions, intracellular ROS levels are tightly controlled by a sophisticated cellular antioxidant system in organism such as some antioxidant enzymes **[21]**. Superoxide dismutase (SOD), as one of the major antioxidant enzymes, provides the most important anti-oxidant via catalyzing the dismutation reaction of superoxide anion to H_2O_2. On the other hand, malondialdehyde (MDA), as an end-product of lipid peroxidation, is regard as an important indicator of the oxidative damage. Therefore, the anti-oxidative stress of **1** was further detected by the level of MDA and the activity of SOD in SH-SY5Y cells. As shown in **Figures 2G-H**, being exposed to H_2O_2 could reduce the SOD activity to 0.45 ± 0.047 units and increase the MDA level to 7.99 ± 0.55 μmol/g compared with control cells (1.52 ± 0.028 units and 3.42 ± 0.037 μmol/g, respectively). However, pretreatment with **1** (1, 10 μM) could effectively improve the SOD activity and decrease the MDA level in a dose-dependent manner, suggesting that **1** could mediate oxidant system to against oxidative stress induced by H_2O_2 in SH-SY5Y cells.

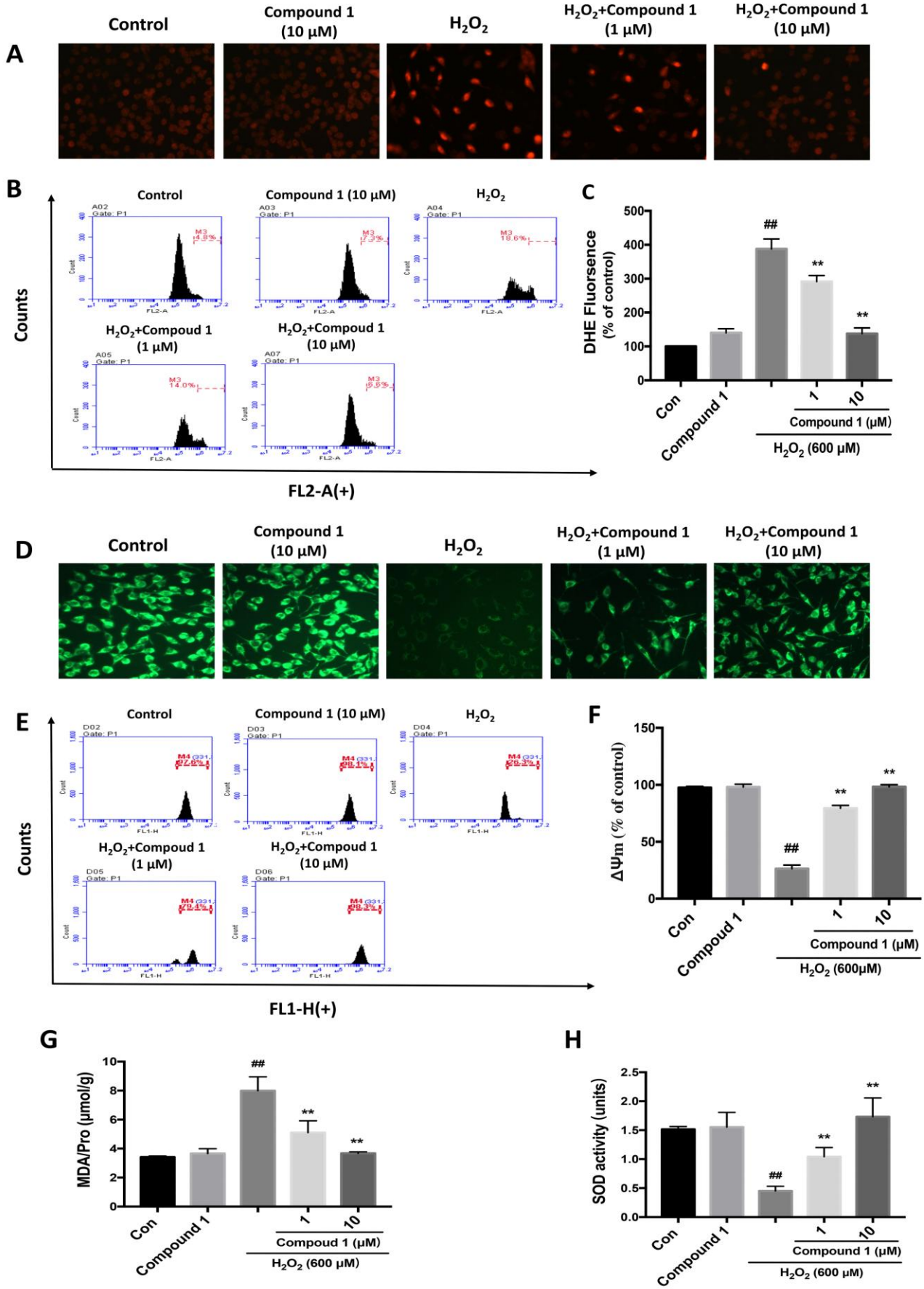

A
Control
Compound 1 (10 μM)
H2O2
H2O2+Compound 1 (1 μM)
H2O2+Compound 1 (10 μM)
B
Control
Compound 1 (10 μM)
H2O2
H2O2+Compoud 1 (1 μM)
H2O2+Compoud 1 (10 μM)
Counts
FL2-A(+)
C
DHE Fluorsence (% of control)
Con
Compound 1
Compound 1 (μM)
H2O2 (600 μM)
D
Control
Compound 1 (10 μM)
H2O2
H2O2+Compound 1 (1 μM)
H2O2+Compound 1 (10 μM)
E
Control
Compound 1 (10 μM)
H2O2
H2O2+Compoud 1 (1 μM)
H2O2+Compoud 1 (10 μM)
Counts
FL1-H(+)
F
ΔΨm (% of control)
Con
Compoud 1
Compound 1 (μM)
H2O2 (600μM)
G
MDA/Pro (μmol/g)
Con
Compound 1
Compoud 1 (μM)
H2O2 (600 μM)
H
SOD activity (units)
Con
Compound 1
Compound 1 (μM)
H2O2 (600 μM)

Figure 2. Anti-oxidative effect of **1** in SH-SY5Y cells. The intracellular ROS production was detected by (A) inverted fluorescence microscope and (B) flow cytometry. (C) Bar graphs showed intracellular ROS production relative to the control group. The levels of ΔΨm was analyzed by (D) inverted fluorescence microscope (E) and flow cytometry. (F) Bar graphs showed the quantitative analysis for MMP. Effects of **1** on (G) the levels of MDA and (H) the activities of SOD in H_2O_2 treated cells. The data were represented as mean ± S.E.M. of three independent experiments. $^{\#\#}p < 0.01$ compared with control and $^{**}p < 0.01$ compared with H_2O_2 treatment group.

Cell apoptosis induced by oxidative stress plays an important role in the pathological process of neurodegenerative disorders **[22]**. To confirm the effects of **1** against the H_2O_2-induced apoptosis in SH-SY5Y cells, Hoechst 33342 staining and Annexin V-PI double staining were performed in this study. For the results of Hoechst 33342 staining, the nuclear fragmentation and DNA condensation were occurred after treatment with H_2O_2. However, pretreatment with **1** could significantly alleviate the typical characteristics of apoptosis (**Figure 3A**). For the results of AnnexinV-PI double staining, the untreated cells showed slight total apoptosis (6.81 ± 0.73%) as well as only treated with **1** at 10 μM (total apoptotic of 4.14 ± 1.12%). Whereas treatment with 600 μM H_2O_2 markedly increased the percentage of total apoptotic (84.17 ± 3.44%) and pretreatment with **1** at 1, 10 μM could decrease the percentage to 24.13 ± 2.21% and 8.86 ± 1.63%, respectively (**Figures 3B-C**). These data proved the anti-apoptotic activities of **1** against H_2O_2-induced neurotoxicity.

Cell apoptosis is primary to mitochondria dependent apoptotic pathways in neurodegenerative diseases **[23]**. As the mitochondrial membrane-associated protein, the anti-apoptotic protein Bcl-2 located in the outer mitochondrial membrane with the anti-apoptotic function and the pro-apoptotic protein Bax promotes cell apoptosis by translocating to the mitochondrial membrane. Cell survival in the initiation phase of apoptosis mostly depends on the balance between the Bcl-2 and Bax. Thus, Bcl-2/Bax ratio could be used to determine whether a cell has undergone apoptosis **[24]**. Moreover, Bcl-2 has been reported to inhibit Bax expression in mitochondria, and inhibit subsequent activation of the caspase-3 **[25]**. In this study, the expressions of these proteins were detected by western-blot assay, β-actin as the internal standard. Results showed that the ratio of Bax/Bcl-2 and the expression of cleaved-caspase-3 were significantly increased by treatment of H_2O_2 compared with control. However, pretreatment with **1** (1,10 μM) could effectively reverse this tendency in a dose-dependent manner. Treated with **1** alone had no obvious effects on the ratio of Bax/Bcl-2 and the expression of cleaved-caspase-3 (**Figures 3D–E**). These results suggested that the anti-apoptosis activity of **1** might be mediated by modulating the expressions of apoptosis-related proteins. Additionally, the

absorption, distribution, metabolism, elimination (ADME) properties of **1-7** were performed well in Discovery Studio 3.0 using ADME descriptors. Results indicated that the compounds have good intestinal absorption, and the penetrating BBB ability of **1** might be better than other candidate compounds (Supporting Information **Table S1**). Taken together, **1**, the optimal one, was chosen to further evaluate its anti-PD effects in vivo.

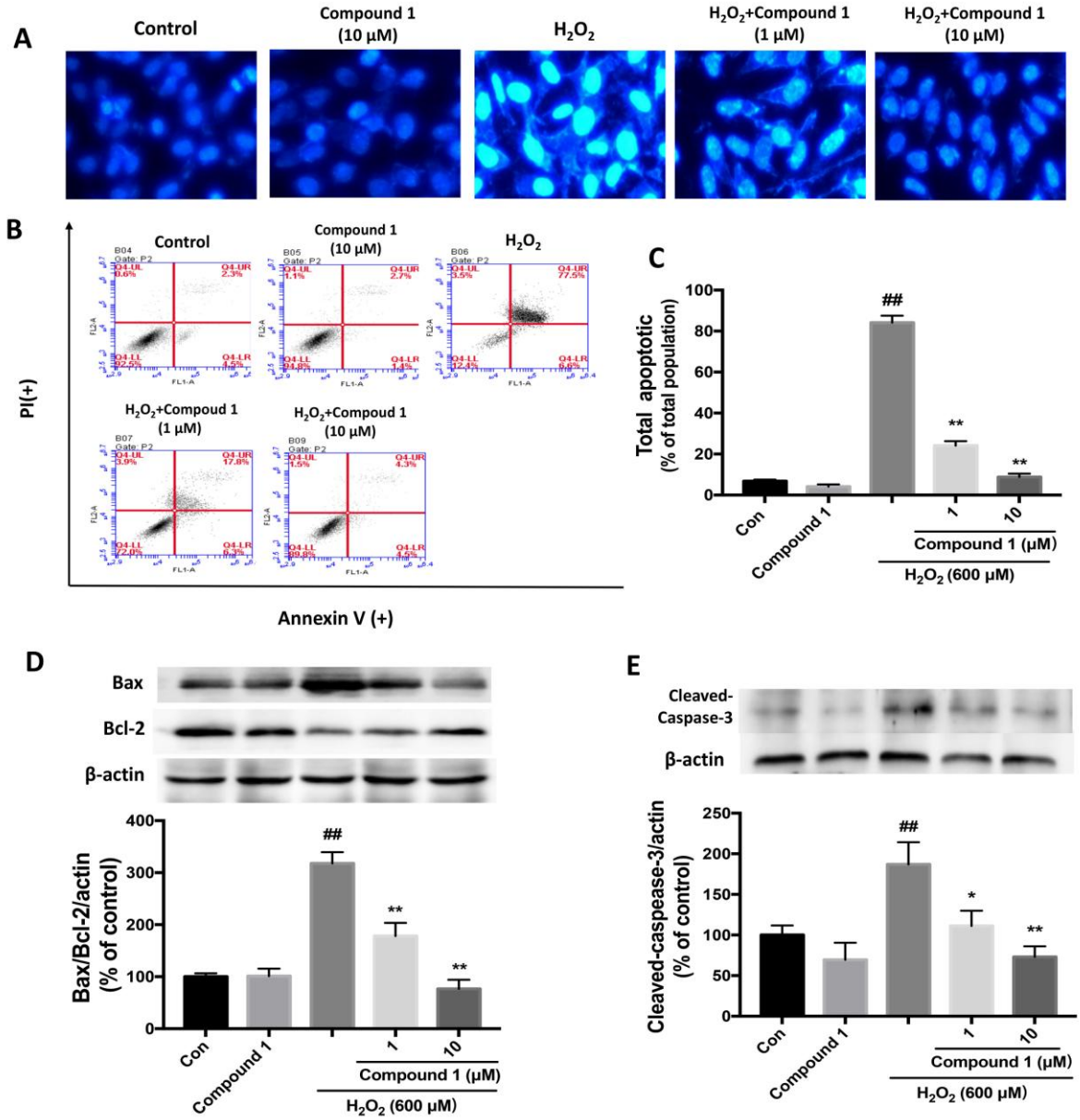


Figure 3. Effects of **1** on apoptotic and the expression of H_2O_2-induced apoptosis-related proteins in SH-SY5Y cells. (A) Hoechst 33342 staining (B) Distribution of viable (lower left, Annexin V^- PI^-) necrotic (upper left, Annexin V^- PI^+) late apoptotic (upper right, Annexin V^+ PI^+) and early apoptotic (lower right, Annexin V^+ PI^-) (C) Bar graphs showed the quantitative results of AV−PI. (D) The original bands of Bax, Bcl-2 and the quantitative analysis of Bcl-2/Bax ratio. (E) The original bands the quantitative analysis of

cleaved-caspase-3 expression. The data were represented as mean ± S.E.M. of three independent experiments. $^{\#\#}p<0.01$ compared with control and $^{*}p<0.05$, $^{**}p<0.01$ and compared with H_2O_2 treatment group.

1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) is an effective dopaminergic neurotoxin in some mouse strains and has been widely used for inducing PD model of animal **[26]**. Evidences indicate that MPTP's deleterious cascade of events include mitochondrial respiration deficit, oxidative stress, and activation of apoptotic genetic programs **[27]**. The above results revealed the anti-apoptosis and anti-oxidative activities of **1** in H_2O_2-induced SH-SY5Y cells, implying **1** may also has the anti-PD activity in MPTP-induced PD model. As mentioned above, the loss of dopaminergic neurons in substantia nigra (SNpc) and striatum (Str) makes patients suffer from different degrees of behavior disorders **[3]**. Therefore, in this study, the motor function of the MPTP intoxicated mice were determined by Rota-rod test (**Figure 4B**). Results showed that the duration time of mice on rotating-stick was significantly decreased compared to control at 4, 24, 48, and 72 h after MPTP treatment. However, oral administration of **1** at 10 or 20 mg/kg markedly increased the retention time compared with MPTP-treated group. Treatment with **1** alone had no influence of motor function. Moreover, tyrosine hydroxylase (TH), as the rate-limiting step in the biosynthesis of DA, is commonly regarded as a marker of dopaminergic neurons and also been used to evaluate the degrees of PD **[28]**. Therefore, in this study, immunohistochemistry (IHC) analysis and western-blot analysis were conducted for TH protein expression. As shown in **Figures 4C-E**, treatment with MPTP could significantly reduce the TH expression both in SNpc and Str compared with control group. However, the loss of TH protein was markedly prevented by **1** at 10 or 20 mg/kg. The above results suggested that **1** could effectively alleviate the behavioral disorders and protect against dopaminergic neuronal loss in MPTP-induced model.

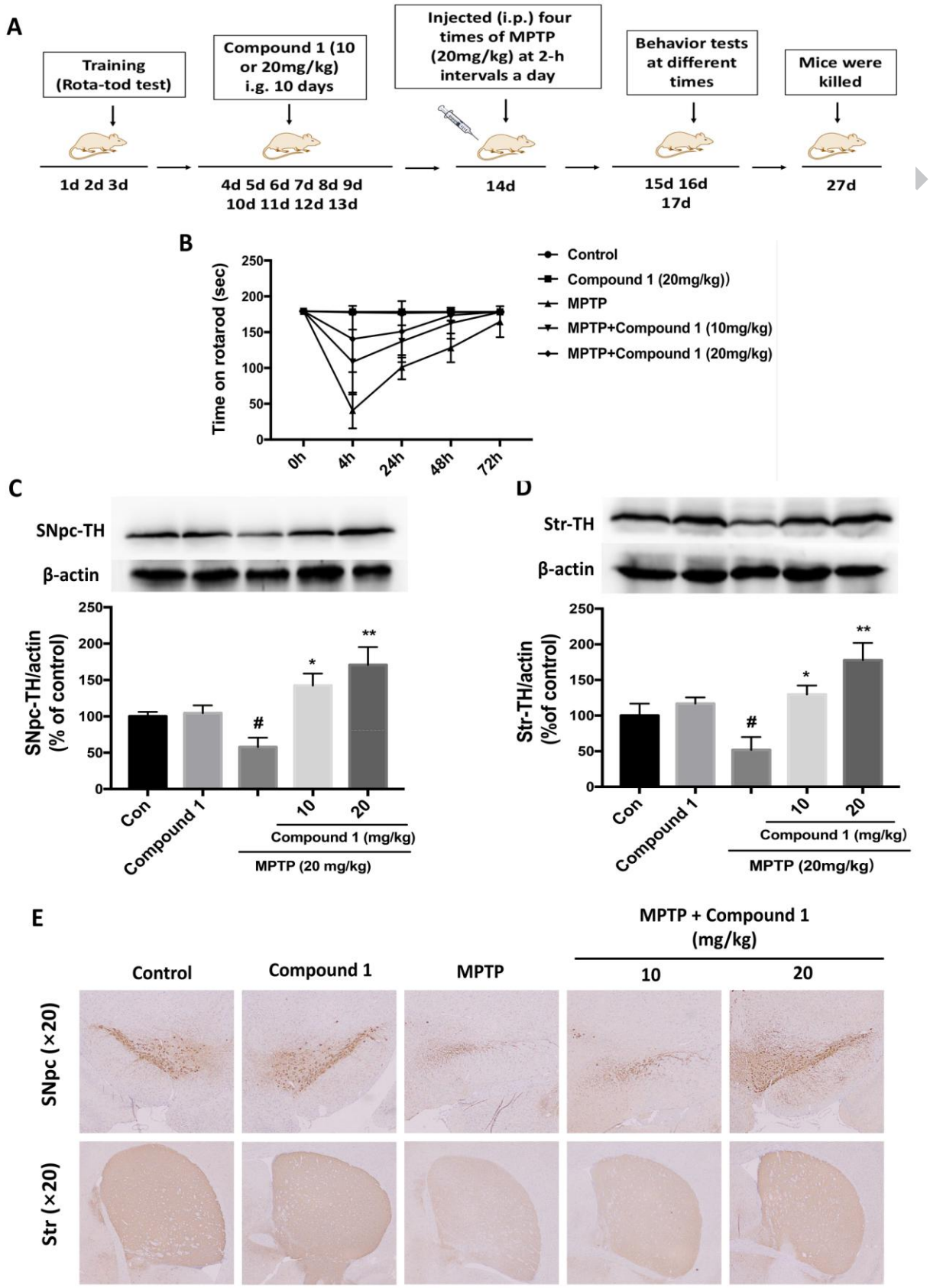


Figure 4. Effects of **1** on motor function and TH-positive cells in the SNpc and Str of MPTP-treated 18 mice. (A) Schematic of experimental procedure. (B) The results of **1** on Rota-rod test. At 10 days after MPTP

injection, DAB staining and western blots were performed. (C) The original bands and the quantitative analysis of TH protein in SNpc. (D) The original bands and the quantitative analysis TH protein in Str; A representative immunoblot was shown from three mice. (E) Representative microphotographs of TH immunostaining of SNpc and Str. All the results were randomly obtained from three of the 3 mice/group and the data were represented as mean ± S.D. (n = 6 mice/group). $^{\#}p < 0.05$ compared with control and $^{*}p < 0.05$, $^{**}p < 0.01$ and compared with MPTP treatment group.

To further investigate the anti-PD activity of **1**, we focused on the anti-apoptotic and anti-oxidative effects of **1** in the brains. Consistent with the in vitro results, pretreatment of **1** at 10 or 20 mg/kg could significantly decrease the ratio of Bax/Bcl-2 and the expression of cleaved-caspase-3 both in SNpc and Str (**Figures 5A-D**). On the other hand, treatment with **1** at 10 or 20 mg/kg dose-dependently increased SOD activity and decreased the level of MDA in the Str and SNpc of the MPTP-induced PD mice (**Figures 6A-D**). Furthermore, pharmacokinetic (PK) assessment of the optimum candidate compound **1** was determined in rats. When **1** was oral administrated (p.o., 25 mg/kg) and injected (i.v., 5 mg/kg), blood samples (100-200 μL) were collected from an orbit at 12 time points within 24 h (0.083, 0.168, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h) (**Figure 7**). The T_{max} of 2 h suggested that **1** was rapidly absorbed with short time of maximum observed concentration after oral administration. The pharmacokinetic (PK) profiles of **1** is projected to have moderate blood clearance ($Cl_{i.v.}$= 25.5 mL/min/kg), moderate volume of distribution (V_{dss} = 2.25 L/kg) and moderate absolute bioavailability (F = 15.52 %) (**Table 2**) , These results highlight the potential of compound **1** as a promising lead in the development of orally active therapies for PD.

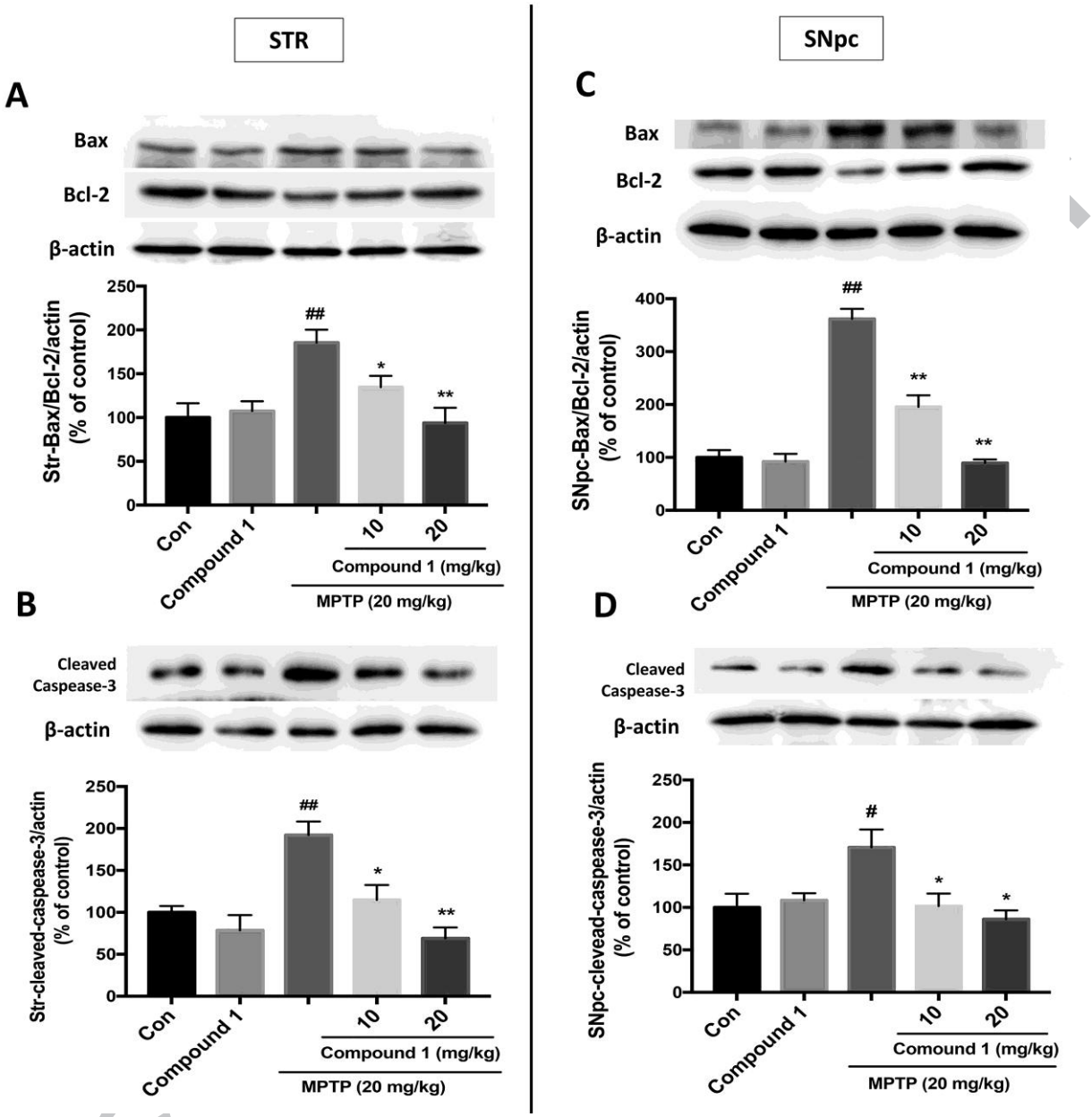


Figure 5. Effects of **1** on anti-oxidative and anti-apoptosis in vivo. Anti-apoptosis activity viewed by the expression of apoptosis-related proteins. (A) The original bands of Bax, Bcl-2 and the quantitative analysis of Bcl-2/Bax ratio in Str. (B) The original bands and the quantitative analysis of cleaved-caspease-3 expression in Str. (C) The original bands of Bax, Bcl-2, and the expression of Bcl-2, Bax and the quantitative analysis of Bcl-2/Bax ratio in SNpc (D) The original bands and the quantitative analysis of cleaved-caspease-3 expression in SNpc. All data were represented as mean ± S.D. (n = 6 mice/group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control and $^{*}p < 0.05$, $^{**}p < 0.01$ and compared with MPTP treatment group.

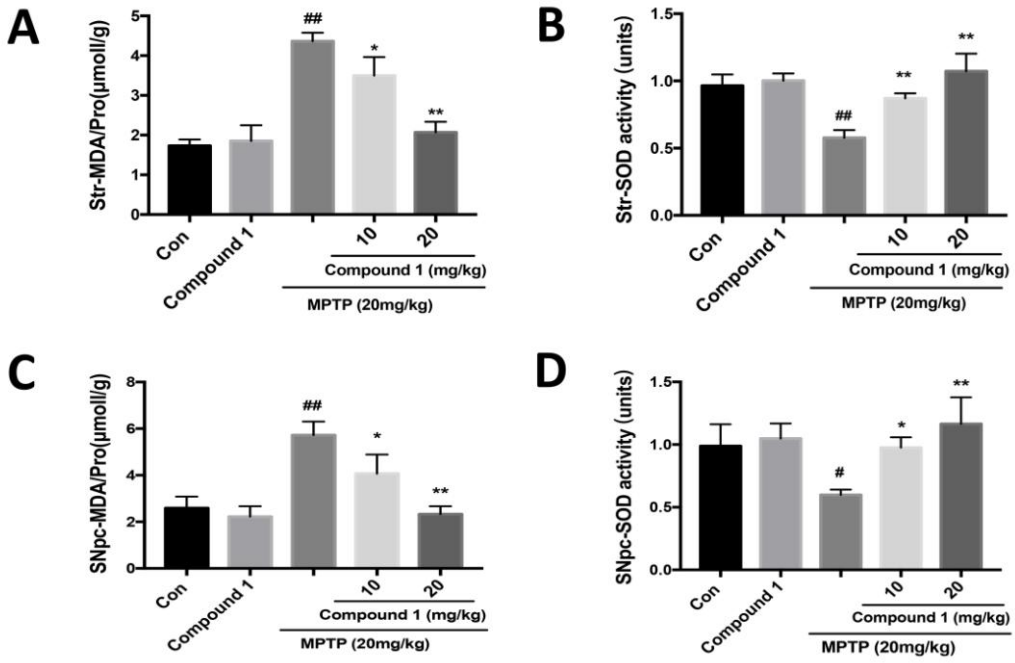


Figure 6. Anti-oxidative activity in vivo viewed by MDA levels and SOD activity. (A) Bar graphs represented the level of MDA in Str. (B) The activity of SOD in Str. (C) The level of MDA in SNpc. (D) The activity of SOD in SNpc. The data were represented as mean ± S.D. (n = 6 mice/group). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control and $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with MPTP treatment group;

Table 2

Pharmacokinetic parameters of **1**

Parameters	p.o. (25 mg/kg)	i.v. (5 mg/kg)
Cl (mL/min/kg)	40.83 ± 8.45	25.5 ± 4.57
V_{dss} (L/kg)	5.98 ± 2.18	2.25 ± 1.46
$AUC_{0-\infty}$ (h · ng/mL)	2495.56 ± 62.32	3272.87 ± 133.41
$T_{1/2}$ (h)	2.70 ± 1.16	0.85 ± 0.74
T_{max}(h)	2.00	——
C_{max} (ng/mL)	807.95 ± 21.32	——
MRT (h)	3.06 ± 0.92	1.45 ± 0.61
F (%)	15.25	

Note：Dose: i.v., 5 mg/kg (5% DMSO +2% F68 + 93% saline)； p.o., 25 mg/kg, dissolved in methylcellulose (0.5 %) solution containing 1% Tween 80. Cl: Plasma clearance; V_{dss} :Volume of distribution; AUC :Area

under curve; $T_{1/2}$: Half-life; T_{max}: time to reach peak plasma concentration; C_{max}: peak plasma concentration; MRT: Mean Retention Time; F: Absolute oral bioavailability.

$$F = \frac{D_{iv} \times AUC_{oral}}{D_{oral} \times AUC_{iv}} \times 100\%$$

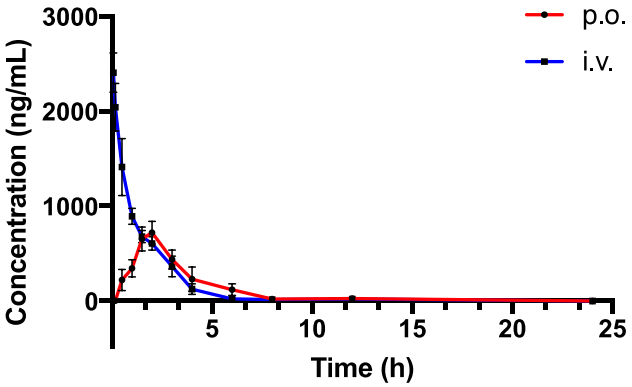


Figure 7. Concentration-time curves in the PK study. Plasma concentration–time profile was evaluated after i.v. (5 mg/kg) and p.o. (25 mg/kg) administration of compound **1** to male Sprague–Dawley rats (mean ± SD, n = 3).

Normally, the level of HO-1 is low in neurons, it can be highly up-regulated during the progression of Parkinson's patients **[29]**. Accumulated evidences suggested that the up-regulation of HO-1 by various stimuli may represent an adaptive and protective cellular response to alleviate the damage caused by these stimuli **[30]**. Fu's study has demonstrated that overexpression of HO-1 protected dopaminergic neurons against MPP^+ induced neurotoxicity in midbrain neuron glia co-cultures, indicating that HO-1 may be involved in the pathogenesis of Parkinsonism **[31]**. Therefore, we were interested in whether or not the neuroprotection of **1** was involved in modulating HO-1 expression. In accordance with the postulation, treatment with **1** at 0.1 μM (111.39 ± 17.30%), 1 μM (154.79 ± 21.83%, $p < 0.05$) and 10 μM (205.82 ± 14.44%, $p < 0.01$) could dose-dependently increase the HO-1 expression of control in SH-SY5Y cells. (**Figure 8A**). Moreover, H_2O_2 (600 μM), as an oxidative stress stimuli, led to an increase of HO-1 expression (181.53 ± 24.67%, $p < 0.05$) of control after 24 h treatment. However, pretreatment with **1** at 1 μM (273.55 ± 18.32%, $p < 0.05$)) or 10 μM (324.80 ± 16.62%, $p < 0.01$) for 6 h, followed by H_2O_2 treatment for 24h, induced a more significant increase of HO-1 expression compared with H_2O_2 treatment alone (**Figure 8B**). These results suggested that **1** could specifically up-regulate the HO-1 expression in SH-SY5Y cells.

To further investigate the potential role of HO-1 in the **1**-mediated neuroprotective activity, cells were pretreated with or without specific HO-1 inhibitor, zinc protoporphyrin-IX (ZnPPIX 10

μM), and followed by **1** plus H_2O_2 treatment. As shown in **Figure 8C**, pretreatment with ZnPPIX cloud significantly alleviate the protective effect of **1**, indicating the induction of HO-1 is essential for **1** to attenuate H_2O_2-induced cytotoxicity. On the other hand, an extremely high level of HO-1 can result in more oxidative stress due to generating excessive free iron **[32]**. Our results showed that **1** could enhance the expression of HO-1 without cytotoxicity in SH-SY5Y cells, suggesting **1** was able to up-regulate HO-1 appropriately.

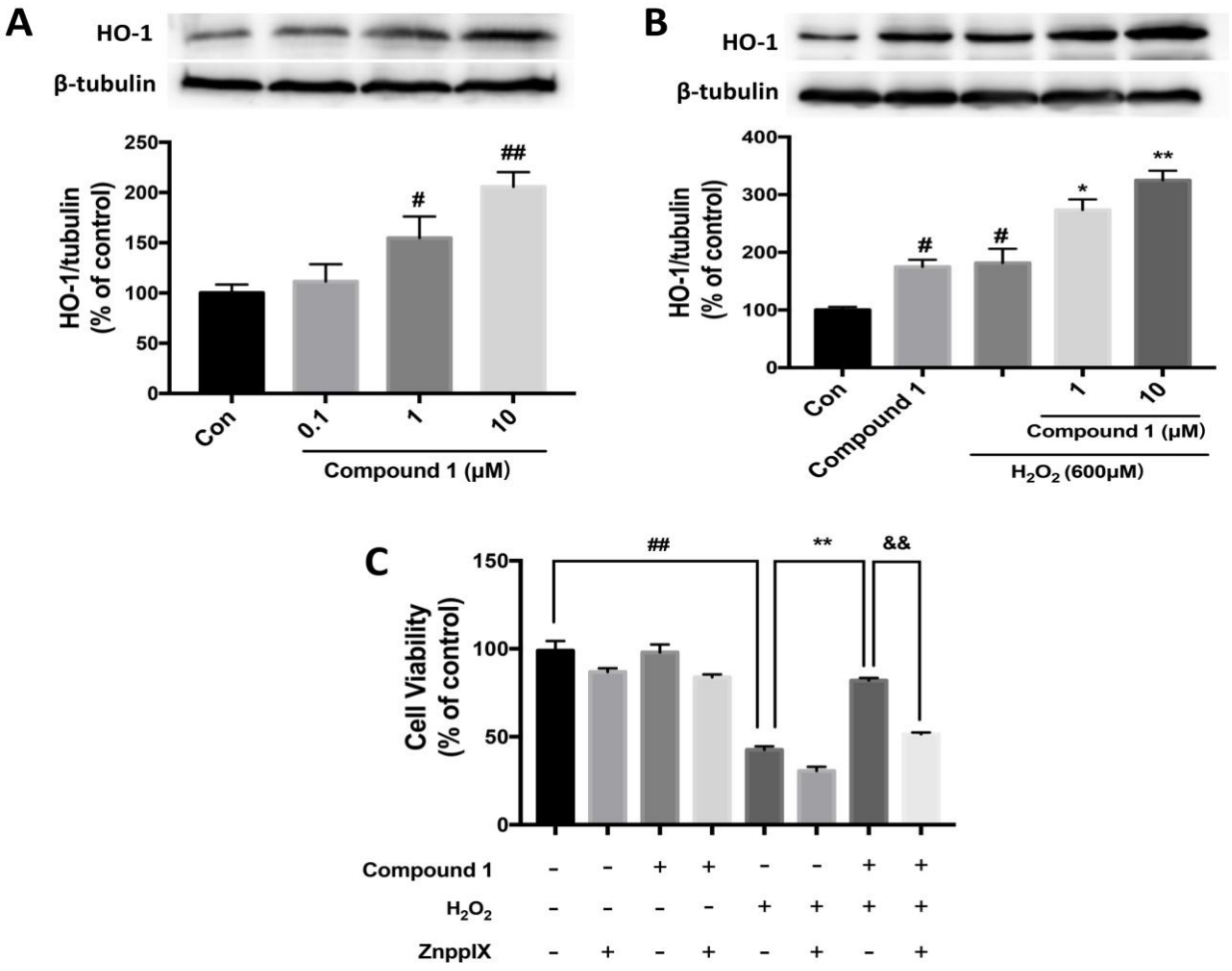


Figure 8. Effect of **1** on the expression of HO-1 in SH-SY5Y cells. (A) The original bands and the quantitative analysis of HO-1 expression in the cells which have been incubated with various concentrations (0.1, 1 or 10 μM) of **1** for 24 h. (B) The original bands and the quantitative analysis of HO-1 expression in the cells which was treated with **1** (1 or 10 μM) and H_2O_2 (600 μM). (C) Cells were pretreated with heme oxygenase-1 inhibitor, ZnPPIX (10 μM) for 1h and **1** (10 μM) for 6 h, then cells were exposed to H_2O_2 (600 μM) for 24 h and the Cell viability was assessed by MTT assay. All the results are expressed as mean ± S.E.M. from three-independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control and $^{*}p < 0.05$, $^{**}p < 0.01$ compared with H_2O_2 treatment and $^{\&\&}p < 0.01$ compared with **1** plus H_2O_2 group.

As mentioned in previous studies, various signaling pathways were related with up-regulation of HO-1, including PI3K/AKT/mTOR **[33, 34]** and MAPKs **[35]**. Therefore, to examine the possible signaling pathways involved in HO-1 induction, in this study, SH-SY5Y cells were respectively pretreated with several inhibitors, including SP600125, SB203580, U0126, LY294002 and Rapmycin for 1 h, and followed by stimulating with **1** and H_2O_2. As shown in **Figure 9A**, pretreatment with the PI3K/AKT/mTOR inhibitors LY294002 and Rapmycin, respectively, could significantly reduce the stimulated HO-1 expression by **1** plus H_2O_2. However, HO-1 expression not affected by MEK1/2 inhibitor, JNK inhibitor or p38 MAPK inhibitor treatment. These results suggested that the PI3K/AKT/mTOR pathway is required for the induction of HO-1 by **1** plus H_2O_2 in SH-SY5Y cells. Moreover, as well known, PI3K/AKT/mTOR pathway plays an important role in cell proliferation and apoptosis in the brain **[36]**. To further test whether the pathway act as a potential signal for neuroprotection of **1**, we examined the effects of the pathway inhibition on the extent of apoptosis in SH-SY5Y cells. As shown in **Figure 9B**, the neuroprotection of **1** was significantly reduced by the pretreatment of PI3K/Akt/mTOR pathway inhibitors, LY294002 (60.53 ± 2.04%, $p < 0.01$) and Rapmycin (65.94 ± 1.53, $p < 0.01$) compared with **1** plus H_2O_2 group (80.77 ± 1.927 %). Furthermore, we investigated the effects of **1** on the expression of phospho-AKT (p-AKT) and its downstream effector phospho-mTOR (p-mTOR) in SH-SY5Y cells. The time course results as shown in **Figures 9C-D**, unstimulated cells appeared a relative low expression of phosphorylated AKT and mTOR, while treatment with **1** in different times (5, 15, 30, 60, 120, 240, 360 min) could increase the phosphorylation of AKT and mTOR, and which both peaked at 30 min. The above results supported that **1** regulates the expression of HO-1, at least in part, through the PI3K/Akt/mTOR pathway, which mainly exert a neuroprotective effect in this experimental model.

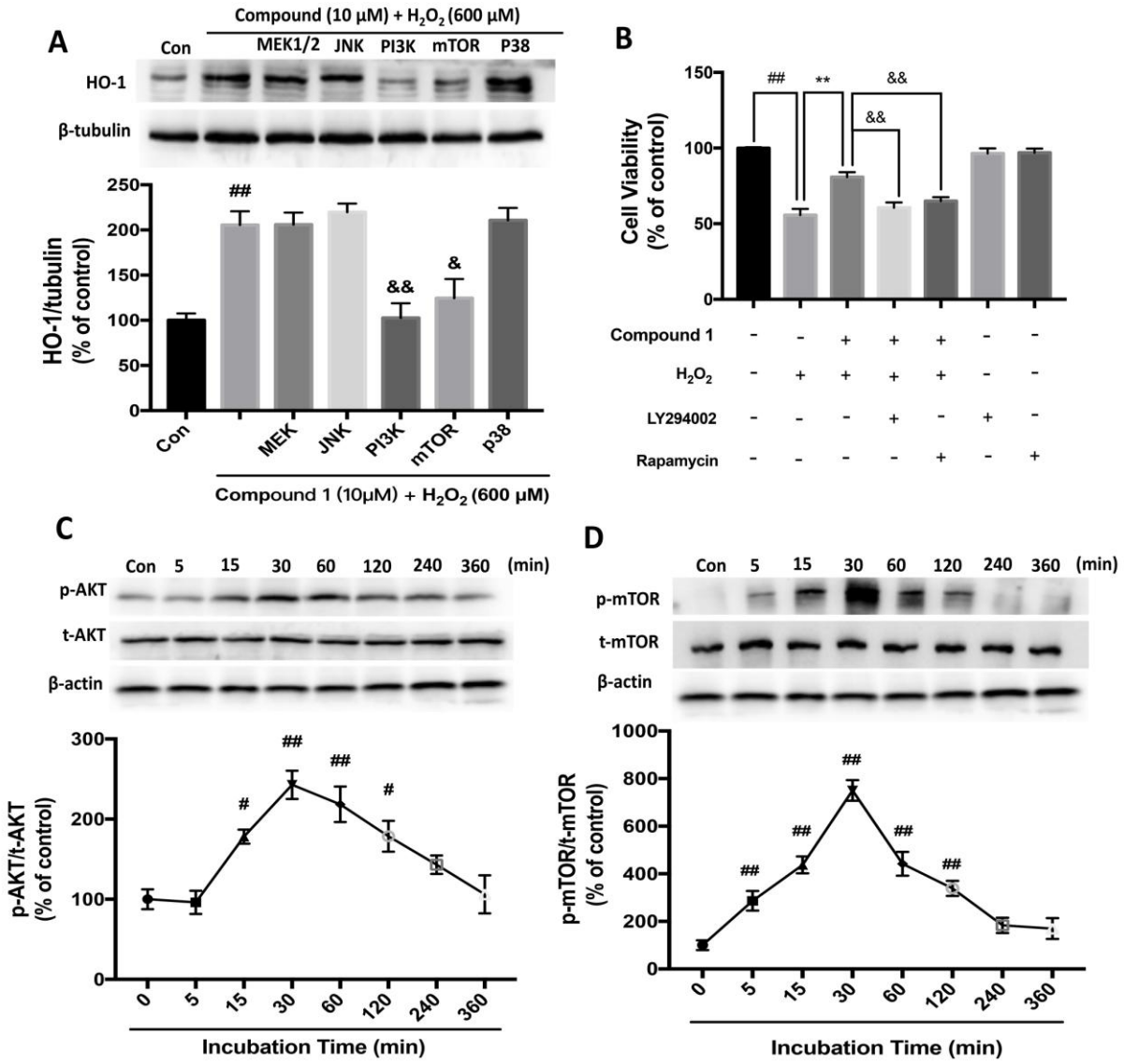


Figure 9. Induction of HO-1 blocked by mTOR inhibition and PI3K inhibition. (A) SH-SY5Y cells were preincubated for 1 h with either SB203580 (p38 inhibitor 10 μM), LY294002 (PI3K inhibitor 5 μM), U0126 (MEK1/2 inhibitor 10 μM), SP600125 (JNK inhibitor 5 μM) and Rapmycin (mTOR inhibitor 100 nM) and then treated with **1** (10 μM) for 6 h before being exposed to 600 μM H_2O_2. The expression of HO-1 were analyzed by Western blot and plotted as a graph after densitometric analysis; (B) Effect of blocking the PI3K/AKT/mTOR pathways on the extent of apoptosis in SH-SY5Y cells. The cell viability was determined by MTT. Effects of **1** on the expression of AKT and mTOR in SH-SY5Y cells. (C) Time courses of phosphorylated AKT proteins, the original bands and the quantitative analysis of p-AKT/t-AKT ratio. (D) Time courses of phosphorylated mTOR proteins, the original bands and the quantitative analysis of p-mTOR/t-mTOR ratio. All the results are expressed as mean ± S.E.M. from three-independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control and $^{*}p < 0.05$, $^{**}p < 0.01$ compared with H_2O_2 treatment and $^{\&}p <$

0.05, $^{\&\&}p < 0.01$ compared with H_2O_2 plus **1** group.

In summary, seven compounds **(1-7)** with the catechol group showed the significant neuroprotection against H_2O_2-induced SH-SY5Y cytotoxicity, compared to those of **8-15** without catechol group, suggesting that catechol group might be the important pharmacophore for their neuroprotective effects against oxidative damage, and their protective effects are independent of the quantity of phenolic hydroxyl groups. Moreover, the optimal candidate, compound **1**, provide neuroprotection involved in reducing the lipid peroxidation and ROS generation, recovering mitochondrial function, and mediating the expression of apoptosis-related proteins. The major finding in our present study is the key role for HO-1 expression-related signals contributing to the neuroprotective effect of **1**. Furthermore, in vivo conditions, oral treatment with **1** could markedly alleviate the behavior impairment and increase TH level both in SNpc and Str of MPTP-induced PD mice. The pharmacokinetic (PK) assessment indicated that **1** has a normal oral bioavailability in the rat. All these promising findings suggested that **1** might be a promising lead in the development of orally active therapies for PD, and might provide a thought for the design of Parkinson's therapeutic agents.

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References

[1] De Virgilio, A., Greco, A., Fabbrini, G., Inghilleri, M., Rizzo, M. I., Gallo, A., Conte, M., Rosato, C., Appiani, M. C., and de Vincentiis, M. (2016) Parkinson's disease: Autoimmunity and neuroinflammation (vol 15, pg 1005, 2016), *Autoimmun Rev 15*, 1210-1210.

[2] Morales-Garcia, J. A., Redondo, M., Alonso-Gil, S., Gil, C., Perez, C., Martinez, A., Santos, A., and Perez-Castillo, A. (2011) Phosphodiesterase 7 Inhibition Preserves Dopaminergic Neurons in Cellular and Rodent Models of Parkinson Disease, *Plos One 6*.

[3] Sveinbjornsdottir, S. (2016) The clinical symptoms of Parkinson's disease, *J Neurochem 139*, 318-324.

[4] Dong, J., Cui, Y., Li, S., and Le, W. (2016) Current Pharmaceutical Treatments and Alternative Therapies of Parkinson's Disease, *Curr Neuropharmacol 14*, 339-355.

[5] Jankovic, J., and Aguilar, L. G. (2008) Current approaches to the treatment of Parkinson's disease, *Neuropsychiatr Dis Treat 4*, 743-757.

[6] Hybertson, B. M., Gao, B. F., Bose, S. K., and McCord, J. M. (2011) Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation, *Mol Aspects Med 32*, 234-246.

[7] Otterbein, L. E., and Choi, A. M. K. (2000) Heme oxygenase: colors of defense against cellular stress, *Am J Physiol-Lung C 279*, L1029-L1037.

[8] Morse, D., and Choi, A. M. K. (2002) Heme oxygenase-1-The "emerging molecule" has arrived, *Am J Resp Cell Mol 27*, 8-16.

[9] Li, R., Zheng, N., Liang, T., He, Q., and Xu, L. (2013) Puerarin attenuates neuronal degeneration and blocks oxidative stress to elicit a neuroprotective effect on substantia nigra injury in 6-OHDA-lesioned rats, *Brain Res 1517*, 28-35.

[10] Lin, H. Y, Yeh, W. L., Huang, B. R., Lin, C., Lai, C. H., Lin, H., and Lu, D. Y. (2012) Desipramine protects neuronal cell death and induces heme oxygenase-1 expression in Mes23.5 dopaminergic neurons, *Plos One 7*, e50138.

[11] Yoshihara, T., Yoshikawa, H, Sakamura, S., Sakuma, T., (1974) Clovamides; L-dopa conjugated with trans- and cis-caffeic acids in red clover (Trifolium pratense), *Agric. Biol. Chem. 38*, 1107–1109.

[12] Zamperone, A., Pietronave, S., Colangelo, D., Antonini, S., Locatelli, M., Travaglia, F., Coisson, J. D., Arlorio, M., and Prat, M. (2014) Protective effects of clovamide against H_2O_2-induced stress in rat cardiomyoblasts H9c2 cell line, *Food Funct* 5, 2542-2551.

[13] Kolodziejczyk, J., Olas, B., Wachowicz, B., Szajwaj, B., Stochmal, A., and Oleszek, W. (2011) Clovamide-rich extract from Trifolium pallidum reduces oxidative stress-induced damage to blood platelets and plasma, *J Physiol Biochem* 67, 391-399.

[14] Heijnen, C. G., Haenen, G. R., Vekemans, J. A., and Bast, A. (2001) Peroxynitrite scavenging of flavonoids: structure activity relationship, *Environ Toxicol Phar 10*, 199-206.

[15] Georgiev, L., Chochkova, M., Totseva, I., Seizova, K., Marinova, E., Ivanova, G., Ninova, M., Najdenski, H., and Milkova, T. (2013) Anti-tyrosinase, antioxidant and antimicrobial activities of hydroxycinnamoylamides, *Med Chem Res 22*, 4173-4182

[16] Hu, X. L., Lin, J., Lv, X. Y., Feng, J. H., Zhang, X. Q., Wang, H., and Ye, W. C. (2018) Synthesis and biological evaluation of clovamide analogues as potent anti-neuroinflammatory agents in vitro and in vivo, *Eur J Med Chem 151*, 261-271.

[17] Jomova, K., and Valko, M. (2011) Importance of Iron Chelation in Free Radical-Induced Oxidative Stress and Human Disease, *Curr Pharm Design 17*, 3460-3473.

[18] Hu, X. L., Gao, L. Y., Niu, Y. X., Tian, X., Wang, J., Meng, W. H., Zhang, Q., Cui, C., Han, L., and Zhao, Q. C. (2015) Neuroprotection by Kukoamine A against oxidative stress may involve N-methyl-D-aspartate receptors, *Bba-Gen Subjects 1850*, 287-298.

[19] Dai, D. F., Chen, T., Szeto, H., Nieves-Cintron, M., Kutyavin, V., Santana, L. F., and Rabinovitch, P. S. (2011) Mitochondrial targeted antioxidant Peptide ameliorates hypertensive cardiomyopathy, *J Am Coll Cardiol 58*, 73-82.

[20] Beal, M. F. (2005) Mitochondria take center stage in aging and neurodegeneration, *Ann Neurol 58*, 495-505.

[21] Matsuda, S., Umeda, M., Uchida, H., Kato, H., and Araki, T. (2009) Alterations of oxidative stress markers and apoptosis markers in the striatum after transient focal cerebral ischemia in rats, *J Neural Transm 116*, 395-404.

[22] Nirmaladevi, D., Venkataramana, M., Chandranayaka, S., Ramesha, A., Jameel, N. M., and Srinivas, C. (2014) Neuroprotective Effects of Bikaverin on H_2O_2-Induced Oxidative Stress Mediated Neuronal Damage in SH-SY5Y Cell Line, *Cell Mol Neurobiol 34*, 973-985.

[23] Lin, M. T., and Beal, M. F. (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, *Nature 443*, 787-795.

[24] Kim, S., Park, S. E., Sapkota, K., Kim, M. K., and Kim, S. J. (2011) Leaf extract of Rhus verniciflua Stokes protects dopaminergic neuronal cells in a rotenone model of Parkinson's disease, *J Pharm Pharmacol 63*, 1358-1367.

[25] Zuo, W., Zhang, W., Han, N., and Chen, N.-H. (2015) Compound IMM-H004, a Novel Coumarin Derivative, Protects against CA1 Cell Loss and Spatial Learning Impairments Resulting from Transient Global Ischemia, *Cns Neurosci Ther 21*, 280-288.

[26] Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983) Chronic parkinsonism in humans due to a product of meperidine-analog synthesis, *Science 219*, 979-980.

[27] Przedborski, S., and Vila, M. (2001) MPTP: a review of its mechanisms of neurotoxicity, *Clin Neurosci Res 1*, 407-418.

[28] Haavik, J., and Toska, K. (1998) Tyrosine hydroxylase and Parkinson's disease, *Mol Neurobiol 16*, 285-309.

[29] Takeda, A., Itoyama, Y., Kimpara, T., Zhu, X. W., Avila, J., Dwyer, B. E., Perry, G., and Smith, M. A. (2004) Heme catabolism and heme oxygenase in neurodegenerative disease, *Antioxid Redox Sign 6*, 888-894.

[30] Hung, S. Y., Liou, H. C., Kang, K. H., Wu, R. M., Wen, C. C., and Fu, W. M. (2008) Overexpression of Heme Oxygenase-1 Protects Dopaminergic Neurons against 1-Methyl-4-Phenylpyridinium-Induced Neurotoxicity, *Mol Pharmacol 74*, 1564-1575.

[31] Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) Biliverdin reductase: A major physiologic cytoprotectant, *P Natl Acad Sci USA 99*, 16093-16098.

[32] Ryter, S. W., Alam, J., and Choi, A. M. K. (2006) Heme oxygenase-1/carbon monoxide: From basic science to therapeutic applications, *Physiol Rev 86*, 583-650.

[33] Hamdulay, S. S., Wang, B., Birdsey, G. M., Ali, F., Dumont, O., Evans, P. C., Haskard, D. O., Wheeler-Jones, C. P., and Mason, J. C. (2010) Celecoxib activates PI-3K/Akt and mitochondrial redox signaling to enhance heme oxygenase-1-mediated anti-inflammatory activity in vascular endothelium, *Free Radic Biol Med 48*, 1013-1023.

[34] Kang, K. W., Lee, S. J., Park, J. W., and Kim, S. G. (2002) Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress, *Mol Pharmacol 62*, 1001-1010.

[35] Ogborne, R. M., Rushworth, S. A., and O'Connell, M. A. (2008) Epigallocatechin activates haem oxygenase-1 expression via protein kinase Cdelta and Nrf2, *Biochem Biophys Res Commun 373*, 584-588.

[36] Annovazzi, L., Mellai, M., Caldera, V., Valente, G., Tessitore, L., and Schiffer, D. (2009) mTOR, S6 and AKT Expression in Relation to Proliferation and Apoptosis/Autophagy in Glioma, *Anticancer Res 29*, 3087-3094

Highlights

1.Fiftten clovamide analogues were design and synthesized

2.Analogues containing catechol functionality exhibited neuroprotective effects against H_2O_2-induced oxidative damage *in vitro*.

3.Compound **1** could up-regulate HO-1 expression via PI3K/AKT/mTOR pathway.

4. Oral administration of compound **1** improve the symptoms of Parkinson's disease in MPTP-induced mice model.

5. Pharmacokinetic (PK) assessment indicated that **1** has a normal oral bioavailability in the rat.