

Original Article

# LLDT-67 attenuates MPTP-induced neurotoxicity in mice by up-regulating NGF expression

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**Aim:** To investigate the neuroprotective effects of LLDT-67, a novel derivative of triptolide, in MPTP-induced mouse Parkinson's disease (PD) models and in primary cultured astrocytes, and to elucidate the mechanisms of the action.

**Methods:** In order to induce PD, C57BL/6 mice were injected MPTP (30 mg/kg, ip) daily from d 2 to d 6. MPTP-induced behavioral changes in the mice were examined using pole test, swimming test and open field test. The mice were administered LLDT-67 (1, 2, or 4 mg/kg, po) daily from d 1 to d 11. On d 12, the mice were decapitated and brains were collected for immunohistochemistry study and measuring monoamine levels in the striatum. Primary cultured astrocytes from the cortices of neonatal C57BL/6 mouse pups were prepared for *in vitro* study.

**Results:** In MPTP-treated mice, administration of LLDT-67 significantly reduced the loss of tyrosine hydroxylase-positive neurons in the substantia nigra, and ameliorated the behavioral changes. LLDT-67 (4 mg/kg) significantly increased the expression of NGF in astrocytes in the substantia nigra and striatum of the mice. Furthermore, administration of LLDT-67 caused approximately 2-fold increases in the phosphorylation of TrkA at tyrosine 751, and marked increases in the phosphorylation of AKT at serine 473 as compared with the mice model group. In the cultured astrocytes, LLDT-67 (1 and 10 nmol/L) increased the NGF levels in the culture medium by 179% and 160%, respectively.

**Conclusion:** The neuroprotective effect of LLDT-67 can be mostly attributed to its ability to enhance NGF synthesis in astrocytes in the midbrain and to rescue dopaminergic neurons indirectly through TrkA activation.

**Keywords:** LLDT-67; triptolide; Parkinson's disease; MPTP; neuroprotection; dopaminergic neurons; substantia nigra; striatum; NGF; TrkA

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## Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by bradykinesia, resting tremor, gait disturbance and postural instability. PD is caused by the progressive degeneration of dopaminergic neurons in the substantia nigra, which results in the depletion of the neurotransmitter dopamine in the striatum. It has been proposed that parkinsonian clinical signs appear at the point when dopaminergic neuronal cell loss exceeds a critical threshold: 70%–80% of striatal nerve terminals and 50%–60% of the substantia nigra par compacta perikaryons<sup>[1, 2]</sup>. L-dopa is the most widely used drug for the treatment of PD, but long-term treatment can produce several adverse side effects. Therefore, the search for novel drugs for the treatment of PD

is an important endeavor. Triptolide is one of the major active components of the Chinese herb *Tripterygium wilfordii* Hook F. It is well known that triptolide has anti-inflammatory, immunosuppressive, contraceptive and antitumor properties<sup>[3–5]</sup>. Some studies indicate that PD is a chronic neuroinflammatory process, and triptolide has been found to possess antiparkinsonian effects<sup>[6]</sup>. However, triptolide's severe toxicity limits its clinical applications. LLDT-67, which is characterized by strong biological activity and low toxicity, is a novel derivative of triptolide.

## Materials and methods

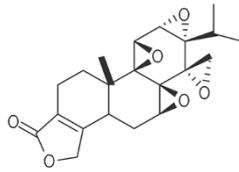
### Reagents

(5*R*)-5*a*-hydroxytriptolide (T8) and LLDT-67 (Figure 1) were synthesized by Prof Yuan-chao LI of the Department of Medicinal Chemistry of Shanghai Institute of Materia Medica. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and L-dopa were purchased from Sigma Chemical Co (St Louis,

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**Figure 1.** Structure of LLDT-67.

MO, USA) and dissolved in saline. Anti-tyrosine hydroxylase (TH) antibodies and anti-gial fibrillary acidic protein (GFAP) were purchased from Chemicon International (Temecula, CA, USA). Anti-NGF was purchased from Sigma Chemical Co (St Louis, MO, USA). Anti-phospho-ERK1/2 and anti-phospho-AKT (473) antibodies were purchased from Cell Signaling Technology, Inc (Boston, USA); the anti-p-TrkA (490) antibody was purchased from Abcam Ltd (Hongkong, China); and the anti-phospho-TrkA (751) antibody was purchased from Invitrogen Inc (Carlsbad, CA, USA). All DyLight<sup>TM</sup>488- and DyLight<sup>TM</sup>59-labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA). Biotinylated secondary antibodies and avidin-biotin peroxidase complexes were purchased from Vector Laboratories (Burlingame, CA, USA). Dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3,4-dihydroxybenzylamine (DHBA), were purchased from Fluka Chemicals (Switzerland). The ChemiKine NGF Immunoassay system and the SuperSignal West Dura Extended Duration Substrate were purchased from Chemicon (Temecula, CA, USA) and Pierce (Rockford, IL, USA), respectively.

### Animals

Male C57BL/6 mice, weighing 20–22 g, were used in the present study. The animals had free access to solid food and water under standard conditions of temperature, humidity and lighting. The study was performed in compliance with the National Research Council's guidelines and was approved by the Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD in mice at a dose of 30 mg/kg. MPTP was administered intraperitoneally for five consecutive days starting from d 2<sup>[7,8]</sup>. Three doses of LLDT-67 were administered intragastrically for 11 consecutive days starting from d 1. On d 12, the animals were anesthetized and brain tissue was collected.

### Behavioral studies

#### Pole test

The pole test was used to evaluate bradykinesia, a typical symptom of parkinsonism<sup>[9,10]</sup>. The mice were placed head up near the top of a vertical rough-surfaced pole (8 mm in diameter and 55 cm in length). The time it took the mice to turn around completely and face the floor (time to turn; T-turn) and the time it took the mice to climb down the pole and place all four feet on the floor (locomotion activity time; T-LA) were

recorded with a cutoff limit of 30 s. The test was performed five times on each mouse.

#### Swimming test

The mice were forced to swim in an acrylic glass cylinder filled with water and from which they could not escape. The test lasted 90 s, and the time that the mice spent swimming was measured. If the time that mice swimming is over 90 s, then the measure is stopped and the time is recorded as 90 s.

#### Open-field test

The open field test is a simple assessment used to determine general activity levels, gross locomotor activity and exploration habits in mice<sup>[11]</sup>. The assessment was conducted on the 7th d in one of two square arenas (40 cm×40 cm) enclosed by a 30-cm wall. Each arena was divided into 16 smaller equal-sized squares (10 cm×10 cm). Each mouse was placed in the center of the open-field arena and allowed to move about freely for 5 min. Four parameters were measured in this experiment: line crossing (the number of lines crossed), cleaning (the number of times the mouse cleaned its fur), hind limb standing (the number of times the mouse stood on its hind legs), and inactive sitting (the duration of inactivity measured in sitting). At the conclusion of each trial, the surface of the arena was cleaned with 90% ethanol. The evaluation was conducted by an investigator who was unaware of the information of grouping and drug administration.

#### Immunohistochemistry

On d 12, after anesthetization with 10% chloral hydrate, the mice were perfused with PBS via the intracardiac route, followed by 4% paraformaldehyde in PBS. The mice were then decapitated, and the brains were removed and immersed for 24 h in 4% paraformaldehyde for fixation. Midbrain coronal sections were then prepared using a cryostat. For DAB staining, the sections were first rinsed with PBS containing 0.1% Triton-X and then immersed in a solution of 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min. After incubating overnight with anti-TH (1:1000) in PBS-T containing 10% goat serum at 4°C, the sections were washed three times with PBS-T and incubated with a biotinylated secondary antibody in PBS-T for 2 h at room temperature. The sections were then incubated with an avidin-biotin peroxidase complex for 1 h. A final incubation with DAB was performed for visualization. The sections were then washed with PBS, mounted on aminopropyltriethoxysilane-coated slides, dried, dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped. For immunofluorescence staining, the sections were immersed in a 1% SDS solution and incubated for 5 min at room temperature. The sections were then incubated in a serum blocking solution for 1 h. After incubating overnight with mouse monoclonal anti-GFAP (1:1000) and anti-NGF (1:1000) in PBS-T containing 10% goat serum at 4°C, the sections were washed three times with PBS-T, incubated with DyLight<sup>TM</sup>488- and DyLight<sup>TM</sup>59-labeled secondary antibodies (1:800) and then examined using laser scanning confocal microscopy (Olympus FV300, IX70).

### HPLC analysis of monoamine neurotransmitter levels in the striatum

On completion of the study, the mice were deeply anesthetized by administering 10% chloral hydrate and rapidly decapitated. Striata were dissected and homogenized by sonication in an ice-cold processing solution (0.2 mol/L perchloric acid, 0.2 mmol/L sodium pyrosulfite, and 0.01% EDTA-2Na). The homogenate was centrifuged at 17 000×g for 15 min at 4°C. The supernatant was analyzed for dopamine and its metabolites, DOPAC and HVA, using reverse-phase HPLC coupled to an electrochemical detector (EC). The mobile phase consisted of 40 mmol/L sodium acetate, 15 mmol/L citric acid, 0.25 mmol/L sodium octanesulfonate, 0.2 mmol/L EDTA-2Na, and 16% methanol (pH 4.3). The filtered supernatant was introduced into the autosampler carousel (Agilent 1100) with a flow rate of 1 mL/min using a C18 column (DICKMA). The concentrations of DA and its metabolites were quantified using a standard curve, generated by determining the ratio between the known concentration of amines and the concentration of an internal standard of DHBA, and expressed in units of µg/g of wet weight.

### Primary astrocyte culture

Astrocytes were prepared from the brains of 1- to 2-d-old neonatal C57BL/6 mouse pups, as described previously by Menet et al<sup>[12]</sup>. Briefly, brain tissues were trypsinized for 15 min, dissociated by gentle trituration, and plated at a density of 5×10<sup>7</sup> cells per 75-cm<sup>2</sup> flask (Corning, USA) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FBS. Cells were maintained in a complete culture medium for 7 d. Between the 8th and 12th d, the cultures were shaken to remove the top layer of cells sitting on top of the astroglial monolayer to yield mainly type-1 astrocytes with a flat morphology. Before experimental treatments, the cultures of astrocytes were passaged twice. Cells were allowed to reach 90% confluence. To investigate the effects of LLDT-67 on NGF expression, astrocytes were maintained in serum-free DMEM and treated with various concentrations of LLDT-67 for the indicated incubation times. Untreated cells were used as controls.

### Enzyme-linked immunosorbent assay (ELISA) for measuring NGF

NGF was detected with the ChemiKine NGF Immunoassay system (Chemicon) according to the manufacturer's instructions. Brain tissue was homogenized in an ice cold homogenization buffer consisting of 100 mmol/L Tris/HCl, pH 7.0, 1 mol/L NaCl, 4 mmol/L EDTA-2Na, 2% Triton X-100, 0.1% sodium azide and protease inhibitors (Sigma) at the following concentrations: 5 µg/mL aprotinin, 0.5 µg/mL antipain, 157 µg/mL benzamidin, 0.1 µg/mL pepstatin A and 17 µg/mL phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 14 000×g for 30 min. The resulting supernatant and mouse astrocyte primary culture medium were used in the NGF assay. Protein concentrations in the supernatant were measured using the mini-Bradford Coomassie Blue colorimetric assay (Bio-Rad). In using the NGF ELISA kit, sheep poly-

clonal antibodies generated against mouse NGF were coated onto a microplate and used to capture NGF from the sample. The sample was incubated overnight at 4°C. After washing the microplate with wash buffer, NGF-specific mouse monoclonal antibodies were added and incubated at room temperature for 2 h. After washing the plate a second time, mouse-specific donkey polyclonal antibodies were added, labeled with peroxidase, and incubated at room temperature for 2 h. After the addition of the substrate and the stop solution, the optical density was measured at 450 nm using a plate reader.

### Western blot analysis

The substantia nigra of decapitated mice were lysed in RIPA lysis buffer and loaded onto a 12% SDS-polyacrylamide gel for electrophoresis. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and incubated with the primary antibodies p-ERK1/2 (1:1000), p-AKT (473) (1:1000), p-TrkA (490) (1:500), and p-TrkA (751) (1:1000, Invitrogen). After washing, the blot was incubated with peroxidase-conjugated secondary antibodies and developed with a SuperSignal West Dura Extended Duration Substrate (Pierce). The bands were digitized, and the optical densities were analyzed using Image-J 1.38.

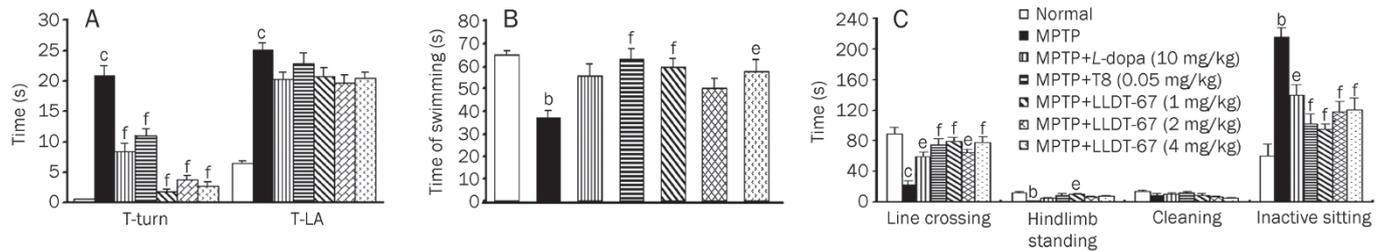
### Statistical analysis

The data are presented as the mean±SEM. Significant differences were determined by paired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparison. In all cases, the differences were considered statistically significant if  $P < 0.05$ .

## Results

### Effect of LLDT-67 on MPTP-induced motor deficits

To ascertain the effect of LLDT-67 on MPTP mouse models, subacute MPTP PD models were used for evaluating the efficacy of LLDT-67. LLDT-67 was administered to C57BL/6 mice (1, 2, and 4 mg/kg, *po*) for 11 d, and the mice were injected with MPTP every day from d 2 until d 6. MPTP injections resulted in significant motor deficits as evaluated by the pole test. Administration of MPTP caused a 33.8-fold increase in the time to turn (T-turn) and a 3.9-fold increase in the locomotion activity time (T-LA), compared with untreated controls. The results were statistically significant ( $P < 0.01$ ). Treatment with LLDT-67 significantly reduced the T-turn, but it did not have a significant effect on the T-LA (Figure 2A). We also used the swimming test to evaluate the efficacy of LLDT-67. Swimming time was reduced by 42.6% in MPTP-treated mice, compared with untreated controls ( $P < 0.01$ ). LLDT-67 (1 and 4 mg/kg) increased the swimming time significantly (Figure 2B). As for the open-field test, MPTP noticeably reduced the frequency of line crossing and increased the time of inactive sitting, compared with untreated controls ( $P < 0.001$  and  $P < 0.01$ , respectively). Treatment with LLDT-67 greatly reduced the magnitude of the MPTP-induced changes in locomotor activity (Figure 2C).



**Figure 2.** Effect of LLDT-67 on MPTP-induced behavioral changes in mice. (A) Pole test results. (B) Swimming test results. (C) Open-field test results. Each column represents the mean $\pm$ SEM of the results from 12 mice. <sup>b</sup> $P$ <0.05, <sup>c</sup> $P$ <0.01 compared with the control group. <sup>b</sup> $P$ <0.05, <sup>c</sup> $P$ <0.01 compared with the model group (one-way ANOVA followed by Dunnett's *post hoc* comparison).

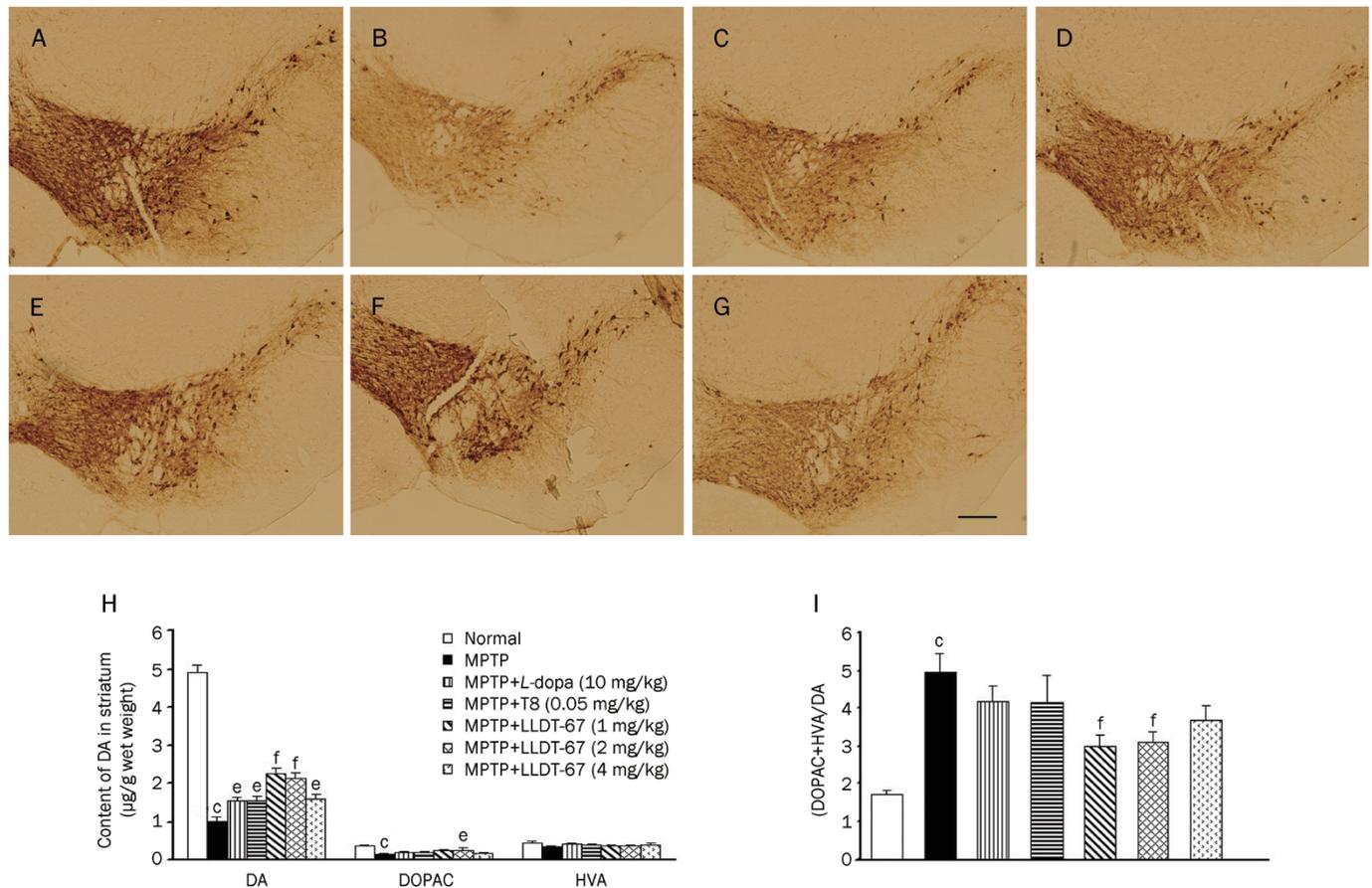
### Effect of LLDT-67 on MPTP-induced dopaminergic neurodegeneration

To elucidate the effect of LLDT-67 on MPTP-induced mouse dopaminergic neurodegeneration, sections of substantia nigra and striatum were stained with anti-TH antibody as a dopaminergic neuron-specific marker. MPTP was administered intraperitoneally for five consecutive days, and dopaminergic neurons in the substantia nigra compacta zone (SNc) were

largely lost (Figure 3B). Treatment with LLDT-67 significantly reduced the loss of dopaminergic neurons in the SNc (Figure 3E–3G). These results indicate that LLDT-67 has a neuroprotective effect when administered to mice with MPTP-induced PD.

### LLDT-67 increases dopamine content in the striatum

The supernatants of homogenates of the striata of mice in each experimental group were analyzed for dopamine and



**Figure 3.** Effect of LLDT-67 on MPTP-induced dopaminergic neuronal loss in the SNc and dopamine-content reduction in the striatum. (A–G) Results of TH histoimmunochemistry, bar=200  $\mu$ m/L.  $\times$ 100. (A) Normal; (B) MPTP; (C) MPTP+L-dopa (10 mg/kg); (D) MPTP+T8 (0.05 mg/kg); (E) MPTP+LLDT-67 (1 mg/kg); (F) MPTP+LLDT-67 (2 mg/kg); (G) MPTP+LLDT-67 (4 mg/kg); (H, I) Results of HPLC analysis of the DA content in the striatum. Each column represents the mean $\pm$ SEM of the results from six mice. <sup>b</sup> $P$ <0.05, <sup>c</sup> $P$ <0.01 compared with the control group; <sup>e</sup> $P$ <0.05, <sup>f</sup> $P$ <0.01 compared with the model group (one-way ANOVA followed by Dunnett's *post hoc* comparison).

its metabolites, DOPAC and HVA. The samples were analyzed using reverse-phase HPLC coupled to an electrochemical detector (EC). The results indicate that MPTP decreased the dopamine content of the striatum by approximately 80%, whereas LLDT-67 significantly increased the striatal dopamine content compared with the model group. The increased ratio of DOPAC+HVA to DA is indicative of an increase in the DA metabolic rate, which is commonly associated with partial neuronal damage. This result indicates that LLDT-67 can slow down the DA metabolic rate in injured neurons and exert a neuroprotective effect (Figure 3H, 3I).

#### LLDT-67 increases NGF expression in astrocytes of the SNc of MPTP-treated mice

To understand the mechanisms underlying the neuroprotective effect of LLDT-67 on mice with MPTP-induced PD, we used fluorescent immunohistochemistry to measure NGF expression in MPTP-treated mice. The results indicate that NGF and GFAP colocalized in astrocytes, and LLDT-67 increased NGF expression in astrocytes of the SNc as shown in Figure 4. In the SNc, 2 mg/kg of LLDT-67 significantly increased the expression of NGF in astrocytes, whereas doses of 1 mg/kg and 4 mg/kg only caused a small increase in NGF expression.

#### LLDT-67 increases the NGF content in the SN and striatum of MPTP-treated mice

We measured NGF concentrations in the SN and the striatum of MPTP-treated mice by performing an ELISA assay. We found that LLDT-67 markedly increased the NGF content in the SN and the striatum of MPTP-treated mice. As shown in Figure 5A and 5B, treatment with a low or moderate dose of LLDT-67 significantly increased NGF concentrations. Interestingly, T8 and L-dopa were ineffective in promoting NGF expression (Figure 5A and 5B).

#### Effect of LLDT-67 on NGF secretion by mouse astrocytes

To confirm the effect of LLDT-67 on NGF secretion in astrocytes, we purified astrocytes from the brains of newborn C57BL/6 mice and cultured the astrocytes in the presence of LLDT-67 ( $1 \times 10^{-11}$ – $1 \times 10^{-7}$  mol/L) for 48 h. NGF levels in the culture medium were measured using an ELISA assay. LLDT-67 treatment increased the NGF levels to some extent in a dose-dependent manner. Notably,  $1 \times 10^{-8}$  and  $1 \times 10^{-9}$  mol/L LLDT-67 increased the NGF levels in the culture medium by approximately 160% and 179%, respectively, compared with controls (Figure 5C).

#### Effect of LLDT-67 on the phosphorylation of the TrkA tyrosine 751 site and AKT in the SN

To study the mechanism of action of LLDT-67 in promoting neuronal survival, we examined several proteins of the NGF signaling pathway. The results indicate that LLDT-67 can activate TrkA in the SN of mice. Therefore, we set out to determine whether TrkA signaling could be activated by three different doses of LLDT-67 in the mouse brain. Phosphorylation

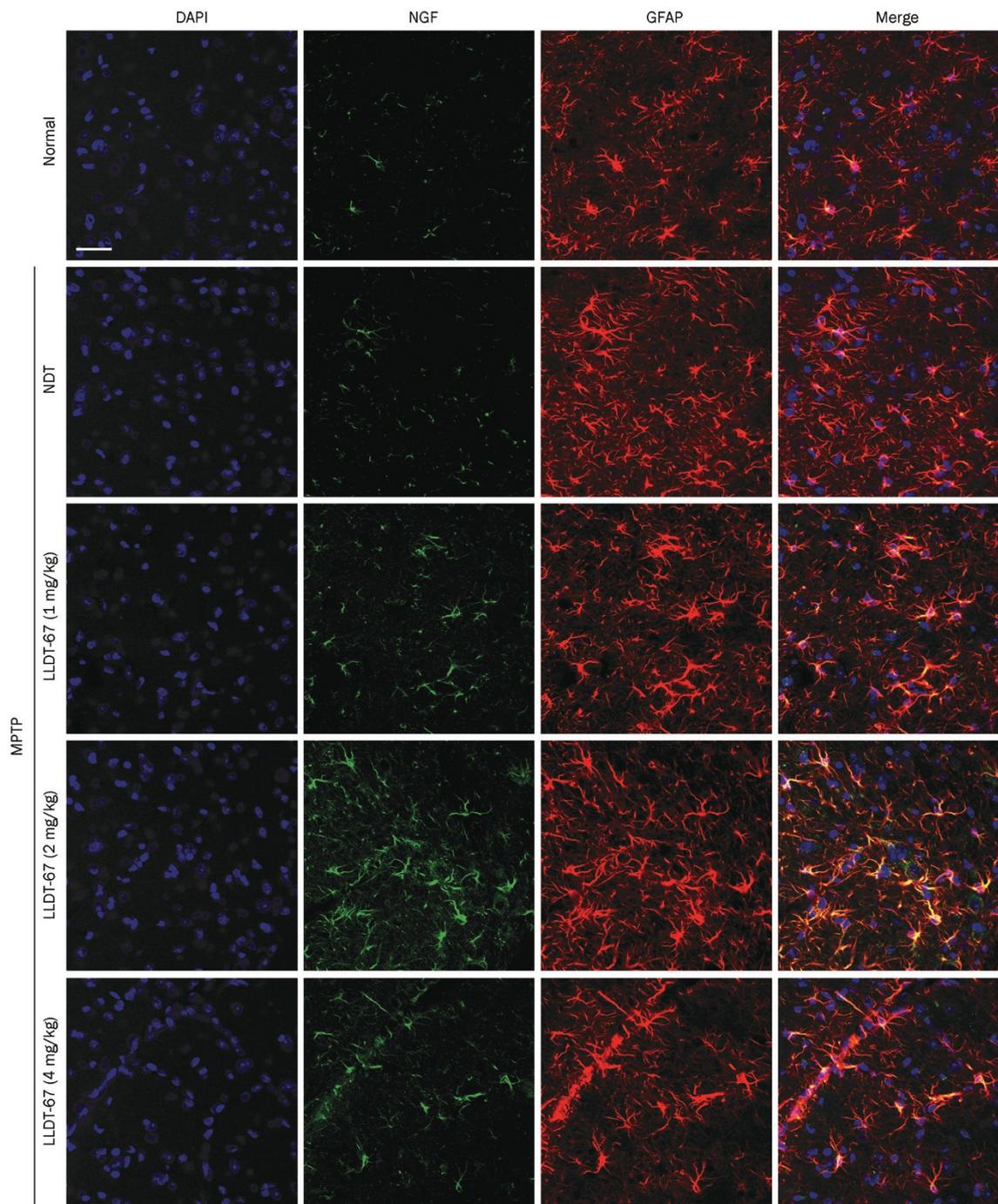
of TrkA on the 751 site was observed. The results indicate that LLDT-67 increased the phosphorylation of the tyrosine 751 site of TrkA in a dose-dependent manner (Figure 6A). Phosphorylation of AKT (Ser473) paralleled that of the TrkA receptors as shown in Figure 6B. We have demonstrated that LLDT-67 indirectly activates NGF-signaling pathways leading to PI3-kinase activation by directly increasing NGF expression. No changes were observed in the phosphorylation of TrkA-490 and its downstream protein, ERK1/2 (data not shown).

#### Discussion

To date, triptolide's mechanism of action has remained largely elusive. Recently, Liu *et al*<sup>[13]</sup> reported that triptolide covalently binds to human XPB (also known as ERCC3), a subunit of the transcription factor TFIIH, and inhibits its DNA-dependent ATPase activity, which leads to the inhibition of RNA polymerase II-mediated transcription and likely nucleotide excision repair. Yu *et al*<sup>[14]</sup> also found that triptolide inhibits global transcription in cancer cells by induction of phosphorylation and subsequent proteasome-dependent degradation of RNA polymerase II (Rpb1), resulting in global gene transcription. Meanwhile triptolide's anti-proliferative and pro-apoptotic effects have been reported as being caused by the inhibition of nuclear factor-kappaB (NF- $\kappa$ B) and nuclear factor of activated T-cells-mediated (NFAT-mediated) transcription and the suppression of HSP70 expression<sup>[15-17]</sup>.

In this report, we demonstrate for the first time that LLDT-67, a novel derivative of triptolide, has a potent and specific effect on the expression of NGF in astrocytes *in vitro* and *in vivo*, which suggests that LLDT-67 can potentially serve as a neuroprotective agent in the treatment of neurodegenerative diseases. NGF is critical for the survival and maintenance of sympathetic and sensory neurons. Without it, these neurons undergo apoptosis<sup>[18]</sup>. NGF causes axonal growth. Studies have shown that it stimulates axonal branching and elongation<sup>[19]</sup>. NGF binds to the high-affinity tyrosine kinase receptor TrkA. TrkA is then phosphorylated, and this leads to the activation of the phosphatidylinositol-3 kinase (PI3K) and PLC signaling pathways. One major pathway leads to the activation of AKT. This pathway begins with the Trk receptor complex recruitment of a second adaptor protein called growth factor-receptor bound protein-2 (Grb2) along with a docking protein called Grb2-associated Binder-1 (GAB1). Subsequently, PI3K is activated, which leads to AKT kinase activation<sup>[20]</sup>. Some studies have shown that inhibiting PI3K or AKT activity results in the death of sympathetic neurons in cultures, regardless of NGF presence<sup>[21]</sup>.

Preclinical and clinical findings suggest that neurotrophins are a promising treatment for peripheral neuropathies<sup>[22]</sup> and neurodegenerative diseases, such as Alzheimer's<sup>[23]</sup> and Parkinson's disease<sup>[24]</sup>. However, neurotrophins do not make good drug candidates because of their poor pharmacokinetic properties and poor bioavailability at desired targets. One of the major hurdles for neurotrophin therapy is the inability of peptide hormones to cross the blood-brain barrier<sup>[25, 26]</sup>. Peripheral administration of peptide hormones only leads to a

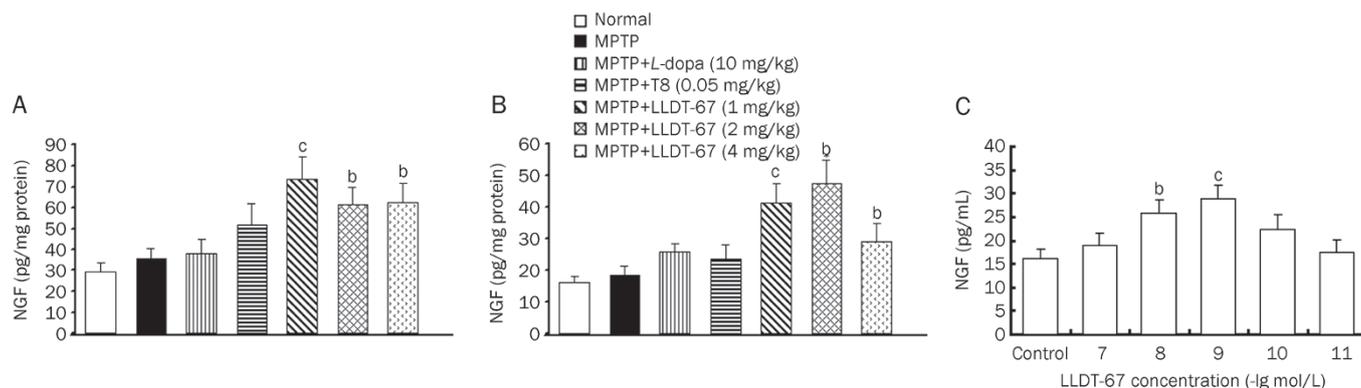


**Figure 4.** Effect of LLDT-67 on NGF expression in astrocytes of the SNc of MPTP-treated mice. bar=30  $\mu$ m.  $\times$ 400.

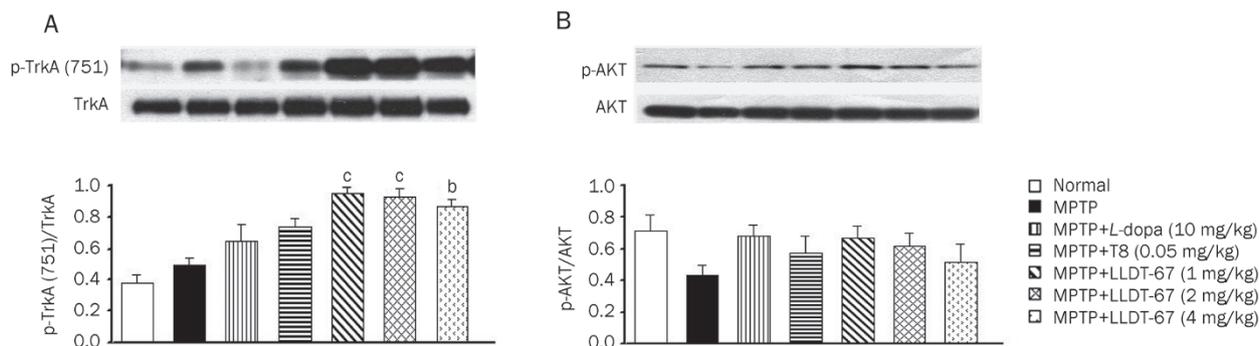
small increase in their intracerebral concentration. Therefore, much effort has been devoted to the search for complicated methods of delivery<sup>[27, 28]</sup> and the development of nonpeptidyl small molecule neurotrophin mimics that can elicit the desired neuroregenerative responses<sup>[29]</sup>.

Our data show that LLDT-67 can up-regulate NGF expression in astrocytes and that PI3K signaling downstream of TrkA contributes to the neuroprotective effects of NGF. The PI3-kinase signaling pathway plays a greater role than the MAPK

pathway in neuronal survival<sup>[24, 30–32]</sup>. The MAPK pathway, however, is essential for NGF-induced neurogenesis<sup>[33]</sup>. This fact may explain why LLDT-67 is more effective in promoting survival than it is in stimulating differentiation because LLDT-67 is a potent activator of AKT but a weak activator of ERK. A low dose of LLDT-67 (1–2 mg/kg) is more effective at activating AKT than a higher dose (4 mg/kg). This result may partially explain the ability of LLDT-67 to attenuate the neurodegeneration induced by MPTP.



**Figure 5.** Effect of LLDT-67 on the NGF content in the SN and striatum of MPTP-treated mice and NGF secretion from primary astrocytes. (A) Results of the ELISA assay measuring the NGF content in the SN. (B) Results of the ELISA assay measuring the NGF content in the striatum. Each column represents the mean±SEM of the results from 6 mice. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared with MPTP-treated mice. (C) Results of the ELISA assay measuring NGF secretion from primary astrocytes. Each column represents the mean±SEM of six measurements. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared with control (one-way ANOVA followed by Dunnett's *post hoc* comparison).



**Figure 6.** Effect of LLDT-67 on the phosphorylation of the TrkA tyrosine 751 site and AKT in the SN. (A) Effect of LLDT-67 on the phosphorylation of the TrkA tyrosine 751 site in the SN of MPTP-treated mice. Each column represents the mean±SEM of the results from six mice. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison). (B) Effect of LLDT-67 on the phosphorylation of AKT in the SN of MPTP-treated mice. Each column represents the mean±SEM of the results from six mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

In conclusion, our study demonstrates that LLDT-67 possesses potent neurotrophic effects and protects dopaminergic neurons from degeneration induced by MPTP. Immunohistochemistry staining and ELISA data show that LLDT-67 can stimulate the expression of NGF, which may contribute to its neuroprotective effects. We propose that LLDT-67 may be a promising drug for the treatment of Parkinson's disease.

#### Acknowledgements

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#### Author contribution

Linyin FENG and Dong-dong WU designed the study; Dong-dong WU, Li HUANG, Lei ZHANG, and Le-yu WU performed the experiments; Yuan-chao LI contributed new reagents; Dong-dong WU analyzed the data; Linyin FENG

and Dong-dong WU wrote the paper.

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