● RESEARCH ARTICLE

(5R)-5-hydroxytriptolide inhibits the inflammatory cascade reaction in astrocytes

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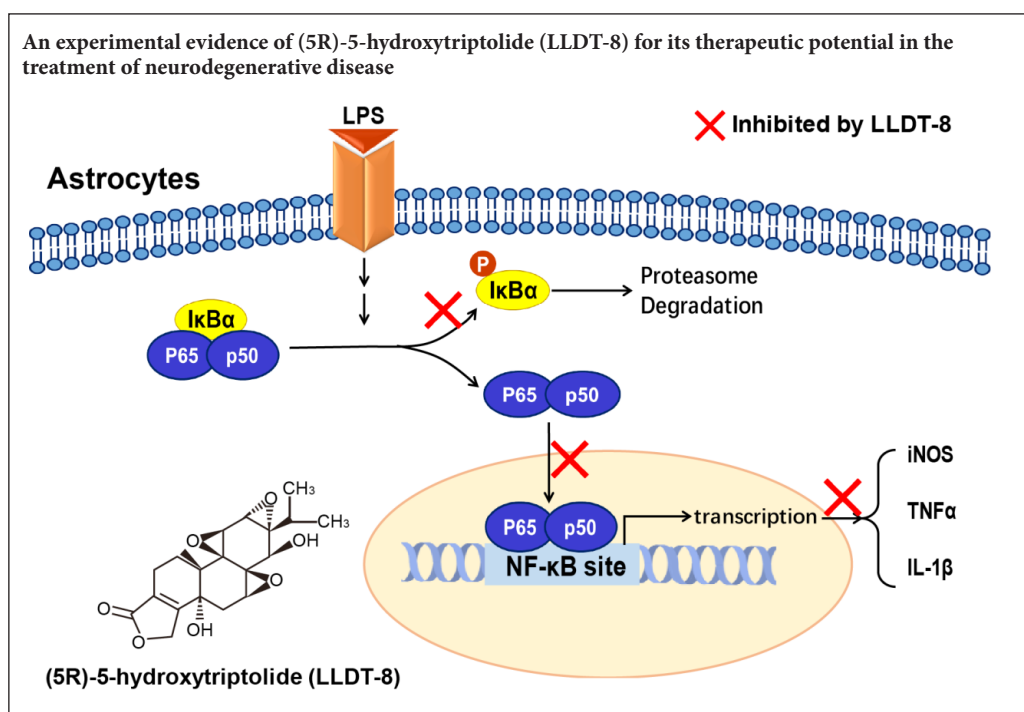3 Department of Physiology, Capital Medical University, Beijing, China
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Graphical Abstract

An experimental evidence of (5R)-5-hydroxytriptolide (LLDT-8) for its therapeutic potential in the treatment of neurodegenerative disease

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Abstract

Many studies have shown that (5R)-5-hydroxytriptolide is the optimal modified analogue of triptolide, possessing comparable immunosuppressive activity but much lower cytotoxicity than triptolide. Whether (5R)-5-hydroxytriptolide has preventive effects on neuroinflammation is unclear. This study was designed to pretreat primary astrocytes from the brains of neonatal Sprague-Dawley rats with 20, 100 and 500 nM (5R)-5-hydroxytriptolide for 1 hour before establishing an *in vitro* neuroinflammation model with 1.0 μg/mL lipopolysaccharide for 24 hours. The generation of nitric oxide was detected by Griess reagents. Astrocyte marker glial fibrillary acidic protein was measured by immunohistochemical staining. The levels of tumor necrosis factor-α and interleukin-1β in the culture supernatant were assayed by enzyme linked immunosorbent assay. Nuclear factor-κB/p65 expression was examined by immunofluorescence staining. The phosphorylation of inhibitor of nuclear factor IκB-α and the location of nuclear factor-κB/P65 were determined using western blot assay. Our data revealed that (5R)-5-hydroxytriptolide inhibited the generation of nitric oxide, tumor necrosis factor-α and interleukin-1β from primary astrocytes activated by lipopolysaccharide, decreased the positive reaction intensity of glial fibrillary acidic protein, reduced the expression of tumor necrosis factor alpha and interleukin-1β in culture supernatant, inhibited the phosphorylation of IκB-α and the translocation of nuclear factor-κB/P65 to the nucleus. These results have confirmed that (5R)-5-hydroxytriptolide inhibits lipopolysaccharide-induced glial inflammatory response and provides cytological experimental data for (5R)-5-hydroxytriptolide in the treatment of neurodegenerative diseases.

Key Words: neuroinflammation; (5R)-5-hydroxytriptolide; tumor necrosis factor-α; interleukin-1β; nitric oxide; nuclear factor-κB/P65; IκB-α; microglia; neural regeneration

Chinese Library Classification No. R453; R364.5; R741

Introduction

Neuroinflammation related to changes in the activity of glia cells has been implicated as a common pathological contributor to neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Rock et al., 2004; Lim et al., 2015; Ransohoff, 2016; Zhou et al., 2017). Glial cell release of cytokines and reactive oxygen species can cause synaptic dysfunction, even damage healthy neurons in pro-inflammatory states, which can lead to irreversible neurodegeneration in the brain (Mosley et al., 2006; Heppner et al., 2015; Gonzalez et al., 2017; Skaper et al., 2018). Accumulating evidence indicates that therapies targeting uncontrolled neuroinflammation produced by an overactive glial reaction might be beneficial in neurodegenerative disorders (Szekely et al., 2004; Van Eldik et al., 2007; Qian et al., 2010; Bronzuoli et al., 2016; Ransohoff, 2016).

Triptolide, an active compound extracted from a traditional Chinese herb, Tripterygium Wilfordii Hook. f., has many pharmacological uses, including immunosuppressive, anti-inflammatory and anti-tumor effects (Han et al., 2012; Zheng et al., 2013; Ziaei and Halaby, 2016). The clinical applications of triptolide have been limited due to its limited therapeutic window and potential biological toxicity (Xi et al., 2017). Zhou et al. (2012) developed many structural derivatives of triptolide that might avoid such disadvantages but retain its beneficial activity. One modified analogue, (5R)-5-hydroxytriptolide (LLDT-8), possesses a relatively higher immunosuppressive activity and much lower biological cytotoxicity than triptolide and other derivatives (Zhou et al., 2005). Many *in vitro* and *in vivo* studies demonstrated that LLDT-8 possesses significant anti-inflammatory and immunosuppressive activities (Zhou et al., 2006a, b, c, 2009; Shen et al., 2015). The China Food and Drug Administration have approved a clinical trial of LLDT-8 as an immunosuppressive drug to treat rheumatoid arthritis. Our recent research indicated that LLDT-8 can prevent 6-hydroxydopamine impairment of dopaminergic neurons in a Parkinson's disease rat model by mechanisms involving peripheral immunosuppression and inhibition of glial reaction in the central nervous system (Su et al., 2017). Although the anti-inflammatory effect of LLDT-8 has been shown in many studies, the question remained of how it could inhibit the production of pro-inflammatory factors.

The present study was designed to explore the mechanism underlying the effect of LLDT-8 on neuroinflammation in a series of *in vitro* studies in lipopolysaccharide (LPS) stimulated primary astrocytes. This would provide experimental evidence of the effects of LLDT-8 that might be the basis of its therapeutic potential clinically in the treatment of neurodegenerative disease.

Materials and Methods

Primary culture of astrocytes

The primary astrocytes were prepared from whole brains of neonatal Sprague-Dawley rats aged 1 to 2 days old (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China; SYXK 2013-0032) (Tallant and Higson, 1997).

Briefly, dissociation of the brains to single cell suspensions was obtained by mild mechanical and physical means. Dissociated cells were then seeded onto 75 cm² culture flasks pre-coated with poly-D-lysine, cultured in Dulbecco's modified Eagle's medium/F12 (Gibco Life Technologies, Rockville, MD, USA) containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (Gibco Life Technologies). The culture medium was replaced twice per week until the cells reached confluence. The confluent monolayers of cells on the flasks were shaken overnight at 180 r/min to remove the remaining microglia and oligodendrocytes. Purified astrocytes were digested and re-suspended with 0.25 trypsin/ethylenediamine tetraacetic acid (Gibco Life Technologies) and replanted onto 6- or 96-well plates, followed by equilibration for 3 days. The purity of the astrocytes was greater than 95%, as determined by glial fibrillary acidic protein (GFAP) (Mouse, 1:500, MAB360; Millipore, Billerica, MA, USA) immunocytochemical staining (**Additional Figure 1**). The protocols were reviewed and approved by the Committee on Animal Care and Usage of Capital Medical University, China (approval number: AEEI-2015-158) on November 15, 2015.

Neuroinflammation model and LLDT-8 groups

Astrocytes were treated with 1.0 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) to establish an *in vitro* neuroinflammation model. Because of the progress of the inflammatory cascade, we chose different incubation times (24, 6 or 1 hour) with LPS to measure different intermediates (Ko et al., 2018).

LLDT-8 is in the form of a white amorphous powder with 99% purity. The LLDT-8 powder was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) to prepare the stock solution (10 mM), which was stored at 4°C. The stock LLDT-8 solution was diluted with culture medium to a desired concentration. Primary astrocytes were pre-incubated with LLDT-8 (20, 50, 100, 200, 500 or 1000 nM) for 1 hour before treatment with LPS.

Measurement of nitric oxide (NO) production

After the establishment of each experimental group, the production of NO was monitored by measuring the amount of nitrite (NO_2^-) with Griess reaction (Green et al., 1982). In brief, 50 μL of the culture supernatant was transferred to a new 96-well plate and mixed with equal volumes of Griess reagent I and reagent II (Beyotime Biotechnology, Nanjing, China). The absorbance was measured at 540 nm by a microplate reader (Thermo scientific, Multiskan MK3, Shanghai, China). The nitrite concentration of each sample was calculated using a standard curve constructed using sodium nitrite.

Cell viability detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

MTT assay was performed to measure cell viability (Mosmann, 1983). Briefly, primary astrocytes were treated with several concentrations of LLDT-8. After 24 hours, 20 μL MTT (5 mg/mL) was added to each well and incubated at

37°C for 2 hours. The medium was then removed carefully, and the water-insoluble formazan crystals were dissolved in dimethyl sulfoxide (100 μL/well). The optical density of each well was measured at 490 nm with a Thermo Scientific microplate reader (Multiskan MK3, Shanghai, China) with a reference wavelength of 630 nm. Cell viability was described as relative percentage of that defined by the control group.

GFAP immunocytochemical staining of astrocytes
When 50–80% confluence was attained, the primary astrocytes seeded onto cover slides were treated with 100 nM of LLDT-8 for 1 hour and then co-incubated with LPS (1.0 μg/mL) for a further 24 hours. The cover slides were removed from the culture medium and the cells were fixed on slides with 4% paraformaldehyde for 30 minutes. Treatment with 0.3% Triton-X 100 at room temperature for 30 minutes was used to permeabilize the cell membranes. Slides were incubated with 3% hydrogen peroxide solution to quench endogenous peroxidase. The slides were incubated with primary antibodies, mouse monoclonal anti-GFAP (Millipore) overnight at 4°C. The cells were then stained using a two-step plus poly-horseradish peroxidase anti-mouse/rabbit IgG detection system (ZhongShan Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's guidelines. Briefly, after being washed, cells were incubated with polymer helper (reagent I) for 30 minutes, followed by polyperoxidase-anti-mouse/rabbit IgG (reagent II, ready to use) for 30 minutes at room temperature. The cells were then visualized with a 3,3′-diaminobenzidine kit (ZhongShan Biotechnology Co., Ltd.). The slides were mounted and images were observed using a light microscope equipped with a digital camera (Olympus, Tokyo, Japan). The active state of primary astrocytes was analyzed by detecting the average optic density value of each slide using Image Pro Plus 6.0 software. All slides were coded and examined blindly.

Quantification of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by enzyme linked immunosorbent assay (ELISA)
The primary astrocytes were pretreated with LLDT-8 for 1 hour and then co-incubated with 1.0 μg/mL LPS. At 6 hours after LPS treatment, the cell culture medium was collected, and the concentrations of TNF-α and IL-1β were measured using the rat ELISA kits (Shanghai ExCell Biology, Inc., Shanghai, China) according to the operational instructions provided. In brief, samples and standards were added to the wells pre-coated with a specific anti-rat TNF-α or IL-1β monoclonal antibody and incubated at 37°C for 90 minutes. After unbound substances were washed off, a biotin-conjugate antibody (1:100) was added into the wells and incubated at 37°C for 60 minutes. Next, the streptavidin-horseradish peroxidase solution was added and incubated at 37°C for 30 minutes. After the substrate solution was used for 10 minutes, the stop solution was added to the wells to stop the color development. The optical density of the 96-well plates was read at 450 nm with a microplate reader (Thermo scientific, Multiskan MK3, Shanghai, China). The concentrations of TNF-α and IL-1β were calculated using standard curves produced from TNF-α or IL-1β standards.

Immunofluorescence assay for nuclear factor (NF)-κB/P65
The primary astrocytes cultured on cover slides were pretreated with LLDT-8 (500 nM) for 1 hour, and then co-incubated with 1.0 μg/mL LPS for an additional 1 hour. After treatment, cells were fixed, perforated and endogenous peroxidase was quenched as described above for immunocytochemical staining. The slides were incubated with blocking buffer at room temperature for 1 hour. Afterwards, the cells were incubated with rabbit anti-NF-κB/p65 polyclonal antibody (1:100; Beyotime Biotechnology) followed by fluorescein (FITC)-conjugated IgG (goat, 1:100; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus for 5 minutes. The fluorescence signals were analyzed by an intelligent laser scanning confocal microscopy (FV10C-PSU; Olympus).

Western blot assay
The cytoplasmic and nuclear extracts of the astrocytes, treated with LLDT-8, with or without 1.0 μg/mL LPS, for 1 hour, were prepared using commercial nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the procedures supplied. In brief, the treated cells were harvested into a microcentrifuge tube and centrifuged. Cytoplasmic extraction reagent I and cytoplasmic extraction reagent II were added to the cell pellet, which caused disruption of cell membranes and release of the cytoplasmic contents. The intact nuclei were separated from the cytoplasmic extracts by centrifugation. Afterwards, the proteins were extracted from the nuclei with a nuclear extraction reagent. The levels of p65 in cytoplasmic and nuclear extracts were analyzed by western blot assay to determine the nuclear translocation of p65. After co-treatment with 1.0 μg/mL LPS for 1 hour or 24 hours, the whole cell lysates were prepared using a lysis buffer (Beyotime Biotechnology), and the protein levels of IKB-α, p-IKB-α, inducible nitric oxide synthase (iNOS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Histone H3 were detected, respectively, by western blot assay. Protease inhibitor and phosphatase inhibitor (Roche, Basel, Switzerland) were added to the cytoplasmic extracts, nuclear extracts and whole cell lysates to depress the activities of proteases and phosphatases. To determine the protein concentrations of samples, bicinchoninic acid protein assay kit (Beyotime Biotechnology) was used in accordance with the manufacturer's instructions. The proteins in each sample were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gels, and then transferred electronically onto nitrocellulose membranes at 100 mV for 1.5 hours. The membranes were blocked with 5% non-fat milk at room temperature for 1 hour, and then incubated overnight at 4°C with the specific antibodies, IKB-α (rabbit, 1:1000; Beyotime Biotechnology), p-IKB-α (mouse, 1:1000; Cell Signaling Technology, Danvers, MA, USA), NF-κB/p65 (rabbit, 1:500; Beyotime Biotechnology),

iNOS (rabbit, 1:800; Abcam, Cambridge, MA, USA), GAPDH (mouse, 1:10,000; Sigma-Aldrich, St. Louis, MO, USA) and Histone H3 (mouse, 1:1000; Beyotime Biotechnology). After three washes, the membranes were incubated with the corresponding secondary antibodies conjugated by horseradish peroxidase (goat anti-mouse/rabbit, 1:1000; Beyotime Biotechnology) at room temperature for 1 hour. Antibody binding of the membranes was visualized by enhanced chemiluminescence western detection system (Beyotime Biotechnology) and detected by a digital chemiluminescence scanner (C-Digit, LI-COR Biosciences, Lincoln, NE, USA). The intensities of the immunoblot bands were detected with Image Studio Version software (5.2, LI-COR Biosciences).

NF-κB/p65 DNA binding activity assay

NF-κB/p65 DNA-binding activity was detected with NF-κB/p65 transcription factor assay kit (Abcam) according to the manufacturer's instructions. In brief, after being treated with LPS for 1 hour, with or without pretreatment with LLDT-8, nuclear protein was extracted from primary astrocytes using the nuclear and cytoplasmic extraction reagents (Thermo Scientific). Nuclear extracts were incubated in plates provided by the kit precoated with a specific double stranded DNA sequence containing the NF-κB response element. NF-κB/p65 in the nuclear extracts combined with the response element on the plates and was detected by a specific primary antibody against NF-κB/p65 and horseradish peroxidase-conjugated secondary antibody. The plate was incubated with the developing solution at room temperature for 30 minutes for the chromogenic reaction and, the absorbance was measured at 450 nm. The NF-κB/p65 DNA-binding activity was determined by normalization to the protein concentration of the nuclear extract of each sample in accordance with the bicinchoninic acid protein assay kit (Beyotime Biotechnology).

Statistical analysis

The given results are expressed as the mean ± SEM. One-way analysis of variance followed by Newman-Keuls' *post hoc* test was used to determine the significance between different groups using Prism5.0 (GraphPad Software, San Diego, CA, USA). A value of *P* < 0.05 was considered statistically significant.

Results

LLDT-8 inhibits NO production and iNOS expression in LPS activated astrocytes

LLDT-8 was used alone in the treatment of astrocytes to test its possible toxic effects. NO production and cell viability were detected by Griess reaction and MTT assay, respectively. We found that nitrite levels were less than 1.0 μM in astrocytes treated with different concentrations of LLDT-8 (20–1000 nM) for 24 hours (**Figure 1A**). Simultaneously, MTT assay indicated that 20–500 nM LLDT-8 did not cause cytotoxicity; however, 1000 nM LLDT-8 reduced cell viability to 86.4 ± 4.1% of control group (*P* < 0.01; **Figure 1B**). Therefore, we restricted the doses to 20 nM, 100 nM and 500 nM LLDT-8 in subsequent experiments. As shown in **Figure 1C**, NO production significantly increased in LPS challenged astrocytes. Nitrite concentrations in supernatants increased after the astrocytes were treated with 0.1, 1.0, and 10.0 μg/mL LPS, respectively, for 24 hours. In subsequent experiments, 1.0 μg/mL LPS was chosen as an effective dose to activate astrocytes.

To determine the effect of LLDT-8 on NO production in LPS activated primary astrocytes, primary astrocytes were pre-incubated with LLDT-8 (20, 100, and 500 nM) for 1 hour, and then co-treated with 1.0 μg/mL LPS for 24 hours. In the control group, the basal concentration of nitrite was barely detectable but this value increased to 9.7 ± 0.2 μM upon LPS challenge (**Figure 1D**). However, pretreatment
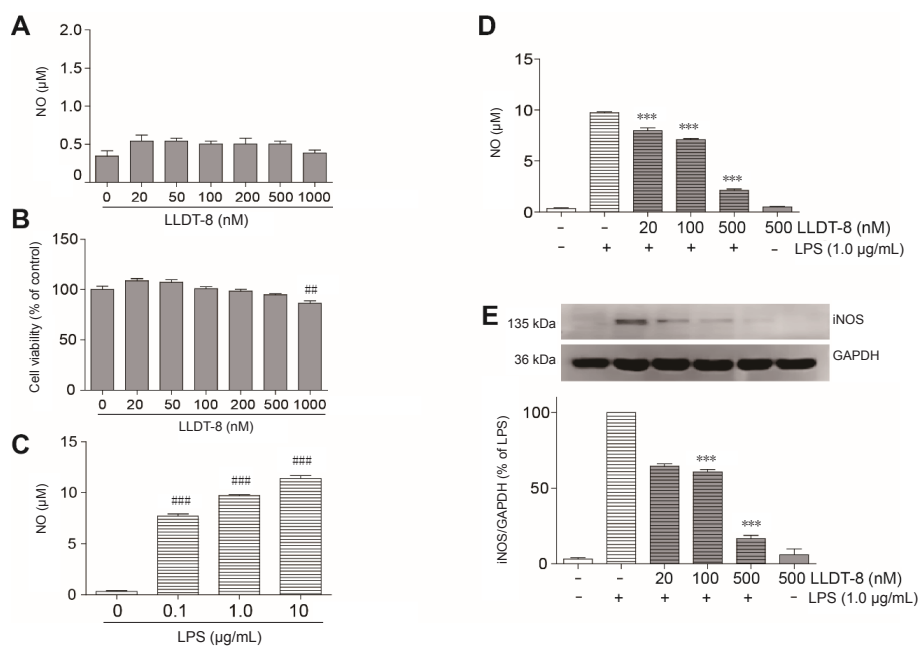
Figure 1 LLDT-8 inhibits NO production and iNOS expression in LPS activated astrocytes.
(A) Effect of 24 hours of LLDT-8 treatment of primary astrocytes on NO production (determined by NO concentration). (B) Cell viability (determined by MTT assay) of astrocytes treated with LLDT-8 for 24 hours. (C) NO production in primary astrocytes treated with LPS for 24 hours. (D) LLDT-8 inhibited NO production in LPS-induced astrocytes. After being incubated with 20, 100, and 500 nM LLDT-8 for 1 hour, astrocytes were then co-treated with 1.0 μg/mL LPS for 24 hours. (E) Effect of LLDT-8 on iNOS expression in LPS activated astrocytes. The intensity ratio of iNOs to GAPDH was further normalized against that of the LPS group. Data are expressed as the mean ± SEM. The experiment was conducted in triplicate. ∗∗∗*P* < 0.001, *vs.* LPS group; ##*P* < 0.01, ###*P* < 0.001, *vs.* control group (one-way analysis of variance followed by Newman-Keuls *post hoc* test). LLDT-8: (5R)-5-hydroxytriptolide; NO: nitric oxide; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

with 20, 100 and 500 nM LLDT-8 significantly reduced nitrite generation ($P < 0.001$) in the LPS-treated groups in a dose-dependent manner. LLDT-8 (500 nM) without LPS had no effect on NO production. iNOS protein levels were detected by western blot assay. **Figure 1E** illustrates that iNOS expression was undetectable in the control or 500 nM LLDT-8 alone groups. LPS treatment increased iNOS expression relative to the control level. LLDT-8 dose dependently suppressed LPS-induced iNOS protein expression. After pre-incubated with 20, 100, and 500 nM LLDT-8, LPS-induced iNOS expression decreased compared with the LPS group ($P < 0.001$). These data indicated that LPS successfully triggered inflammatory stress of astrocytes and LLDT-8 inhibited the LPS effect in a dose-dependent manner.

LLDT-8 diminishes TNF-α and IL-1β generation and attenuates astrocyte activation induced by LPS

TNF-α and IL-1β generation were analyzed using ELISA. **Figure 2A** shows that the levels of TNF-α in both the control group and the 500 nM LLDT-8 only treated group were comparably low. LPS treatment significantly increased TNF-α concentration to 301.8 ± 15.4 pg/mL. However, 20, 100 and 500 nM LLDT-8 pre-incubation significantly diminished TNF-α generation induced by LPS ($P < 0.01$ or $P < 0.001$), respectively. **Figure 2B** illustrates that LLDT-8 had similar results with IL-1β generation in LPS activated astrocytes. 20, 100 and 500 nM LLDT-8 treatment significantly reduced LPS induced IL-1β production ($P < 0.001$), respectively.

The activated state of astrocytes was assessed by GFAP immunocytochemical staining and the degree of GFAP immunopositivity was determined by the average optical density value of GFAP-positive signals. **Figure 2C** shows that LPS treatment significantly increased the GFAP immunoposi-

tivity to $134.3 \pm 4.1\%$ of that in the control group. However, 100 nM LLDT-8 pre-incubation reduced the GFAP immunopositivity to $106.2 \pm 1.7\%$ of that in control group. The results above suggested that glial response to LPS-induced inflammation was significantly suppressed by LLDT-8 in primary astrocytes.

LLDT-8 suppresses the phosphorylation and degradation of IκB-α in LPS activated astrocytes

As a crucial transcription factor, NF-κB participates in the gene expression of most proinflammatory cytokines. The phosphorylation and subsequent degradation of IκB-α were required for the NF-κB activation (Shih et al., 2015). Therefore, we detected the levels of total IκB-α and phosphorylated IκB-α in LPS activated astrocytes by western blot assay. Our data indicate that LPS treatment induced IκB-α degradation to $58.6 \pm 0.7\%$ of the control group. However, LLDT-8 pre-incubation suppressed the degradation of IκB-α. High doses of LLDT-8 (500 nM) increased IκB-α to $113.1 \pm 11.7\%$ of control group ($P < 0.01$, *vs.* LPS group; **Figure 3A**). We also studied the effects of LLDT-8 on the phosphorylation level of IκB-α in LPS induced astrocytes. As shown in **Figure 3B**, LPS treatment significantly increased IκB-α phosphorylation in astrocytes, while 20, 100 and 500 nM LLDT-8 pre-incubation reduced the LPS induced IκB-α phosphorylation to $84.2 \pm 15.6\%$ ($P > 0.05$), $73.3 \pm 10.2\%$ ($P > 0.05$) and $42.4 \pm 14.8\%$ ($P < 0.01$), respectively.

LLDT-8 decreases nuclear translocation and DNA binding activity of NF-κB/p65 in LPS activated astrocytes

To investigate the exact role of LLDT-8 on NF-κB activation, we investigated NF-κB/p65 nuclear translocation, which is the event downstream of IκB-α degradation (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). Western
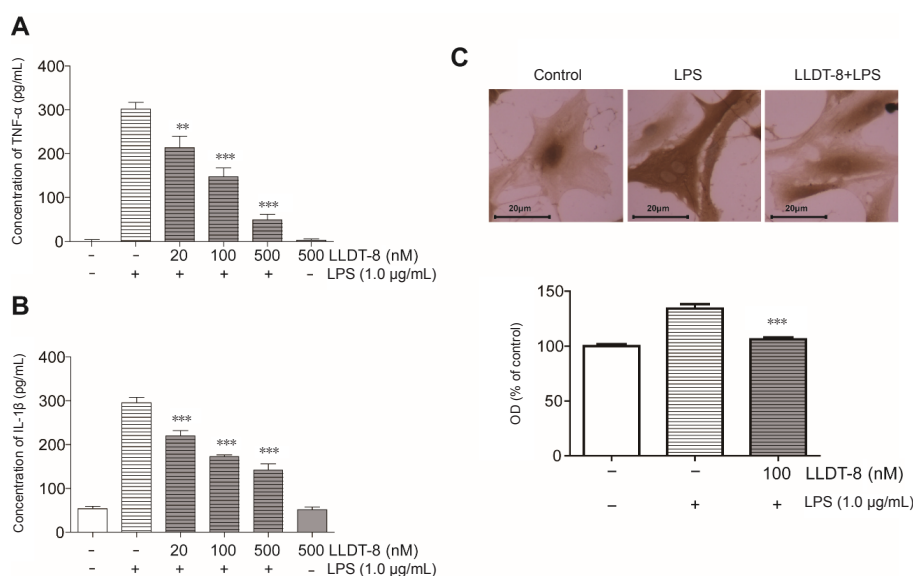

Figure 2 LLDT-8 diminishes TNF-α and IL-1β generation and attenuates the activation in LPS activated astrocytes.
(A, B) LLDT-8 inhibited LPS-induced TNF-α (A) and IL-1β (B) release in astrocytes. Astrocytes were incubated in the medium containing 20 nM, 100 nM or 500 nM LLDT-8 for 1 hour and then activated by 1.0 μg/mL LPS for 6 hours. The concentrations of TNF-α and IL-1β were detected using commercial enzyme linked immunosorbent assay kits. (C) LLDT-8 attenuated astrocyte activation (evaluated by GFAP immunocytochemical staining) induced by LPS. Representative images of control (normal medium), LPS (1.0 μg/mL LPS), LLDT-8 + LPS (100 nM LLDT-8 pretreatment for 1 hour and then co-incubated with 1.0 μg/mL LPS) were shown. Scale bars: 20 μm. The average OD value of each group was normalized against control group. Data are expressed as the mean ± SEM. The experiment was conducted in triplicate. $**P < 0.01$, $***P < 0.001$, *vs.* LPS group (one-way analysis of variance followed by Newman-Keuls *post hoc* test). LLDT-8: (5R)-5-hydroxytriptolide; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; GFAP: glial fibrillary acidic protein; OD: optical density.

blot assay of the nuclear extracts confirmed that nuclear p65 level was increased by LPS stimulation but this was prevented by LLDT-8 treatment at final concentrations of 20 ($P <$ 0.05), 100 ($P < 0.05$) and 500 nM ($P < 0.05$) (**Figure 4A** and **B**). However, the protein level of cytosolic p65 showed no marked change after LPS and/or LLDT-8 treatment. The results above were also confirmed by immunofluorescence assay for NF-κB/p65 (**Figure 4C**), which showed that LPS treatment increased the intranuclear accumulation of NF-κB/p65 immunosignals. LLDT-8 pre-treatment significantly reduced the nuclear location of NF-κB/p65 positive signals. In addition, the nuclear extracts of primary astrocytes were analyzed using a commercial NF-κB/p65 DNA binding activity assay kit (**Figure 4D**). Our findings confirmed that LPS treatment remarkably increased the activity of NF-κB/p65 binding to DNA. Although there was no effect of LLDT-8 at a final concentration of 20 nM, pretreatment with 100 or 500 nM LLDT-8 significantly inhibited the NF-κB/p65 DNA binding activity ($P < 0.05$), respectively. All these results imply that LLDT-8 possesses the ability to block NF-κB/p65 nuclear translocation under inflammatory stress.

Discussion

Neuroinflammation plays a major role in the cause and progression of neurodegenerative diseases and relies on the complex integration of all cells present in the central nervous system, including neurons, microglia and astrocytes (Lopategui Cabezas et al., 2014; Acosta et al., 2017; González-Reyes et al., 2017). When there is a brain injury (*e.g.*, brain trauma, ischemia, and Aβ accumulation), microglia and astrocytes are activated to remove the primary injury (Li et al., 2011; Minter et al., 2016; Schwartz and Deczkowska, 2016). However, the long-lasting and uncontrolled activation of microglia and astrocytes continue to produce and secrete a wide range of cytokines and chemokines (including NO, TNF-α, IL-β, and reactive oxygen species). These may lead to chronic inflammation and finally cause further neurodegeneration (Bronzuoli et al., 2016; González-Reyes et al., 2017). For these reasons, the depression of the activation of astrocytes and microglia is being considered as a new therapeutic approach to treat neurodegenerative disorders.

LLDT-8 is a modified synthesized analogue of triptolide that has much lower cytotoxicity and relatively higher immunosuppressive activity. Many previous studies have shown that LLDT-8 possesses obvious anti-inflammatory and immunosuppression activities in peripheral tissue (Zhou et al., 2006a, b, c, 2009; Shen et al., 2015). In the central nervous system, it was confirmed that LLDT-8 treatment suppressed the generation of pro-inflammatory factors in both 6-hydroxydopamine-induced hemi Parkinson rats (Su et al., 2017) and middle cerebral artery occlusion mice (Chen et al., 2016). These data indicate that LLDT-8 has a remarkably inhibitory action on neuroinflammation and a clear neuroprotective effect *in vivo*.

In the central nervous system, microglia and resident macrophage cells play a crucial role in neurodegenerative diseases (Colonna and Butovsky, 2017; Martinez and Peplow, 2017;

Bisht et al., 2018; Perea et al., 2018). A recent report about the effect of LLDT-8 on activated microglia indicates that LLDT-8 decreased NO production and pro-inflammatory cytokines released in LPS-stimulated primary microglia and BV-2 microglia cells (Chen et al., 2016). This is consistent with our findings on microglia (**Additional Figure 2**). However, in response to brain injury, astrocytes also play a crucial role and become a key cellular component both in neuroinflammation and later neurodegenerative disorders (Medeiros and LaFerla, 2013; Colombo and Farina, 2016). Therefore, in our current study, we investigated the effect of LLDT-8 on astrocyte-mediated neuroinflammation. Our data clarified that LLDT-8 treatment potently suppressed LPS induced NO generation and proinflammatory cytokines (TNF-α and IL-1β) production in primary cultured astrocytes.

NF-κB is a crucial transcription factor in inflammatory reaction, therefore both its activation and subsequent transcription of inflammatory factors are relevant to the inflammatory process in several neurodegenerative diseases (Tak and Firestein, 2001; Medeiros and LaFerla, 2013; Yue et al., 2018). In the cytoplasm, NF-κB dimers, composed of p65 and p50 subunits, are commonly sequestered by associating with IκB-α. Cell activation by inflammatory stimulation leads to phosphorylation and subsequent degradation of IκB-α. Consequently, released NF-κB translocates to the nucleus and then modulates the expression of a variety of target genes for a series of related factors. There is growing evidence to suggest that astrocyte NF-κB regulates neuroinflammation and neurotoxicity, and plays a central role in neurodegenerative disorders (Colombo and Farina, 2016). Therefore, we asked whether or not LLDT-8 can affect the NF-κB signaling pathway in LPS activated primary astrocytes. Our results indicated that LLDT-8 inhibited the phosphorylation of IκB-α and reduced the translocation of NF-κB/p65 to the nucleus of LPS stimulated astrocytes. These suggest that the NF-κB pathway is the potential target through which LLDT-8 inhibits neuroinflammation. Some previous studies demonstrated similar results in RAW264.7 cells: LLDT-8 markedly suppressed the phosphorylation of IκB induced by a receptor activator of NF-κB ligand (Shen et al., 2015) and decreased LPS induced NF-κB binding activity (Zhou et al., 2006b). Taken together, these data indicate that LLDT-8 may reduce neuroinflammation *via* down-regulating NF-κB signaling pathway in a variety of cells activated by different stimulators.

In addition to NF-κB, LLDT-8 has been confirmed to act on some other signaling molecules, adding to its anti-inflammatory and immunosuppressive activities. A previous study indicated that LLDT-8 treatment significantly decreased the phosphorylation of SAPK/JNK, and partially reduced p38 and ERK1/2 phosphorylation. This suggests that mitogen-activated protein kinase is a prime target of LLDT-8 (Zhou et al., 2006b). The activation of mitogen-activated protein kinases mediates the IκB-α phosphorylation and leads to the downstream nuclear translocation of NF-κB in astrocytes (Colombo and Farina, 2016). Further research is required to clarify whether the inhibitory action of LLDT-

8 on IκB-α/NF-κB pathway involves mitogen-activated protein kinases or not. Microglia, as well as astrocytes, play a central role in neuroinflammation. Our recent study indicated that LLDT-8 decreased NO generation, TNF-α and IL-1β release in LPS-stimulated primary microglia (**Additional Figures 1** and **2**). However, the effect of LLDT-8 on the IκB-α/NF-κB pathway in microglia has not been studied because of the low yield of primary microglia using extraction techniques. Further animal experiments should be carried out to determine whether the IκB-α/NF-κB pathway in glia is a specific target for LLDT8 anti-inflammatory effects *in vivo*.

Additional files:
Additional Figure 1: Identification of primary astrocytes (immunocytochemical staining).
Additional Figure 2: (5R)-5-hydroxytriptolide reduced lipopolysaccharide-induced nitric oxide generation, tumor necrosis factor-α and interleukin-1β release, and inhibited microglia activation by detecting CD11b.
Additional file 1: Open peer review report 1.

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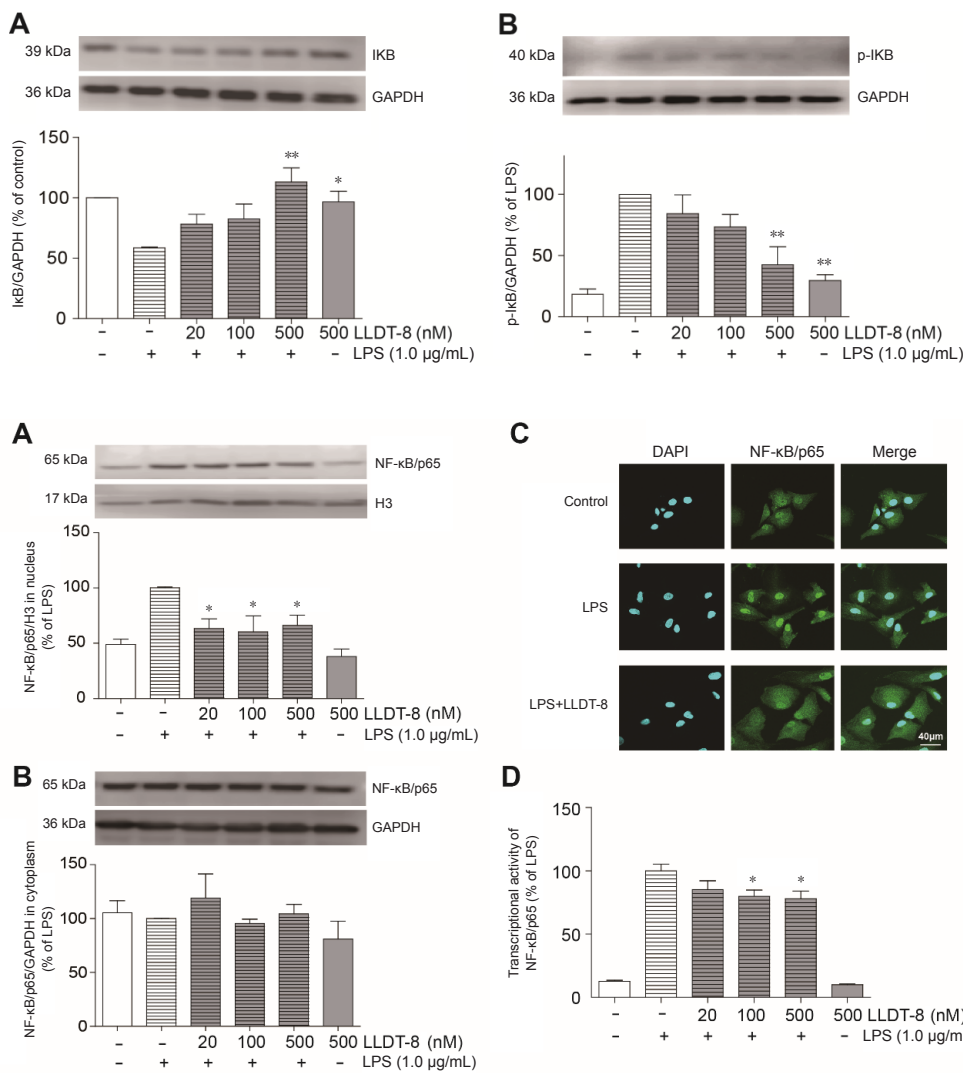
Figure 3 LLDT-8 inhibits the phosphorylation and degradation of IκB-α in LPS activated astrocytes.
Representative western blot bands and densitometric quantification of IκB-α (A) and phosphorylated IκB-α (B) in LPS activated astrocytes with or without LLDT-8 pretreatment. Values were expressed percentage of control group or LPS group. Data are expressed as the mean ± SEM. The experiment was conducted in triplicate. *P < 0.05, **P < 0.01, *vs.* LPS group (one-way analysis of variance followed by Newman-Keuls *post hoc* test). LLDT-8: (5R)-5-hydroxytriptolide; LPS: lipopolysaccharide.

(A, B) Cultured astrocytes were incubated with 1.0 μg/mL LPS with or without pre-incubation of LLDT-8 for 1 hour, respectively. Western blot assay was used to detect the levels of NF-κB/p65 in cytoplasm (A) or nucleus (B). The internal controls for cytosolic and nuclear extracts were GAPDH and Histone H3 respectively. The gray value of each band was scanned, and the ratio of p65 to Histone H3 or GAPDH was calculated and normalized to that of LPS group. (C) The nuclear localization of NF-κB/p65 was shown with FITC fluorescence signals detected by immunofluorescence assay. The nuclei appeared blue and the p65 protein appeared green under fluorescence microscopy. Scale bar: 40 μm. (D) LLDT-8 decreased transcriptional activity of NF-κB/p65 in astrocytes induced by LPS. Data are expressed as the mean ± SEM. The experiment was conducted in triplicate. *P < 0.05, *vs.* LPS group (one-way analysis of variance followed by Newman-Keuls *post hoc* test). LLDT-8: (5R)-5-hydroxytriptolide; NF: nuclear factor; LPS: lipopolysaccharide; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FITC: fluorescein.

Figure 4 LLDT-8 decreases nuclear translocation and DNA binding activity of NF-κB/p65 in LPS activated astrocytes.

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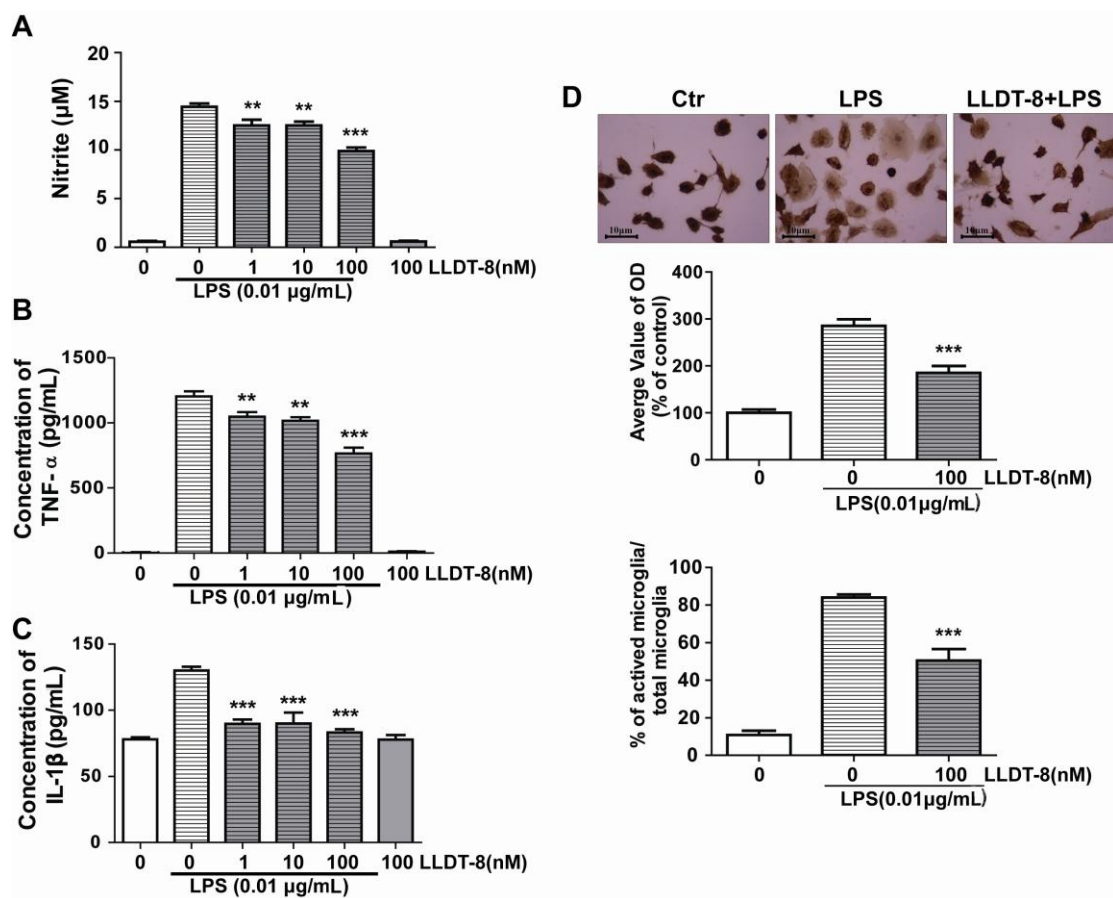
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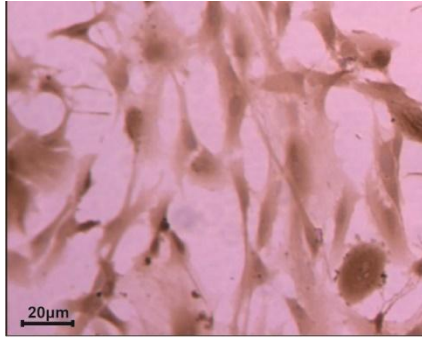
Additional Figure 1 Identification of primary astrocytes (immunocytochemical staining).

Primary astrocytes were determined by glial fibrillary acidic protein. Bar:20 μm.

Additional Figure 2 LLDT-8 reduced LPS-induced NO generation, TNF-α and IL-1β release, and inhibited microglia activation by detecting CD11b.

(A) NO generation; (B) TNF-α expression; (C) and IL-1β expression; (D) CD11b expression. Data represent the mean ± SEM, and analyzed by one-way analysis of variance followed by Newman-Keuls *post hoc* test. The experiment was repeated three times. **$P < 0.01$, ***$P < 0.001$, *vs*. LPS group. LLDT-8: (5R)-5-hydroxytriptolide; LPS: lipopolysaccharide; NO: nitric oxide; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β.