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

Yan-Qiu Cui1,2, Yan Zheng1, Gui-Lian Tan1, Dong-Mei Zhang1, Jun-Ya Wang1, Xiao-Min Wang2,*,

1 Functional Laboratory of Experiment Teaching Center for Basic Medical Sciences, Capital Medical University, Beijing, China
2 Department of Neurobiology, Key Laboratory for Neurodegenerative Disorders of the Ministry of Education, Capital Medical University, Beijing, China
3 Department of Physiology, Capital Medical University, Beijing, China
4 Department of Basic Medicine, Yanjing Medical College, Capital Medical University, Beijing, China
5 Beijing Institute for Brain Disorders, Beijing, China

Funding: This study was supported by the National Natural Science Foundation of China, No. 81402932 (to YQC).

Graphical Abstract

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

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; Bronzutzki 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 (NO₂⁻) 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 for an additional 1 hour. After treatment, 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 IκB-α, p-IκB-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, IκB-α (rabbit, 1:1000; Beyotime Biotechnology), p-IκB-α (mouse, 1:1000; Cell Signaling Technology, Danvers, MA, USA), NF-κB/p65 (rabbit, 1:500; Beyotime Biotechnology), and 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 IκB-α, p-IκB-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, IκB-α (rabbit, 1:1000; Beyotime Biotechnology), p-IκB-α (mouse, 1:1000; Cell Signaling Technology, Danvers, MA, USA), NF-κB/p65 (rabbit, 1:500; Beyotime Biotechnology), and the concentrations of TNF-α and IL-1β were calculated using standard curves produced from TNF-α or IL-1β standards.
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
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 immunopositivity to 134.3 ± 4.1% of that in the control group. However, 100 nM LLDT-8 pre-incubation reduced the GFAP immunopositivity to 106.2 ± 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 ± 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 ± 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 ± 15.6% \((P > 0.05)\), 73.3 ± 10.2% \((P > 0.05)\) and 42.4 ± 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.
Nervous system, microglia and resident macrophage cells play a crucial role in neurodegenerative diseases (Colonna and Butovsky, 2017; Martinez and Peplow, 2017; 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; 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; Bish 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 IκBα 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 stimuliators.

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.

Acknowledgments: We would like to thank Professor Jian-Ping Zuo from Shanghai Institute of Materia, Chinese Academy of Science, China for kindly providing LLDT-8.

Author contributions: Study design, experimental implementation, and manuscript writing: YQC, experimental implementation: GLT, DMZ and JYW; study design and manuscript modification: YZ and XMW. All authors approved the final version of the manuscript.

Conflicts of interest: The authors have no conflict of interest to declare.

Financial support: This study was supported by the National Natural Science Foundation of China, No. 81502932 (to YQC). The conception, design, execution, and analysis of experiments, as well as the preparation of and decision to publish this manuscript, were made independent of this funding organization.

Institutional review board statement: The study was approved by the Committee on Animal Care and Usage of Capital Medical University, China (approval number: AEEI-2015-158) on November 15, 2015.

Copyright license agreement: The Copyright License Agreement has been signed by all authors before publication.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non-Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

Open peer review: Jin-Tao Li, Kunming Medical University, China.

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-a and interleukin-1β release, and inhibited microglia activation by detecting CD11b.

Additional file 1: Open peer review report 1.

References


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 as 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: (3R)-5-hydroxytriptolide; LPS: lipopolysaccharide.

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


