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ORIGINAL RESEARCH

Therapeutic Potential of TPT-260 in Ischemic Stroke: An Investigation Into Its Anti-Inflammatory Effects and Impact on Microglial Activation

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Background: Ischemic stroke is characterized by a high incidence and elevated mortality. Ischemic events trigger neuroinflammation, leading to severe brain edema and neuronal necrosis. Microglia are the primary mediators of neuroinflammation. Inhibition of M1 microglia effectively alleviate neuronal damage in mild stroke. TPT-260 is a minimally cytotoxic, small molecule chaperone of the retromer complex, which mediates the recycling and trafficking of membrane protein receptors. This study explores the therapeutic effects and related mechanisms of TPT-260 in stroke model mice from an anti-inflammatory perspective, aiming to evaluate the efficacy and mechanism of TPT-260 in treating stroke.

Methods: In this study, a middle cerebral artery occlusion (MCAO) animal model was established to simulate ischemic stroke. Primary microglia were cultured for lipopolysaccharides treatment to construct M1 microglia. Both animals and cells were treated with TPT-260. Nuclear factor-κB (NF-κB) nuclear translocation and the expression of downstream pro-inflammatory factors Interleukin 1β (IL-1β) and Tumor necrosis factor-α (TNF-α) were determined.

Results: In vivo results revealed that TPT-260 significantly reduced the brain infarct area and inflammation as well as improved the neurological function of the stroke model mice. The potential mechanism of TPT-260 involved the marked inhibition of the lipopolysaccharides-induced M1 microglia by suppressing NF-κB nuclear translocation and attenuating the expression IL-1β and TNF-α. Moreover, TPT-260 inhibited NOD-like receptor protein 3 and reduced inflammasome formation, thereby decreasing the release of mature IL-1β and alleviating neuroinflammation.

Conclusion: TPT-260 attenuated M1 microglia via repression of NF-κB signaling, thus preventing neuroinflammation and neuronal injuries in stroke model mice.

Keywords: TPT-260, ischemic stroke, microglia, NF-κB, inflammation

Introduction

Stroke has emerged as one of the leading causes of death in recent years. According to the Global Burden of Disease Study Group, the estimated lifetime global risk of stroke is 24.9%. Ischemic stroke, which is the most common stroke type, is associated with inflammatory damage to the nervous system that can even induce severe symptoms such as cerebral edema. Ischemic stroke triggers an inflammatory response involving the activation of immune cells (such as microglia and astrocytes) and the release of inflammatory mediators (including cytokines and chemokines). These inflammatory factors further exacerbate brain injury by promoting cell death, disrupting the blood-brain barrier, and causing cerebral edema, oxidative stress, and neuronal dysfunction.[1,2] Therefore, anti-inflammatory treatment is crucial in managing ischemic stroke. Certain anti-inflammatory drugs, such as aspirin and cottonseed oil, have been utilized in the clinical treatment of ischemic stroke.[3–5] However, the existing approaches for the use of anti-inflammatory drugs in

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ischemic stroke treatment have several limitations, including an increased risk of bleeding, drug interactions, gastrointestinal reactions, allergic reactions, and other side effects.[5–8]

The inflammatory response of the nervous system is mainly mediated by microglia. In patients with ischemic stroke, extreme hypoxia in the brain tissue causes hypoxic injury in the neurons, which in turn releases damage-related proteins that activate microglia.[9] In patients with hemorrhagic stroke, exogenous substances in the blood, including leukocytes, albumin, and other substances, can also activate microglia.[10,11] The activated microglia then undergo M1 polarization, which leads to the increased production of pro-inflammatory factors. This elevated level of pro-inflammatory factors exacerbates neuronal injury[12,13] and causes alterations in astrocytes that result in their swelling and consequently induces cerebral edema.[14–16] Therefore, the targeted inhibition of the microglial pro-inflammatory response and enhancement of waste metabolism in brain microglia are valuable strategies for alleviating neuronal injury and aiding recovery in patients with stroke.

Microglial activation involves multiple signaling pathways, among which the nuclear factor-κB (NF-κB) signaling is a closely related pathway. NF-κB plays a critical role in the regulation of the human immune system.[17] In immune cells, NF-κB usually exists as a homodimer of p65/p50 or a heterodimer complex of the components of p65 and p50.[18] In resting immune cells, NF-κB is retained in the cytoplasm by binding to IκB.[18] Upon stimulation of these resting cells, a signal transduction pathway is triggered that leads to the nuclear translocation of the NF-κB p65 component, which contains the main transcription regulatory domain that activates NF-κB-responsive genes. Ultimately, the expression of downstream pro-inflammatory factors is induced.[18] After microglial activation, NF-κB p65 undergoes nuclear translocation that results in the expression and release of various pro-inflammatory genes and proteins, including interleukin-1β (IL-1β), IL-18, and tumor necrosis factor-α (TNF-α).[18,19] Furthermore, the nuclear translocation of NF-κB p65 leads to the expression of NOD-like receptor family pyrin domain-containing protein 3 (NLRP3), which recruits the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) to form an inflammasome. The formation of the NLRP3 inflammasome then results in the cleavage of the pro-inflammatory factor IL-1β to generate its mature IL-1β form that is secreted extracellularly, thereby further stimulating the microglia and amplifying the inflammatory response.[20,21]

TPT-260 (CAS: 2076–91-7) is a small molecule chaperone of the retromer complex, which mediates the recycling and circulation of cell membrane protein receptors. TPT-260 has been reported to reduce amyloid plaque deposition in Alzheimer's disease (AD).[22] However, no research or clinical applications have been published on the use of TPT-260 in mitigating the anti-inflammatory response, particularly in stroke treatment. In this study, we revealed the regulatory role of TPT-260 in inhibiting the pro-inflammatory microglial activation via the NF-κB pathway and effectively alleviating ischemic stroke. This study aims to provide more therapeutic strategies and primary data for improving the clinical treatment of ischemic stroke.

Materials and Methods

Generation of Middle Cerebral Artery Occlusion (MCAO) Model in Mice and the Bederson Scoring System

To establish the MCAO model, the external carotid artery (ECA) and its branches were blocked first, and then the pterygopalatine artery was blocked to cut off the lateral circulation from extracranial sources. A thread was inserted from ECA through the internal carotid artery to the anterior cerebral artery to mechanically block the blood supply from the middle cerebral artery. The ischemia time was 90 minutes.

Fifteen two-month-old C57BL/6 mice were divided into three groups and each group comprised five mice. In the TPT-260 treatment group, the mice were intraperitoneally injected with 5 mg/kg of TPT-260 24 h before the MCAO modeling surgery, whereas the sham surgery group and the MCAO model group were injected with an equal volume of saline. At 3 h post-surgery, the cerebral injury of the mice was assessed using the Bederson score.[23] Briefly, the mice were lifted by the tail to a height of 10 cm above the platform. In normal mice, the forepaws are held in an extended state, and the mice are given a corresponding score of 0. However, mice with neurological dysfunction may exhibit distinct behaviors and are scored as follows: a score of 1 is assigned when the forelimb on the paralyzed side is retracted and bent

under the abdomen, whereas the limb on the normal side extends towards the platform; a score of 2 is given when the mice lie prone on the platform, and the resistance to pushing the mice towards the paralyzed side is significantly reduced compared to the normal side; a score of 3 is allocated when the mice rotate towards the paralyzed side when walking; and finally, a score of 4 is defined when the mice cannot walk independently and show signs of loss of consciousness.

All animal studies were reviewed and approved by the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (approval ID: SYXK[SU]2007–0021). Laboratory animals—General code of animal welfare (GB/T 42011–2022) issued by National Laboratory Animal Standardization Committee (TC281) was followed for the welfare of the laboratory animals.

2, 3, 5-Triphenyltetrazolium Chloride (TTC) Staining

After the MCAO modeling procedure, the mice were anesthetized, and their brains were removed after perfusion with 4% paraformaldehyde. Using a brain-positioning system, the brain tissue was sliced every 2 mm into 4 slices. The obtained brain sections were immediately immersed in a 2% solution of TTC at 37°C. The slices were then incubated in the TTC solution for 15–20 min, with gentle agitation every 5 min to ensure even staining. TTC solution stains viable brain tissue with a deep red color, while infarcted or damaged tissue remains unstained or appears pale. A digital camera was used to capture images of the stained brain slices, and the infarct areas distinguished by their pale appearance were measured utilizing ImageJ software. Finally, the infarct size was calculated based on the formula shown below:

Infarct volume =the sum of areas of the white infarct area in all slices / the sum of areas of the hemisphere in all slices×100%.

ELISA of Brain IL-1β and TNF-α

Brain IL-1β and TNF-α were determined by kits of ELISA MAX™ Standard Set Mouse IL-1β (432601, Biolegend) and ELISA MAX™ Standard Set Mouse TNF-α (430901, Biolegend). Briefly, the weighed brain tissue was resuspended in PBS after liquid nitrogen grinding. Subsequently, after centrifugation at 12,000g, the supernatant was subjected to Elisa assay and standardized using brain tissue weight.

Primary Microglia Culture and Treatment Protocols

Primary microglia were acquired from the cerebral cortices of 2-day-old neonatal mice as described previously.[24] After removing the meninges, the cortical tissue was digested into a single-cell suspension. In the microglia culture process, the primary microglial cells were cultured in a DF-12 media supplemented with 10% fetal bovine serum, 5 ng/mL of GM-CSF (78017, STEMCELL), and penicillin/streptomycin (100 U/mL and 100 mg/mL, respectively) at 37°C in a 5% CO_2 humidified incubator. After culturing for 14 days, the microglial cells were harvested from the supernatant and seeded in 12-well plates. The purity of the primary microglia was determined, as indicated in a previous publication.[25] Lipopolysaccharide (LPS) or nigericin (Nig) induction of the microglia to generate M1 microglia was performed by treating the cells with 100 ng/mL of LPS for 3 h, followed by 10 μM of Nig for 0.5 h. The resulting M1 microglial cells were then treated with different concentrations of TPT-260. After TPT-260 treatment, the total RNA and protein were extracted from the cells. Subsequently, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted to detect the expression levels of *Il1b, Tnfa*, and *Nlrp3*, while Western blotting was performed to detect the levels of precursor and mature forms of IL-1β and the expression levels of the NF-κB p65 protein.

TPT-260 Treatment

TPT-260 (CAS: 2076–91-7, HY-23769A, MedChemExpress) was initially dissolved in DMSO to a concentration of 20 mm. For cell treatment, the solution was further diluted in culture medium to concentrations of 5, 10, and 20 nM and directly incubated in the primary microglia cultures.

Western Blotting

Cells and tissue were lysed using the radioimmunoprecipitation assay buffer. Protein concentration was estimated via the bicinchoninic acid assay. Additionally, proteins were isolated by SDS-PAGE and transferred to PVDF membranes. The

membranes were then blocked with 5% nonfat milk and incubated with primary antibodies including anti-Toll-like receptor 4 (TLR4) (14358s, Cell Signaling), anti-p-Ikkβ (inhibitory kappa B kinase beta) (2078T, Cell Signaling), anti-Ikkβ (8943s, Cell Signaling), anti-ASC (67824T, Cell Signaling), anti-NLRP3 (PA5-20838, AdipoGen), anti-p65 (8242, Cell Signaling), anti-p-p65 (3033S, Cell Signaling), anti-IL-1β (ab9722, Abcam), and anti-β-actin (A5316, Sigma) overnight at 4°C. The binding of the primary antibodies was visualized with secondary antibodies, including goat anti-rabbit-HRP (115–035-033, Jackson) or goat anti-mouse-HRP (111–035-003, Jackson).

Detection of Cell Viability

Cell viability was determined using a CCK-8 cell counting kit (A311-01, Vazyme), while lactate dehydrogenase (LDH) release was measured using an LDH Cytotoxicity Assay Kit (40209ES76, Yeasen).

Immunofluorescence Imaging

Microglia were fixed and permeabilized with 0.3% Triton X-100. After blocking with 10% bovine serum albumin, the samples were probed with anti-p65 (8242, Cell Signaling) and anti-ASC (67824T, Cell Signaling) primary antibodies. The binding of the primary antibodies was detected with Alexa Fluor 555-conjugated donkey anti-rabbit IgG (A31572, Thermo). Lastly, the samples were counterstained with DAPI, and images were procured using a Leica SP8 confocal microscope with a 63× objective.

qRT-PCR

Total RNA was isolated using TRIzol, and a 1-μg sample of purified total RNA was reverse transcribed using a HiScript 1st Strand cDNA Synthesis Kit (R323-01, Vazyme). Subsequently, real-time PCR was performed utilizing a SYBR premix (Roche, USA) according to the following conditions: 95°C for 5 min, followed by 40 two-step cycles of 95°C for 30s and 60°C for 30s. All the primers employed in the real-time PCR are listed in Table 1.

Statistical Analyses

Data analyses were conducted using GraphPad Prism v.9, including Student's *t*-test or one- or two-way ANOVA followed by Tukey's multiple comparisons. Data were presented as mean ± standard error of the mean for in vivo experiments or mean ± standard deviation for in vitro experiments. Statistical significance was indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, with n.s denoting no significance.

Table 1 Primer Information

Name	Direction	Sequence (5′—3′)
Pycard	Forward	CTGCTCAGAGTACAGCCAGAAC
	Reverse	CTGTCCTTCAGTCAGCACACTG
TLR4	Forward	ATGGCATGGCTTACACCACC
	Reverse	GAGGCCAATTTTGTCTCCACA
Nlrp3	Forward	TCACAACTCGCCCAAGGAGGAA
	Reverse	AAGAGACCACGGCAGAAGCTAG
Il18	Forward	GTGAACCCCAGACCAGACTG
	Reverse	CCTGGAACACGTTTCTGAAAGA
Il1b	Forward	TGCCACCTTTTGACAGTGATG
	Reverse	TGATGTGCTGCTGCGAGATT
Tnfa	Forward	AAGCCTGTAGCCCACGTCGTA
	Reverse	GGCACCACTAGTTGGTTGTCTTTG
Actb	Forward	CATCCGTAAAGACCTCTATGCCAAC
	Reverse	ATGGAGCCACCGATCCACA

Results

TPT-260 Alleviates Cerebral Infarction in MCAO Mice

In this study, we investigated the therapeutic potential of TPT-260 in a MCAO mouse model of cerebral infarction. Two-month-old C57BL/6 mice were intraperitoneally injected with 5 mg/kg of TPT-260. Then, MCAO surgery was performed on the mice 24 h after treatments with TPT-260 as well as saline (control). At 3 h following the MCAO surgery, the Bederson score of the mice was assessed. The Bederson score results indicated that TPT-260 significantly alleviated brain injury in the MCAO mice (Figure 1A). Furthermore, the brain slices of the mice were stained using the TTC method, with the infarct area identified as pale tissue. The staining results revealed that TPT-260 significantly reduced the infarct area on the affected side of the brain (Figure 1B and C). By examining the levels of pro-inflammatory factors IL-1β and TNF-α in brain tissue, we found that TPT-260 significantly suppressed neuroinflammation in the brain (Figure 1D and E).

TPT-260 Reduces the Inflammatory Response in Microglia After LPS/Nig Administration

Next, we examined the effect of TPT-260 on the microglial inflammatory response. In this experiment, primary cultured microglia were treated with LPS for 3 h, followed by Nigericin (Nig) treatment for 0.5 h. After the treatment protocol, the expression levels of pro-inflammatory factors such as NLRP3, IL-1β, and ASC were significantly increased, while the phosphorylation level of p65 was notably upregulated (Figure 2A and B). These findings suggested that LPS/Nig administration induced the activation of the NF-κB signaling pathway in the microglia, triggering a pro-inflammatory response. Subsequently, the microglia were treated with a series of LPS concentrations, and the expression of *Il1b, Tnfa*, and *Il18* was assessed at the mRNAs level. The results indicated that the most suitable dose of LPS for inducing an adequate pro-inflammatory response was 100 ng/mL (Figure 2C), exhibiting significant induction of *Il1b* and *Tnfa* transcription in the microglial cells at this concentration.

Additionally, the potential therapeutic effects of TPT-260 were further investigated by treating primary microglia with different concentrations of TPT-260. The results indicated that TPT-260 administration did not affect cell viability or cause cell damage (Figure 3A and B). Moreover, the combined administration of LPS/Nig led to decreased microglial vitality and damage

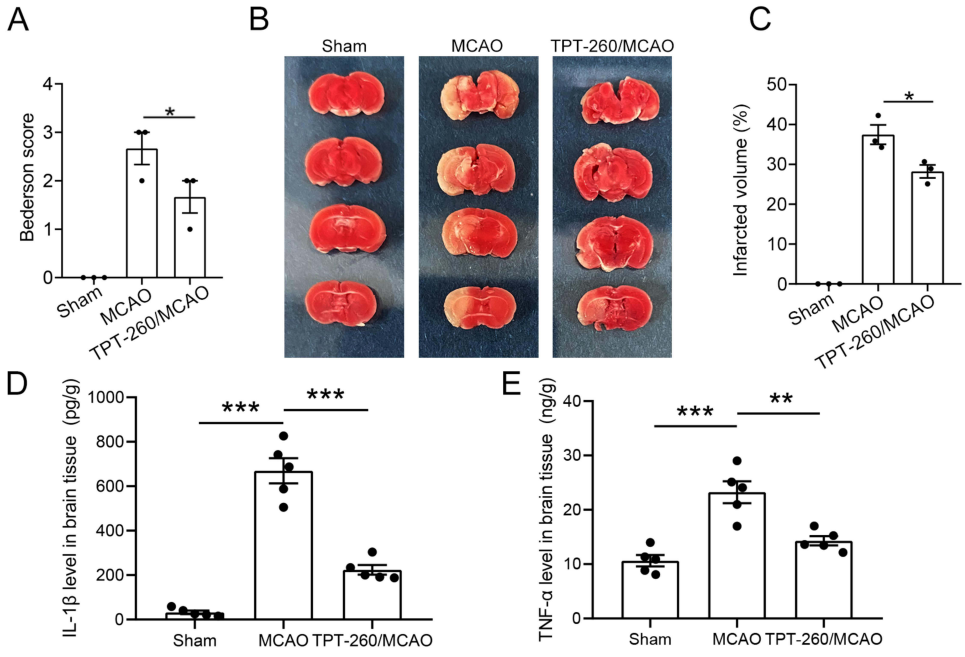

Figure 1 Therapeutic effect of TPT-260 on the MCAO mice model of cerebral infarction. TPT-260 treatment improved the Bederson score (**A**), reduced the infarcted brain area (**B** and **C**), and inhibited the proinflammatory factors IL-1β (**D**) and TNF-α (**E**) levels in brains of MCAO mice compared to that in the control mice injected with saline. Data were analyzed using two-way ANOVA (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). N=3 in (**A** and **C**). Representative images from one mouse were shown in (**B**) N=5 in (**D** and **E**).
Abbreviations: IL-1β, interleukin-1β; MCAO, middle cerebral artery occlusion; TNF-α, tumor necrosis factor-α.

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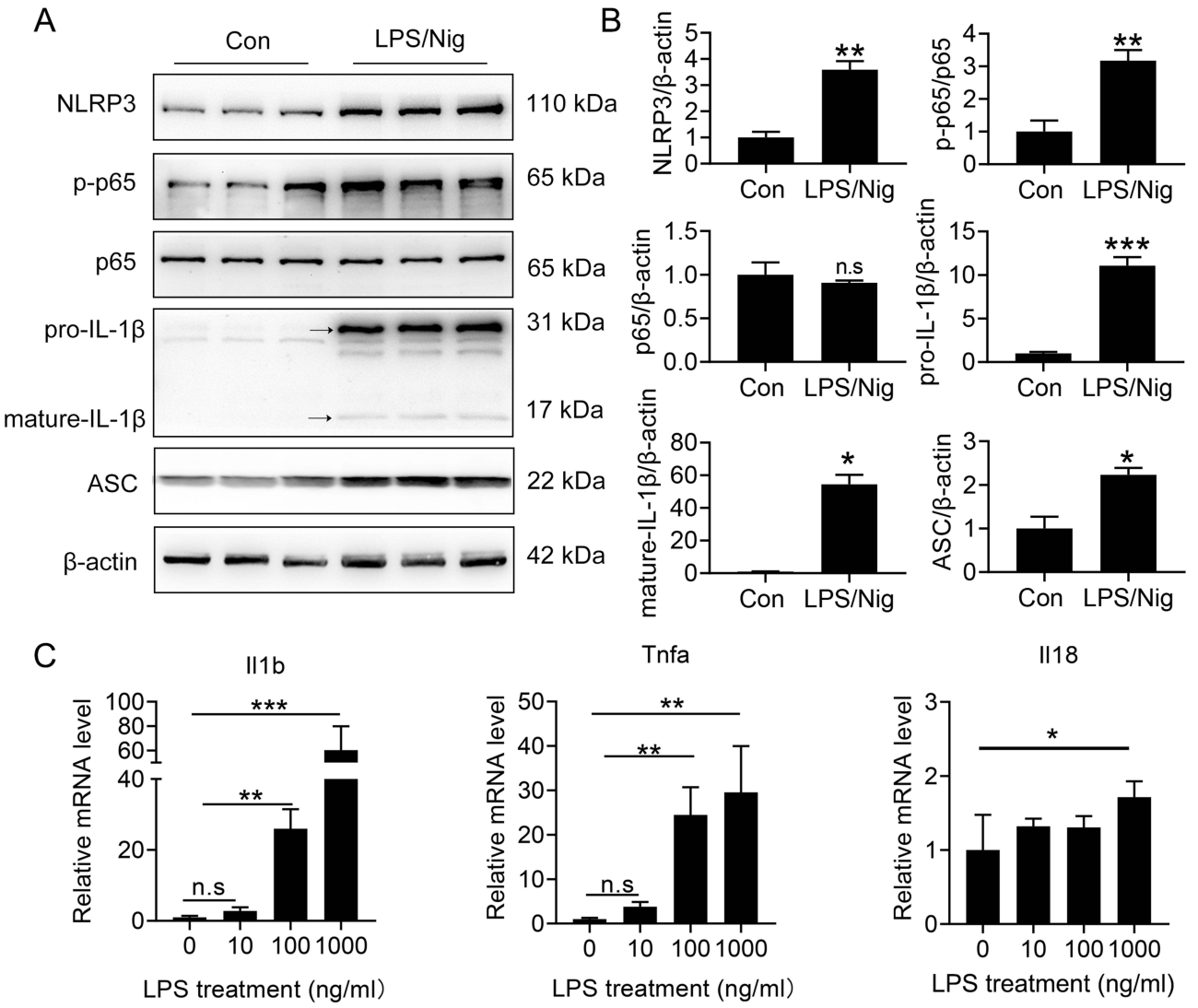

Figure 2 LPS/Nig administration activates the NF-κB signaling pathway in microglia. The levels of NLRP3, phosphorylated p65, the precursor and mature forms of IL-1β, and ASC increased after LPS/Nig administration in the microglial cells (**A** and **B**). The expression levels of *Il-1β, Tnf-α*, and *Il-18* were determined using qRT-PCR (**C**). Data were analyzed using one-way ANOVA (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; n.s=no significance).

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; Con, control; IL-1β, interleukin-1β; IL-18, interleukin-18; LPS, Lipopolysaccharide; Nig, nigericin; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; TNF-α, tumor necrosis factor-α.

to the cell membrane integrity (Figure 3C and D). TPT-260 treatment was shown to effectively alleviate the impaired cell activity induced by LPS/Nig administration and was observed to play a protective role in maintaining cell membrane integrity.

We also investigated whether TPT-260 treatment could inhibit the microglial inflammatory response. In this investigation, microglia were pre-treated with TPT-260 before the administration of LPS/Nig. Our results suggested that TPT-260 effectively inhibits inflammasome formation in the microglia (Figure 4A). Simultaneously, TPT-260 pre-treatment in the LPS/Nig-administered microglia significantly suppressed the expression levels of the pro-inflammatory factors *Nlrp3, Tnfa*, and *Il1b* compared to the LPS/Nig-treated microglia without TPT-260 pre-treatment (Figure 4B and C). All these findings suggest that TPT-260 effectively inhibits the microglial inflammatory response induced by LPS/Nig.

TPT-260 Reduces NF-κB Signaling by Inhibiting the TLR4-IKKβ Pathway in LPS/Nig-Treated Microglia

We further elucidated the impact of TPT-260 treatment on the NF-κB signaling pathway. In this investigation, the effect of TPT-260 treatment on the LPS/Nig-induced nuclear translocation of NF-κB p65 in primary microglia cells was assessed. For

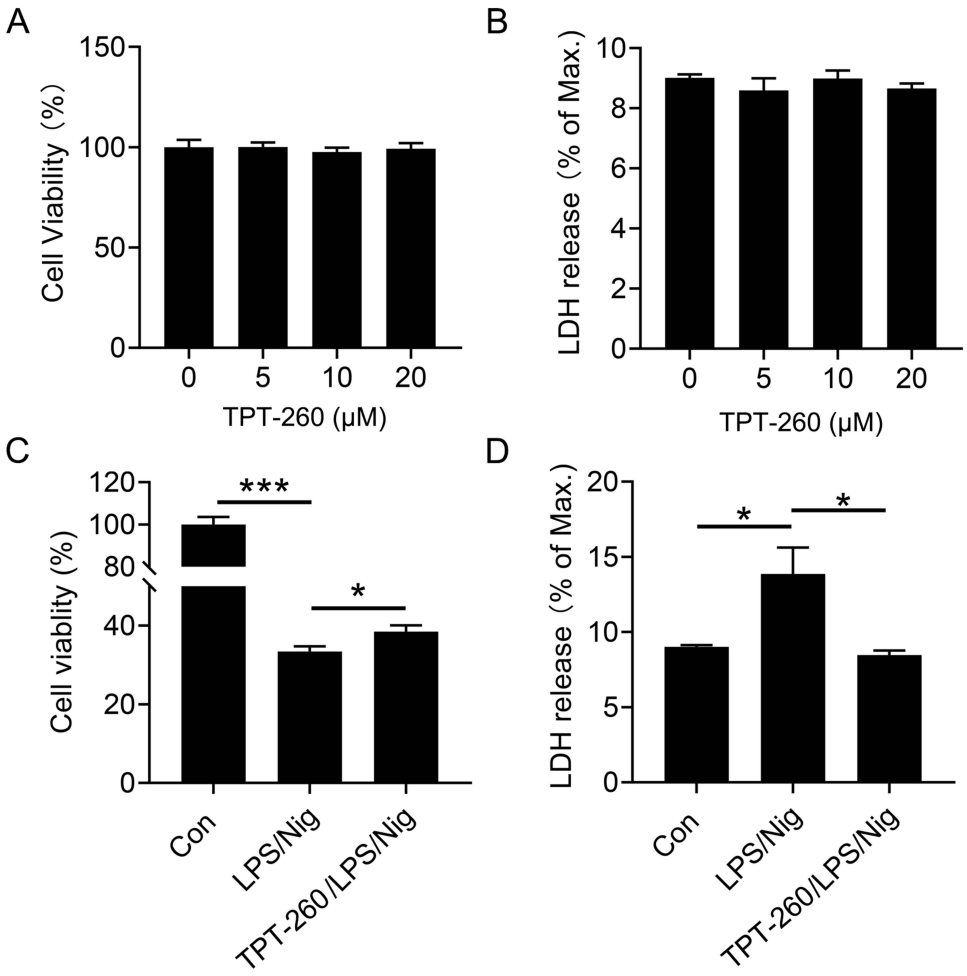

Figure 3 Effect of TPT-260 treatment on cell viability and membrane integrity in microglia and LPS/Nig-treated microglia. TPT-260 treatment did not affect cell viability (**A**) and did not cause cell damage (**B**). Data were analyzed using one-way ANOVA (**A** and **B**). The combined administration of LPS/Nig led to diminished microglial viability and damage to cell membrane integrity, whereas TPT-260 treatment effectively alleviated the impairment of cell activity induced by LPS/Nig administration (**C** and **D**). Data were analyzed using Student's *t*-test (*$p < 0.05$ and ***$p < 0.001$).
Abbreviations: LDH, lactate dehydrogenase; LPS, Lipopolysaccharide; Nig, nigericin.

this purpose, microglia were pre-treated with TPT-260 before the administration of LPS/Nig. The staining intensity of p65 in the TPT-260-co-treated cells was significantly reduced compared to that in the LPS/Nig cells (Figure 5A and B). Based on this result, TPT-260 may effectively inhibit the LPS/Nig-induced nuclear translocation of NF-κB p65 in the primary microglia (Figure 5B). Next, we evaluated the mechanism by which TPT-260 inhibits the NF-κB signaling pathway. Our results showed that LPS/Nig administration significantly upregulated TLR4, an upstream NF-κB agonist, and increased the phosphorylation levels of IKKβ. Conversely, TPT-260 treatment inhibited the upregulation of TLR4 and downregulated the phosphorylation of IKKβ and p65 in the microglia treated with LPS/Nig. Finally, TPT-260 treatment also led to the significant downregulation of NF-κB downstream factors NLRP3 and IL-1β in LPS/Nig-treated microglia (Figure 5C). All these results imply that TPT-260 attenuates the LPS/Nig-induced pro-inflammatory response of microglia by inhibiting TLR4-IKKβ activation.

Discussion

In this study, we revealed evidence that TPT-260 exerts a crucial inhibitory effect on the NF-κB signaling pathway, impedes the pro-inflammatory polarization of microglia, and decreases the secretion of inflammatory factors. Our findings suggest that TPT-260 reduces the brain infarct area, thereby serving as a potential therapeutic option in ischemic stroke. Anti-inflammatory treatment can mitigate the harmful effects of inflammation by inhibiting immune cell activation, suppressing the production of inflammatory mediators, reducing cerebral edema, improving blood flow,

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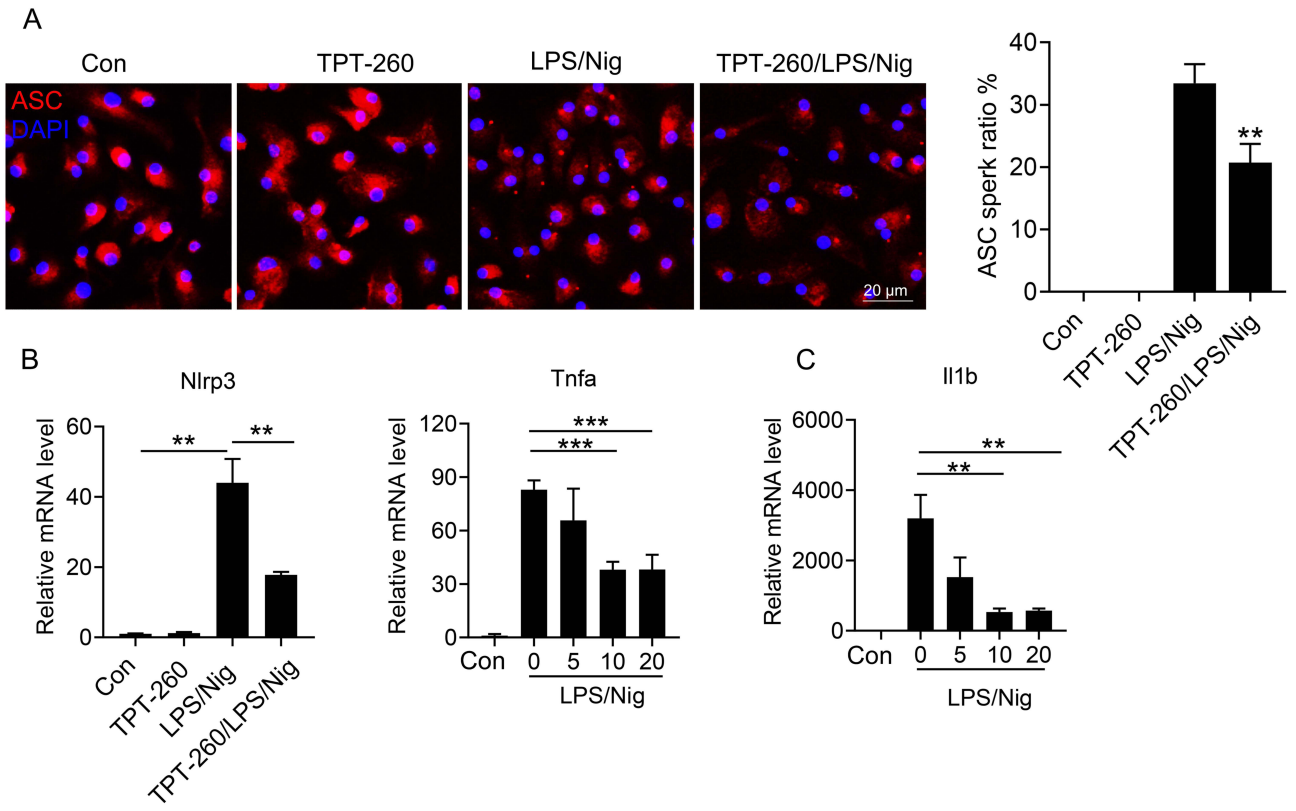

Figure 4 Effect of TPT-260 treatment on the inflammatory response of pro-inflammatory M1 microglia. TPT-260 treatment reduced LPS/Nig-induced inflammation formation (ACS speck) (**A**) and decreased the expression of pro-inflammatory genes *Nlrp3*, *Tnf-α*, and *Il-1β* in M1 microglia (**B** and **C**). Data were analyzed using two-way ANOVA (**$p < 0.01$ and ***$p < 0.001$).

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; Con, control; IL-1β, interleukin-1β; LPS, Lipopolysaccharide; Nig, nigericin; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; TNF-α, tumor necrosis factor-α.

protecting neurons and vascular endothelial cells, and preventing or delaying ischemic stroke-related complications, such as cognitive impairment, depression, and infection.[6,7,26] Furthermore, the activation of microglia alters its phagocytic function, leading to the ineffective clearance of waste generated in brain tissue and further exacerbation of brain injury.[12] Targeting microglia has been identified as a promising strategy to develop anti-inflammatory therapies[27] and novel molecules capable of resolving the neuroinflammation mediated by M1 microglia are in urgent need.[28,29] Therefore, the anti-inflammatory effect of TPT-260 that might result from inhibiting M1-like microglia in our study suggests its potential for treating stroke since it not only aids in reducing neuroinflammation in the brain and alleviating inflammatory neuronal death but also can effectively lead to long-term improvements in ischemic stroke prognosis, such as the recovery of cognitive and motor abilities and anxiety reduction.

Stroke includes hemorrhagic and ischemic stroke[10,30] and is characterized by a sudden onset and narrow treatment window. In both ischemic and hemorrhagic stroke, the golden period of treatment is often missed, resulting in severe sequelae in affected patients. Moreover, certain patients with ischemic stroke may experience vascular leakage due to necrosis and collapse in the local ischemic area or thrombolytic treatment failure, eventually leading to hemorrhagic transformation. Severe hemorrhagic transformation has a high rate of disability and mortality, thus posing a challenge to clinical treatment in such populations. The inflammatory response plays a vital role in the pathological process of both hemorrhagic and ischemic stroke, encompassing various processes such as inflammation, immune cell recruitment, and microglia activation.[7] A variety of leukocytes, including neutrophils, macrophages, microglia, and T cells, participate in the immune response following hemorrhagic stroke with an increment of neurotoxic cytokines such as TNF and IL-1β.[11] Likewise, numerous peripheral immune cells accumulate in the ischemic lesion and microglia contribute to either the exacerbation or alleviation of immune responses depending on their polarization and differentiation during ischemic

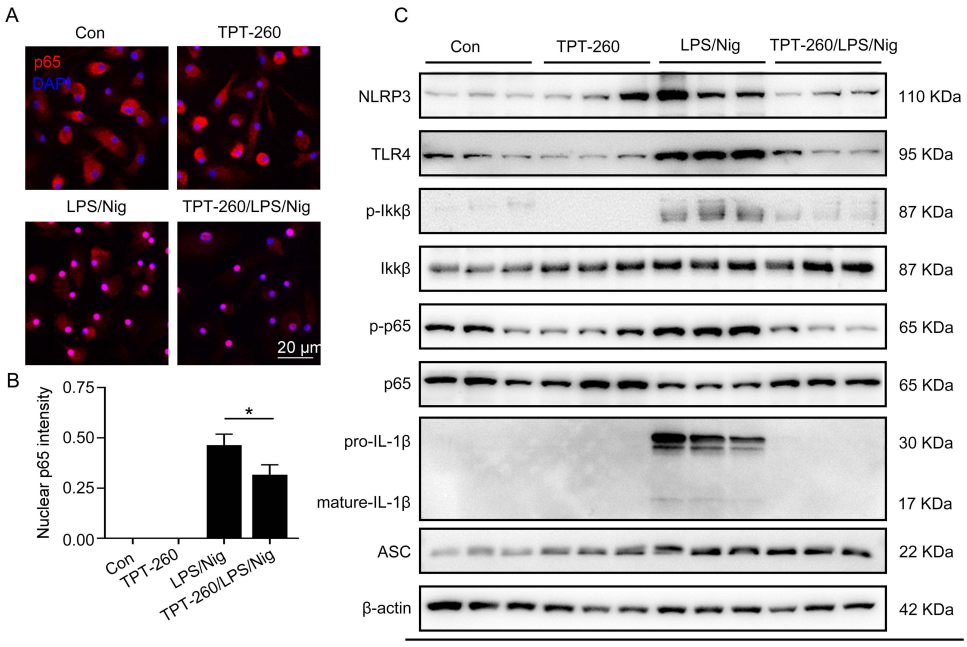

Figure 5 Effect of TPT-260 treatment on the nuclear localization of NF-κB p65 in pro-inflammatory M1 microglia. TPT-260 treatment decreased the LPS/Nig-induced nuclear localization of NF-κB p65 in M1 microglia (**A** and **B**). TPT-260 treatment resulted in decreased levels of NLRP3, TLR4, phosphorylated IKKβ, phosphorylated p65, and IL-1β in LPS/Nig-treated microglia (**C**). Data were analyzed using two-way ANOVA (*$p < 0.05$).

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; Con, control; Ikkβ, inhibitory kappa B kinase beta; IL-1β, interleukin-1β; LPS, Lipopolysaccharide; Nig, nigericin; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; TLR4, Toll-like receptor 4.

stroke.[7,10] Considering that the disease mechanisms of both stroke types involve strong inflammatory responses, TPT-260 may be used as a therapeutic drug for both ischemic and hemorrhagic stroke.

TPT-260 has been proven to exhibit excellent therapeutic effects in animal models of degenerative diseases of the central nervous system; however, its mechanism remains unclear.[22] Microglia are macrophages located in the brain and possess the fundamental characteristics of innate immune cells. Recent studies have shown that microglia are critically involved in the pruning of dendritic spines/synapses.[31,32] In adult brains, activated microglia also wrap around dendritic spines and even enclose part of the presynaptic structure and clear them by phagocytosis, thereby reducing the number of dendritic spines.[33] Conversely, the inhibition of M1 microglia can significantly reduce dendritic spine loss,[34] thus affecting the long-term potentiation of related neurons. The dendritic spines of hippocampal neurons are known to play a pivotal role in information processing and storage.[35] A recent prominent study found that hippocampal microglia could cause the impairment of memory encoding by phagocytizing the synaptic structures of hippocampal neurons.[36] Suppressing neuroinflammation by targeting microglia has been indicated as an important mechanism in treating neurodegenerative diseases.[12,27,37,38] Reducing M1 microglia and pro-inflammatory factors could prevent damage on neurons in the lesions and induce neuroprotective effects.[12,27,37,38] Our study also proposes that one of the possible mechanisms of TPT-260 in the treatment of degenerative diseases of the central nervous system could at least partly involve the reduction in the degenerative neuronal lesions via the inhibition of neuroinflammation. Similar to the anti-inflammatory effects observed in treating ischemic stroke with cottonseed oil,[3] TPT-260 has the potential to reduce the neurotoxicity caused by microglia in stroke.

TPT-260 is a small molecule chaperone of the retromer complex that mainly mediates the recycling and trafficking of membrane protein receptors. However, no research and clinical application studies have been published on the effect of TPT-260 on the anti-inflammatory response, particularly in stroke treatment. The core component of the retromer complex, the vacuolar sorting protein 35 (VPS35), is an essential binding protein for maintaining the function of the anti-inflammatory triggering receptor expressed on myeloid cells 2 (TREM2). Previous studies have demonstrated that TREM2 can downregulate the PI3K/NF-κB signaling pathway in microglia and inhibit neuroinflammation.[39] Additionally, LPS-induced imbalance in TLR4/TREM2 has been found to cause elevated neuroinflammation and

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aggravate cognitive impairment in AD mice.[40] A functional defect in VPS35 can also lead the microglial cells to show characteristic pro-inflammatory polarization.[41] Given that TPT-260 is a small molecule chaperone of VPS35, it can effectively stabilize the basic function of the retromer. Consequently, we postulate that the VPS35-dependent TREM2 may be involved in the upstream regulation of the NF-κB signaling pathway, suggesting the regulatory role of VPS35-retromer in innate immunity. Therefore, TPT-260 may exert an anti-inflammatory effect by augmenting VPS35 function and increasing the intracellular circulation of TREM2.

Several limitations in the current study should be acknowledged. 1) Conclusions drawn from the small sample size of the animals in the current study should be considered with caution. Future studies on TPT-260 can be improved based on the experimental designs in other studies with larger sample sizes and more analytical methods.[3,42–44] 2) Different animal models of ischemic stroke with different methods to induce MCAO could be investigated in the future to evaluate TPT-260 in a model that more closely resembles the pathology of ischemic stroke in humans compared to the current model.[30,45,46] Although MCAO models built by inserting a thread through the artery have been commonly used in mice stroke models, approaches such as photochemically initiated thrombosis, injections of fibrin and in situ clot formation have been suggested to model ischemic stroke in ways that are more similar to the conditions observed in stroke patients.[30] Rodent models of MCAO, rats in particular, are used most extensively in the preclinical studies on stroke; however, the white matter content in their brains is much lower than that in humans.[45,46] Despite practical reasons, rabbits, dogs, pigs and non-human primates have emerged as models to assess novel treatments in stroke to promote translation of basic research into clinics because their brains mimic human brain better than rodents.[45,46] Therefore, validation of the effects of TPT-260 in rats and rabbits could be considered. 3) Efforts are needed to assess the impact of timing of administration on the anti-inflammatory properties of TPT-260 before and after MCAO. Administration of anti-inflammatory agents in mice varied among different studies on treatments for ischemic stroke; for example, treatments were initiated 5 days,[43] 1 h,[44] or 24 h before MCAO[7] in several studies while mice were treated 4 to 72 h after MCAO.[7,42] The mechanism of actions and biological pharmacokinetics of different types of treatments may account for the various timing of administration. Besides, to optimize the therapeutic effects in treating ischemic stroke, multiple doses of TPT-260 may be necessary, as a single dose may not suffice. How to translate the optimal timing and dosing determined in animal models to that in patients will also need to be taken into consideration.

Conclusions

In summary, this study established a MCAO animal model to simulate ischemic stroke and investigated the therapeutic effects and underlying mechanisms of TPT-260 in the stroke model mice from an anti-inflammatory perspective. Our study provided further evidence on the effects of anti-inflammatory treatment on stroke, thereby effectively expanding our understanding of targeted therapeutic strategies for managing ischemic stroke.

Abbreviations

AD, Alzheimer's disease; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; Con, control; ECA, external carotid artery; Ikkβ, inhibitory kappa B kinase beta; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; LPS, Lipopolysaccharide; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor-κB; Nig, nigericin; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; TNF-α, tumor necrosis factor-α; TTC, 2, 3, 5-triphenyltetrazolium chloride; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TLR4, Toll-like receptor 4; TREM2, triggering receptor expressed on myeloid cells 2; VPS35, vacuolar sorting protein 35.

Data Sharing Statement

The data extracted for analyses are available by the corresponding author upon reasonable requests.

Ethics

All animal studies were reviewed and approved by the Animal Care and Use Committee of Nantong University (approval ID: S20210318-003) and the Jiangsu Province Animal Care Ethics Committee (approval ID: SYXK[SU]2007-0021).

Laboratory animals—General code of animal welfare (GB/T 42011-2022) issued by National Laboratory Animal Standardization Committee (TC281) was followed for the welfare of the laboratory animals.

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Disclosure

The authors declare no conflicts of interest in this work.

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