A self-developed MyD88 inhibitor across the blood-brain barrier fully reverses acute cerebral ischemia-reperfusion injury

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Abstract

Background: The acute ischemia-reperfusion injury that occurs after an ischemic stroke is almost inevitable. In treating ischemic stroke, the prevention and treatment of cerebral ischemia-reperfusion injury (CIRI) is an undeniable difficulty clinically. Despite increasing studies being done on the subject, medicines showing effective neurological improvements after reperfusion is still minimal. This study aims to investigate the effects of a self-developed MyD88 inhibitor (TJ-M2010-5) on CIRI and its mechanism.

Methods: The middle cerebral artery occlusion (MCAO) model was used to simulate CIRI in mice. BV-2 cells were cultured for the mechanism study by LPS stimulation in vitro. Neurological deficit score and cerebral infarction volume were studied. Immunofluorescence staining was used to measure neuronal damage and apoptosis in the brain. The anti-inflammation effect of TJ-M2010-5 was evaluated by analyzing the expression of inflammatory cytokines, activation of microglia and infiltration of peripheral myeloid cells. The expression of TLR4/Myd88/NF- κ B signaling pathway protein was detected by Simple Western. The concentrations of TJ-M2010-5 in blood and brain were analyzed by LC-MS methods.

Results: The cerebral infarction volume decreased in mice treated with TJ-M2010-5, with the most prominent decrease in approximately 80% of the original infarction volume. Moreover, the downregulation of apoptosis and NeuN protein expression in the brain of infarction area further indicated that neuronal damage was alleviated. TJ-M2010-5 treatment also reduced the infiltration ratio of peripheral myeloid cells in the cerebral infarction area and increased the proportion of inactive microglia. The inflammatory cytokines decreased after TJ-M2010-5 treatment in infarction area and supernatant. TJ-M2010-5 downregulated the expression of iNOS and inhibited TLR4/MyD88/NF- κ B signaling pathway in vivo and in vitro. In addition, TJ-M2010-5 could freely pass through the blood-brain barrier.

Conclusions: TJ-M2010-5 has shown to have an excellent therapeutic effect on CIRI by inhibiting TLR4/Myd88/NF- κ B signaling pathway to decrease excessive inflammatory response, showing the potential to change the history that there is no effective medicine for the treatment of CIRI. and also has the potential to be utilized in brain neuroinflammatory diseases.

1. Introduction

Stroke is an acute cerebrovascular disease in which focal neurological loss suddenly occurs in relevant parts of the brain due to infarction or hemorrhage [1]. The most common type of stroke is ischemic stroke, accounting for about 80%. In recent years, vascular recanalization techniques such as thrombolysis and endovascular therapy have made great progress in the treatment of stroke, but the contradiction between the high rate of vascular recanalization and the low clinical benefit ratio of patients is still a prominent issue in clinical practice. There is some evidence that shows the acute immune-inflammatory reaction related to reperfusion can lead to secondary brain injury and expand the scope of brain injury [2,3]. Although increasing studies have been conducted regarding cerebral ischemia-

reperfusion injury (CIRI) in the medical field, targeted therapeutic drugs are still very scarce clinically. Studies have shown that myeloid differentiation factor 88 (Myd88) plays a vital role in the development of CIRI[4–9].

MyD88 is a ligand-protein that plays a key role in the toll-like receptors (TLRs) signaling pathway [10]. After identifying pathogen-related and danger-related molecular patterns (DAMPs and PAMPs), TLRs activate the downstream pathway and lead to a series of inflammatory injuries [11]. Cerebral ischemia-reperfusion (I/R) followed by infiltration of peripheral leukocytes and activation of endogenous microglia trigger a series of inflammatory responses [12]. The TLRs of these cells identify DAMPs and then activate downstream MyD88/NF- κ B signaling, resulting in increased expression of pro-inflammatory factors [13,14]. Furthermore, the inflammatory responses induce more immune cells to activate and secrete increasing amounts of pro-inflammatory factors, leading to a cascade amplification of the inflammatory response, expanding the scope of damage and finally resulting in more neuron loss in CIRI. Recent studies have shown that blocking TLR/MyD88/NF- κ B signaling pathway can downregulate inflammatory response and reduce CIRI [15–17]. Therefore, the activation of the TLR/MyD88/NF- κ B pathway contributes to the neuroinflammatory response of CIRI, which causes an excessive inflammatory reaction.

However, many studies have only shown the effects of inhibiting the inflammatory pathway by gene knockout or biomacromolecules that are difficult to cross the BBB, which is not applicable in clinical settings. By analyzing the structural domain of MyD88, and using computer-aided systems such as drug design and virtual screening, small molecule aminothiazole derivative MyD88 inhibitor, TJ-M2010 series (WIPO Patent Application Number: PCT/CN2012/070811) has been innovatively developed, which can specifically bind to the TIR domain of MyD88 and prevent homodimerization of MyD88 [18]. The chemical structure of MyD88 and its interaction with the MyD88 TIR domain was provided in the previous study(Fig. 1) [19]. The previous studies have shown that MyD88 inhibitor can inhibit the activation of peripheral innate immune cells such as macrophages and dendritic cells (DCs) [19–21], but it is unknown whether MyD88 inhibitor can directly inhibit the activation of microglia across the BBB, which is important for assessing the potential to treat diseases caused by neuroinflammation. Here, we focused on the inflammation inhibition effects of TJ-M2010-5 (TJ-5), a member of MyD88 inhibitor in mice of CIRI and BV-2 microglial cells. The neuroprotective potential of TJ-5 for treating CIRI was evaluated.

2. Methods

2.1. Animals

Male C57BL/6 mice (Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) weighing 22-28 g and aging 8-10 weeks were used. All the animal experiments were approved by the Institutional Animal Care and Use Committee at the Tongji Hospital, Wuhan, China. All breeding, management, and procedures with animals were in strict accordance with the SPF standard and protocols for the care and use of animals in research. Mice were randomly divided into the following seven experimental groups:

sham group, I/R group, vehicle (I/R + saline) group, I/R + TJ-5 5 mg/kg group, I/R + TJ-5 10 mg/kg group, I/R + TJ-5 (I/R + TJ-5 15 mg/kg) group and I/R + Edaravone (I/R + Edaravone 3mg/kg) group.

2.2. CIRI model

The mice were anesthetized with 1% pentobarbital sodium solution by intraperitoneal injection, and the body temperature was maintained at 37.0-37.5 °C. The MCAO model was performed according to the Longa method as described previously [22]. Briefly, the right carotid artery was carefully separated and exposed. The distal end of the external carotid artery (ECA) was ligated with a silk ligature, and a silicon-coated embolic suture (Doccol Corporation, MA, USA) was inserted slowly from ECA into the internal carotid artery (ICA) and reached into the middle cerebral artery (MCA). After one hour of ischemia, the embolic suture was withdrawn, the ECA was ligated, and mice were injected with TJ-5 or edaravone intravenously immediately. Then, the mice were placed in a 32 °C incubator, and 1 ml of normal saline was injected intraperitoneally after they were awake. The surgical procedure in the sham group was the same as that of the ischemic model group, but the MCA was not obstructed.

2.3. Neurological deficit score

After one hour of ischemia and 24 hours of reperfusion, each group of mice was scored blindly according to the scoring system of the Longa method [22]. The score was 0 for no obvious neurological deficit; 1 for inability to fully extend the left forelimb; 2 for turning to the left; 3 for leaning to the left side while walking; and 4 for inability to walk spontaneously and impaired consciousness.

2.4. TTC Staining

The mice were sacrificed after 24 h I/R. The mice brains were harvested for the measurement of cerebral infarct volume. The brains were frozen at -80°C for 5 min and cut into 5 slices of 2 mm thick brain slices. The slices were then placed with the front side facing down in a small dish, 2% TTC solution (Sigma-Aldrich, USA) was added and then 4% paraformaldehyde was used for fixation. After that, the slices were carefully taken out, the liquid was absorbed with filter paper, and pictures were taken with a digital camera. Image J, a professional image analysis software, was used to measure the infarct area of each brain tissue slice and the volume of brain infarction was calculated.

2.5. Cell culture of BV-2 cell line and Stimulation

The culture of BV-2 microglial cells was as previously described [23]. BV-2 cells were seeded on a 6-well plate at a density of 2×10^5 cells/well. After 2 days of culture, lipopolysaccharide (LPS #L2880, Sigma-Aldrich, USA) was added at 1 µg/ml to stimulate BV-2 cells. BV-2 cells were pretreated with different

concentrations of TJ-5 for 2 h before LPS stimulation. Twenty-four hours later, BV-2 cells and the culture supernatant were harvested for subsequent experiments.

2.6. CCK-8 cell viability assay

BV-2 cells were seeded on a 96-well plate at a density of 1×10^4 cells/well. Twenty-four hours later, different concentrations of TJ-5 (0 μ M, 1 μ M, 5 μ M, 10 μ M, 20 μ M and 30 μ M) were added and co-cultured with cells for 24 h. Then, a Cell Counting Kit-8 (CCK8, Sigma-Aldrich, Shanghai, China) was used to measure cell viability. Experiments were conducted according to the manufacturer's manual. After incubation at 37 °C for 2 h, absorbance was measured at 450 nm with a microplate reader (Synergy 2, BioTek Instruments, USA).

2.7. Fluorescence staining

As mentioned earlier, immunofluorescence staining was carried out on paraffin-embedded brain slices[24]. After standard histological procedures, the brain slices were treated with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture to detect apoptosis according to the manufacturer's protocol (Roche, Germany). Furthermore, the brain slices were also used for immunofluorescence with rabbit anti-NeuN antibody (1:300, CST) and rabbit anti-Iba-1 antibody (1:500, CST). After incubating overnight at 4 °C, the goat anti-rabbit IgG (1:1000, Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated antibody) secondary antibodies were used and the brain slices were incubated for 2 h at room temperature. Finally, the slices were washed and labeled with 4', 6-diamidino-2-phenylindole(DAPI) for 10 min at room temperature. The images were captured using a fluorescence microscope.

2.8. Quantitative real-time PCR

Total mRNA was extracted from the brain cortex using trizol reagent and reversed transcription into cDNA by the PrimeScript™ RT reagent kit (Takara, Japan), following the manufacturer's instructions. Then, RT-qPCR assay was performed using SYBR Green Realtime PCR Master Mix (Takara, Japan) with Step One System (Life Technologies). The results of mRNA levels were expressed as a fold of untreated control and analyzed using the $2^{-\Delta\Delta C_t}$ method. The primers were as follows (5'-3'): MyD88 forward: TTTATCTGCTACTGCCCCAACG, reverse: GCGGCGACACCTTTTCTCA; TLR4 forward: ATGCTGCAACTGATGTTCTTC, reverse: GATGTTAGACCTTTCTTCCTCCC; GAPDH forward: TGTTCTACCCCCAATGTGTCC, reverse: GGAGTTGCTGTTGAAGTCGCAG; TNF- α forward: ATGGCCTCCCTCTCATCAGT, reverse: TGGTTTGCTACGACGTGGG; IL-6 forward: AGTGGCTAAGGACCAAGAC, reverse: ATAACGCACTAGGTTTGCCGA; iNOS forward: ATTCACAGCTCATCCGGTACG, reverse: GGATCTTGACCATCAGCTTGC; IL-1 β forward: GCACTACAGGCTCCGAGATGAA, reverse: GTCGTTGCTTGGTTCTCCTTGT.

2.9. Isolation of immune cells

After the mice were anesthetized, 0.9% saline was used for transcardial perfusion. Right brain hemispheres were homogenized in a 6-well plate using 2 ml Hank's balanced salt solution (HBSS, Salarbio) per well. Collagenase IV (1 mg/ml) was added for myelin removal. Then, brain homogenate was filtered through a 70 µm cell strainer and centrifuged at 300 g for 5 min at 4 °C. Afterward, 2 ml of 30% Percoll (Sigma) were added to resuspend brain cells precipitate, The resuspended cells were slowly added to a 15ml centrifuge tube with 3 mL of 70% Percoll. Intermediate layer cells were taken for flow cytometry after density-gradient centrifugation.

2.10. Flow cytometry

Approximately 5×10^5 cells were suspended in 200 µl HBSS and anti-mouse CD16/ CD32 (5 ng/µl) was used to block Fc receptor binding. The cells were stained using APC-conjugated CD45 antibody (1 ng/µl), FITC-conjugated CD11b antibody (1 ng/µl), PE-conjugated Ly6G antibody (1 ng/µl), and incubated in the dark at 4 °C for 30min. Finally, the cells were washed with HBSS buffer, resuspended in 200 µl of HBSS, and analyzed with the flow cytometer (BD FACSCalibur).

2.11. ELISA assay

ELISA kits were used to detect the levels of TNF-α, IL-1β, and IL-6 in the mice brain. The brain tissue was then homogenized in 1 mL of phosphate-buffered saline (PBS) by using a tissue homogenizer. Then, the homogenates were centrifuged at 14000 g for 10 min at 4 °C. The supernatant was immediately transferred for measuring, following the manufacturer's instructions. In vitro assay, BV-2 cells were incubated with different treatments. Then, the supernatant was collected and measured.

2.12. Western Blot analysis

The total proteins, nuclear proteins, and cytoplasmic proteins in BV-2 cells were extracted and quantified using the commercial bicinchoninic acid (BCA) protein quantification kit. The prepared proteins were loaded onto an SDS- PAGE to separate the proteins. Then, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% non-fat milk for 2 h at room temperature. The membrane was immunoblotted with primary antibodies (GAPDH, TLR4, MyD88, iNOS, p-p38, p-JNK, p-ERK, NF-κB p65) at 4 °C overnight and detected with horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (ECL) method. The band densities were analyzed with Image J.

2.13. Simple Western assay

The total proteins, nuclear proteins, and cytoplasmic proteins in brain were extracted and quantified using the BCA protein quantification kit. Here, Simple Western assay, a new method to detect proteins was used as previously described [25]. Briefly, the prepared proteins were diluted to a concentration of 0.5mg/ml by using a sample preparation kit. Then, according to the instructions of Protein Simple Kit, the prepared reagents were added to the detection plate in turn for an automated capillary western blot system, the WES System. The primary antibodies (iNOS, TLR4, MyD88, NeuN, Cleaved-cas-3, p-ERK, NF- κ B p65) were diluted with the antibody diluter at a ratio of 1:50 and the dilution ratio of GAPDH was 1:1000. Compass software (Protein Simple, USA) was used to quantitatively analyze the signal intensity (area) of the protein.

2.14. Detection of drug concentration by LC-MS

The mice were injected 15mg/kg TJ-5 intravenously at the time of reperfusion after 1 h of MCAO. Then, mice were anesthetized at 5 min, 10 min, 30 min, 1 h, 2 h, or 6 h post-dose. The blood was collected to prepare serum samples and then right ipsilateral brain (IL brain), left contralateral brain (CL brain), heart, and liver samples were obtained after transcardial perfusion using 0.9% saline. All samples were frozen at -80 °C for later analysis. The liquid chromatography-mass spectrometry (LC-MS) method was performed to detect the concentration of TJ-5 in the samples. Time–concentration curve, area under the curve (AUC), and pharmacokinetic (PK) parameters of TJ-5 were analyzed using PKslover 2.0 PK software [26]. The BBB permeability was evaluated as the brain-to-serum partition coefficient (K_p) which was calculated as $AUC_{\text{brain}}/AUC_{\text{serum}}$.

2.15. Statistical analysis

All experimental data were statistically analyzed using the professional analysis software GraphPad 8.0. The data obtained were expressed as Mean $\pm$ standard error of the mean (SEM) values. The paired comparison was conducted using a t-test and a one-way analysis of variance (ANOVA) was used for comparison among multiple groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. TJ-5 improves neurological function, reduces the infarct volume and neuronal loss in CIRI mice

To investigate the therapeutic effects of TJ-5 in cerebral I/R induced acute injury, the neurological deficit test of mice was evaluated after 24 h reperfusion. The results showed that the neurological deficit score of the TJ-5 15 mg/kg group was significantly reduced compared with the I/R group (Fig. 2a). The infarct volume was evaluated by TTC staining. We found that TJ-5 significantly reduced the infarct volume, showing more effective neuroprotection, especially on the cortex with the increase in dosage amounts

(Fig. 2b). The percentage of infarct volume was 33.24% in the I/R group, while the most effective TJ-5 15 mg/kg group was only 6.02%, reducing by approximately 80%. Furthermore, compared with 18.59% in the Edaravone group, TJ-5 at 15 mg/kg achieved a better effect (Fig. 2c). We regarded TJ-5 at 15 mg/kg as the I/R + TJ-5 group in subsequent trials because of its best effect. Immunofluorescence staining of the neuron-specific nuclear protein (NeuN) in the brain showed that the number of neurons on the injury hemisphere in the I/R + TJ-5 group significantly increased when compared to that in the Vehicle group (Fig. 2d). Furthermore, the expression of NeuN protein in the I/R group was significantly lower than that in the Sham group, and the results demonstrated that TJ-5 could inhibit this decrease (Fig. 2e and 2f). Together, these data indicate that TJ-5 has powerful neuroprotective effects and reduces neuron loss caused by CIRI.

3.2. TJ-5 inhibits apoptosis and alleviates the excessive inflammatory response in CIRI mice

To investigate the protective effects of TJ-5 on CIRI-induced inflammatory responses, the mRNA expression and protein content of TNF- α , IL-1 β and IL-6 in brain were detected by RT-qPCR and ELISA, respectively. The results showed that I/R injury significantly increased the expression and production of inflammatory factors in the brain tissue, while TJ-5 reduced the expression of TNF- α , IL-1 β and IL-6 (Fig. 3a and 3b). The expression of iNOS in brain was detected via western blotting and RT-qPCR, and TJ-5 was found to inhibit the upregulation of iNOS expression caused by CIRI (Fig. 3c and 3e). TUNEL fluorescence staining was used to detect apoptosis. As shown in Fig. 3d, the scope of apoptosis in brain tissue and the apoptotic cells in the ischemic penumbra were significantly reduced with the treatment of TJ-5. And the expression level of cleaved caspase-3 was significantly upregulated in the I/R group, while the treatment of TJ-5 significantly downregulated the activation of caspase-3 (Fig. 3f). These results suggest that TJ-5 reduces the scope of damage by inhibiting the excessive inflammatory response and apoptosis.

3.3. TJ-5 inhibits activation of microglia and infiltration of peripheral myeloid cells in CIRI mice

This study further explored how TJ-5 inhibits the excessive inflammatory response at the level of cellular mechanism. Microglia in brain were labeled with Iba-1 staining and the results indicated that the number of Iba-1 positive cells in the I/R group increased, while that in the I/R + TJ-5 group significantly decreased (Fig. 4a). Flow cytometry results showed that the proportions of CD11b⁺CD45hiLy6G⁺ neutrophils (PMNs), CD11b⁺CD45hiLy6G⁻ mononuclear macrophages (Mo/M Φ) increased and the proportion of CD11b⁺CD45int inactive microglia decreased in CIRI mice. However, TJ-5 reversed this proportional change and inhibited the activation and infiltration of these inflammatory cells (Fig. 4b). Injecting of TJ-5 at the time of reperfusion reduced brain-infiltrating CD11b⁺CD45hi myeloid cells and increased the

proportion of inactive microglia (Fig. 4c and 4d). Thus, we clarify the level of cellular mechanism at which TJ-5 inhibits both infiltration of myeloid cells and activation of microglia to alleviate neuroinflammation.

3.4. TJ-5 inhibits the activation of BV-2 cells after LPS stimulation

To evaluate the anti-inflammatory effect of TJ-5 in vitro, neuroinflammation was induced by LPS in BV-2 cells. The CCK8 experiment confirmed that TJ-5 did not affect the viability of BV2 cells below a concentration of 20 μ M (Fig. 5a). Observation of cell morphology under the microscope after 24 h of LPS stimulation showed that after LPS stimulation, the activation of BV-2 cells became amoebic-like and had shown more protrusions, while the cells in the control group were spherical with a small number of protrusions and the activation of BV2 cells were inhibited with TJ-5 intervention (Fig. 5b). The expression of iNOS in LPS-stimulated BV-2 cells was reduced with TJ-5 intervention (Fig. 5c). The levels of TNF- α and IL-6 in the supernatant were detected by ELISA, and treatment with TJ-5 markedly reduced the levels of these pro-inflammatory cytokines in LPS-stimulated BV-2 (Fig. 5d). These results demonstrate that TJ-5 inhibits the activation of BV-2 cells and attenuates the inflammatory reaction induced by LPS.

3.5. TJ-5 down-regulates the TLR4/MyD88/NF- κ B pathway signaling in CIRI mice and LPS-induced BV-2 cells

TLR4/MyD88/NF- κ B signaling pathway played a key role in neuroinflammation induced by CIRI, we investigated this pathway in CIRI mice and LPS-induced BV-2 cells with the treatment of TJ-5. In vivo, the results showed that TJ-5 down-regulated the expression levels of TLR4 and MyD88 and inhibited the phosphorylation of ERK (Fig. 6a-f). Nuclear translocation of NF- κ B p65 activated the excessive expression of pro-inflammation factors, and the results indicated that nuclear translocation of NF- κ B p65 was obstructed with TJ-5 treatment in CIRI mice (Fig. 6g-i). In vitro, under the stimulation of LPS, the expressions of TLR4 and MyD88, the phosphorylation level of the MAPK pathways and the nuclear translocation of NF- κ B p65 increased, but these signals were down-regulated with TJ-5 treatment (Fig. 6j). Therefore, in molecular mechanism, TJ-5 inhibits the TLR/MyD88 signaling pathway to reduce neuroinflammation injury.

3.6. The blood-brain barrier permeability and pharmacokinetics of TJ-5

To investigate whether TJ-5 could directly inhibit microglia activation across the blood-brain barrier, the concentration of TJ-5 in brain was measured and compared with those in serum, liver, and heart. The concentrations of TJ-5 were summarized in Table 1 and the calculated PK parameters were summarized in Table 2. The time-concentration curves of TJ-5 in different tissues indicated that TJ-5 was eliminated

according to first-order kinetics. Surprisingly, there was no significant difference between the injury hemisphere and normal hemisphere of brain in BBB permeability of TJ-5. Besides, the drug was quickly distributed from blood to organ after intravenous injection (Fig. 7a and 7b). The concentration ratio of brain to serum at each time point was displayed (Fig. 7c). Thus, we found that TJ-5 may freely pass through the BBB and directly inhibit microglial activation in CIRI mice.

4. Discussion

This study is the first to prove that TJ-5 can alleviate cerebral ischemia-reperfusion injury and inhibit the excessive immune-inflammatory reaction in CIRI mice. In addition, current data and pharmacologic evidence demonstrate that TJ-5, a small molecule Myd88 inhibitor, can pass through the BBB and directly inhibit the activation of microglia in CIRI mice. These results may indicate that TJ-5 has the potential for treating ischemic stroke and brain diseases caused by neuroinflammation in clinical settings, opening a novel field for the clinical treatment of CIRI.

In recent decades, ischemic stroke has become one of the most common causes of disability and death worldwide [27]. Recanalization as soon as possible is the primary treatment after ischemic stroke, but the ensuing reperfusion injury will aggravate the brain injury and expand the infarct size. Unfortunately, there is a lack of effective treatment use in clinical settings currently [2]. At present, there has been a breakthrough in many aspects of the research on the mechanism of CIRI, involving multiple complex pathological processes such as oxidative stress, amino acid toxicity, the release of endogenous substances, inflammation and apoptosis [28]. The interaction between these factors forms a complex regulatory network, which leads to a series of pathological cascade reactions [29]. Although the pathogenesis of CIRI is multifactorial, many experimental studies suggest that the inflammatory response of immune system is essential to the occurrence and progression of CIRI [30]. Although the relationship is still unclear, recent studies continue to elucidate the role of immune-inflammatory response in CIRI. TLR4 has been found to be involved in the development of neuroinflammation in CIRI, which initiates a cascade of inflammatory responses through the TLR4/MyD88/NF- κ B signaling pathway [31–33]. Furthermore, MyD88 is the core transduction protein in the toll-like receptor signaling pathway [34], and the immune-inflammatory response induced by CIRI in the MyD88 knockout mice is alleviated [35]. Considering that Myd88 activation may enhance neuroinflammation caused by ischemic stroke, we hypothesized that TJ-5 can potentially inhibit neuroinflammation to protect against CIRI (Fig. 8).

BBB is the interface that controls the exchange of substances between the central nervous system (CNS) and blood, which brings difficulty in developing drugs that can cross such barrier [36]. Currently, not many drugs can enter the CNS efficiently through the BBB, which limits the development of therapies for brain diseases [37,38]. Microglia are resident macrophages in the brain. Studies have shown that the activation of microglia in CNS is an important factor that contributes to the occurrence and development of CIRI [39–41]. In CIRI mice, activation of endogenous microglia and infiltration of exogenous immune cells promote the cascade of inflammation in the focal brain and enlarge the scope of injury [42]. Inhibiting the activation of microglia and its transformation to M1 type is the main focus in the research of drugs for

treating ischemic stroke [23,40]. However, many studies lack evidence for the BBB permeability of drugs, which is an essential factor as it determines whether drugs can directly affect microglia in the brain. Therefore, the inhibitory effect of TJ-5 on microglia activation and its BBB permeability is examined in the present study.

In this research, we investigated the protective effects of edaravone and different concentrations of TJ-5 in CIRI mice and found that TJ-5 at 15mg/kg was the most effective as the infarction volume was reduced by approximately 80%, which achieved better neuroprotection effects compared with that of edaravone, a medicine currently in use clinically for treating ischemic stroke. This indicates that TJ-5 has significant clinical application value for treating CIRI. The findings also prove that TJ-5 reduces the infiltration ratio of peripheral myeloid cells in the cerebral infarction area, increases the proportion of inactive microglia, and decreases the expression levels of iNOS, TNF- α , IL-1 β and IL-6 in infarction areas after 24 h of reperfusion, which suggests that TJ-5 could interrupt the inflammatory cascade to inhibit excessive inflammation. Furthermore, we found that TJ-5 was able to cross the BBB and the activation of LPS-induced BV-2 cells were effectively inhibited with the intervention of TJ-5, demonstrating that TJ-5 can directly inhibit the activation of microglia in brain to alleviate neuroinflammation. In addition, the results showed that TJ-5 inhibited the TLR4/MyD88/NF- κ B signaling pathway in brain of CIRI mice and BV-2 cells, which were consistent with previous studies that focused on peripheral innate immune cells [19,43].

This study affirms the potency of TJ-5 in treating CIRI as it demonstrates a better neuroprotective effect in the early stage of cerebral I/R and the ability to cross the blood-brain barrier, as compared with other treatments. Moreover, we verify that TJ-5 not only can act on peripheral innate immune cells but also directly on brain tissue cells, which may be an influential factor contributing to its exceptional anti-inflammatory and neuroprotective effects. However, this study is only limited to microglia alone, so further complementary studies are needed to demonstrate the effects of TJ-5 on the cell interaction and molecular mechanism of CIRI. The transformation mechanism from the acute inflammation induced by CIRI to the chronic inflammation in the entity of the brain and the mechanisms of the adaptive immune system in CIRI both need to be further explored [44]. On the other hand, the inflammatory response in CIRI is a "double-edged sword". The excessive inflammatory response causes the injury to expand, but the inflammatory response also promotes the immune cells to devour the necrotic tissue, which can promote tissue repair [45]. MyD88 inhibitor regulates the immune response in CIRI from its origin and has efficacy in the acute phase, but the efficacy in the chronic phase still requires further investigation. Taken together, for aseptic inflammatory reactions like CIRI, it can be concluded that the key to the treatment of CIRI is to balance the regulation of the immune system and minimize neuron loss.

5. Conclusion

In summary, we confirm that the self-developed MyD88 inhibitor TJ-5 presented in this study has an impressive therapeutic effect on CIRI after acute ischemic stroke and the possible mechanisms behind its potency are that TJ-5 reduces neuroinflammation by inhibiting TLR4/MyD88/NF- κ B signaling pathway

and crosses the BBB to directly inhibit the activation of microglia, providing new insight for the potential of TJ-5 treating acute ischemic stroke and other neuroinflammatory diseases in clinical settings.

Abbreviations

CIRI: Cerebral ischemia-reperfusion injury; MCAO: Middle cerebral artery occlusion; LPS: Lipopolysaccharide; NeuN: Neuron-specific nuclear protein; BBB: Blood-brain barrier; Myd88: Myeloid differentiation factor 88; TLRs: Toll-like receptors; DAMPs: Danger-related molecular patterns; PAMPs: Pathogen-related molecular patterns; I/R: ischemia-reperfusion; DCs: Dendritic cells; TJ-5: TJ-M2010-5; ECA: External carotid artery; ICA: Internal carotid artery; MCA: middle cerebral artery; TTC: Triphenyltetrazolium chloride; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; AUC: Area under the curve; PK: Pharmacokinetic; HMGB1: High mobility group box1; HSPs: Heat shock protein; HA: Hyaluronic acid.

Declarations

Ethics approval and consent to participate

All animal experiments and protocols have been reviewed and approved by the Institutional Animal Care and Use Committee at the Tongji Hospital, Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

PZ and FCJ have the patents of TJ-M2010 series. ZYL designed and performed the experiments, analyzed the results, and wrote the manuscript. YRL and XQZ helped with the experiments in vivo and manuscript editing. MHZ, LMZ, YY and YLX assisted with the experiments in vitro. ZMZ, LZ and RSS helped with data acquisition and analysis. DFD helped with the study design. All authors approved the final manuscript.

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Tables

Table 1. Distribution of TJ-5 in tissues during the different time points (ng/ml or ng/g, mean ± SD)

Sample	5min	10min	30min	1h	2h	6h
Serum	1187.80±2	979.85±2	471.74±1	284.90±2	79.60±2	2.08±1.
	08.63	02.81	51.14	9.10	6.93	12
IL brain	6646.07±5	5798.80±	2893.70±	1747.40±	327.29±	11.65±6
	90.77	29.80	611.00	163.95	57.19	.43
CL brain	5877.53±9	5364.30±	2480.87±	1662.40±	347.65±	10.43±5
	89.32	749.83	985.41	117.03	55.25	.69
Heart	5914.10±8	4591.93±	4729.27±	1655.33±	684.13±	51.60±1
	24.68	351.85	2061.45	335.19	198.89	1.87
Liver	7162.10±5	5890.47±	4713.00±	2961.40±	750.37±	169.90±
	93.97	528.302	1130.68	1031.41	249.40	36.06

Table 2. PK parameters of TJ-5 in tissues of mice. ^aThe tissue density was assumed to be 1 g/ml. K_p: Brain-serum ratio was calculated by the mean of AUC_{0-6h} ratios. t_{1/2}: elimination half-life.

Sample	Pharmacokinetic parameters	
Serum	AUC _{0-6h} (ng/ml*h)	919.3±82.24
	t _{1/2} (h)	0.38
IL brain	AUC _{0-6h} (ng/ml*h) ^a	5136±239.3
	t _{1/2} (h)	0.41
	K _p	5.58
CL brain	AUC _{0-6h} (ng/ml*h) ^a	4792±352.8
	t _{1/2} (h)	0.41
	K _p	5.21
Heart	AUC _{0-6h} (ng/ml*h) ^a	6485±768.9
	t _{1/2} (h)	0.67
Liver	AUC _{0-6h} (ng/ml*h) ^a	8238±852.1
	t _{1/2} (h)	0.70

Figures

Formula: $C_{23}H_{26}ON_4S$

Exact Mass: 406.18

Mol. Wt.: 406.55

m/z: 406.18(100.0%); 407.34(31.4%); 408.27(5.5%)

Figure 1

The molecular structure of MyD88 inhibitor TJ-M2010-5, named as 3-(4-benzylpiperazin-1-yl)-N-(4-phenyl-1,3-thiazol-2-yl)propanamide.

Figure 2

Neuroprotective effect of TJ-5 treatment in CIRC model. **a** Effects of TJ-5 on neurological deficit score ($n = 6/\text{group}$; $*P < 0.05$, $**P < 0.01$ vs I/R group). **b c** Representative TTC-stained slices at 24 h after reperfusion and statistical analyses of infarct volume ($n = 6/\text{group}$; $**P < 0.01$, $****P < 0.0001$ vs I/R group; $##P < 0.01$ vs I/R + TJ-5 15 mg/kg group). **d** Representative NeuN immunofluorescence staining

image (bar = 50 μ m). **e** NeuN protein levels in brain were analyzed. Values are mean $\pm$ SEM, **P < 0.01, ***P < 0.001.

Figure 3

Effect of TJ-5 treatment on the inflammatory response and apoptosis in CIRI model. **a** TNF- α , IL-6 and IL-1 β mRNA levels were downregulated with TJ-5 treatment in CIRI mice. **b** The content of TNF- α , IL-6 and IL-

1 β were reduced with TJ-5 treatment in CIRI mice. **c** The mRNA level of iNOS in brain was detected. **d** Representative TUNEL immunofluorescence staining image of brain slices (bar = 500 μ m). **e** TJ-5 downregulated the expression of iNOS in CIRI mice. **f** The expression of cleaved caspase-3 was analyzed. Values are mean $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 4

Effect of TJ-5 treatment on microglia and peripheral infiltrating myeloid cells in CIRI model. **a** Representative Iba-1 immunofluorescence staining image (bar = 50 μ m). **b** Representative plots of CD11b+CD45+, CD11b+CD45+Ly6G+ myeloid cells and CD11b+CD45int microglia in ischemic hemisphere. **c** Percentage of brain-infiltrating CD11b+CD45hi myeloid cells was decreased in TJ-5-treated mice at 24 h after reperfusion. **d** Percentages of PMNs, Mo/M Φ and microglia in CD11b+CD45+ cells were statistically analyzed.

Values are mean $\pm$ SEM, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5

Effect of TJ-5 intervention on BV-2 cells induced by LPS. **a** Cell viability of BV-2 cells at 24 h after the intervention of TJ-5. **b** The morphology of BV-2 cells was observed under the microscope. TJ-5 inhibited the activation of LPS-stimulated BV-2 cells (original magnification $\times 200$). **c** TJ-5 downregulated the expression of iNOS in LPS-stimulated BV-2 cells. **d** The secretions of TNF- α and IL-6 in LPS-stimulated BV-2 cells were inhibited with TJ-5 intervention. Values are mean $\pm$ SEM, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6

TJ-5 downregulates the TLR/MyD88/NF-κB signaling pathway in vivo and in vitro. **a b** The mRNA levels of TLR4 and Myd88 in brain were detected. **c-f** TLR4, Myd88 and p-ERK protein levels in brain were assessed and analyzed. **g-i** Cytoplasmic protein and nuclear protein were extracted from brain to detect the NF-κB p65 protein levels and analyze NF-κB p65 nuclear translocation. **j** TLR4, Myd88, p-p38, p-JNK and p-ERK

protein levels in BV-2 cells were analyzed and NF-κB p65 protein levels in cytoplasmic and nuclear of BV-2 cells were also detected. Values are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7

Pharmacokinetic profile of TJ-5. The concentrations of TJ-5 were analyzed in serum, IL brain, CL brain, liver and heart at specified time points after intravenous injection of TJ-5 at 15 mg/kg. **a** The concentration-time profiles of TJ-5 in serum, IL brain and CL brain. TJ-5 was quickly distributed into the brain after intravenous injection. **b** The concentration-time profiles of TJ-5 with expressed in logarithmic ordinate. Elimination of TJ-5 followed the first-order elimination kinetics. **c** Concentration ratios of IL brain or CL brain to serum at 5 min, 10min, 30 min, 1 h, 2 h and 6 h.

Figure 8

Schematic model of the present study. The activation of microglia and infiltration of myeloid cells promote the neuroinflammation and brain tissue injury after acute ischemic stroke. TJ-5 obstructs the cascade of inflammatory responses by inhibiting TLR/MyD88/NF-κB signaling pathway and protects against acute cerebral ischemia-reperfusion injury.