Antioxidants and Redox Signaling
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Abbreviated title **：** A Sirt3 agonist cerebral ischemia injury

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Abstract

Aims: Neuroinflammation and oxidative stress are deemed the prime causes of brain injury after cerebral ischemia-reperfusion (I/R). Since Sirt3 pathway plays an imperative role in protecting against neuroinflammation and oxidative stress and has been verified as a target to treat ischemia stroke. Therefore, we attempted to seek novel Sirt3 agonist and explore its underlying mechanism for stroke treatment both in *vivo* and in *vitro*.

Results: Trilobatin (TLB) not only dramatically suppressed neuroinflammation and oxidative stress injury after middle cerebral artery occlusion (MCAO) in rats, but also effectively mitigated oxygen and glucose deprivation/reoxygenation (OGD/R) injury in primary cultured astrocytes. These beneficial effects along with the reduced pro-inflammatory cytokines *via* suppressing TLR4 signaling pathway, as well as lessened oxidative injury *via* activating Nrf2 signaling pathways, in keeping with the findings in *vivo*. Intriguingly, the TLB-mediated neuroprotection on cerebral I/R injury was modulated by reciprocity between TLR4-mediated neuroinflammatory responses and Nrf2 antioxidant responses as evidenced by molecular docking and silencing TLR4 and Nrf2, respectively. Most importantly, TLB not only directly bond to Sirt3, but also increased Sirt3 expression and activity, indicating that Sirt3 might be a promising therapeutic target of TLB.

Innovation: TLB is a naturally-occurring Sirt3 agonist with potent neuroprotective effects *via* regulation of TLR4/NF-κB and Nrf2/Keap-1 signaling pathways both in *vivo* and in *vitro*,.

Conclusion: Our findings indicate that TLB protects against cerebral I/R-induced neuroinflammation and oxidative injury through the regulation of neuroinflammatory and oxidative responses *via* TLR4, Nrf2 and Sirt3, suggesting that TLB might be a promising Sirt3 agonist against ischemic stroke.

Keywords: trilobatin; cerebral ischemia; reperfusion; TLR4; Nrf2; Sirt3

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Introduction

Ischemic stroke is a major cause of death and disability worldwide. The outcomes of ischemic stroke damage are far-reaching, generating immense burden to both the family and society(5). To date, ideal drugs or strategies for ischemic stroke are still unavailable. Present strategies for treatment of stroke include recanalization by means of pharmacologic or mechanical thrombolysis and neuroprotective agents(2). So far, recombinant tissue plasminogen activator (rtPA), known as a thrombolytic agent, is the only FDA-approved medical therapy for ischemic stroke(40). However, clinical application of rtPA is limited because of its rigid narrow therapeutic time window, and especially latent ischemia/reperfusion (I/R) injury, which plays a crucial role in aggravating succeeding ischemic brain lesion(35,42). Therefore, it is of significance to elucidate the mechanisms of cerebral I/R injury and develop more effective strategies or agents to treat cerebral I/R injury.

Emerging evidence suggests that cerebral I/R injury causes a sophisticated cascade of pathophysiologic events, especially neuroinflammation and oxidative stress, which ultimately lead to neuronal injury, even demise(12,17). Toll-like receptors (TLRs) are known as a trans-membrane pattern-recognition receptor family with crucial roles in the mediation of inflammatory responses(49). Up to now, thirteen TLRs have been identified in mammals, of which TLR4 is expressed on the cell surface, and is identified as the most important pattern recognition receptor involved in cerebral I/R injury. TLR4 is activated in response to cerebral I/R injury, and subsequently directly promote its pivotal adapter protein myeloid differentiation factor 88 (MyD88) recruitment, then results to the activation of downstream nuclear factor-kappa B (NF-κB) and thereby releases proinflammatory cytokines to aggravate cerebral I/R injury in turn(18,20). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcriptional factor involved in anti-oxidative stress insults(37). Under quiescent condition, Nrf2 localizes to the cytoplasm and is restrained by Kelch-like ECH-associated protein 1 (Keap-1)(27). When cells were subjected to oxidative stress or any other deleterious insults such as cerebral I/R injury, Nrf2 liberates from Keap1 and translocates into the nucleus and then binds to antioxidant response element (ARE), including hemeoxygenase-1(HO1), NAD(P)H-quinone

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oxidoreductase 1 (NQO1) and glutathione peroxidase (GSH-Px)(1,33). Intriguingly, accumulating evidence indicates that the Nrf2 signaling pathway is activated during inflammation, which induces the downstream genes of Nrf2 to restrain the inflammatory response, and the Nrf2 signaling pathway cross talks with TLR4 and its downstream genes (e.g NF-κB)(41); However, whether reciprocity between TLR4 and Nrf2 is involved in the cerebral I/R injury remains still a mystery.

Trilobatin (TLB),a major active constituent of *Lithocarpus polystachyus* Rehd., is a folk medicine which is used to prophylaxis and treatment of multiple diseases in China with a long history(46). Of note, previous report demonstrated that TLB exerted attenuation effect on LPS-induced inflammatory response through hindering the NF-κB signaling pathway(6). Moreover, our previous study revealed that TLB also presented apparent potential neuroprotective effect on hydrogen peroxide-induced oxidative injury in a neuron-like PC12 cell *via* regulating Nrf2/silent mating-type information regulation 2 homolog 3 (Sirt3) signaling pathway(8). Whereas, whether TLB can protect against cerebral I/R injury and its underlying mechanisms are associated with reciprocity between the Nrf2 and TLR4 pathways remains still unclear.

Consequently, the focus of this study was designed to investigate whether TLB treatment can elicit neuroprotection against MCAO-induced cerebral I/R injury in rats and oxygen and glucose deprivation/reoxygenation (OGD/R)-induced injury in primary cultured rat astrocytes through mediating TLR4/Nrf2 signaling pathway.

Results

TLB suppressed MCAO-induced injury through decreasing neurologic deficits, infarct volume and cerebral edema

The inhibitory effects of TLB on cerebral I/R-induced outcome 3 d after 2 h MCAO were measured by neurologic deficits, cerebral edema and infarct volume in rats. Firstly, regional cerebral blood flow (rCBF) was reduced to below 20% and recoverd to greater than 80% of baseline, indicating that a successful MCAO model was accepted (Fig. 1A, B). The results showed that TLB ameliorated cerebral I/R-induced neurological deficits (Fig. 1C), cerebral edema (Fig. 1D) and reduced infarct volume (Fig. 1E, F), respectively. These

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findings suggested that TLB dose-dependently inhibited cerebral I/R-induced injury. Furthermore, the time window for TLB treatment after MCAO was explored, due to a appropriate time window for treating cerebral I/R injury was crucial for the therapeutic effects of TLB in the clinic. TLB was administrated at 1, 2, 3, 4 and 6 h after MCAO and neurological deficits, cerebral edema and infarct volume were also measured in rats, respectively. The results showed that TLB (20 mg/kg) significantly reduced the neurologic deficits, cerebral edema and infarct volume after MCAO within 4 h, while, these effects were decreased at 6 h after MCAO (Fig. 2).

TLB restored long-term neurological functions at 28 days after MCAO in rats

To further explore the effect of TLB on long-term (28 days after MCAO) neurological function recovery, an attery of sensorimotor and cognitive tests including neurological scores, rotarod, adhesive tape removal, Y-Maze and NOR tests were performed at 28 days after MCAO in rats. The results showed that the neurological scores of rats treated with TLB were markedly lower than those of rats after MCAO at day 28 (Fig. 3A). TLB obviously improved the performance in rotarod and adhesive tape removal from 28 days after MCAO in rats (Fig. 3B, C). Additionally, TLB conspicuously increased percentage of correct spontaneous alternations in Y maze test (Fig. 3D) and the discrimination index of novel from familiar objects in the NOR test (Fig. 3E). Collectively, these findings indicated that TLB effectively restored long-term neurological functions after MCAO in rats.

Microarray Data Analyses

Differentially expressed genes (DEGs) were determined in the condition of both *P*-value < 0.05 and FC > 1.5. As shown in Fig. 4A, 1014 DEGs were identified in MCAO versus sham groups, while 363 DEGs were identified in TLB versus MCAO groups. Furthermore, 52 out of the 415 DEGs responding to TLB treatment were associated with DEGs elicited by cerebral I/R injury as evidenced by Venn diagram. Moreover, Hierarchical clustering analysis showed that the expression profiles of the 415 DEGs in Sham and TLB groups were significantly different to that of the MCAO group (Fig. 4B). Moreover, the KEGG pathway and enrichment of GO terms for the selected 415 DEGs involved were depicted. We revealed that the top three pathways enriched from KEGG database were positive

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regulation of MAPK cascade, negative regulation of endopeptidase activity and negative regulation of inflammatory response (Fig. 4C). As shown in Fig. 4D-G, top 10 biological process, cellular component and molecular functions for up-regulated or down-regulated DEGs were listed in bubble plots, which showed that beneficial effect such as positive regulation of MAPK cascade, positive regulation of angiogenesis and negative regulation of apoptotic process were included. As present in Fig. 4H, protein-protein interactions in the 415 DEGs identified after TLB treatment were depicted. There were 742 interaction pairs among in these DEGs-encoded proteins, the size of circles represents that the degree of protein connection to others. Notably, TLR4 was involved in 19 interactions, suggesting that TLR4 might be the dominant relevant signaling molecule. Furthermore, we also found that TLR4 interacted with Nrf2 and Nrf2 interacted with Sirt3 using ClusterONE analysis. Of note, to validate the microarray results, the expressions of TLR4, Nrf2 and Sirt3 were determined by qRT-PCR. The results showed that TLB down-regulated TLR4, up-regulated Nrf2 and Sirt3 than those of MCAO group in accordance with the array data (Fig. 4I-K).

TLB reduced MCAO-induced astrocyte and microglial activation

The effect of TLB on glial activation was measured by immunohistochemical (IHC) staining using anti-glial fibrillary acidic protein (GFAP) antibody to mark astrocyte and anti-Iba-1 antibody to mark microglia. The results showed that the number of GFAP-positive cells and Iba-1-positive cells were markedly elevated after MCAO than those of sham group; However, the increased number of activated astrocyte and microglia were attenuated by TLB (Fig. 5).

TLB inhibited inflammatory cytokine and TLR4 signaling pathway after MCAO

Inflammatory cytokine levels and inducible nitric oxide synthase (iNOS), TLR4, MyD88, TRAF6, NF-κBp65 in the brain tissues of rats after MCAO were detected using according inflammatory cytokine ELISA kits and Western blot, respectively. The results indicated that the levels of IL-1β, IL-6, TNF-α and iNOS expression were markedly augmented in MCAO group than those of sham group, as well as expressions of TLR4, MyD88, TRAF6 and phosphorylation level of NF-κBp65. Whereas, TLB reduced IL-1β, IL-6, TNF-α and iNOS

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expression (Fig. 6A-D), as well as expression of TLR4, MyD88, TRAF6 and phosphorylation level of NF-κBp65 (Fig. 6E-I).

TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after MCAO

Reactive oxygen species (ROS) and malondialdehyde (MDA) levels, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were detected using the according kits, and Nrf2, Keap1, HO-1, NQO1 expressions were detected by Western blot. The results indicated that ROS and MDA levels were elevated and SOD and GSH-Px activities were reduced after MCAO; However, these change were suppressed by TLB (Fig. 7A-D). Furthermore, nuclear Nrf2 level was increased and cytosol Nrf2 level was decreased after MCAO than those of sham group; However, TLB significantly upregulated Nrf2 expression of the nucleus and decreased it in the cytoplasm than those of MCAO group (Fig. 7E-G). Notably, TLB not only downregulated the Keap-1 expression and upregulated NQO-1 and HO-1 expressions than those of MCAO group (Fig. 7E, H-J), but also increased Sirt3 expression (Fig. 5K).

Prediction of drug targets of TLB against MCAO-induced injury

Additionally, the results further exhibited the strong binding affinity between TLB and Sirt3 with binding energy of -5.44 kcal/mol. To investigate the activity and structure relationship of TLB, we synthesized an analog of TLB, which was named as Tr1. TLB and Tr1 have a similar structure, except for the glucose of TLB. Tr1 and Sirt3 with binding energy of -4.09 kcal/mol, which indicated that Tr1 failed to bound with Sirt3. Thereafter, the presumptive binding modes and the pocket of amino acid were tested by a molecular docking, including LYS205, SER152, ARG365, PRO355, HIS354, LEU 173 and ASP172, which further confirmed that TLB bound to the hydrophobic pocket of Sirt3, consisting with the results both in *vivo* and in *vitro* (Fig. 8). These findings indicated that TLB might directly bind to Sirt3 to exert its pharmacological activities.

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TLB protected against OGD/R-induced injury in astrocytes via inhibiting inflammatory cytokine and TLR4 signaling pathway

To further explore the role of TLB during cerebral I/R, the OGD/R model in primary cultured rat astrocytes was applied to mimic the cerebral I/R. We first detected the cytotoxicity of TLB and Tr1, an analog of TLB (Fig. 9A) in astrocytes to determine suitable *in vitro* treatment concentrations by a 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TLB or Tr1 exerted no effect on astrocytes below 50 μM within 48 h (Fig. 9B, C). Therefore, 50 μM or less of TLB and Tr1 was used in the following experiments. Next, the cell viability and cytotoxicity were determined using MTT assay and lactate dehydrogenase (LDH) assay, respectively. The results showed that TLB protected against OGD/R-induced astrocyte injury in a concentration-dependent manner, however, 50 μM Tr1 did not improve the cell viability (Fig. 9D). Moreover, TLB also reduced the amount of LDH release in a concentration-dependent manner. However, 50 μM Tr1 did not alter the LDH level (Fig. 9E). Additionally, OGD/R resulted in astrocytes shrink, depletion in cell numbers, even death. Whereas, TLB reversed these change after OGD/R, as evidenced by observation of light converted microscopy. However, 50 μM Tr1 did not alter these change (Fig. 9F). Furthermore, inflammatory cytokine levels and iNOS, TLR4, MyD88, TRAF6, NF-κBp65 in the astrocytes after OGD/R were detected using according inflammatory cytokine ELISA kits and Western blot, respectively. The results demonstrated that the levels of IL-1β, IL-6, TNF-α and iNOS expressions were markedly enhanced in OGD/R group than those of control group, as well as expressions of TLR4, MyD88, TRAF6 and phosphorylation level of NF-κBp65. Whereas, TLB mitigated IL-1β, IL-6, TNF-α and iNOS expression (Fig. 9G-J), as well as expressions of TLR4, MyD88, TRAF6 and phosphorylation level of NF-κBp65 (Fig. 9K-M).

TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after OGD/R insult in astrocytes

MDA level, SOD, GSH-Px activities, intracellular ROS and mitochondrial superoxide anions ($O_2^{\bullet-}$) generation were detected using according kits and mito-SOX staining, respectively, and NRF2, Keap1, HO-1, NQO1 expressions were evaluated by Western blot.

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The results indicated that the red fluorescence was enhanced after OGD/R than that of control group, which suggested that OGD/R accelerated $O_2^{\bullet-}$ generation in mitochondria. Moreover, the generation of intracellular ROS and MDA were also elevated and SOD and GSH-Px activities were reduced after OGD/R; However, these change were restrained by TLB (Fig. 10A-E). Furthermore, nuclear Nrf2 level was increased and cytosol Nrf2 level was decreased after OGD/R than that of control group; However, TLB upregulated Nrf2 expression of the nucleus and downregulated it of the cytoplasm (Fig. 10F-H). Notably, TLB not only downregulated the Keap-1 expression and upregulated NQO-1 and HO-1 expressions than those of OGD/R group (Fig. 10I-K), but also increased Sirt3 expression and its activity (Fig. 10L, M). Whereas, 50 μM Tr1 did not alter Sirt3 expression and its activity (Fig. 11A-C).

TLB protected against OGD/R-induced injury in primary cortical neurons

The effect of TLB on OGD/R-induced injury in primary cortical neurons was also investigated. TLB or Tr1, an analog of TLB, exerted no effect on neurons below 25 μM within 24 h (Fig. 12A, B). Therefore, 25 μM or less of TLB and Tr1 was used in the following experiments. Next, the cell viability and cytotoxicity were determined using MTT assay and lactate dehydrogenase (LDH) assay, respectively. The results showed that TLB concentration-dependently protected against OGD/R-induced neuronal injury, however, 25 μM Tr1 did not improve the cell viability (Fig.12C). Moreover, TLB also reduced the amount of LDH release in a concentration-dependent manner. However, 25 μM Tr1 did not alter the LDH level (Fig. 12D). In addition, OGD/R lead to neurons shrink, reduction in cell numbers, even death. Whereas, TLB reversed these change after OGD/R, as evidenced by observation of light converted microscopy. However, 25 μM Tr1 did not alter these change (Fig. 12E).

TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after OGD/R insult in primary cortical neurons

MDA level, SOD, GSH-Px activities, intracellular ROS and mitochondrial superoxide anions ($O_2^{\bullet-}$) generation were determined using according kits and mito-SOX staining, respectively, and NRF2, Keap1, HO-1, NQO1 expressions were detected by Western blot.

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The red fluorescence was augmented after OGD/R than that of control, which suggested that OGD/R accelerated $O_2^{\bullet-}$ accumulation in mitochondria. Moreover, the generation of intracellular ROS and MDA were also increased and SOD and GSH-Px activities were decreased after OGD/R; However, these change were reversed by TLB (Fig. 13A-E). Furthermore, nuclear Nrf2 level was increased and cytosol Nrf2 level was decreased after OGD/R than that of control group; However, TLB upregulated Nrf2 expression of the nucleus and downregulated it of the cytoplasm (Fig. 13F, G). Notably, TLB not only downregulated the Keap-1 expression and upregulated NQO-1 and HO-1 expressions than those of OGD/R group (Fig. 13F, H), but also increased Sirt3 expression and its activity (Fig. 13I, J). Whereas, 25 μM Tr1 did not alter Sirt3 expression and its activity (Fig. 11D-F).

The effect of TLB in Sirt3-knockout (Sirt3-KO) rats and Sirt3-KO astrocytes or neurons

Sirt3-KO rats and Sirt3-KO astrocytes or neurons were generated using the CRISPR/Cas9 system resulted in > 80% loss in Sirt3 mRNA levels relative to sham group (Fig. S1A) or control cells (Fig. S1B). To further confirm that TLB works *via* binding to and activating Sirt3, we conditionally deleted Sirt3 both in rats and in neurons and astrocytes using CRISPR-Cas9 Sirt3 lentivirus. The results showed that Sirt3 KO rats showed more severe injury after MCAO insulted than that of WT rats, which was keeping with the previous report(39). Whereas, TLB treatment partly lost its ability to reduction of MCAO-induced injury in Sirt3-KO rats than those of wild type (WT) rats (Fig. 14A-C). Interestingly, consistent with the results in *vivo*, Sirt3-KO neurons or astrocytes also showed more severe injury after OGD/R insult than that of WT neurons or astrocytes. However, the protective effects of TLB on OGD/R-induced injury were partially occluded in Sirt3-KO astrocytes (Fig. 14D, E), and Sirt3-KO neurons (Fig. 14F, G) than those of WT astrocytes or neurons. These findings highlighted that TLB-mediated protection is, at least partly, dependent on the the presence of Sirt3, and Sirt3 might be a potential target of TLB, which was consistent with the findings of molecular docking mentioned above.

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Reciprocity between TLR4 and Nrf2 was involved in the beneficial effects of TLB on OGD/R-induced injury

Furthermore, siRNA was applied to verify whether TLB regulated TLR4/Nrf2 signaling pathway to attenuate OGD/R-induced injury in astrocytes. Firstly, the levels of TLR4 and Nrf2 in TLR4 and Nrf2 siRNA treated group dramastically decreased than those of scrambled siRNA transfected group (Fig. S2). Without any OGD/R stimulus, silencing of TLR4 or Nrf2 exerted no effect on cell viability and cytotoxicity. Whereas, upon OGD/R stimulus, the cell viability and cytotoxicity of TLR4 siRNA transfected cells were significantly increased and decreased, respectively, than those of scrambled siRNA transfected cells; However, the beneficial effect of TLB was markedly promoted by the TLR4 siRNA. Meanwhile, the cell viability and cytotoxicity of Nrf2 siRNA transfected cells were significantly reduced and augmented, respectively, than those of scrambled siRNA transfected cells; However, the beneficial effect of TLB was significantly abolished by the Nrf2 siRNA (Fig. S3). Next, the effect of TLB on inflammatory cytokine in primary rat astrocytes were also further examined by measuring the levels of IL-1β, IL-6, TNF-α and iNOS in the serum of rats with siTLR4 and siNrf2 by ELISA. The results indicated that the inhibitory effects of TLB in inflammatory cytokines were enhanced by TLR4 siRNA and partially abolished by Nrf2 siRNA (Fig. S4A-D). Moreover, the inhibitory effects of TLB in ROS and MDA levels were increased by TLR4 siRNA and almost abolished by Nrf2 siRNA; while, the elevation effects of TLB in antioxidant enzymes including SOD and GSH-Px were elevated by TLR4 siRNA and almost abolished by Nrf2 siRNA, respectively (Fig. S4E-H).

Of note, the results further demonstrated that knockdown of Nrf2 enhanced TLR4 expression in response to OGD/R, while, the attenuative effects of TLB on OGD/R-induced TLR4 increase were almost abolished by Nrf2 siRNA (Fig. 15A, B). Moreover, knockdown of TLR4 significantly increased Nrf2 expression in the nucleus and decreased it in the cytoplasm after OGD/R, while, the promotion effects of TLB were also elevated by TLR4 siRNA (Fig. 15C, D). Furthermore, the docking of Nrf2 and TLR4 was carried out by ZDOCK and RDOCK method as previous study. The results showed that there were strong interactions between Nrf2 and TLR4 as evidenced by the output values of score of E_RDOCK (Tab.1). Of note, the results further demonstrated that hydrogen bond and

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charge interactions were generated through the amino acid residues including HIS3, ASN409, HIS5, PHE263, PHE429, GLN430, SER9 and HIS7; and Pi interactions with the residues such as TRP332 and PHE330, TRP332 and PHE313, TRP332 and PHE330, TRP332 and PHE313, PHE408 and HIS431 (Fig. 15E-G). Notably, we further verified the direct interaction between Nrf2 and TLR4 by surface plasmon resonance (SPR). The results demonstrated that Nrf2 directly bound to TLR4 in a concentration-dependent manner with a KD value of $2.139e^{-9}$ M (Fig. 15H). These findings suggested that there existed potent affinity between Nrf2 with TLR4.

Discussion

The present study, for the first time, discovered that: (1) TLB, a naturally-occurring Sirt3 agonist, derived from herbal *Lithocarpus polystachyus* Rehd. exerted neuroprotection against MCAO-induced injury in rats and OGD/R-induced injury in primary cultured astrocytes or neurons *in vitro*; (2) The inhibitory effects of TLB were due to inhibition of neuroinflammation through suppressing TLR4 signaling pathway, as well as reduction of oxidative stress injury *via* activating Nrf2 signaling pathway; (3) Reciprocity between TLR4 and Nrf2 was involved in the neuroprotection of TLB against cerebral I/R injury (Fig.16).

On account of lacking effective prophylaxis and treatment strategies for ischemic stroke, a mass of pharmacological neuroprotectant have been researched and developed, unfortunately, though with limitation of clinical success or failure of preclinical due to a complicated pathological process with manifold mechanisms during cerebral I/R injury(19,26). Thus, exploring effective neuroprotectant is an extreme clinical demand. In the current study, we explored the neuroprotection of TLB, a natural small molecule monomer, against cerebral I/R injury and endeavored to elucidate its underlying mechanisms. Firstly, our findings demonstrated that TLB effectively protected against outcomes of cerebral I/R injury as evidenced by reduced MCAO-induced neurological deficits, cerebral edema and infarction volume and in *vivo*. Next, of note, TLR4, Nrf2 and Sirt3 were identified as DEGs as proved by Microarray Data Analyses (Venn diagram, Hierarchical clustering analysis, GeneMANIA analysis, GO terms) and TLB also reduced TLR4 mRNA level, increased Nrf2 and Sirt3 mRNA levels in line with the array data as

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affirmed by qRT-PCR. Therefore, it is reasonable to speculate TLR4-mediating neuroinflammation and Nrf2/Sirt3-mediating oxidative stress were involved in TLB-induced neuroprotection against cerebral I/R injury. As noted, neuroinflammation is a crucial step and a secondary damage mechanism in the cerebral I/R injury(34). When neuronal cell are challenged by cerebral I/R, neurogliocyte (astrocyte and microglia) are activated and release pro-inflammatory factors including IL-1β, IL-6, TNF-α, and iNOS thereby exacerbating brain injury(28). As we expected, MCAO-induced activation of astrocyte and microglia, and also promoted release of inflammatory cytokine (IL-1β, IL-6, TNF-α, and iNOS), which are the premier triggers of activate astrocytes in cerebral I/R injury; whereas, TLB reversed these change after MCAO, suggesting that TLB-mediated neuroprotection against cerebral I/R injury, at least partially, through inactivation of neurogliocyte and inhibition of pro-inflammatory factors release. Furthermore, due to TLR4 is expressed by neurogliocyte and contributes to cerebral I/R-induced inflammatory injuries(13), the role of TLR4 signaling pathway during the beneficial effects of TLB on cerebral I/R injury were determined. The results revealed that the expressions of TLR4, MyD88 and TRAF6 significantly increased after MCAO, which suggested that TLR4-regulated signaling pathway was activated after cerebral I/R and heightened inflammatory responses and further aggravated brain injury, in keeping with previous study; However, TLB inhibited MCAO-elicited these change. Moreover, TLR4 signaling was stimulated by cerebral I/R injury results in activating the transcription factors NF-κB which are involved in activation of pro-inflammatory genes and cytokines(30). Our findings also demonstrated that phosphorylation level of NF-κBp65 was up-regulated by MCAO, while, TLB obviously repressed phosphorylation level of NF-κBp65 after MCAO. These findings suggested that the attenuative neuroinflammation effects of TLB on cerebral I/R injury was mainly due to inactivation of TLR4/MyD88/TRAF6/NF-κB pathway.

Additionally, mounting evidence suggests that oxidative stress, termed as an imbalance between the antioxidase system and the generation of reactive oxygen species (ROS), is known as an early event during cerebral I/R injury for that the brain is very suggestible to oxidative stress(22). Moreover, accumulation of ROS is primary in activating TLR4-regulated signaling pathways and induced pro-inflammatory factors release in

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cerebral I/R injury(23). Our results manifested that MCAO-induced increase in ROS generation, level of MDA, which is derived from lipid peroxidation and considered as a biomarker of oxidative stress, consistent with theory that cerebral I/R injury can attract a remarkable amount of MDA generation in the ischemic brain hemisphere(25); also, MCAO-induced decrease in SOD and GSH-Px activities, which were the anti-oxidant enzymes to maintain redox homeostasis and affect the inflammatory response(9). Conversely, TLB reversed these change by MCAO insulted, inferring that TLB-mediated neuroprotection against cerebral I/R injury, partially, was *via* suppressing oxidative lesions. Thereafter, our results further clarified that MCAO promoted Nrf2 translocate from cytoplasm into nucleus; however, TLB significantly facilitated nuclear translocation of Nrf2. Whereas, the change of Nrf2 protein expression after MCAO was not consistent with the the results of the mRNA level of Nrf2, which did not change. The reason might be due to that there is a more intrinsic and complex dependence between mRNA and protein, such as translation and transcription of gene expression existed time-space span, and there would be post-transcriptional processing, degradation of transcriptional products, translation, post-translational processing and modification after transcription. Furthermore, MCAO also decreased ARE including HO-1 and NQO1 expressions, while, TLB increased HO-1 and NQO1 expressions after MCAO, indicating that TLB prompted Nrf2 dissociated from Keap1 and then translocated into the nucleus, thereby activating ARE to protected against oxidative stress in cerebral I/R injury. Thus, we believed herein, TLB alleviated neuroinflammatory responses and oxidative stress during cerebral I/R injury. Nevertheless, what is the potential target of TLB and whether reciprocity between TLR4 and Nrf2 was involved in the TLB-mediated neuroprotection against on cerebral I/R injury were still unclear. Therefore, we further explore the uncovered problem in neurons and astrocytes in *vitro*. Notably, astrocytes are the most plentiful non-neuronal cell type in the central nervous system and have been indicated to have intensive resistance to cerebral I/R injury compared to neurons(10,45); while, susceptibility of neurons to cerebral I/R will be augmented once astrocyte dysfunction(14). Most importantly, astrocytes were not only expressed TLR4, but also enriched transcription factor Nrf2(21). Moreover, since in ischemic stroke, a dramatically decrease in regional cerebral blood flow elicits deprivation of oxygen and glucose and eventually leads to cerebral injury. Thus, we used OGD/R-

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induced astrocyte and neuron injury to mimic cerebral I/R injury in *vitro* to further investigate the mechanism of TLB-mediated neuroprotection. The results showed that TLB effectively increased cell viability and decreased cytotoxicity after OGD/R insult both in neurons and astrocytes, respectively. Furthermore, TLB also restrained pro-inflammatory factors release as well as inactivated TLR4/MyD88/TRAF6/NF-κB pathway after OGD/R insult in astrocytes. Meanwhile, some oxygen free radicals and their derivatives are accumulated after cerebral I/R injury, including $O_2^{\bullet -}$, which generated in mitochondria. The results indicated that both intracellular and mitochondrial ROS generation exhibited significant decrease by TLB after OGD/R, as well as activated Nrf2 signaling pathway accompanied with elevated ARE genes and enzymes both in neurons and astrocytes. Our findings further verified that TLB effectively attenuated cerebral I/R injury through inactivation of TLR4-mediated pathway and activation of Nrf2-mediatied pathway to suppress neuroinflammation and oxidative stress, in keeping with the findings in *vivo*.

Although multiple studies demonstrated that TLR4 signaling pathway and Nrf2 signaling are all activated during neuroinflammation(11), little is known about whether reciprocity between TLR4 and Nrf2 was involved in cerebral I/R injury. Interestingly, our results revealed that knockdown of TLR4 with siRNA evidently partly strengthened the protective roles of TLB in promoting astrocyte survival, decreasing proinflammatory cytokines production and ROS generation as well as facilitating Nrf 2 nuclear translocation in astrocytes after OGD/R insult. Moreover, knockdown of Nrf 2 with siRNA apparently partly abolished the protective roles of TLB in facilitating astrocyte survival, decreasing inflammatory cytokines and ROS generation as well as reducing TLR4 protein expression in astrocytes under OGD/R condition. Thus, it is speculated that there might be a direct reciprocity between TLR4 and Nrf2. Of particular interest was that, TLR4 directly bound to Nrf2 as confirmed by ZDOCK and RDOCK as well as SPR. These findings disclosed the effect of TLB against cerebral I/R injury *via* reciprocity between the Nrf2 and TLR4 signaling pathways. Of note, Sirt3 is a mitochondrial nicotinamide adenine dinucleotide-dependent protein, which localized to the mitochondrial matrix(38). Recently, Sirt3 has been deemed to not only mediated oxidative stress and mtROS homeostasis, but also orchestrate suppression of inflammation(15,29). Noteworthily, the results in this study demonstrated

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that Sirt3 was apparently decreased after MCAO and OGD/R, in line with the theory of Sirt3 deficiency impairs neurovascular recovery in ischemic stroke(4); whereas, TLB increased Sirt3 expression both in *vivo* and in *vitro* consistent with our previous study(8). Moreover, TLB also significantly enhanced Sirt3 activity after OGD/R insult. Intriguingly, in fact, a direct interaction between TLB and Sirt3 was evidenced by molecular docking. Furthermore, the beneficial effects of TLB on cerebral I/R injury were paritially abolished in Sirt3 deficiency rats although Sirt3-KO rats showed normal under common conditions, which indicated that Sirt3 plays a vital role under stress conditions, and further verified that TLB might be a naturally-occurring Sirt3 agonist. Thus, it is plausible to suppose that Sirt3 was likely to be a promising therapeutic target of TLB against cerebral I/R injury. Emerging evidence demonstrates that a mass of neuroprotective agents exhibited valid effects *in vivo* experiments, but did not display significant effects in clinical trial due to these narrow therapeutic window. Therefore, it is necessary to explore the therapeutic window of neuroprotective agents *in vivo* experiments. Our findings indicated that TLB exerted significantly therapeutic effects on cerebral I/R injury in rats within 4 h, although its beneficial effects partially lost after 6 h, it still effectively attenuated the injury after MCAO in rats. Thus, whether TLB can induce extended window of ischemic tolerance in the rat brain will be investigated in our next story. It should be noted that the present study has evaluated the neuroprotection of TLB against cerebral I/R injury and its preliminary mechanisms. Whether TLB can cross blood-brain barrier and mitigate cerebral I/R-induced astrocyte injury further reinforce the resistance of neurons to neuroinflammation and oxidative injury still need to be in-depth explored. In fact, these problems could be solved by means pharmacokinetics, BBB-3D model, co-cultured astrocytes and neurons in trans-well in our next story.

Collectively, our findings afford new insight into that TLB inhibited cerebral I/R-induced neuroinflammation and oxidative injury *via* TLR4/Nrf2/Sirt3 signaling pathway. These findings highlight the feasibility that TLB might be a promising Sirt3 agonist against ischemic stroke.

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Innovation

The present study, for the first time, discovers that TLB, a novel naturally-occurring Sirt3 agonist from *Lithocarpus polystachyus* Rehd., mitigates cerebral ischemia/reperfusion injury through regulation of TLR4/NF-κB and Nrf2/ Keap-1 signaling. Hence TLB is a novel lead toward the development of a neuroprotective agent.

Materials and Methods

Chemicals and Reagents

Trilobatin was from Guangdong Kedi Medical Technology Corporation (purity ≥ 98%). The syntheses of Tr1 was implemented as described in Fig. 9A. The NMR spectra and MS data of the TLB and Tr1 were showed in Supplementary Fig.S5-7. 2,3,5-Triphenyltetrazolium chloride (TTC) and MTT were purchased from Sigma-Aldrich), Neurobasal™-A Medium, B-27™ Supplement (50X) and MitoSOX Red were obtained from Invitrogen (Eugene, OR, USA). The lactate dehydrogenase (LDH) Cytotoxicity Assay Kit, IL-1β, IL-6, TNF-α, ROS, MDA, SOD, GSH-Px assay kits were obtained from Shanghai Renjie Bioengineering Institute (Shanghai, China). The primary antibodies used in present study, including GFAP, Iba-1, iNOS, TLR4, MyD88, TRAF, NF-κBp65, Nrf2, Keap1, HO-1, NQO1, Sirt3 and Sirt3 activity assay kit (Fluorometric) were purchased from Abcam (Cambridge, UK). TLR4 siRNA (r) and Nrf2 siRNA (r) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Lipofectamine™ RNAiMAX transfection reagent and scrambled siRNA were obtained from Invitrogen (Eugene, OR, USA).

Animal

Male adult Sprague-Dawley (SD) rats (8-10 weeks old, 250-280 g), were purchased from the Experimental Animal Center of Daping Hospital (Certificate No. SCXK 2014-0011). All rats were housed in five to six per cage with a 12 h light/dark cycles and provided free access to standard rodent diet and tap water and kept under temperature (23 ± 1 °C) and humidity (55 ± 5%)-controlled environment. Randomization was used to allocated animals to various experimental groups and the data analysis were performed by a blinded investigator. All animal experimental protocols in the present study were operated

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according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication 85-23, revised 1996), and were approved by the Experimental Animal Ethics Committee of the Zunyi Medical University (Guizhou, China).

Induction of focal cerebral ischemia and drug treatments

Focal cerebral ischemia was induced by MCAO as described in previous study(43). In brief, the rats were anaesthetized with 1% sodium pentobarbital (45 mg/kg, i.p.) and the right common carotid artery, external carotid artery and internal carotid artery were separated carefully. Then, a piece of 5/0 monofilament nylon suture with diameter 0.36 mm was inserted into the internal carotid artery through the external carotid artery stump to occlude the origin of middle cerebral artery. After 2 h, the filament was withdrawn to establish reperfusion and the rat body temperature was kept at 37 °C during surgery. Laser Doppler flowmetry (Moor Instruments, UK) was applied to monitor MCAO severity rCBF during the surgical procedure and at the reperfusion as described previously. A successful MCAO model was accepted when rCBF lowered to below 20% and recovered to higher than 80% of baseline. In the first study of TLB treatment on cerebral I/R, the rats were randomly divided into five groups: sham group, sham + TLB (20 mg/kg) group, MCAO group, MCAO + TLB (5 mg/kg) group, MCAO + TLB (10 mg/kg) group, MCAO + TLB (20 mg/kg) group. Sham group underwent the same operation as mentioned above except MCAO. Animals were treated TLB by gavage at doses of 5, 10 and 20 mg/kg at the onset reperfusion twice a day for 3 days, and the rats of sham and model groups were given volume-matched saline, instead. The second group was designed to evaluate the time window of TLB for the treatment of cerebral I/R, TLB was administered at the doses of 20 mg/kg at 1, 2, 3, 4 and 6 h after MCAO. The sham group and MCAO group of rats were received volume-matched saline. In the third group, to discover the effect of TLB on functional recovery after MCAO, TLB was administered at the dosage of 5, 10 and 20 mg/kg at the onset reperfusion twice daily for 28 days after MCAO. The sham group and MCAO group of rats were administrated volume-matched saline. In the final group, to determine the effects of TLB on the MCAO model, rats were divided into the following four groups 3 weeks after CRISPR-Cas9 Sirt3 lentivirus microinjection: Wild type (WT) + sham,

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WT + sham + TLB (20 mg/kg), WT + MCAO, WT + MCAO + TLB (20 mg/kg), Sirt3-knockout (Sirt3-KO) + sham, Sirt3-KO + sham +TLB (20 mg/kg), Sirt3-KO + MCAO, Sirt3-KO + MCAO + TLB (20 mg/kg).

Determination of Neurological deficit scores

Neurological injury after MCAO was determined by a neurobehavioral test that was scored on a five-point scale: grade 0, observable deficit; grade 1, failure to fully extend left forepaw; grade 2, circling to the left; falling 3, falling to the left; 4, inable to walk spontaneously accompanied with a depressed level of consciousness. Neurobehavioral test was carried out 3 days after MACO in a double-blind manner as described previously(16).

Assessment for long term functional recovery after cerebral I/R injury in rats

Neurological deficit scores were determined after day 28 as mentioned above. In addition, rats were trained in the sensorimotor tests for successive 3 days before MCAO. Rotorod and adhesive tape removal tests were performed as described in previous reports(31,48). The rotarod was applied to determined general fitness and motor co-ordination. In brief, rats were placed on an accelerating rotating beam (acceleration from 4 rpm to 40 rpm within 5 min) and a stop-clock was started. The latency for each rat to fall off the rotarod onto the sensing platform below was registered. Each rat performed three trials. Moreover, the sensorimotor impariment-induced by MCAO were detected by adhesive tape removal tests. In brief, a piece of adhesive tape (3 × 1 cm) were applied as bilateral tactile stimuli occupying the distal-radial area on the wrist of each forelimb in rats. Then, the rats were placed bace to their cages. The mean latency to remove adhesive tapes from the forepaws was registered for the lesioned forepaws. The times to detect and remove the tapes were recorded with a maximum limition of 120 s. Furthermore, the spatial memory and learning were detected using Y-Maze test as previous study(44). In brief, three same arms of the maze apparatus (30 cm × 10 cm× 20 cm) were randomly assigned as the begin arm, novel arm, or other arm. Firstly, the rats were subjected to investigate the begining arm and the other arm for 8 min. Thereafter, rats were returned back in the maze in the same begining arm and allowed to investigate for 5 min with

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access to all three arms at liberty. The number of entries and percentage of time spent in the novel arm that rats detected were recorded. Spontaneous alternation (%) = [(number of actual alternations)/(number of total arm entries-2)] × 100, was applied as an index to evaluate spatial memory. Moreover, NOR tasks were use to determine learning and memory after MCAO as described in previous report(42). In Brief, rats were accustomed to an open-field box (50 × 50 × 50 cm) at 28 days after MCAO. During the first phase (10 min), two of the same objects (A1 and A2, green cubes) were placed symmetrically from the wall. During familiarization phase (10 min), two dissimilar objects (object A2 was replaced with a novel object B1, a brown box) were placed in the same box for 1 h after the first trial. The total time of an rat spent detecting each object during two phases was recorded. The objects discrimination index was caculated using a discrimination index (DI) as described in previous report(42).

Measurement of infarct volume

After neurological test, the rats were anaesthetized using 1% sodium pentobarbital and decapitated under anesthesia. Thereafter, the rat brains were promptly removed and frozen at -20 °C for 15 min. Then, five coronal brain sections of 2 mm thickness were stained with TTC at 37 °C for 15 min in darkness, and fixed with 4% formaldehyde for 24 h in the dark. the red color area and the pale gray color area of the slices were evaluated by Image J software in a double-blind manner as described previously(14).

Microarray Processing and Data Analysis

The total RNA was extracted from the brain tissues of rats in Sham, MCAO + MCAO group and MCAO + TLB (20 mg/kg) groups using TRIzol buffer on the basis of experimental protocol, and then quantified them using an Agilent 2100 (Agilent Technologies Co. Ltd., Palo Alto, CA, United States) analysis meter. Qualified RNA transcriptome was sequenced on the platform of BGISEQ-500RS RNA-Seq supported by Beijing Genomics Institute (Shenzhen, China). The gene expression was calculated using FPKM value of each sample, gene expression with a fold change (FC) greater than 1.5 and *P*-value less than 0.05 were identified as DEGs in this study. OmicShare tools (www.omicshare.com/tools) was used to plotted volcano plot and advanced bubble diagram, Venny 2.1.0[3] was applied to draw

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Venn diagram. Protein-protein interactions (PPIs) network was constructed by Cytoscape 3.6.0 software. DAVID web-based tools (www.david.ncifcrf.gov) was employed to carried out the functional enrichment analysis, the enrichment of pathways and functional processing were formed based on KEGG and annotations of Gene Ontology (GO), respectively. Furthermore, STRING web-based tools (www.string-db.org) was adopted to analyzed PPIs.

Immunohistochemical (IHC) staining

IHC was used to determined the activation of astrocyte and microglia using GFAP staining and Iba-1 staining as described previously(3). In brief, the brain sections with paraffin-embedded (4 μm) were rinsed with 3% H_2O_2 for 10 min to block endogenous peroxide activity and incubated with 10% goat serum albumin for 30 min to block nonspecific binding. Then, the sections were incubated with the primary antibodies anti-GFAP (1:100) and anti-Iba1 (1:200) overnight at 4 °C, after incubation, sections were incubated with corresponding secondary antibody (anti-goat IgG-HRP, 1:200). Immunoreactions were visualized using 3,30-diaminobenzidine tetrahydrochloride. Counterstaining was performed using hematoxylin. Negative control sections were stained only with secondary antibody to control for possible on specific staining. For the semiquantitative analysis of the immunohistochemical results, three sections from each brain, with each section containing three microscopic fields from the ischemic boundary zone (penumbra) in the cerebral cortex, were digitized under a 40 × objective. The immunoreactivity of the target proteins was quantified based on the integrated optical density of immunostaining per field using Image Pro Plus 6.0 software.

Primary rat astrocytes and cortical neurons culture and drug treatment

Primary rat astrocytes culture were obtained as described in previous study(3). In briefly, the astrocytes were collected and identified using anti-GFAP antibody. The astrocytes were cultured on 96-well plates or 6-well plates were washed with HBSS, the culture medium was shifted with a glucose-free DMEM/F12, and then the astrocytes were cultured for 1.5 h in oxygen-free N_2/CO_2 (95%/5%) gas. The 1.5-h time point was chosen because greater than 50% cell death occurred. Then, the OGD/R group medium was

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replaced with standard culture medium, or treated with various concentrations of TLB (12.5, 25, 50 μM) for another 48 h.

Primary rat cortical neurons were obtained from new born SD rats as mentioned in previous study(47). In brief, the primary cortical neurons were cultured in 96-well plates or 6-well plates with poly L-lysine-coated and resuspended in neurobasal medium containing 10% FBS and 2% B27 supplement, then the cells were cultured in a humidified incubator with 5% CO_2 at 37 °C for successive 10 days. Cultures contain > 95% neurons were identified by neuron-specific enolase. Thereafter, neurons were insulted by OGD for 2 h to mimic ischemic damage *in vitro* as mentioned in previous report(32). Brief, neurons were incubated for 2 h in oxygen-free N_2/CO_2 (95%/5%) gas, and the neurons of control group was cultured in EBSS with 10 mM glucose. Then the OGD/R group medium was replaced with standard culture medium, or treated with various concentrations of TLB (6.25, 12.5, 25, 50 μM) for another 24 h.

Determination of cell viability and neurotoxicity

The primary rat astrocytes or cortical neurons were treated as mentioned above. In briefly, at the end point of the treatment, each well was cultured with MTT (5 mg/ml) for another 4 h. Then the medium was discarded and DMSO (150 ml) was added to dissolve the formazan. whereafter, the absorbance of formazan formation was evaluated by a microplate reader at 490 nm wavelength. Additionally, in parallel, neurotoxicity was also detected by a LDH kit as described previously(7). In briefly, at the end point of the treatment as described above, the supernatants were obtained with centrifuged for 5 min at 400 × g. The amount of LDH released from astrocytes were lysed in 1% Triton X-100 following to the manufacturer's introduction. Moreover, cellular morphologic change were observed by a phase contrast microscopy.

Determination of inflammatory factors

The astrocytes were treated as mentioned above. Briefly, the tissues or cells were collected and homogenized using 0.1 M PBS (pH 7.4). Thereafter, the tissue homogenates were centrifuged at 3000 × g for 20 min at 4 °C. Then, levels of inflammatory factors

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including IL-1β, IL-6 and TNF-α were detected according ELISA kits complied with the manufacturer's protocol.

Determination of ROS level

The astrocytes or cortical neurons were treated as mentioned above. In brief, the tissues or cells were collected and homogenized using 0.1 M PBS (pH 7.4). Thereafter, the tissue homogenates were centrifuged at 3000 × g for 20 min at 4 °C. Then, levels of ROS were determined using related ELISA kits according to the manufacturer's protocol. Additionally, the mitochondrial $O_2^{\bullet-}$ generation was measured by MitoSOX Red staining, a peculiar fluorescent probe for detecting mitochondrial $O_2^{\bullet-}$ generation (7). In brief, after astrocytes or cortical neurons were treated as mentioned above and then were washed with balanced salt solution and stained with 5 μM MitoSOX Red in the dark at 37 °C for 20 min. Then, the astrocytes were observed using fluorescence microscopy (Olympus IX73; Olympus, Tokyo, Japan) with excitation/emission (510/580 nm) filters. ROS level was quantified by the Image Pro Plus software.

Measurement of SOD and GSH-Px activities

The astrocytes or cortical neurons were treated as mentioned above. Briefly, the tissues or cells were collected and homogenized using 0.1 M PBS (pH 7.4). Thereafter, the tissue homogenates were centrifuged at 3000 × g for 20 min at 4 °C. Then, activities of SOD and GSH-Px were determined using related ELISA kits according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

Total RNA was obtained with the Trizol Reagent, which was reverse transcribed to cDNA with the PrimeScript™ RT Reagent Kit. The CFX96 real-time PCR detection system (Bio-Rad Laboratories Ltd, Hertfordshire, UK) was used to perform qRT-PCR. The specific primers and according sequences were displayed as follows: GAPDH, forward 5'-AACGACCCCTTCATTGACCT-3' and reverse 5'- CCCCATTTGATGTTAGCGGG -3'; Nrf2, forward 5'- GTTCAGTCGGTGCTTTGACA -3' and reverse 5'- CTCTGATGTGCGTCTCTCCA -3'; TLR4, forward 5'- CTGGGTGAGAAAGCTGGTAA -3' and reverse 5'-

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AGCCTTCCTGGATGATGTTGG -3'; Sirt3, forward 5'- TACTTCCTTCGGCTGCTTCA -3' and reverse 5'- AAGGCGAAATCAGCCACA -3'. In the reaction, 1 μl cDNA of each sample was mixed with SYBR®GREEN PCR Master Mix according to the manufacture's protocol. The PCR conditions were listed as follow: 30 s at 95 °C, then 40 cycles at 95 °C for 5 s, followed by 56 °C for 30 s. Results were normalized to GAPDH mRNA level and presented as the fold change ($2^{-\Delta\Delta Ct}$).

Western blot analysis

In *vivo*, at 3 day after reperfusion, the rats were sacrificed 1% sodium pentobarbital after treatment with TLB, and then the ischemic penumbra was collected as described previously(24). In *vitro*, primary rat astrocytes or cortical neurons were washed three times with ice-cold PBS and then for following protein extraction after treatment with TLB as above-mentioned. Protein extracts from nuclear and cytosolic fraction were extracted by a nuclear extraction kit according to the manufacturer introduction, and the protein concentration was measured using BCA assay. The brain tissues and primary rat astrocytes or cortical neurons were homogenized with RIPA buffer. Thereafter, the lysates were normalized to equal amounts of protein, and 10 μg protein from tissue lysates or 20 μg protein of cell lysates were divided in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and transferred to a nitrocellulose membrane, with blocked with 5% nonfat milk in TBST for 1 h at room temperature. Then, the membranes were incubated with primary antibodies including iNOS (1:1000), TLR4 (1:1000), MyD88 (1:1000), TRAF6 (1:1000), p-NF-κBp65 (1:1000), NF-κBp65 (1:1000), Nrf2 (1:1000), Keap1 (1:1000), HO-1 (1:1000), NQO1 (1:1000) and Sirt3 (1:1000) overnight at 4°C. whereafter, proteins were determined with according species-specific HRP-conjugated secondary antibodies for 2 h at room temperature. Representative bands then were visualized by ECL Western blot detection reagents and Image J software was applied to quantify the band optical intensity.

Transient silencing by small interfering RNAs (siRNAs)

Transfection was implemented when the primary rat astrocytes were achieved to 70–80% confluence in 96-well or six-well plates. The Nrf2-targeted siRNA or TLR4-targeted

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siRNA was diluted with Opti-MEM and balanced for 15 min at room temperature. Then astrocytes were transfected with Nrf2 siRNA, TLR4 siRNA or scrambled siRNA by lipofectamine™ RNAiMAX transfection reagent following the manufacturer's protocol. The knockdown of endogenous Nrf2 or TLR4 with siRNA was verified using qRT-PCR and Western blot, respectively. After transfected for 24 h, the transfected astrocytes were exposed to OGD/R and treated with TLB as described above. Thereafter, cell viability, LDH release, inflammatory factors, ROS, MDA, anti-oxidant enzymes, expression of TLR4, levels of cytoplasmic Nrf2 and nuclear Nrf2 were also detected.

Molecular docking analysis

Molecular docking analysis between TLB and Sirt3 were performed using Autodock 4.2 and Autodock Tools (ADT). The human X-ray crystal structure of Sirt3 (PDB ID: 3GLS) was obtained from the Protein Data Bank (PDB) archives and used as target for molecular docking. The molecular docking results were written as a pose viewer file, and the protein-ligand complex interactions were studied using the PyMOL molecular graphics system. The interactions between TLR4 (PDB ID: 2Z63) and Nrf2 (PDB ID: 2LZL) were detected using ZDOCK and RDOCK, which was an widespread admissive method to implement detailed prediction of protein-protein docking. Subsequently, the poses of predicted protein were scored from ZDOCK, then RDOCK was used to refine and re-rank the scores. Thereafter, the binding affinity of TLR4 and Nrf2 was predicted by E_RDOCK, which was used as the default scoring function. At last, the 18 poses of docked conformations that had lower E_RDOCK were chose for subsequent analysis.

SPR

Biacore X100 instrument (GE Healthcare, Uppsala, Sweden) with Biacore X100 and sensor chip CM5 (GE, BR-1003-99) was performed to further confirm the interaction between TLR4 and Nrf2(36). Briefly, TLR4 (Abcam, ab233665) was dissolved in 10 mM sodium acetate (PH 5.0) at a concentration of 20 μg/mL, and absorbed as immobilization. Nrf2 (Abcam, ab132356) was diluted at 10, 20, 40, 80 and 160 μM in HBS-EP buffer. The protein interaction time was set to 120 s and 300 s for dissociation. Glycine-HCl (PH 2.0) (GE Healthcare, BR-1003-55) was applied for regeneration. The Biacore evaluation 3.1

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analysis software (GE Healthcare) was used to analyzed the data for evaluating binding affinity between TLR4 and Nrf2. Equilibrium dissociation constants (K_D) was determined by global fitting of the kinetic data from various concentrations of Nrf2 with a 1:1 Langmuir binding model.

Measurement of Sirt3 activity

The SIRT3 activity was determined using Sirt3 activity assay kit. Briefly, the astrocytes or neurons were treated with TLB or Tr1 as described above. Then the samples were added double-distilled water, Sirt3 assay buffer, flour-substrate peptide and NAD, then developer to each well of the microtiter plate and mix well and initiate reactions by adding 5 µL of enzyme sample or buffer of enzyme sample or recombinant Sirt3 to each well and mixing thoroughly at room temperature according to the manufacturer's introduction. Thereafter, Sirt3 activity was determined by kinetic measurements at 2 min intervals by a microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm for 30 min.

Generation of Sirt3-KO rats and Sirt3-KO primary rat astrocytes or cortical neurons

Sirt3-KO rats and Sirt3-KO primary rat astrocytes or cortical neurons were generated by a CRISPR-Cas9 system. The lentivirus-based CRISPR/Cas9 KO plasmid, pHBLV-U6-gRNA-EF1-CAS9-PURO, with the Sirt3 gRNA sequences 5'-TGGTAGTCATGCGTGTTGGG-3' (forward) and 5'-CTCAGAACCCAGAAGGTGTG-3' (reverse) primers. Promoter U6 drived gRNA and CMV drived Cas9. In brief, the lateral ventricles of 6-week-old SD rats were microinjected with either a lentiviral-packed CRISPR-Cas9 Sirt3 (5 µl, MOI of 100) (Hanbio Biotechnology Co., Ltd., Shanghai, China) at a constant rate (1 µl/min) using a syringe pump system at the subsequent microinjection coordinates: 1.0 mm behind the bregma and 1.0 mm lateral from the sagittal midline, at a depth of 3.5 mm from the skull surface. 3 weeks after lentivirus microinjection, the rats were treated with or without TLB for 3 days after MCAO. Thereafter, the neurological function and infarct volume were determined using five-point scale and TTC staining, respectively. Moreover, primary rat astrocytes or cortical neurons were transfected with lentiviral-packed CRISPR-Cas9 Sirt3 at an MOI of 100 for 48 h. Thereafter, the knockout of endogenous Sirt3 by CRISPR-Cas9 Sirt3 lentivirus

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were confirmed by RT-PCR and Western blot, respectively. Then, the transfected cells were cultured and treated with or without TLB after OGD/R insults for further analysis.

Statistical analysis

Data are expressed as mean ± SD, and 'n' represents the number of independent experiments and not replicates. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze all data. Some data were normalized to control for unwanted sources of variation as follows. Firstly, the data was normalized to bring all of the variation into proportion with one another after deleting any outliers in various groups. Then, the coefficients associated with each variable will scale properly to adjust for the disparity in the variable sizes. The cell viability of control group was deemed to be 100%, and the cell viability of TLB-treated group was displayed as a percentage of the control group. The iNOS, TLR4, MyD88, TRAF6, Keap1, HO-1 and NQO1expression were normalized to β-actin, and Sirt3 expression was normalized to GAPDH. Additionally, cytoplasmic Nrf2 level was normalized to cytoplasmic β-actin and nuclear Nrf2 level was normalized to nuclear PCNA. The protein expressions or levels of TLB-treated group were presented as fold change of the sham or control group, which expression was set to 1. Two or multiple groups were compared using Student's unpaired *t*-test or one-way ANOVA followed by Bonferroni *post hoc* test with F at $P < 0.05$ and no significant variance inhomogeneity. $P < 0.05$ was considered statistically significant.

Author Disclosure Statement

The authors declare no conflict of interest.

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List of Abbreviations

ARE, antioxidant response element; DCF-DA, 2',7'-dichlorofluorescin diacetate; DMEM, Dulbecco's Modified Eagle Medium; DEGs, differentially expressed genes; GSH-Px, glutathione peroxidase; GFAP, glial fibrillary acidic protein; HO-1, heme oxygenase-1; IHC, immunohistochemical; I/R, ischemia-reperfusion; IL-1β, interleukin-1β; IL-6, interleukin-6; Keap-1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; MCAO, Middle cerebral artery occlusion; MDA, malondialdehyde; MTT, 3-(4,5-22dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MyD88, myeloid differentiation factor 88; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H-quinone oxidoreductase 1; NF-κB, nuclear factor-kappa B; OGD/R, oxygen–glucose deprivation followed by reperfusion; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction; rtPA, recombinant tissue plasminogen activator; ROS, reactive oxygen species; SOD, superoxide dismutase; SD, Sprague-Dawley; Sirt3, silent mating-type information regulation 2 homolog 3; TLB, trilobatin; TTC, 2,3,5-triphenyltetrazolium chloride; TLR4, Toll-like receptor4; TNF-α, tumor necrosis factor α.

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References

1. Alfieri A, Srivastava S, Siow RCM, Cash D, Modo M, Duchen MR, Fraser PA, Williams SCR, Mann GE. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. *Free Radic Biol Med* 65: 1012-1022, 2013.

2. Alim I, Caulfield JT, Chen Y, Swarup V, Geschwind DH, Ivanova E, Seravalli J, Ai Y, Sansing LH, Ste Marie EJ, Hondal RJ, Mukherjee S, Cave JW, Sagdullaev BT, Karuppagounder SS, Ratan RR. Selenium Drives a Transcriptional Adaptive Program to Block Ferroptosis and Treat Stroke. *Cell* 177: 1262-1279 e25, 2019.

3. Bachstetter AD, Webster SJ, Goulding DS, Morton JE, Watterson DM, Van Eldik LJ. Attenuation of traumatic brain injury-induced cognitive impairment in mice by targeting increased cytokine levels with a small molecule experimental therapeutic. *J Neuroinflammation* 12: 69, 2015.

4. Chen T, Dai SH, Li X, Luo P, Zhu J, Wang YH, Fei Z, Jiang XF. Sirt1-Sirt3 axis regulates human blood-brain barrier permeability in response to ischemia. *Redox Biol* 14: 229-236, 2018.

5. Collaborators GBDCoD. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 392: 1736-1788, 2018.

6. Fan X, Zhang Y, Dong H, Wang B, Ji H, Liu X. Trilobatin attenuates the LPS-mediated inflammatory response by suppressing the NF-kappaB signaling pathway. *Food Chem* 166: 609-15, 2015.

7. Gao J, Deng Y, Yin C, Liu Y, Zhang W, Shi J, Gong Q. Icariside II, a novel phosphodiesterase 5 inhibitor, protects against H_2O_2 -induced PC12 cells death by inhibiting mitochondria-mediated autophagy. *J Cell Mol Med* 21: 375-386, 2017.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

8. Gao J, Liu S, Xu F, Liu Y, Lv C, Deng Y, Shi J, Gong Q. Trilobatin Protects Against Oxidative Injury in Neuronal PC12 Cells Through Regulating Mitochondrial ROS Homeostasis Mediated by AMPK/Nrf2/Sirt3 Signaling Pathway. *Front Mol Neurosci* 11: 267, 2018.

9. Gupta S, Gupta YK. Combination of Zizyphus jujuba and silymarin showed better neuroprotective effect as compared to single agent in MCAo-induced focal cerebral ischemia in rats. *J Ethnopharmacol* 197: 118-127, 2017.

10. Hind WH, Tufarelli C, Neophytou M, Anderson SI, England TJ, O'Sullivan SE. Endocannabinoids modulate human blood-brain barrier permeability in vitro. *Br J Pharmacol* 172: 3015-27, 2015.

11. Ikram M, Muhammad T, Rehman SU, Khan A, Jo MG, Ali T, Kim MO. Hesperetin Confers Neuroprotection by Regulating Nrf2/TLR4/NF-kappaB Signaling in an Abeta Mouse Model. *Mol Neurobiol*, 2019.

12. Kahles T, Brandes RP. Which NADPH oxidase isoform is relevant for ischemic stroke? The case for nox 2. *Antioxid Redox Signal* 18: 1400-17, 2013.

13. Kuang X, Wang LF, Yu L, Li YJ, Wang YN, He Q, Chen C, Du JR. Ligustilide ameliorates neuroinflammation and brain injury in focal cerebral ischemia/reperfusion rats: involvement of inhibition of TLR4/peroxiredoxin 6 signaling. *Free Radic Biol Med* 71: 165-75, 2014.

14. Liu L, Vollmer MK, Ahmad AS, Fernandez VM, Kim H, Dore S. Pretreatment with Korean red ginseng or dimethyl fumarate attenuates reactive gliosis and confers sustained neuroprotection against cerebral hypoxic-ischemic damage by an Nrf2-dependent mechanism. *Free Radic Biol Med* 131: 98-114, 2019.

15. Liu T, Ma X, Ouyang T, Chen H, Xiao Y, Huang Y, Liu J, Xu M. Efficacy of 5-aminolevulinic acid-based photodynamic therapy against keloid compromised by downregulation of SIRT1-SIRT3-SOD2-mROS dependent autophagy pathway. *Redox Biol* 20: 195-203, 2019.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

16. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20: 84-91, 1989.

17. Ludewig P, Winneberger J, Magnus T. The cerebral endothelial cell as a key regulator of inflammatory processes in sterile inflammation. *J Neuroimmunol* 326: 38-44, 2019.

18. Luo Y, Wang C, Li WH, Liu J, He HH, Long JH, Yang J, Sui X, Wang S, You Z, Wang YA. Madecassoside protects BV2 microglial cells from oxygen-glucose deprivation/reperfusion-induced injury via inhibition of the toll-like receptor 4 signaling pathway. *Brain Res* 1679: 144-154, 2018.

19. Muzzi M, Gerace E, Buonvicino D, Coppi E, Resta F, Formentini L, Zecchi R, Tigli L, Guasti D, Ferri M, Camaioni E, Masi A, Pellegrini-Giampietro DE, Mannaioni G, Bani D, Pugliese AM, Chiarugi A. Dexpramipexole improves bioenergetics and outcome in experimental stroke. *Br J Pharmacol* 175: 272-283, 2018.

20. Nalamolu KR, Smith NJ, Chelluboina B, Klopfenstein JD, Pinson DM, Wang DZ, Vemuganti R, Veeravalli KK. Prevention of the Severity of Post-ischemic Inflammation and Brain Damage by Simultaneous Knockdown of Toll-like Receptors 2 and 4. *Neuroscience* 373: 82-91, 2018.

21. Narayanan SV, Dave KR, Saul I, Perez-Pinzon MA. Resveratrol Preconditioning Protects Against Cerebral Ischemic Injury via Nuclear Erythroid 2-Related Factor 2. *Stroke* 46: 1626-32, 2015.

22. Nash KM, Schiefer IT, Shah ZA. Development of a reactive oxygen species-sensitive nitric oxide synthase inhibitor for the treatment of ischemic stroke. *Free Radic Biol Med* 115: 395-404, 2018.

23. Parada E, Casas AI, Palomino-Antolin A, Gomez-Rangel V, Rubio-Navarro A, Farre-Alins V, Narros-Fernandez P, Guerrero-Hue M, Moreno JA, Rosa JM, Roda JM, Hernandez-Garcia BJ, Egea J. Early TLR4 blockade reduces ROS and inflammation triggered by microglial pro-inflammatory phenotype in rodents and human brain ischemia models. *Br J Pharmacol*, 2019.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

24. Peng L, Yin J, Ge M, Wang S, Xie L, Li Y, Si JQ, Ma K. Isoflurane Post-conditioning Ameliorates Cerebral Ischemia/Reperfusion Injury by Enhancing Angiogenesis Through Activating the Shh/Gli Signaling Pathway in Rats. *Front Neurosci* 13: 321, 2019.

25. Pinchuk I, Weber D, Kochlik B, Stuetz W, Toussaint O, Debacq-Chainiaux F, Dolle MET, Jansen E, Gonos ES, Sikora E, Breusing N, Gradinaru D, Sindlinger T, Moreno-Villanueva M, Burkle A, Grune T, Lichtenberg D. Gender- and age-dependencies of oxidative stress, as detected based on the steady state concentrations of different biomarkers in the MARK-AGE study. *Redox Biol* 24: 101204, 2019.

26. Pu H, Shi Y, Zhang L, Lu Z, Ye Q, Leak RK, Xu F, Ma S, Mu H, Wei Z, Xu N, Xia Y, Hu X, Hitchens TK, Bennett MVL, Chen J. Protease-independent action of tissue plasminogen activator in brain plasticity and neurological recovery after ischemic stroke. *Proc Natl Acad Sci USA* 116: 9115-9124, 2019.

27. Sivandzade F, Prasad S, Bhalerao A, Cucullo L. NRF2 and NF-B interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. *Redox Biol* 21: 101059, 2019.

28. Song S, Wang S, Pigott VM, Jiang T, Foley LM, Mishra A, Nayak R, Zhu W, Begum G, Shi Y, Carney KE, Hitchens TK, Shull GE, Sun D. Selective role of Na^+/H^+ exchanger in $Cx3cr1^+$ microglial activation, white matter demyelination, and post-stroke function recovery. *Glia* 66: 2279-2298, 2018.

29. Traba J, Geiger SS, Kwarteng-Siaw M, Han K, Ra OH, Siegel RM, Gius D, Sack MN. Prolonged fasting suppresses mitochondrial NLRP3 inflammasome assembly and activation via SIRT3-mediated activation of superoxide dismutase 2. *J Biol Chem* 292: 12153-12164, 2017.

30. Vartanian KB, Stevens SL, Marsh BJ, Williams-Karnesky R, Lessov NS, Stenzel-Poore MP. LPS preconditioning redirects TLR signaling following stroke: TRIF-IRF3 plays a seminal role in mediating tolerance to ischemic injury. *J Neuroinflammation* 8: 140, 2011.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

31. Wang J, Shi Y, Zhang L, Zhang F, Hu X, Zhang W, Leak RK, Gao Y, Chen L, Chen J. Omega-3 polyunsaturated fatty acids enhance cerebral angiogenesis and provide long-term protection after stroke. *Neurobiol Dis* 68: 91-103, 2014.

32. Wang Q, Gong Q, Wu Q, Shi J. Neuroprotective effects of Dendrobium alkaloids on rat cortical neurons injured by oxygen-glucose deprivation and reperfusion. *Phytomedicine* 17: 108-15, 2010.

33. Wang Y, Huang Y, Xu Y, Ruan W, Wang H, Zhang Y, Saavedra JM, Zhang L, Huang Z, Pang T. A Dual AMPK/Nrf2 Activator Reduces Brain Inflammation After Stroke by Enhancing Microglia M2 Polarization. *Antioxid Redox Signal* 28: 141-163, 2018.

34. Wang Y, Jin H, Wang W, Wang F, Zhao H. Myosin1f-mediated neutrophil migration contributes to acute neuroinflammation and brain injury after stroke in mice. *J Neuroinflammation* 16: 77, 2019.

35. Yagi K, Kitazato KT, Uno M, Tada Y, Kinouchi T, Shimada K, Nagahiro S. Edaravone, a free radical scavenger, inhibits MMP-9-related brain hemorrhage in rats treated with tissue plasminogen activator. *Stroke* 40: 626-31, 2009.

36. Yang L, Zhang XY, Li K, Li AP, Yang WD, Yang R, Wang P, Zhao ZH, Cui F, Qin Y, Yang JH, Tao HL, Sun T, Chen S, Yu PH, Liu HJ, Yang C. Protopanaxadiol inhibits epithelial-mesenchymal transition of hepatocellular carcinoma by targeting STAT3 pathway. *Cell Death Dis* 10: 630, 2019.

37. Yang T, Sun Y, Mao L, Zhang M, Li Q, Zhang L, Shi Y, Leak RK, Chen J, Zhang F. Brain ischemic preconditioning protects against ischemic injury and preserves the blood-brain barrier via oxidative signaling and Nrf2 activation. *Redox Biol* 17: 323-337, 2018.

38. Yang W, Nagasawa K, Munch C, Xu Y, Satterstrom K, Jeong S, Hayes SD, Jedrychowski MP, Vyas FS, Zaganjor E, Guarani V, Ringel AE, Gygi SP, Harper JW, Haigis MC. Mitochondrial Sirtuin Network Reveals Dynamic SIRT3-Dependent Deacetylation in Response to Membrane Depolarization. *Cell* 167: 985-1000 e21, 2016.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

39. Yang X, Geng KY, Zhang YS, Zhang JF, Yang K, Shao JX, Xia WL. Sirt3 deficiency impairs neurovascular recovery in ischemic stroke. *CNS Neurosci Ther* 24: 775-783, 2018.

40. Zaidi SF, Castonguay AC, Jumaa MA, Malisch TW, Linfante I, Marden FA, Abraham MG, Chebl AB, Novakovic R, Taqi MA, Nogueira RG, Martin CO, Holloway WE, Mueller-Kronast N, English JD, Dabus G, Bozorgchami H, Xavier A, Rai AT, Froehler MT, Badruddin A, Nguyen TN, Yoo AJ, Shaltoni H, Janardhan V, Chen PR, Britz GW, Kaushal R, Nanda A, Gupta R, Zaidat OO. Intraarterial Thrombolysis as Rescue Therapy for Large Vessel Occlusions. *Stroke* 50: 1003-1006, 2019.

41. Zeng J, Chen Y, Ding R, Feng L, Fu Z, Yang S, Deng X, Xie Z, Zheng S. Isoliquiritigenin alleviates early brain injury after experimental intracerebral hemorrhage via suppressing ROS- and/or NF-κB-mediated NLRP3 inflammasome activation by promoting Nrf2 antioxidant pathway. *J Neuroinflammation* 14: 119, 2017.

42. Zhang G, Zhang T, Li N, Wu L, Gu J, Li C, Zhao C, Liu W, Shan L, Yu P, Yang X, Tang Y, Yang GY, Wang Y, Sun Y, Zhang Z. Tetramethylpyrazine nitrone activates the BDNF/Akt/CREB pathway to promote post-ischaemic neuroregeneration and recovery of neurological functions in rats. *Br J Pharmacol* 175: 517-531, 2018.

43. Zhang K, Zhang Q, Deng J, Li J, Li J, Wen L, Ma J, Li C. ALK5 signaling pathway mediates neurogenesis and functional recovery after cerebral ischemia/reperfusion in rats via Gadd45b. *Cell Death Dis* 10: 360, 2019.

44. Zhang L, Seo JH, Li H, Nam G, Yang HO. The phosphodiesterase 5 inhibitor, KJH-1002, reverses a mouse model of amnesia by activating a cGMP/cAMP response element binding protein pathway and decreasing oxidative damage. *Br J Pharmacol* 175: 3347-3360, 2018.

45. Zhang M, Ma Y, Chai L, Mao H, Zhang J, Fan X. Storax Protected Oxygen-Glucose Deprivation/Reoxygenation Induced Primary Astrocyte Injury by Inhibiting NF-κB Activation in vitro. *Front Pharmacol* 9: 1527, 2018.

46. Zhang Q, Wu HH, Wang Y, Gu GJ, Zhang W, Xia R. Neural stem cell transplantation decreases neuroinflammation in a transgenic mouse model of Alzheimer's disease. *J Neurochem* 136: 815-825, 2016.

47. Zhang W, Liu J, Hu X, Li P, Leak RK, Gao Y, Chen J. n-3 Polyunsaturated Fatty Acids Reduce Neonatal Hypoxic/Ischemic Brain Injury by Promoting Phosphatidylserine Formation and Akt Signaling. *Stroke* 46: 2943-50, 2015.

48. Zhang X, Tang X, Liu K, Hamblin MH, Yin KJ. Long Noncoding RNA Malat1 Regulates Cerebrovascular Pathologies in Ischemic Stroke. *J Neurosci* 37: 1797-1806, 2017.

49. Zhang Z, Qin P, Deng Y, Ma Z, Guo H, Guo H, Hou Y, Wang S, Zou W, Sun Y, Ma Y, Hou W. The novel estrogenic receptor GPR30 alleviates ischemic injury by inhibiting TLR4-mediated microglial inflammation. *J Neuroinflammation* 15: 206, 2018.

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Table 1. ZDOCK and RDOCK score of TLR4 and Nrf2. [a]Lower values of ZRANK score and E_RDOCK and higher ZDOCK score indicate top docking of Nrf2 and TLR4. [b]Clash '0' indicates no stearic clash between the proteins after refined using RDOCK.

Pose No.	ZDOCK Score[a]	ZRANK Score[a]	E_RDOCK Score[a]	Clash[b]
Pose6	19.1	-35.321	0.26327	0
Pose7	18.96	-52.831	15.6914	0
Pose3	19.82	-33.752	25.8673	0
Pose2	20.06	-75.092	26.6702	0
Pose4	19.8	-12.517	27.9418	0
Pose8	18.64	-10.121	35.0065	0
Pose1	21.04	-19.287	40.575	0
Pose5	19.44	-15.909	42.6009	0

[a]Lower values of ZRANK score and E_RDOCK and higher ZDOCK score indicate top docking of Nrf2 and TLR4.

[b]Clash '0' indicates no stearic clash between the proteins after refined using RDOCK.

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Figure legends

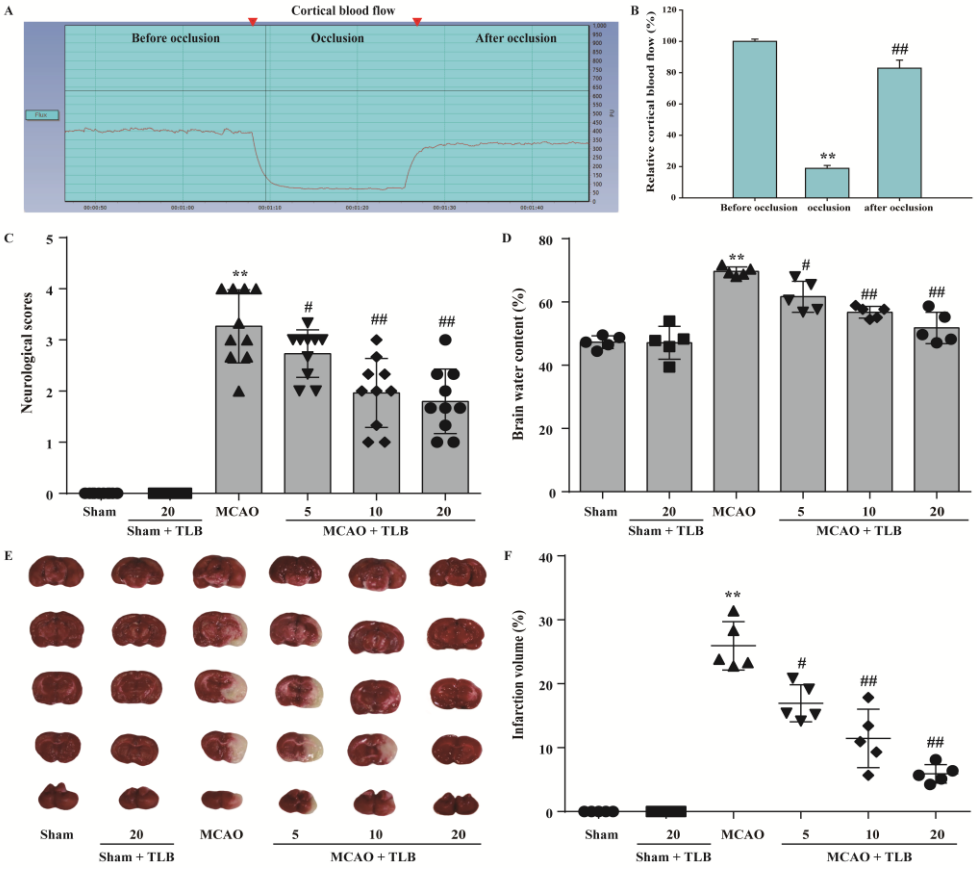

FIG. 1. TLB suppressed MCAO-induced injury through inhibiting neurologic deficits, infarct volume and cerebral edema. (A) laser-doppler flowmeter indicated that regional cortical blood flow (rCBF) reduced to < 20% and recovered to >80% of baseline in MCAO model. (B) Quantitation of rCBF. (C) Neurological deficits were measured using a five-point scale (n=10). (D) Brain water content was evaluated (n=5). (E) Representative images of TTC stained serial brain sections at day 3. (F) Quantification of infarct size at day 3 (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

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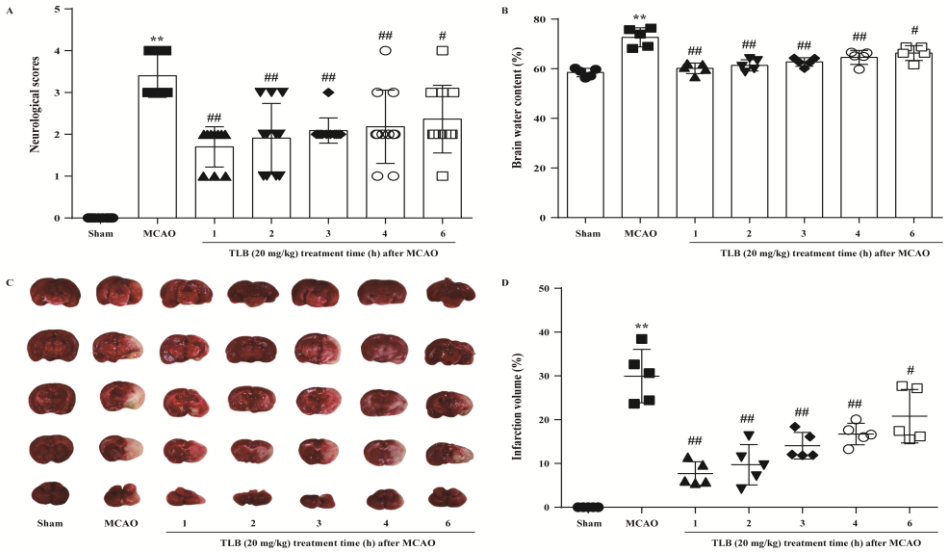

Fig. 2. Treatment protocols of the therapeutic time window of TLB. 20 mg/kg of TLB was administrated once at different times as follows: 1, 2, 3, 4, or 6 h after the initiation of reperfusion in rats. (A) Neurological deficits were measured using a five-point scale (n=10). (B) Brain water content was evaluated (n=5). (C) Representative images of TTC stained serial brain sections at day 3. (D) Quantification of infarct size at day 3 (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* MCAO group.

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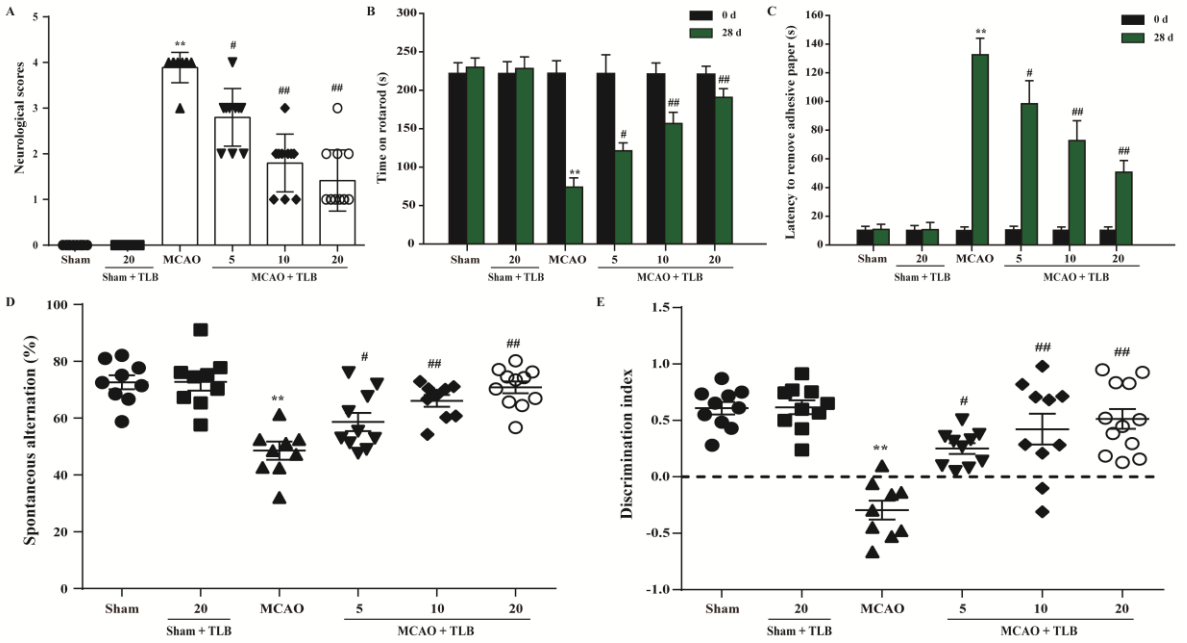

FIG. 3. TLB restores long-term neurological functions at 28 days after MCAO in rats. 20 mg/kg of TLB was administrated at the onset of reperfusion after MCAO in rats for 28 days. (A) Neurological deficit score. (B) Rotarod performance. (C) Latency to remove adhesive paper. (D) Percentage of rats performing a correct spontaneous alternation. (E) Objects discriminated memory was evaluated by a discrimination index. One-way ANOVA with Bonferroni post hoc test (A, D, E) and two-way ANOVA with Bonferroni post hoc test (B, C). The data were presented as the mean ± SEM (n = 9 - 12 per group). $^{*}P < 0.05$ *versus* sham group; $^{\#}P < 0.05$ *versus* MCAO group.

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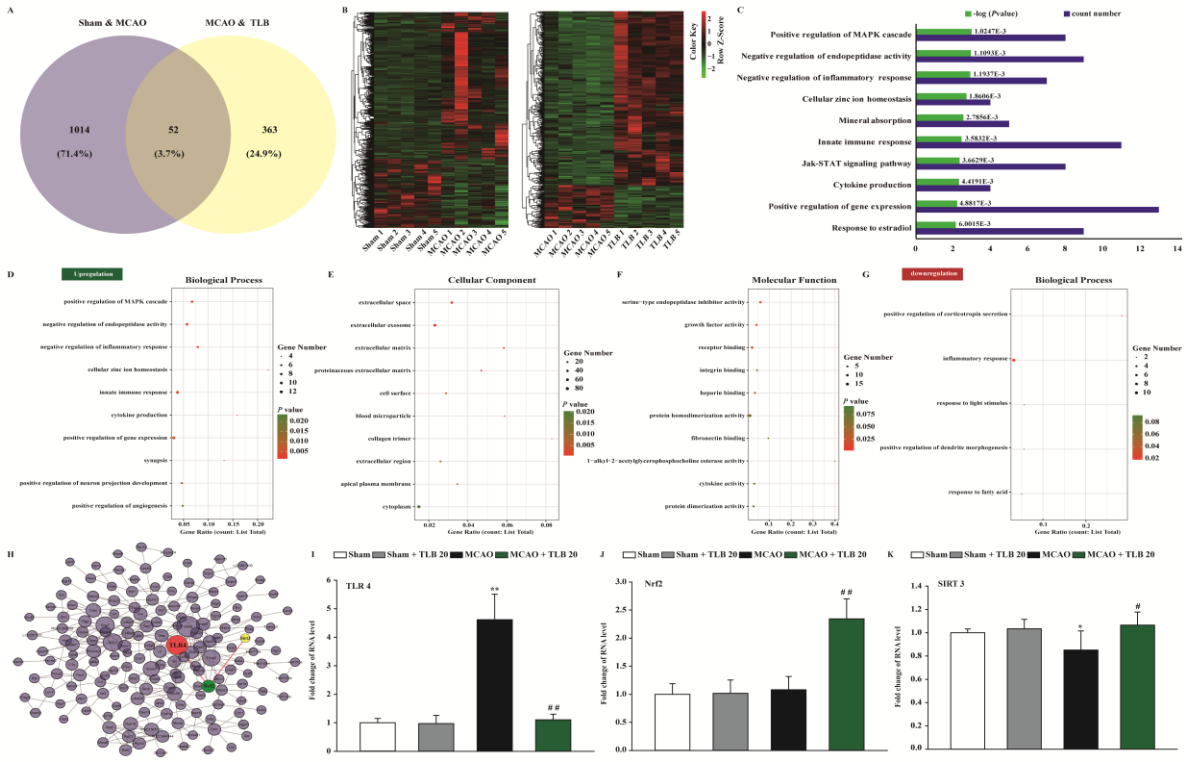

FIG. 4. Analysis of DEGs profiling. (A) The DEGs between sham versus MCAO groups and TLB versus MCAO groups were summarized by Venn diagram. (B) The correlation coefficients between altered gene expression profiles were analyzed using Hierarchical clustering analysis. (C) Top KEGG pathways enriched with DEGs and their matching *P*-values. (D-F) Dot plot showed the fold enrichment values of the top 10 most significantly enriched terms for the upregulation of genes were analyzed by significant GO terms using *P* values. (G) Dot plot showing the fold enrichment values of the top 10 most markedly enriched terms for the downregulation of genes were analyzed by significant GO terms using *P* values. (H) GeneMANIA analysis was used to generate protein-protein interaction network. The size of respective node presented the degree of connectivity in protein-protein interaction network. Larger node (TLR4, Nrf2, Sirt3) shares more connection to other nodes. qRT-PCR was used to validate for microarray data. The expression of (I) TLR4, (J) Nrf2, and (K) Sirt3 in rat brains were detected using qRT-PCR (n=5). The data were presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

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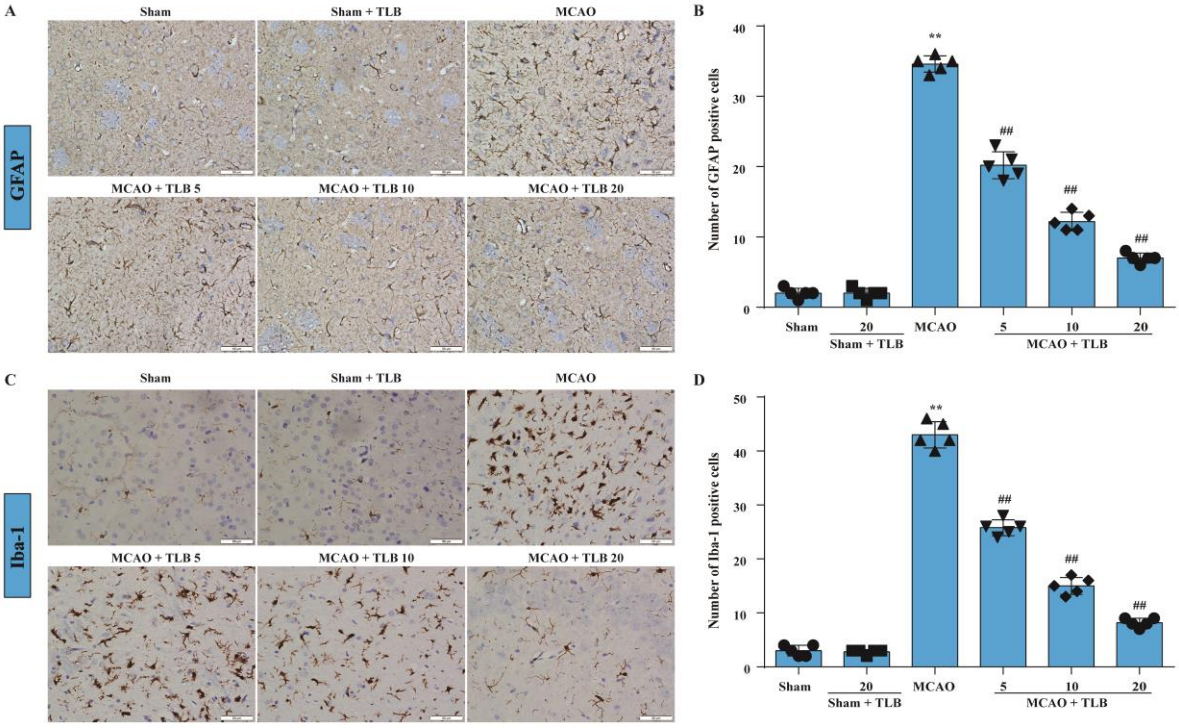

FIG. 5. TLB reduced MCAO-induced astrocyte and microglial activation. Treatment with or without of TLB after MCAO for 3 days and glial activation was measured by IHC using anti-GFAP antibody to mark astrocyte and anti-Iba-1 antibody to mark microglia. (A) Representative images of GFAP stained astrocytes. (B) Quantitation of GFAP positive cells (n=5). (C) Representative images of Iba-1 stained astrocytes. (D) Quantitation of Iba-1 positive cells (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ versus sham group; $^{\#\#}P < 0.01$ versus MCAO group.

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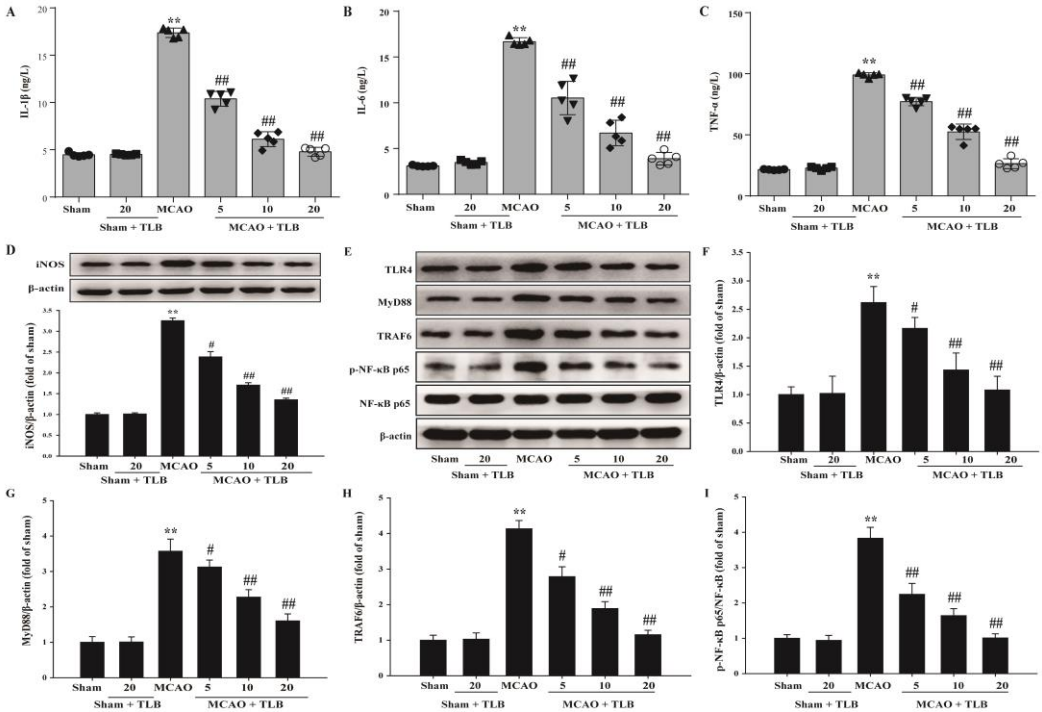

FIG. 6. TLB inhibited inflammatory cytokine and TLR4 signaling pathway after MCAO. (A) IL-1β level (n=5). (B) IL-6 level (n=5). (C) TNF-α level (n=5). (D) Expression and quantitation of iNOS (n=5). (E) Representative Western blots of TLR4, MyD88, TRAF6 expressions and phosphorylation level of NF-κBp65. (F) Quantitation of TLR4 expression (n=5). (G) Quantitation of MyD88 expression (n=5). (H) Quantitation of TRAF6 expression (n=5). (I) Quantitation of phosphorylation level of NF-κBp65 (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

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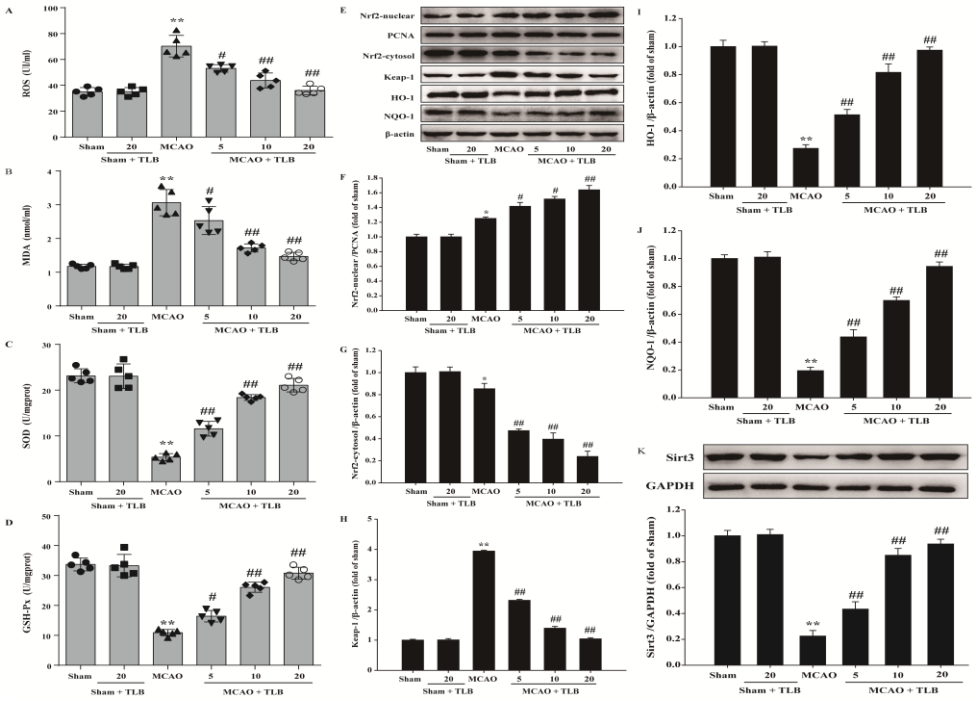

FIG. 7. TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after MCAO. (A) ROS level (n=5). (B) MDA level (n=5). (C) SOD activity (n=5). (D) GSH-Px activity (n=5). (E) Representative Western blots of nuclear and cytosol Nrf2 levels, Keap-1, NQO-1 and HO-1 expressions. (F) Quantitation of nuclear Nrf2 level (n=5). (G) Quantitation of cytosol Nrf2 level (n=5).(H) Quantitation of Keap-1 expression (n=5). (I) Quantitation of HO-1 expression (n=5). (J) Quantitation of NQO-1 expression (n=5). (K) Expression and quantitation of Sirt3 (n=5). The data were presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

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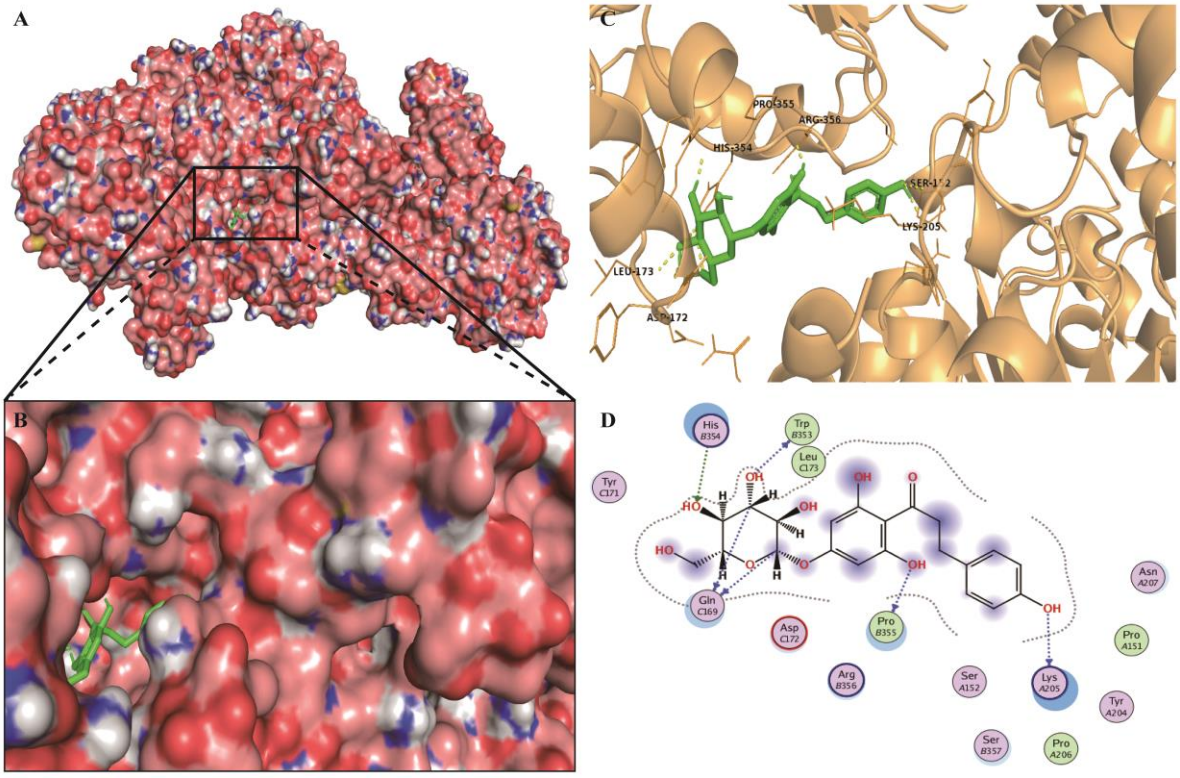

FIG. 8. The molecular docking results of TLB with the Sirt3 complex. (A) The whole view of the TLB dimer and Sirt3 displaying the molecular binding pocket. (B) A close-up amplification of the molecule binding pocket from the side. (C) Crystal structure of TLB (green) displaying Sirt3 (yellow) bound to the docking pocket. (D) Residues of amino acids between TLB with the Sirt3 complex.

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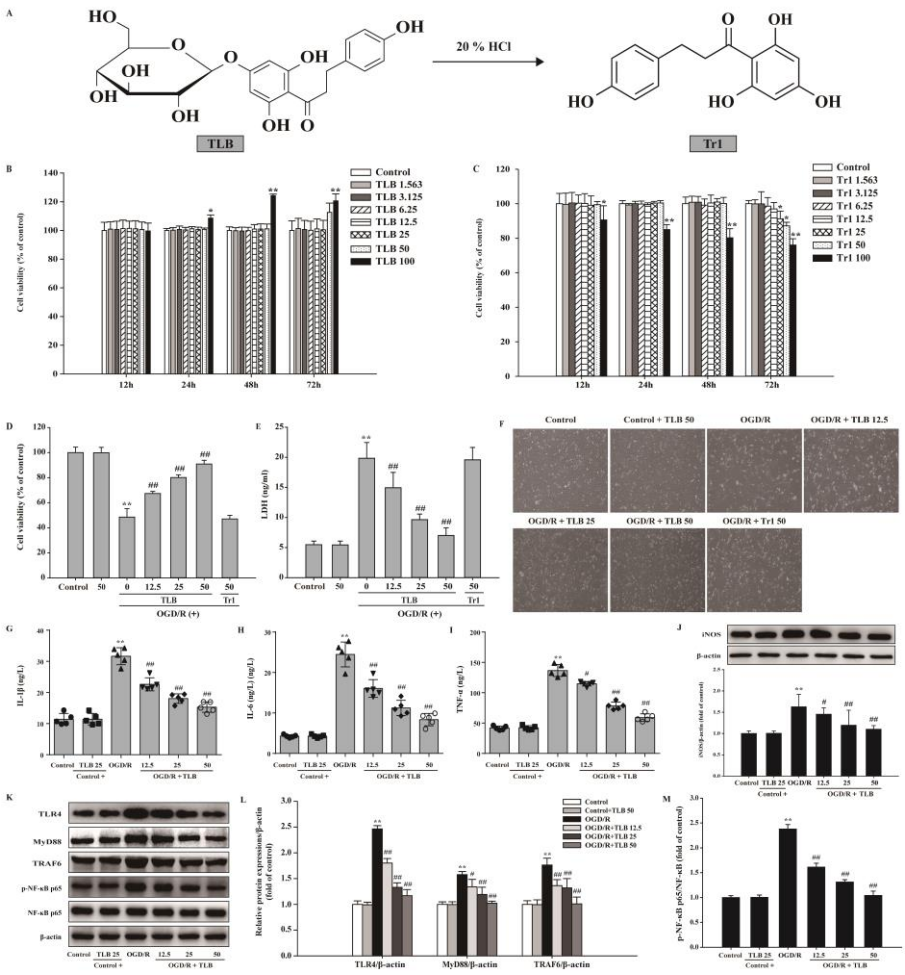

FIG. 9. TLB protected against OGD/R-induced injury *via* inhibiting inflammatory cytokine and TLR4 signaling pathway after OGD/R in astrocytes. (A) Chemical structures of TLB and Tr1. (B, C) TLB and Tr1 up to 50 μM did not cause cytotoxicity (n=5). Astrocytes were treated with or without TLB (12.5, 25, 50 μM) or Tr1 (50 μM) for 48 h after OGD/R. (D) Cell viability were determined using MTT assay (n=5). (E) Cytotoxicity was determined by the LDH release assay (n=5). (F) The morphology of astrocytes was observed by reverse-phase microscope. (G) IL-1β level (n=5). (H) IL-6 level (n=5). (I) TNF-α level (n=5). (J) Expression and quantitation of iNOS (n=5). (K) Representative Western blots of TLR4, MyD88, TRAF6 expressions and phosphorylation level of NF-κBp65 (n=5). (L) Quantitation of TLR4, MyD88 and TRAF6 expressions (n=5). (M) Quantitation of phosphorylation level of NF-κBp65 (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus OGD/R group.

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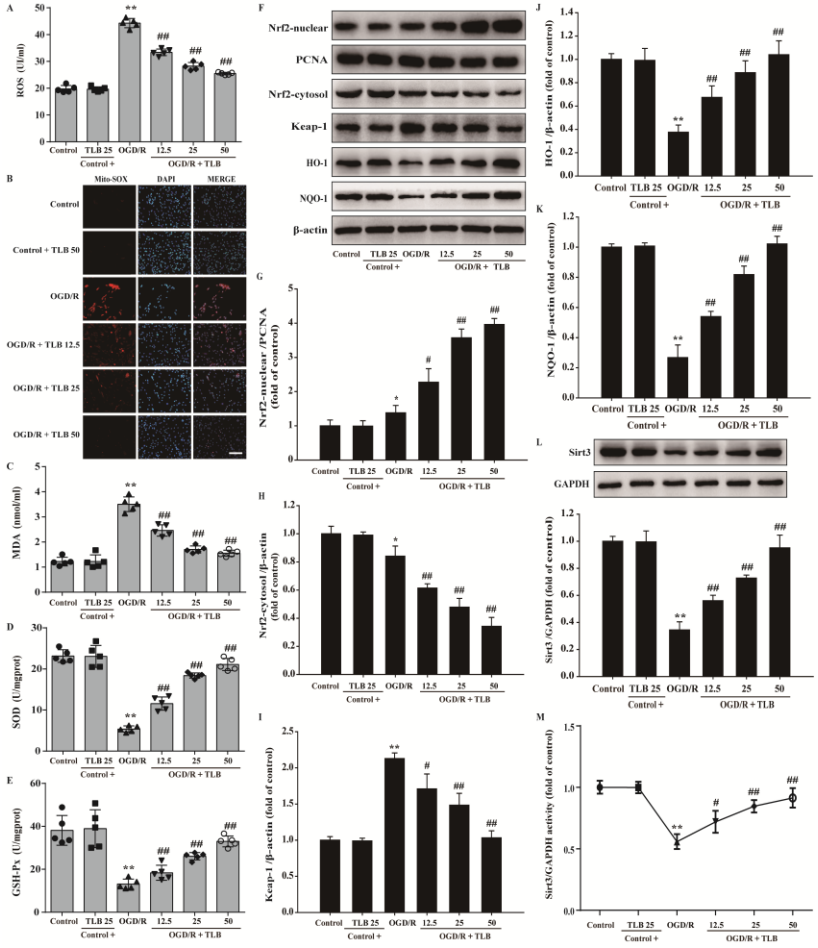

FIG. 10. TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after OGD/R in astrocytes. (A) Intracellular ROS level (n=5). (B) Mitochondrial ROS level. (C) MDA level (n=5). (D) SOD activity (n=5). (E) GSH-Px activity (n=5). (F) Representative Western blots of nuclear and cytosol Nrf2 levels, Keap-1, HO-1 and NQO-1 expressions. (G) Quantitation of nuclear Nrf2 level (n=5). (H) Quantitation of cytosol Nrf2 level (n=5). (I) Quantitation of Keap-1 expression (n=5). (J) Quantitation of HO-1 expression (n=5). (K) Quantitation of NQO-1 expression (n=5). (L) Expression and quantitation of Sirt3 (n=5). (M) Sirt3 activity (n=5). The data were presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus OGD/R group.

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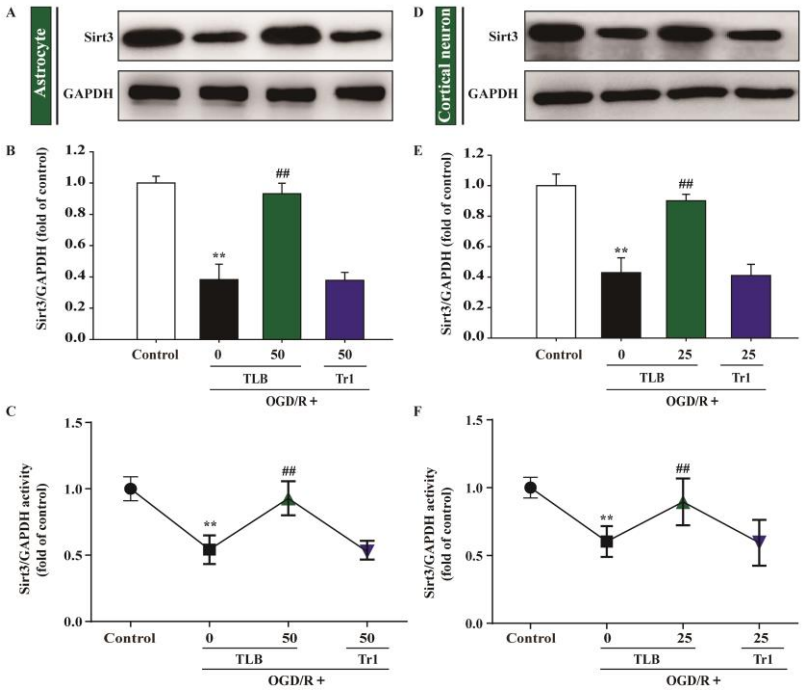

FIG. 11. The effects of TLB or Tr1 on Sirt3 expression and its activity after OGD/R in primary rat astrocytes or cortical neurons. (A) Expression of Sirt3 in primary rat astrocytes. (B) Quantitation of Sirt3 in primary rat astrocytes (n=5). (C) Sirt3 activity in primary rat astrocytes (n=5). (D) Expression of Sirt3 in primary rat cortical neurons. (E) Quantitation of Sirt3 in primary rat cortical neurons (n=5). (F) Sirt3 activity in primary rat cortical neurons (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ versus control group; $^{\#\#}P < 0.01$ versus OGD/R group.

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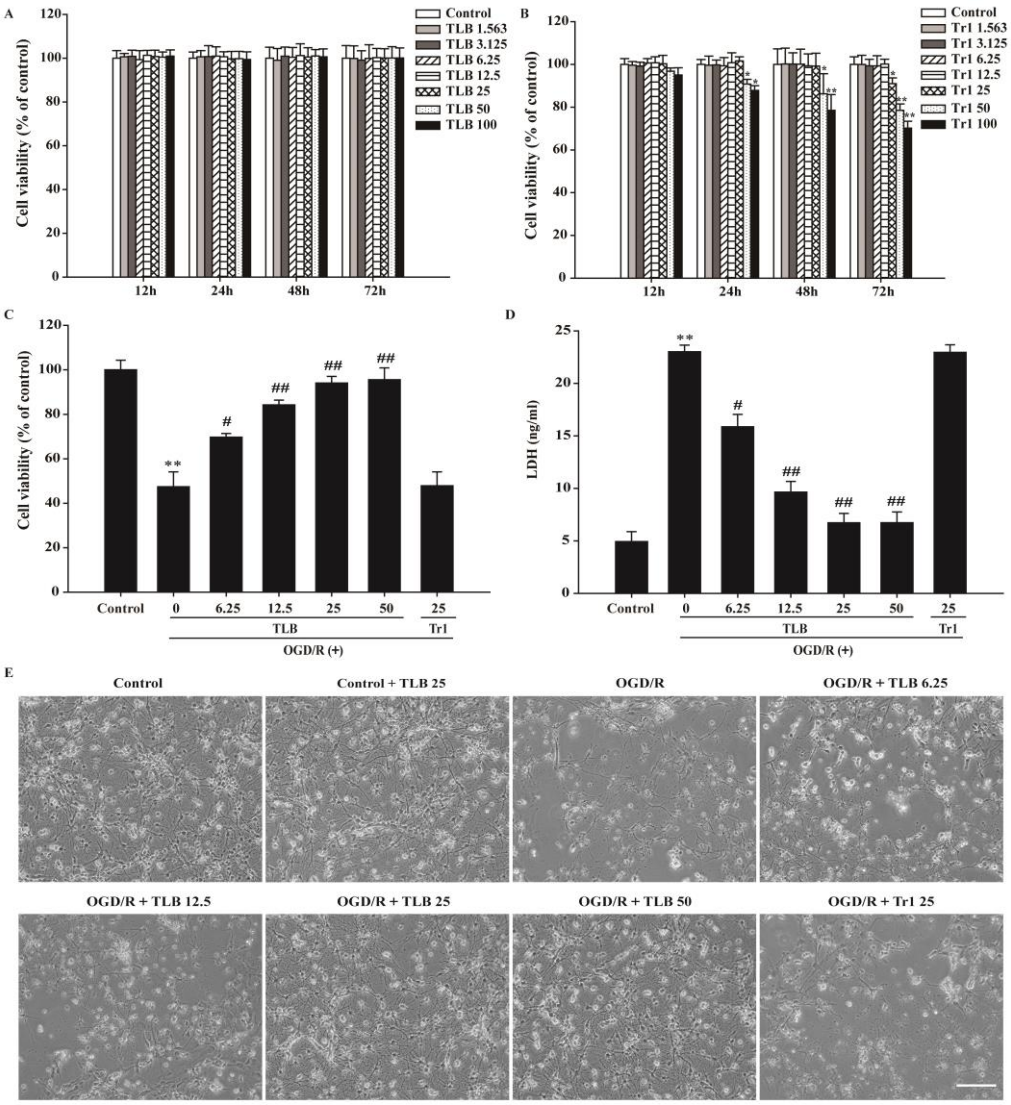

FIG. 12. TLB protected against OGD/R-induced injury in primary cortical neurons. Primary cortical neurons were treated with or without different concentrations of TLB or Tr1. (A) TLB up to 100 μM did not cause cytotoxicity within 24 h (n=5). (B) Tr1 up to 25 μM did not cause cytotoxicity within 24 h (n=5). Primary cortical neurons were treated with or without TLB (6.25, 12.5, 25, 50 μM) or Tr1 (25 μM) for 24 h after OGD/R. (C) Cell viability were determined using MTT assay (n=5). (D) Cytotoxicity was determined by the LDH release assay (n=5). (E) The morphology of astrocytes was observed by reverse-phase microscope. The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus OGD/R group.

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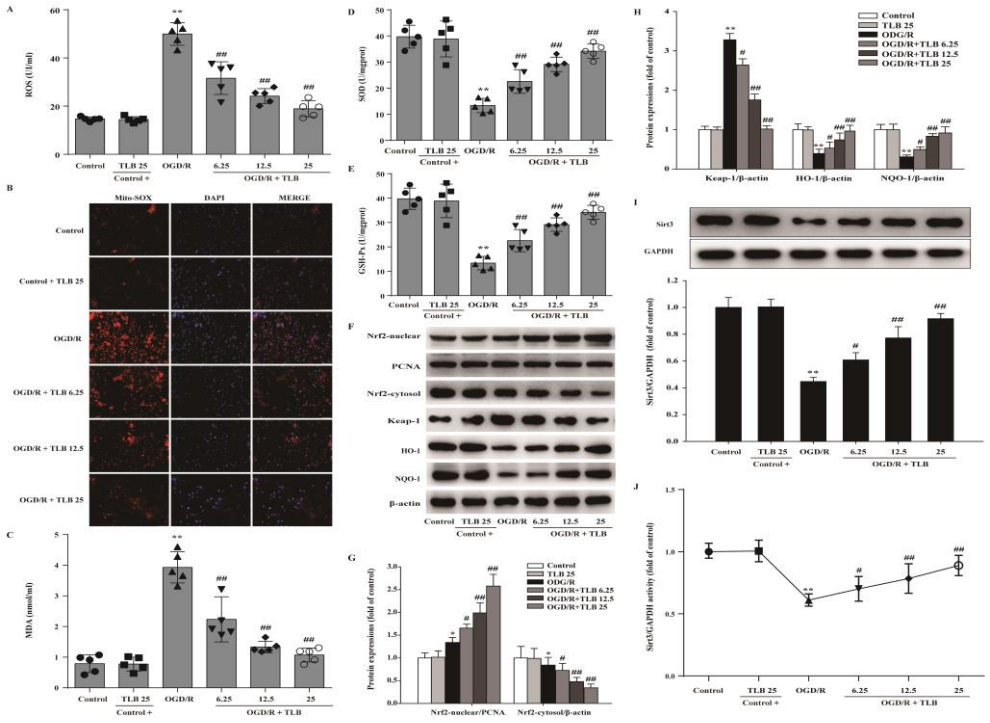

FIG. 13. TLB attenuated oxidative injury and activated Nrf2/Sirt3 signaling pathway after OGD/R in primary cortical neurons. (A) Intracellular ROS level. (B) Mitochondrial ROS level (n=5). (C) MDA level (n=5). (D) SOD activity (n=5). (E) GSH-Px activity (n=5). (F) Representative Western blots of nuclear and cytosol Nrf2 levels, Keap-1, HO-1 and NQO-1 expressions. (G) Quantitation of nuclear Nrf2 and cytosol Nrf2 levels (n=5). (H) Quantitation of Keap-1, HO-1, and NQO-1 expressions (n=5). (I) Expression and quantitation of Sirt3 (n=5). (J) Sirt3 activity (n=5). The data were presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* OGD/R group.

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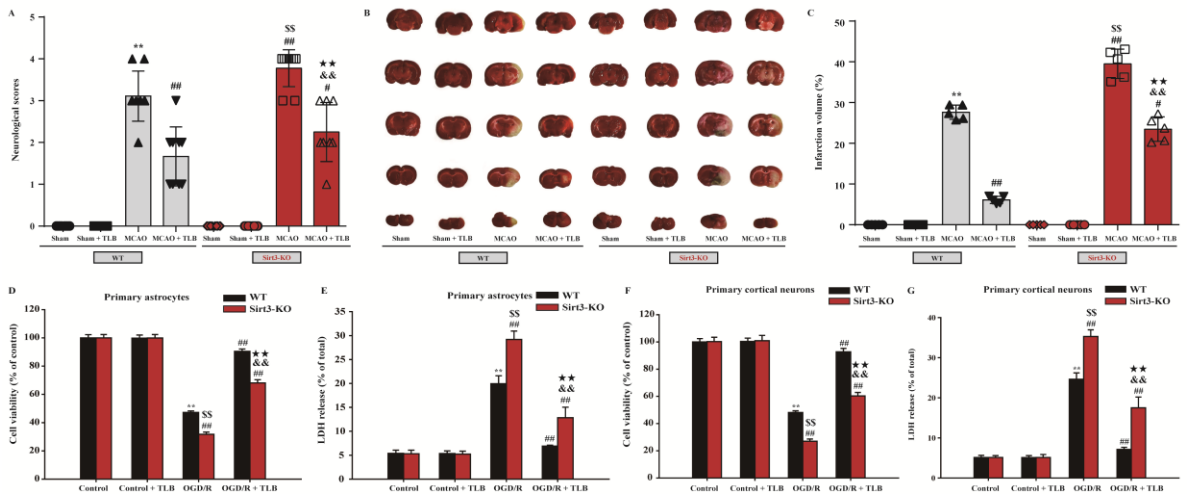

FIG. 14. The effect of TLB in Sirt3-KO rats and Sirt3-KO astrocytes or neurons. Treatment with or without TLB (20 mg/kg) after MCAO Sirt3-KO rats and WT rats for 3 days. (A) Neurological deficit score (n=5-10). (B) Pale areas manifest infarcted brain. (C) Quantification of infarct size (n=5). Treatment with or without TLB (50 μM) after OGD/R-induced injury in Sirt3-KO astrocytes and WT astrocytes. (D) Cell viability (n=5). (E) LDH level (n=5). Treatment with or without TLB (25 μM) after OGD/R-induced injury in Sirt3-KO neurons and WT neurons. (F) Cell viability (n=5). (G) LDH level (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* sham (WT) or control group (WT); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* MCAO (WT) or OGD/R group (WT); $^{\$\$}P < 0.01$ *versus* sham (SIRT3-KO) or control group (SIRT3-KO); $^{\&\&}P < 0.01$ *versus* MCAO (SIRT3-KO) or OGD/R group (SIRT3-KO); $^{\star\star}P < 0.01$ *versus* MCAO + TLB (WT) or OGD/R + TLB group (WT).

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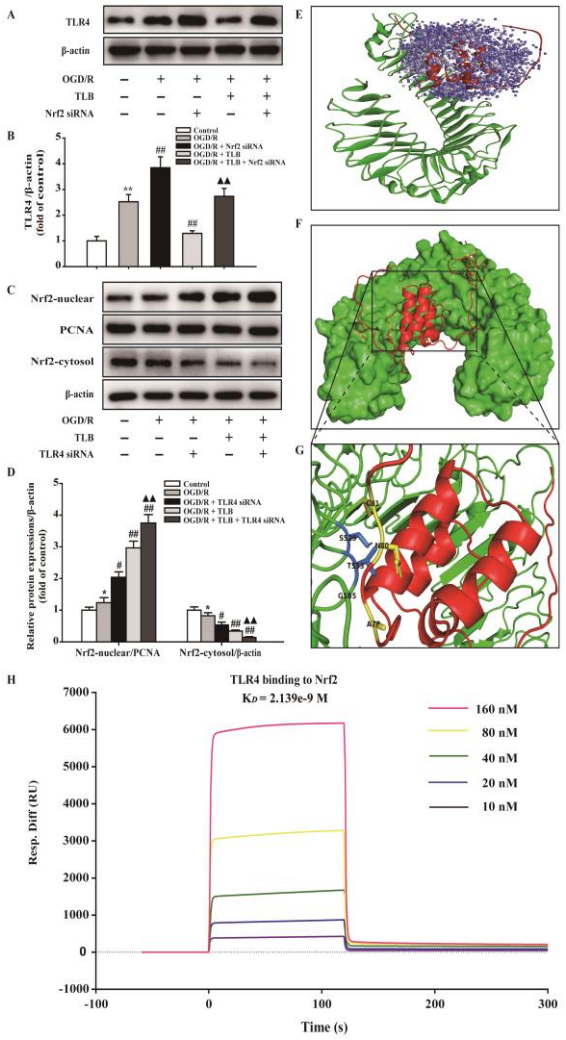

FIG. 15. Reciprocity between TLR4 and Nrf2 was involved in the beneficial effects of TLB on OGD/R-induced injury. (A) Representative Western blots of TLR4 expression with or without Nrf2 siRNA. (B) Quantitation of TLR4 expression with or without Nrf2 siRNA (n=5). (C) Representative Western blots of nuclear and cytosol Nrf2 level with or without TLR4 siRNA. (D) Quantitation of nuclear and cytosol Nrf2 level with or without TLR4 siRNA (n=5). The interaction between TLR4 and Nrf2 were displayed using ZDOCK. (E) The substrate binding sites. (F) The substrate binding surface. (G) Interactions of TLR4 with active amino acids sites and Pi of Nrf2. The Nrf2 protein was shown in red color solid ribbon while TLR4 protein was in green. (H) The binding affinity of Nrf2 with TLR4 was determined using a SPR assay. Various concentrations of Nrf2 were mixed with TLR4, and binding was detected. The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* control group; $^{\#\#}P < 0.01$ *versus* OGD/R group; $^{\blacktriangle\blacktriangle}P < 0.01$ *versus* OGD/R + TLB group.

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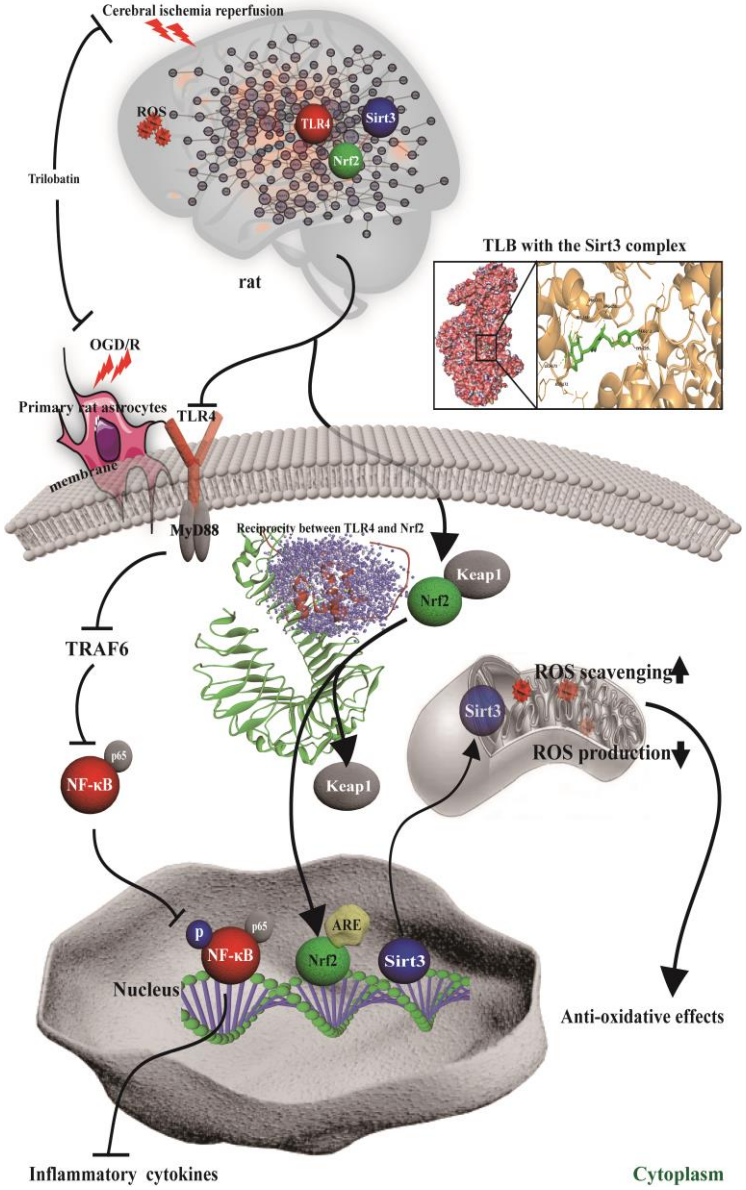

FIG. 16. Schematic presentation of a proposed mechanism for the protective role of TLB against cerebral I/R-induced injury. The inhibitory effects of TLB due to restriction of neuroinflammation through suppressing TLR4 signaling pathway, as well as reduction of oxidative injury *via* activating Nrf2 signaling pathway; Interestingly, reciprocity between TLR4 and Nrf2 was involved in the neuroprotection of TLB against cerebral I/R injury.

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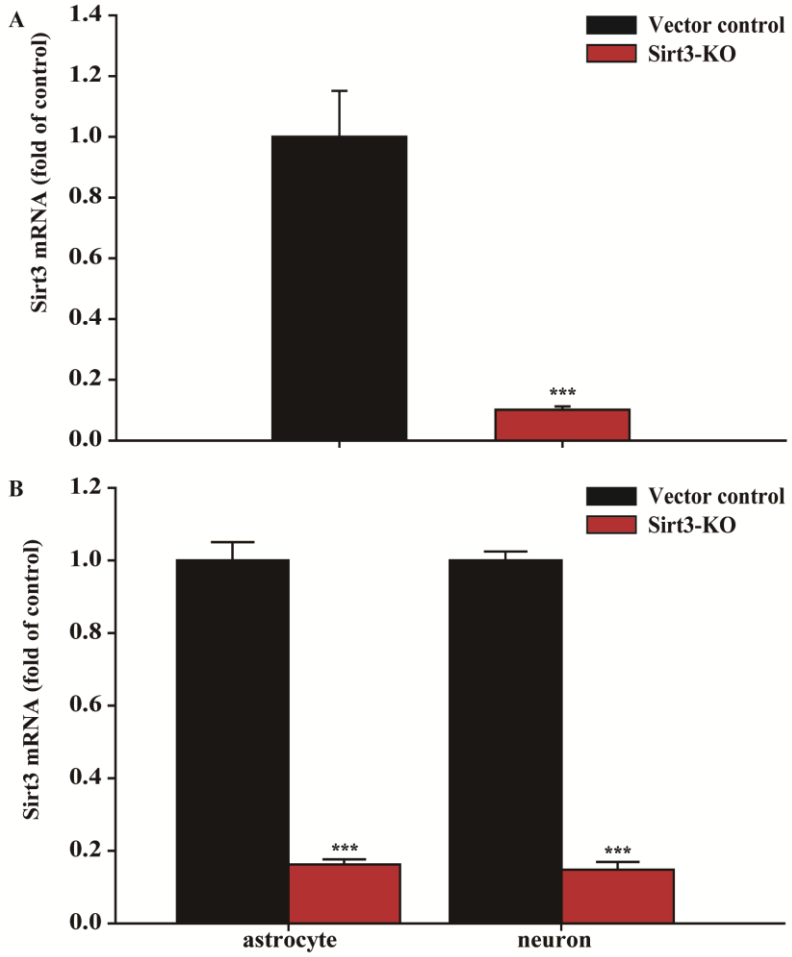
Supplementary information

Supplementary figure legends



Supplementary Fig. S1 Loss of Sirt3 in CRISPR/Cas9-dependent knockout rats, and primary rat astrocytes and cortical neurons. Quantitative qPCR analysis showing > 80% reduction in Sirt3 mRNA *versus* control lentivirus-infected primary rat astrocytes or cortical neurons (n=5). The data were presented as the mean ± SD. $^{***}P < 0.001$ *versus* sham group.

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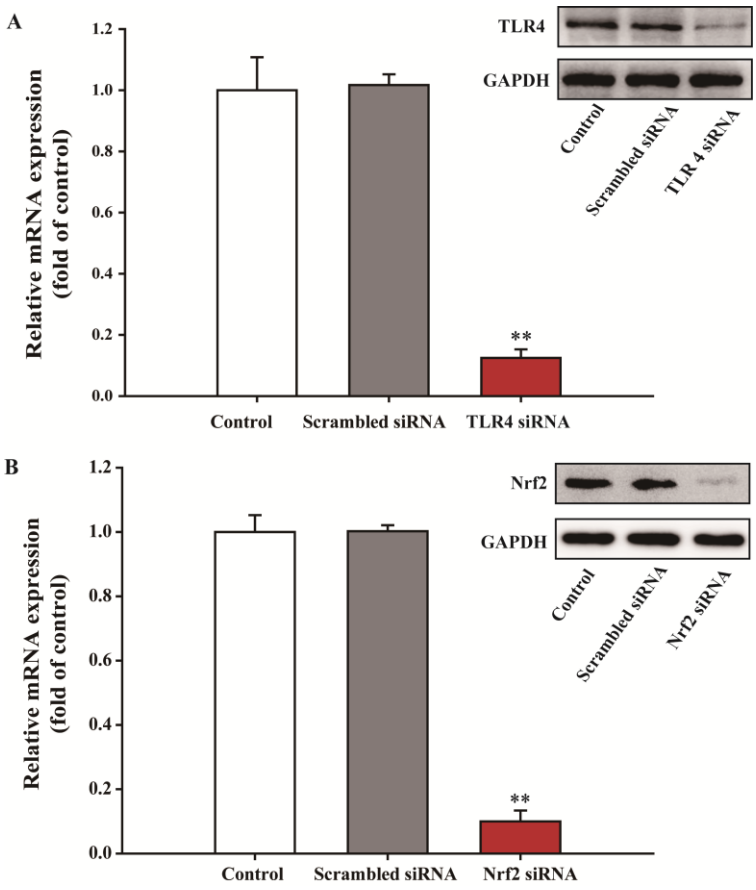

Supplementary Fig. S2 Knockdown of TLR4 and Nrf2 by small interfering RNAs (siRNAs). (A) Quantitation of qRT-PCR and representative Western blot were shown for TLR4 siRNA (n=5). (B) Quantitation of qRT-PCR and representative Western blot were shown for Nrf2 siRNA (n=5). Data were expressed as mean ± SD of five independent experiments. The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* scrambled siRNA group.

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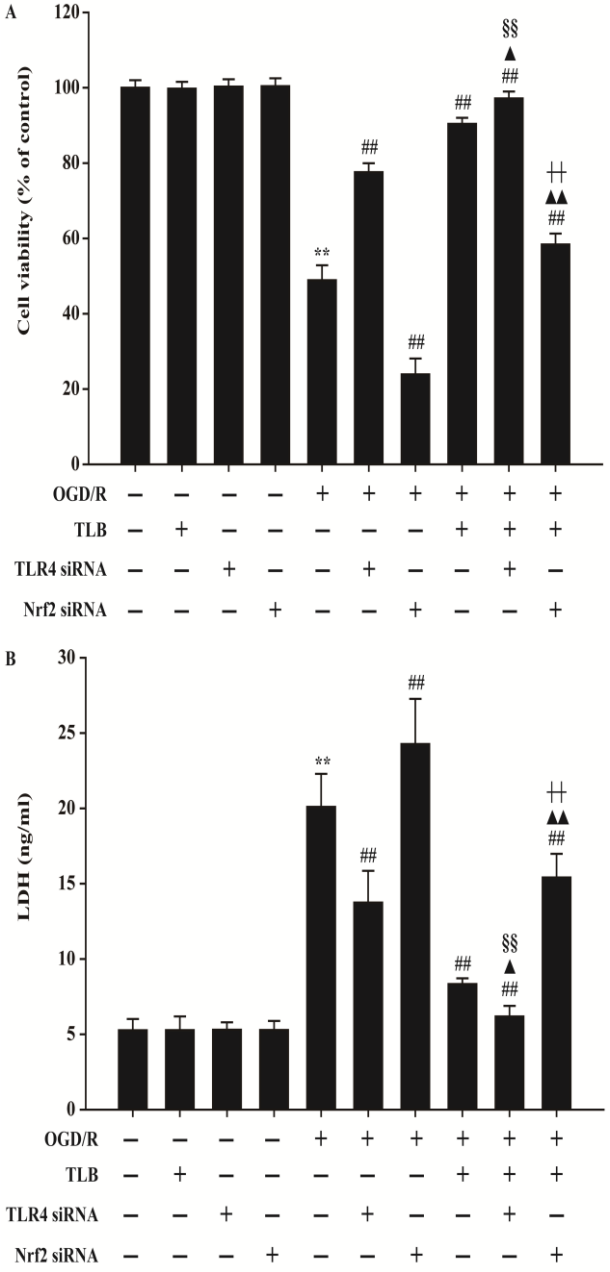

Supplementary Fig. S3 The effects of TLB on OGD/R-induced injury with or without TLR4 siRNA or Nrf2 siRNA. (A) Cell viability was detected in astrocytes transfected with or without TLR4 siRNA or Nrf2 siRNA (n=5). (B) LDH release was determined in astrocytes transfected with or without TLR4 siRNA or Nrf2 siRNA (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* control group; $^{\#\#}P < 0.01$ versus OGD/R group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *versus* OGD/R + TLB group. $^{\S\S}P < 0.01$ *versus* OGD/R + TLR4 siRNA group; $^{\ddagger\ddagger}P < 0.01$ *versus* OGD/R + Nrf2 siRNA group.

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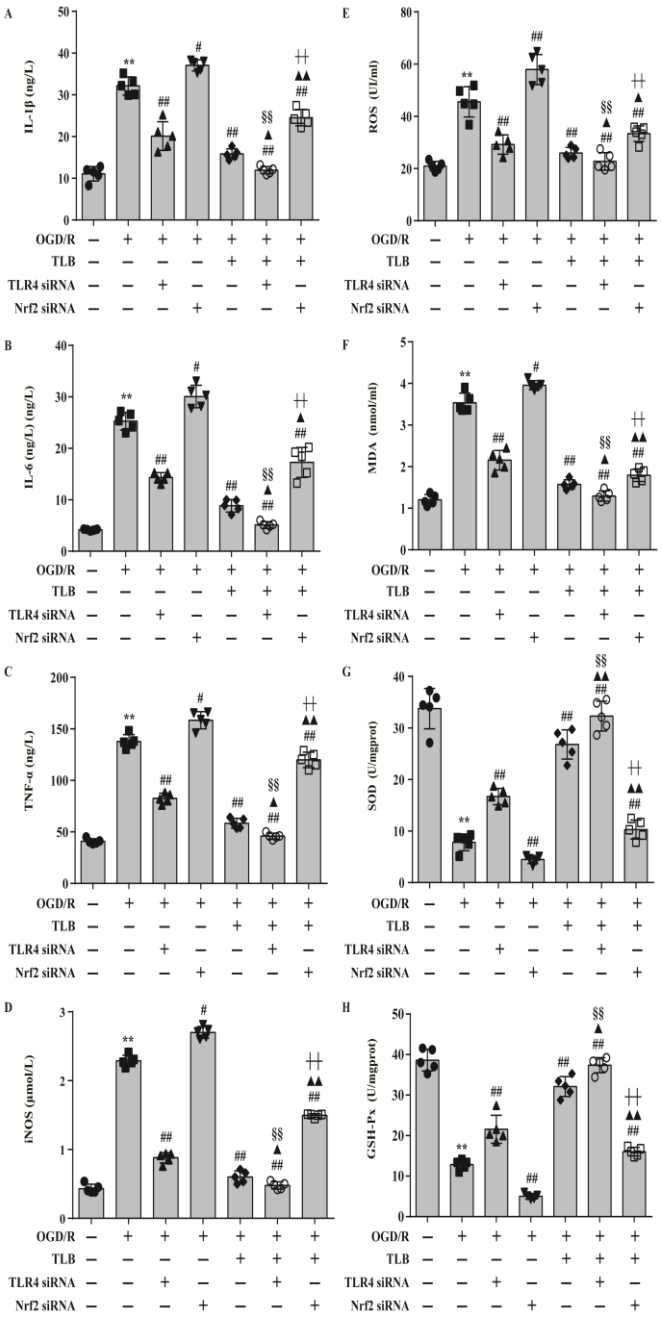

Supplementary Fig. S4 The effects of TLB on inflammatory cytokine and anti-oxidative enzymes with or without TLR4 siRNA or Nrf2 siRNA. (A) IL-1β level (n=5). (B) IL-6 level (n=5). (C) TNF-α level (n=5). (D) iNOS level (n=5). (E) ROS level (n=5). (F) MDA level (n=5). (G) SOD activity (n=5). (H) GSH-Px activity (n=5). The data were presented as the mean ± SD. $^{**}P < 0.01$ *versus* control group; $^{\#\#}P < 0.01$ versus OGD/R group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *versus* OGD/R + TLB group. $^{\S\S}P < 0.01$ *versus* OGD/R + TLR4 siRNA group; $^{\dagger\dagger}P < 0.01$ *versus* OGD/R + Nrf2 siRNA group.

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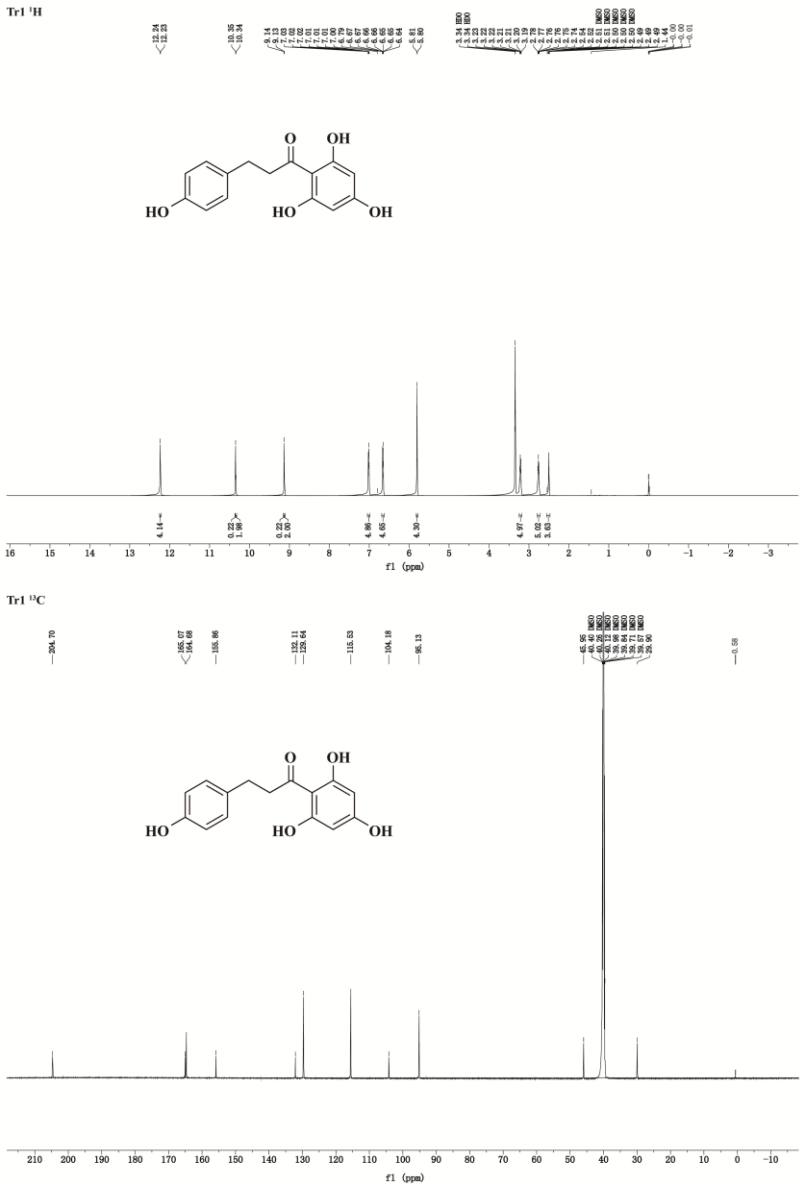

Supplementary Fig.S5 ^{1}H and ^{13}C NMR spectra for Tr1.

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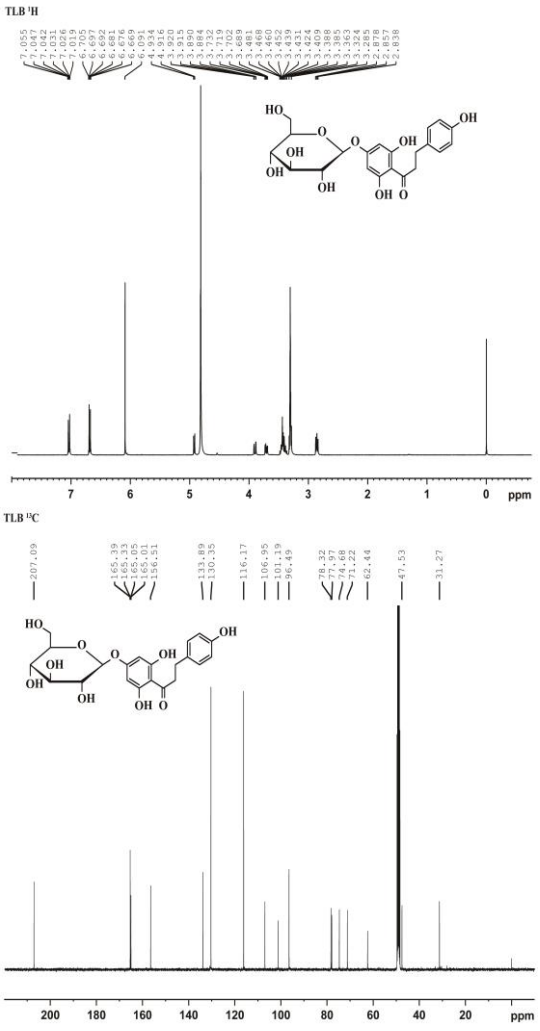

Supplementary Fig.S6 ^{1}H and ^{13}C NMR spectra for TLB.

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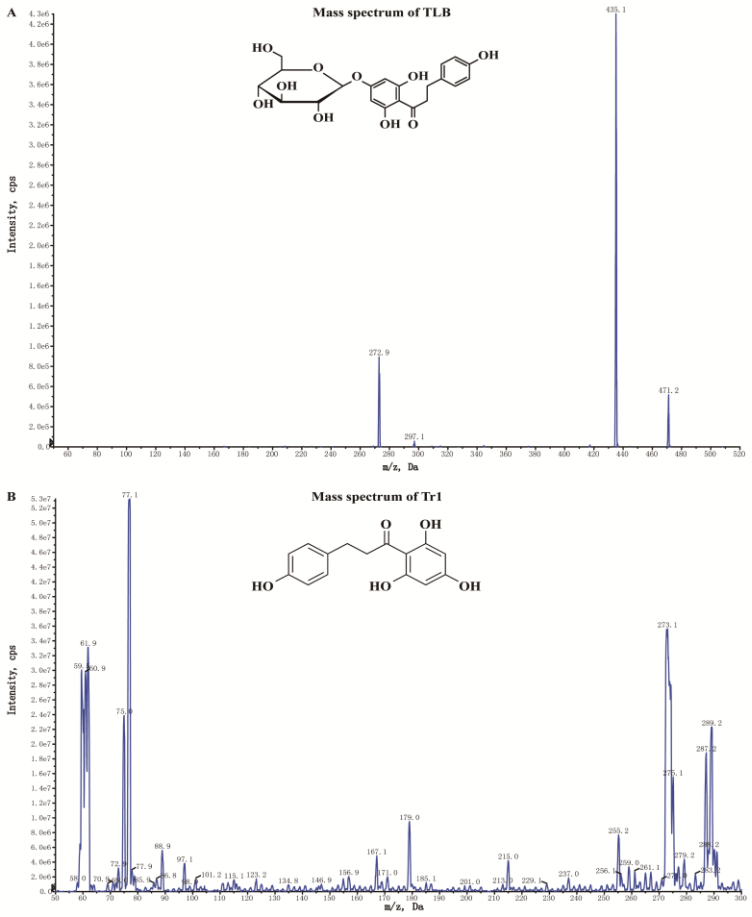

Supplementary Fig.S7 Mass spectra for TLB and Tr1.

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Supplementary data

^{1}H and ^{13}C NMR spectra for Tr1

The NMR data of Tr1, ^{1}H-NMR (400MHz, DMSO) δ 2.74 (2H, t, J=7.6Hz, β-H), 3.19 (2H, t, J=7.6Hz, α-H), 5.80 (2H, s, H-3',5'), 6.65 (2H, d, J=8.5Hz, H-3,5), 7.00 (2H, d, J=8.5Hz, H-2,6), 9.13 (1H, s, 4-OH), 10.34 (1H, s, 4'-OH), 12.23 (2H, s, 2',6'-OH).

^{13}C-NMR (400MHz, DMSO) δ 204.70 (C=O), 165.07 (C-4'), 164.68 (C-2',6'), 155.86 (C-4), 132.11 (C-1), 129.64 (C-2,C-6), 115.53 (C-3,C-5) , 104.18 (C-1'), 95.13 (C-3',C-5'), 45.95 (C-α), 29.90 (C-β).

^{1}H and ^{13}C NMR spectra for TLB

^{1}H-NMR (400MHz, CD_3OD) δ 7.01 (1H, d, J=8.4Hz, H-2,6), 6.67 (1H, d, J=8.4Hz, H-3,5), 6.66 (1H, dd, J=7.2, 1.8Hz, H-6), 6.09 (1H, d, J=2.1Hz, H-5'), 4.91 (2H, d, J=8.5Hz, H2,6), 3.28 (2H, t, m, H-α), 3.28-3.92 (5H, m, H-2''-6''), 2.83 (2H, t, J=7.2Hz, H-β).

^{13}C-NMR (400MHz,CD_3OD) δ 207.09 (C=O), 165.39 (C-4'), 165.05 (C-2'), 165.01 (C-6'), 156.51 (C-4), 133.89 (C-1), 130.35 (C-2, C-6), 116.17 (C-3, C-5), 106.95 (C-1'), 101.19 (C-1''), 96.49 (C-3', C-5'), 78.32 (C-5''), 77.97 (C-3''), 74.68 (C-2''), 71.22 (C-4''), 62.44 (C-6''), 47.53 (C-α), 31.27 (C-β).