Introduction

Microglial activation-mediated neuroinflammation plays an important role in the process of several neuroinflammation-associated diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), and multiple sclerosis (MS) (1, 2). Microglia cells, resident macrophages in the central nervous system (CNS), exist in a resting state characterized by ramified morphology in physiological conditions (3, 4). However, the cells are rapidly activated in response to various immunological stimuli and neuronal injuries (3, 5). Activated microglia turn into an amoeboid type in morphology and release neurotoxic factors including pro-inflammatory cytokines, nitric oxide (NO), prostaglandin E2 (PGE2), and reactive oxygen species (ROS) (6 – 9). Overproduction of these neurotoxic molecules is believed to contribute to neuronal death and neurodegenerative processes (10, 11). Therefore, suppression of microglial activation would be a valuable therapeutic option for treatment of different neurodegenerative diseases.

The fruit of *Melia toosendan* is a well-known traditional Chinese medicine recorded in Chinese Pharmacopoeia. Limonoids are a class of highly oxygenated nortriterpenoids and are abundant in plants of *Rutaceae* and *Meliaceae* families. These compounds have a variety of biological activities such as being antiviral, antibacterial, antifungal, antimalarial, and antineoplastic (12 – 14). It has been reported that two limonoids, isotoosendanin and 1-O-tigloyl-1-O-deacetyl-nimbolinin B inhibit LPS-stimulated inflammatory responses by suppressing NF-κB and JNK activation in microglia cells.
1-O-tigloyl-1-O-debenzoylochinal, which were extracted from *Melia toosendan*, inhibited acetic acid-induced vascular permeability and carrageenan-induced paw edema in ICR mice and displayed significant anti-inflammatory effect without causing acute toxicity in vivo (15). It has been recently reported that several novel limonoids had inhibitory effects against lipopolysaccharide (LPS)-induced NO production in RAW264.7 mouse macrophage cells (16, 17). However, the anti-inflammatory effect of nimbinolins in brain microglia has not been investigated. In the present study, we determined the effects of three nimbinolin-type limonoids, nimbinolin A, nimbinolin B, and 1-O-tigloyl-1-O-deacetyl-nimbinolin B (TNB) (Fig. 1), which were isolated from the fruits of *Melia toosendan*, on brain microglia. Nimbinolin A and nimbinolin B are known compounds, whereas TNB is a newly identified compound with its structure characterized by nuclear magnetic resonance (NMR) (18).

Our data showed that TNB inhibited LPS-induced production of NO, ROS, TNF-α, and their gene expression in cultured microglia. TNB also attenuated NF-κB and JNK activation in LPS-stimulated microglia cells. Furthermore, TNB showed neuroprotective effects by inhibiting microglial neurotoxicity in a microglia-neuron co-culture system. Taken together, our findings suggest that TNB is a potent inhibitor in microglia-mediated inflammation via suppression of NF-κB and the JNK pathway.

**Material and Methods**

**Sample preparation**

Fruits of *Melia toosendan* were collected from Sichuan province, China. Three limonoids, Nimbinolin A, Nimbinolin B, and TNB (> 95%, usually requires 87% or more), were isolated from *Melia toosendan* and identified by extensive spectroscopic methods including UV, IR, NMR, and ESI-MS, as described previously (18). The compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock solution. The final concentration of DMSO in the culture media was less than 0.2%.

**Reagents and cell culture**

Bacterial lipopolysaccharide (LPS) (*E. coli* serotype 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Murine BV-2 microglia cells and HT-22 hippocampal cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, and streptomycin, at 37°C in a humidified incubator with 5% CO₂.

**Isolation of mouse primary microglia**

Mouse primary microglial cultures were prepared by mild trypsinization as described previously with minor modifications (19). Briefly, mixed glial cultures were prepared from cerebral cortices of 1 – 2 day old Institute of Cancer Research (ICR) mice. The cortices were chopped and dissociated by mechanical disruption using a nylon mesh. The dissociated cells were seeded in poly-d-lysine-coated flasks and cultured at 37°C, 5% CO₂. Cultures were replaced every 3 – 4 days with DMEM/F12 with 10% FBS. After culture for 14 days, microglia cells were isolated from mixed glial cultures following shaking of cultures at 150 rpm for 2 h at 37°C. The purity of the cultured microglia cells were more than 95% as determined by CD11b immunostaining (data not shown). Microglia cells were collected for NO and MTT assay.

**Animals used in the current research were acquired and cared for in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by Institutional Review Board of Soochow University.**

**Nitrite assay**

The production of NO by microglial cells was measured using Griess assay (20, 21). Cells (2 × 10⁴) were seeded in 96-well plates in triplicate and pretreated with compounds at different concentrations for 30 min and then stimulated with 200 ng/ml LPS for 24 h. Cell supernatant was collected for measurement of NO production. Samples of 5 μl each were mixed with 50 μl of Griess reagent (1% sulfanilamide / 0.1% naphthylethylene diamine dihydrochloride / 2% phosphoric acid) and the absorbance at 550 nm was measured. Sodium nitrite was used to prepare a standard curve to calculate the concentration of NO.

**Cell viability assay**

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After removing the medium for Griess assay, 30 μl of...
0.5 mg/ml MTT was added to each well and incubated at 37°C for 2 h; then, culture media were discarded, 100 μl DMSO was added to dissolve the formazan dye, and the absorbance was taken at 570 nm.

Enzyme-linked immunosorbent assay (ELISA)

The secretion of TNF-α in culture supernatants was measured as previous described (21, 22) by specific ELISA using rat monoclonal anti-mouse TNF-α antibody as the capture antibody and goat biotinylated polyclonal anti-mouse TNF-α antibody as the detection antibody (ELISA development regents; R&D Systems, Minneapolis, MN, USA). The biotinylated anti-TNF-α antibody was detected by sequential incubation with streptavidin–horseradish peroxidase conjugate and chromogenic substrates. Cells were plated in a 96-well plate at a density of 2 × 10^4 cells/ml and pretreated with compounds for 30 min, followed by stimulation with 200 ng/ml LPS for 24 h. Cell culture media were collected for measurement of TNF-α.

Isolation of total RNA and quantitative real-time PCR

Cells were cultured in a 6-well plate at a density of 2 × 10^5 cells/well. After incubation for 24 h, cells were pretreated with TNB at different concentrations for 30 min, and then stimulated with 100 ng/ml LPS for 8 h. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Takara Bio, Dalian, China). cDNA was amplified using specific primers iNOS, COX-2, TNF-α, IL-1β, or GAPDH: iNOS forward, TAG GCA GAG ATT GGA GGC CTT G, and iNOS reverse, GGG TTG CTG AAC TTC CAG TC; COX-2 forward, CAG GAG GCC ACT GAT ACC TA; TNF-α forward, CAG GAG GGA GAA CAG AAA CTC CA, and GAPDH reverse, CCT GGT TGG CTG CTT CCT GCT T; IL-1β forward, TCC AGG ATG AGG ACA TGA GCA C, and IL-1β reverse, GAA CGT CAC ACA CCA GGA GGT TA; GAPDH forward, TGT GTC CGT GTG GGA TCT GA, and GAPDH reverse, TTGCGT TTG AAG TCG CAG GAG. Quantitative real-time PCR was performed using SYBR Premix II on CFX96 PCR instrument (Bio-Rad, Hercules, CA, USA). The values of obtained for target gene expression on CFX96 PCR instrument (Bio-Rad, Hercules, CA, USA). The values of obtained for target gene expression were normalized to GAPDH and quantified relative to the expression in control samples. \(2^{-([-\Delta \Delta CT])}\) formula was used in the calculations of relative quantification, and \(-\Delta \Delta CT = (CT, target-CT, GAPDH)\) experimental sample – (CT, target-CT, GAPDH) control sample.

Immunofluorescence assay

Nuclear translocation of p65 subunit of NF-κB was determined by immunofluorescence assay as described (23, 24). Microglia cells (2 × 10^4 cells/well in 24-well plates) were cultured on sterile cover slips in 24-well plates and treated with compounds and LPS. After treatment with LPS for 60 min, the cells were fixed with ice-cold methanol for 20 min and washed with PBS for 5 min. The fixed cells were then treated with 1% Triton X-100 in PBS and blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. The permeabilized cells were then incubated with 1 μg/ml of rabbit polyclonal IgG NF-κB p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Cells were washed with 0.05% Tween-20 / 1% BSA in PBS for 5 min. Cells were then incubated in a 1:2000 dilution of Alexa Fluor 488–labeled goat anti-rabbit antibody (Molecular Probes Inc., Eugene, OR, USA) for 60 min at room temperature and washed with 0.05% Tween-20 / 1% BSA in PBS for 5 min. Cells were then stained with 0.2 μg/ml of Hoechst staining solution (Molecular Probes) for 30 min at 37°C and then washed. Finally, the cover slips with cells were dried at 37°C in an oven for 45 min and mounted in a 1:1 mixture of xylene and malinol. More than 50 cells per field were counted under a fluorescence microscope.

Western blot analysis

Cells were lysed in a triple-detergent lysis buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium azide, 1% Nonidet P-40 (Sigma-Aldrich) 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride]. Protein concentration in cell lysates was determined using a protein assay kit (Bio-Rad). Forty micrograms of protein from each sample was separated by SDS–polyacrylamide gel electrophoresis (10% gel) and transferred to polyvinylidene difluoride membrane nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk and sequentially incubated with primary antibodies: polyclonal rabbit anti-mouse COX-2 (Santa Cruz Biotechnology), polyclonal rabbit anti-mouse COX-2 (Santa Cruz Biotechnology); IL-1β antibody (R&D Systems); monoclonal anti-α-tubulin clone B-5-1-2 mouse ascites fluid (Sigma); and antibodies against signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38 mitogen-activated protein kinase (MAPK), p-p38 MAPK, c-Jun N-terminal kinase (JNK), p-JNK, which were purchased from Cell Signaling Technology Inc., Beverly, MA, USA. Secondary antibodies were purchased from Sigma-Aldrich.

Measurement of intracellular ROS

Intracellular ROS was measured with CellROX® Deep Red Reagent (Molecular probes) using FACS analysis. BV-2 microglia cells were plated in a 6-well plate at a...
density of 2 × 10^5 cells/well. The cells were pretreated with 20 μM TNB for 30 min before 200 ng/ml LPS treatment. After 4 h of LPS stimulation, cells were stained with 5 μM of CellROX reagent and Hoechst by adding the probe to the complete medium and incubating the cells for 30 min at 37°C. The cells were then washed with PBS and analyzed by flow cytometry (FACScalibur, 640/665 nm; Becton-Dickinson, Franklin Lakes, NJ, USA).

**Microglia and HT-22 cell co-culture**

The neuroprotective effect of TNB using primary microglia cells were tested by a transwell insert system. Primary microglia cells were cultured at the density of 5 × 10^4 on the transwell insert (pore size 0.4 μm; Corning, NY, USA) in 24-well plates and incubated overnight. Microglia cells were pretreated with TNB for 30 min, and then treated with LPS for 6 h, and after stimulation, inserts were washed with fresh DMEM and then placed into wells in which HT-22 cells were cultured. After cells were co-cultured for 36 h, the inserts were removed and HT-22 cell viability was assessed by MTT assay.

**Statistical analysis**

Results were expressed as the mean ± S.D. The data were analyzed by one-way ANOVA following the Student Newman Keul’s post hoc analysis, using GraphPad Prism 5.0. A value of P < 0.05 was considered statistically significant.

**Results**

**TNB inhibits LPS-induced NO and TNF-α production in microglia**

NO production has been used as a representative measure of inflammatory activation of microglia (25, 26), to test the anti-inflammatory effect of 3 limonoids compounds, we first measured the NO production in LPS-stimulated BV-2 cells. Cells were incubated with different concentrations of limonoid compound (5 – 20 μM) for 30 min prior to LPS treatment. After stimulation with 200 ng/ml LPS for 24 h, the content of nitric oxide in the culture medium was determined. Among the compounds detected, TNB showed strongest inhibitory effect on NO production in LPS-stimulated BV-2 cells (Fig. 2A). The potency order for IC50 of NO inhibition: TNB (7.892 ± 0.897 μM) < nimbolinin A (11.613 ± 1.650 μM) < nimbolinin B (43.439 ± 1.638 μM). To exclude the possibility that the decrease of NO was due to cell death, we determined the cell viability by MTT assay. Results showed that the 3 compounds at the indicated concentrations (5 – 20 μM) did not alter the cell viability (Fig. 2B). TNF-α is another pro-inflammatory cytokine

![Fig. 2.](image-url)
that can initiate the inflammatory response and mediate the development of chronic inflammatory diseases (27 – 29). We measured the production of TNF-α in the culture supernatants by ELISA. In agreement with the results of NO, TNB markedly inhibited LPS-induced production of TNF-α in a dose dependent manner. Nimbinin B and nimbinin A only showed weak inhibitory effect at the indicated concentrations compared with TNB in LPS-stimulated BV-2 cells (Fig. 2C). Based on its strongest inhibitory effect on NO and TNF-α production in microglia cells, we thus focused on the anti-inflammatory activity of TNB in the following experiments. A similar result was observed in primary microglia cells that was pretreated with TNB (20 μM) for 30 min prior to LPS treatment for 24 h, TNB significantly inhibited the content of NO and TNF-α in the supernatants (Fig. 2: D and E). Again, TNB showed no significant cytotoxicity on primary microglia cells (data not shown).

TNB suppresses LPS-induced expression of pro-inflammatory genes and generation of ROS in microglia

We further investigated the effects of TNB on the gene expression of pro-inflammatory cytokines by quantitative real-time PCR. As shown in Fig. 3A, TNB inhibited the gene expression of iNOS, COX-2, TNF-α, and IL-1β in a dose-dependent manner in BV-2 cells. The effect of the TNB on iNOS, COX-2, and IL-1β expression was further assessed at the protein levels by western blot analysis. As shown in Fig. 3B, TNB also suppressed LPS-induced iNOS, COX-2, and IL-1β expression. LPS stimulated microglia cells to produce ROS that is associated with the neuroinflammation and neurodegeneration (10). Thus, we examined the effects of TNB on generation of intracellular ROS using CellROX® Deep Red probe. TNB markedly diminished LPS-induced ROS generation in BV-2 microglia cells (Fig. 3C).

TNB inhibited LPS-induced NF-κB and JNK activation

Since NF-κB plays an important role in the modulation of various pro-inflammatory mediators such as iNOS, COX2, IL-1β, and TNF-α in microglia cells (21, 30 – 33), we thus determined whether TNB affected NF-κB activation in microglia by immunofluorescence analysis and western blotting. As shown in Fig. 4, A and B, TNB inhibited the LPS-induced p65 subunit of NF-κB translocation into the nucleus. This is further confirmed by the result of western blotting analysis that indicated that TNB inhibited LPS-induced nuclear translocation of p65 in BV-2 microglia cells (Fig. 4C). As expected, TNB attenuated LPS-induced IκBα degradation and increase of IκBα phosphorylation (Fig. 4D). Since it is known that LPS induced phosphorylation of IκBα is dependent on the activation of IκB kinase (IKK) (34), we also demonstrated that LPS-stimulated phosphorylation of IκBα/β was markedly attenuated by TNB (Fig. 4D).

To confirm that TNB suppressed LPS-induced gene expression via the NF-κB pathway, we next examined the synergic effect of TNB and NF-κB inhibitor on LPS-induced gene expression. As shown in Fig 4E, the effect of TNB-inhibited mRNA expression of iNOS and TNF-α was consistent and showed synergistic action when co-treated with the specific NF-κB inhibitor PDTC on LPS-stimulated BV-2 microglia cells (Fig. 4E). Along with NF-κB, MAPKs have been shown to play an important role in signaling pathways that regulate pro-inflammatory cytokines, iNOS, and COX-2 expression in microglia cells (21, 35, 36). Thus, the effect of TNB on MAPKs (ERK, p38, JNK) pathways was investigated. Interestingly, TNB selectively inhibited LPS-induced phosphorylation of JNK without alteration of LPS-activated ERK and p38 (Fig. 5A). We also found that TNB and the specific JNK inhibitor, SP600125, synergically inhibited iNOS and TNF-α expression in LPS-stimulated BV-2 microglia cells (Fig. 5B). Taken together, these results indicated that inhibitory effects of TNB on pro-inflammatory mediators and cytokines were mediated by inhibiting NF-κB and the JNK signaling pathway in LPS-stimulated microglia cells.

TNB protects the HT-22 hippocampal cells from activated microglia-mediated toxicity

It is well-known that the various neuro-inflammatory mediators released by activated microglial cells are involved in neuronal cell death and promoting progression of neuronal degeneration (37). Pretreatment of TNB markedly attenuated the cytotoxicity of LPS-stimulated microglia cells toward HT-22 cells (Fig. 6A). Moreover, TNB alone did not affect HT-22 cell viability without co-culture in the presence of LPS (Fig. 6B). The results suggested that the protective effect of TNB is most likely attributed to the inhibition of microglia activation, but not the direct protection on HT-22 cells.

Discussion

In the present study, we first compared the anti-inflammatory effects of three limonoid-type compounds in microglia cells. We demonstrated that TNB inhibited the activation of NF-κB and JNK signaling pathways that may be the potential mechanism for the drug’s inhibitory effects on the production of pro-inflammatory mediators in LPS-stimulated microglia cells. Furthermore, TNB elicited neuronal protection via suppression of microglia activation.
Fig. 3. Effects of TNB on the expression of proinflammatory genes and ROS generation in LPS-stimulated microglia cells. A) BV-2 microglia cells were pre-treated with TNB (5 – 20 μM) for 30 min and then stimulated with 200 ng/ml LPS for 8 h. Total RNA was prepared for quantitative real-time PCR assay. GAPDH were used as the internal control for iNOS, COX-2, TNF-α, and IL-1β. B) After 16 h of LPS stimulation, the cell lysates (40 μg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-iNOS or COX-2 or IL-1β antibody (left). The α-tubulin was used as an internal control. Quantification of iNOS, COX-2, and IL-1β protein expression was performed by densitometric analysis (right). The data are the mean ± S.D. of 3 independent experiments. *P < 0.05, **P < 0.01, significantly different from the value treated with LPS only, which was set to 100% (lane 3). C) BV-2 cells were pre-treated with TNB for 30 min, followed by treatment with 200 ng/ml LPS for 4 h. After incubation with 5 μM CellROX reagent, the generation of intracellular ROS was measured by flow cytometry.
Microglia-mediated neuroinflammation is considered to play a critical role in the progression of neurodegenerative diseases such as AD and PD (38–40). Excessive and chronic activation of microglia leads to increased secretion of various pro-inflammatory molecules that may contribute neuronal damage and neurodegeneration (39, 41, 42). Therefore, inhibition of microglia activation could be a potential therapeutic target in neurodegenerative diseases. The fruit of *Melia toosendan* is a well-known traditional Chinese medicine that has been used in the treatment of cholelithiasis, stomachache, and various inflammations such as gastritis, mastitis, and cholecystitis, and also for ascariasis (12–14). Although anti-inflammatory effects of some limonoids isolated from fruits of *Melia toosendan* in the periphery have been previously reported (15), the effects of nimbin...
Anti-inflammatory Effect of TNB

Type limonoids on the microglia-mediated inflammatory response has not been investigated. We found that the 3 limonoid-type compounds exhibited different potencies in inhibiting NO production in microglia cells. NO, ROS, and cytokines such as TNF-α, IL-1β are considered key factors involved in neuroinflammatory responses and in microglia-mediated neurotoxicity (43–45). Our data showed that TNB strongly inhibited the release of NO and TNF-α without cytotoxicity in LPS-stimulated BV-2 cells, but nimbolinin B only had weak inhibitory effects on NO production at the tested concentration (Fig. 2). These results suggested that the anti-inflammatory activities of the 3 limonoids are related to their chemical structure; the R₁ and R₂ substituents of nimbolinin A are the acetyl group and Bz group and for nimbolinin B, the acetyl group and tigloyl group, respectively, while both the R₁ and R₂ substituent of TNB is the tigloyl group, which has a high liposolubility (Fig. 1). It is conceivable that the high liposolubility the tigloyl group of TNB rendered the compound easier to penetrate into the cells. These results indicated that limonoid TNB may provide a potential candidate for drug discovery in neuroinflammatory diseases. Supporting this, TNB inhibited the generation of ROS and suppressed the gene expression of iNOS, COX-2, TNF-α, and IL-1β in LPS-induced BV-2 microglia cells.

In our experiments with microglia cells, we observed that 20 μM TNB pretreatment led to almost complete inhibition in LPS-stimulated NO production, while TNB led to only a 50% inhibition of LPS-stimulated TNF-α production in microglia cells (Fig. 2E). This phenomenon was possibly due to the following reasons: 1) TNF-α belongs to early primary responsive genes that usually contain CpG island promoter and are expressed rapidly (within 1h of stimulation) and independently of nucleosome remodeling (46, 47). 2) In our present study,
microglia cells were pretreated with TNB for 30 min before LPS stimulation, which was not enough time for the limonoids to act completely on these primary response genes, whereas a secondary response gene such as iNOS is not affected (Figs. 2 and 3A).

The transcription factor NF-κB is a key regulator of various genes such as iNOS, COX-2, TNF-α, and IL-1β involved in the inflammatory responses (32, 33, 48). It has been well studied that the NF-κB activation results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB proteins followed by translocation from the cytoplasm to nucleus (49, 50). Recent studies have demonstrated that several agents confer anti-inflammatory effects through blockade of NF-κB activation in LPS-stimulated BV-2 microglia cells (21, 37, 51). In agreement with this study, we found that TNB inhibited the p65 subunit of NF-κB translocation from the cytosol to the nucleus by blocking IKKα/β activation in LPS-stimulated BV-2 cells. Additionally, TNB inhibited LPS-induced activation of JNK, which has been implicated in the signal transduction pathways responsible for increased proinflammatory genes expression in LPS-stimulated microglia cells (53, 54). Moreover, we demonstrated the synergic effects of TNB and a specific inhibitor of NF-κB or JNK on LPS-induced gene expression. Taken together, these results suggested that blockade of NF-κB and JNK signaling may be a molecular mechanism underlying the anti-inflammatory effect of TNB in CNS microglia.

Excessively activated microglia contributes to neurodegenerative processes through producing a number of neurotoxic molecules including free radicals and proinflammatory cytokines. Therefore, inhibition of microglial activation will be beneficial to neurodegenerative diseases (37, 55). In fact, many anti-inflammatory agents, which inhibited microglial activation or production of proinflammatory mediators under CNS disease conditions, attenuated neuronal degeneration (37, 56, 57). The present study showed that TNB protected neuroblastoma cells against microglial neurotoxicity in a neuron/microglia co-culture (Fig. 6). Although the co-culture of the LPS-stimulated microglia with neuroblastoma cell line may not be the same as the in vivo conditions, it partially reflects the pathological conditions where activated microglia influences the death and survival of neuronal cells in neurodegenerative diseases. It is required to further evaluate the precise mechanism of anti-inflammatory activity of TNB and investigate the neuroprotective effects using an in vivo model in future studies. Nevertheless, this is the first study that demonstrated TNB elicited its neuroprotective effect via inhibition of microglial activation.

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