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2-cyclohexylamino-5, 8-dimethoxy-1, 4-naphthoquinone inhibits LPS-induced BV2 microglial activation through MAPK/ NF-kB signaling pathways

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Abstract

Aims: To verify the effects of several 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) derivatives on LPS-induced NO production, cellular ROS levels and cytokine expression in BV-2 microglial cells.

Main methods: An MTT assay and FACS flow cytometry were performed to assess the cellular viability and apoptosis and cellular ROS levels, respectively. To examine the expression of pro-inflammatory cytokines and cellular signaling pathways, semi-quantitative RT-PCR and Western blotting were also used in this study.

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Key findings: Among the six newly synthesized DMNQ derivatives, 2-cyclohexylamino-5,8-dimethoxy-1,4-naphthoquinone (R6) significantly inhibited the NO production, cellular ROS levels and the cytokines expression in BV-2 microglial cells, which stimulated by LPS. Signaling study showed that compound R6 treatment also significantly down-regulated the LPS-induced phosphorylation of MAPKs (ERK, JNK and p38) and decreased the degradation of IκB-α in BV2 microglial cells.

Significance: Our findings demonstrate that our newly synthesized compound derived from DMNQ, 2-cyclohexylamino-5,8-dimethoxy-1,4-naphthoquinone (R6), might be a therapeutic agent for the treatment of glia-mediated neuroinflammatory diseases.

Keywords: Neuroscience, Immunology, Cell biology, Medicine, Biochemistry

1. Introduction

Inflammation plays an important role in the pathology of neurodegenerative disorders in the brain. Microglia are glial cells that function as the prime effector cells in immune defense and inflammatory responses in the central nervous system (CNS) [1, 2, 3]. Increased evidences showed, activated microglia was involved in pathological processes for diseases such as Alzheimer's disease [4], Parkinson's disease [5], and multiple sclerosis [6], through producing the cytokines and superoxides. In response to pro-inflammatory triggers, microglia exhibited active phenotype, resulting in a shift of cellular function and subsequent release of cytotoxic factors (e.g., tumor necrosis factor-alpha [TNF-α], nitric oxide [NO], and reactive oxygen species [ROS]) aimed at destroying the invading pathogens. All of these evidences suggest that microglia can become a major source of cytokines and ROS production to drive progressive neuronal damages, and these damages are implicated in the chronic nature of neurodegenerative diseases.

In response to environmental changes, such as neuronal damage, microglial cells proliferate and become phagocytic, and up-regulate the expression of various molecules (cytokines, adhesion molecules, and transcription factors) [7, 8, 9, 10]. Inflammatory agonists, such as bacterial lipopolysaccharides (LPS), the β-amyloid-related peptides, and human immunodeficiency virus (HIV) coat protein gp120, could induce the microglial cells activation by producing many inflammatory factors, such as TNF-α, interleukin-1betaβ (IL-1β) and NO, which involves in glia-mediated neurotoxicity [11, 12].

Thus, finding new compounds to control the activity of microglial cells may be critical to limiting glia-mediated neurotoxicity and its consequences and delaying the progression of neurodegenerative diseases and neuroinflammation in the CNS.

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Naphthoquinones are widely distributed in nature and play important physiological roles in animals and plants. Their derivatives have exhibited a variety of biological responses, which include anti-allergic, anti-bacterial, anti-fungal, anti-inflammatory, anti-thrombotic, anti-platelet, anti-viral, apoptosis, lipoxygenase, radical scavenging, and anti-ringworm activities [13, 14]. As a consequence, the molecular framework of many pharmaceuticals and biologically important compounds contain a quinine moiety. The 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) used as common start compound to synthesize Naphthoquinone derivatives, and it was reported that DMNQ derivatives exhibit the anti-tumor activity in breast cancers [15, 16], and could also prevent cell proliferations through regulating the cellular MAPK and PI3 K signaling pathways [17, 18]. But the inhibitory effect of naphthoquinone derivatives on the LPS-induced activation of microglial cells is not yet understood.

In the present studies, we investigated the potency of serial derivatives synthesized from DMNQ as inhibitors of BV2 microglia activation by probing NO production, the expression of cytokines (e.g., IL-6, TNF-α and IL-1β), and cellular ROS levels and their mechanism of action including MAPK and NF-kB signaling pathways.

2. Materials and methods

2.1. Reagents

Lipopolysaccharides (LPS, from *Escherichia coli* serotype 0111:B4) were purchased from Sigma (St. Louis, MO, USA), and the iNOS inhibitor S-methylisothiourea sulfate (SMT) was obtained from Calbiochem (San Diego, CA, USA). A classical Michael addition reaction was used to synthesize 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) derivatives.

2.2. Synthesis of 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) derivatives

The synthetic schemes for 2-substituted amino-DMNQ derivatives are summarized in Suppl. 1. The starting material was 5-dihydroxynaphthalene (Fig. 1A), and it was reacted with sodium hydroxide and dimethyl sulfate under nitrogen to produce 5,8-dimethoxynaphthalene (Fig. 1B). This compound was then brominated with *N*-bromosuccinimide (NBS) at room temperature for 3 h to yield 1,5-dibromo-4,8-dimethoxynaphthalene (Fig. 1C). After methoxylation with sodium methoxide and copper (I) iodide in a *N,N*-dimethyl formamide/methanol solution, oxidative demethylation of the 1,4,5,8-tetramethoxynaphthalene (Fig. 1D) was performed with cerium (IV) ammonium nitrate (CAN) to produce the key intermediate, DMNQ (Fig. 1E). The direct 1,4- addition of various alkylamines to the quinone moiety of DMNQ (Fig. 1F) synthesized the appropriated 2-alkylamino-DMNQs,

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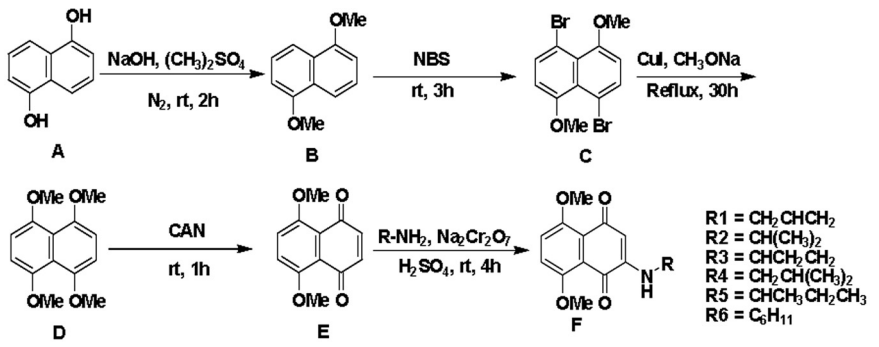

Fig. 1. Simplified diagram for synthesis of the DMNQ derivatives.

with yields varying from 23.6 to 55.5%. The compounds used in this study are marked as R1 to R6, and their full names are summarized in Table 1.

2.3. Cell culture

BV2 microglial cells were cultured in Dulbucco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and penicillin-streptomycin (100 U/ml, 100 μg/ml). The BV2 microglial cells were pre-treated with 30 μM of the DMNQ derivatives, followed by treatment with 1 μg/mL of LPS.

2.4. Cell viability assay

Cell viability was quantitatively determined using a 3-[4][4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, BV2 microglial cells were grown in 96-multi-well plates in DMEM in the presence of only the R6 compound at the indicated concentration ranges (0 μM to 30 μM) for 24 h. The produced formazan was quantified by measuring the absorbance of the

Table 1. The full name of DMNQ derivatives used in the experiments.

R	No	Full Name
Propene-	R1	2-Vinylamino-5,8-dimethoxy-1,4-naphthoquinone
i-propyl-	R2	2-i-Propylamino-5,8-dimethoxy-1,4-naphthoquinone
Cyclopropyl-	R3	2-Cylopropylamino-5,8dimethoxy-1,4-naphthoquinone
i-Butyl-	R4	2-i-Butylamino-5,8-dimethoxy-1,4-naphthoquinone
l-methylpropyl-	R5	2-(1-Methylpropylamino-5,8-dimethoxy-1,4-naphthoquinone
Cyclohexyl-	R6	2-cyclohexylamino-5,8-dimethoxy-1,4-naphthoquinone

R: substituent group; No: Number.

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dye solution at 490 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA).

2.5. Biochemical assay for the production of NO

NO production was assessed based on the accumulation of nitrite in the medium using a colorimetric reaction with Griess reagent (0.1% N-[1][1-naphthyl] ethylenediamine dihydrochloride, 0.1% sulfanilamide, and 2.5% H_3PO_4). Briefly, the culture supernatants were collected and mixed with an equal volume of Griess reagent for 10 min and by measuring absorbance at 540 nm with a UV MAX kinetic microtiter plate reader.

2.6. Western blotting analysis

Protein lysates (30 μg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted with antibodies against IκB-α (Santa Cruz Biotechnology, USA), iNOS (Upstate Biotech, Charlottesville, VA, USA), pERK, pJNK (Santa Cruz Biotechnology, USA), and NAPDH (Sigma, St. Louis, MO, USA) at 4 °C overnight and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) or anti-mouse IgG (Sigma) for 1 h at room temperature (RT). The specific binding was detected using a chemiluminescence detection system (Amersham, Berkshire, UK) according to the manufacturer's instructions.

2.7. RNA isolation and semi-quantitative RT-PCR analysis

To isolate the total RNA, the cells were lysed with Trizol (Invitrogen). After chloroform was added at 1/5 volume of Trizol used, the cell lysates were mixed thoroughly by vortexing and centrifuged at 15,000 g for 15 min at 4 °C. Upper phase solution was harvested and mixed by equal volume of isopropanol. After centrifugation at 12,000 g for 8 min at RT, the precipitated RNA was washed with 75% EtOH and melted with DDW. The first-strand cDNA was synthesized from 0.5 μg of DNase-treated total RNA using 0.5 μg random hexamers (Invitrogen), and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a volume of 20 μl at 37 °C for 60 min. The first strand cDNA (1 μl) was used for PCR amplification in a 25 μl reaction mixture. PCR was performed under the following conditions: 94 °C for 30 sec, 55–60 °C for 30 sec, and 72 °C for 30 sec, with additional incubation for 10 min at 72 °C after cycle completion. Primers for iNOS forward:5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3'; reverse:5'-GGC TGT CAG AGC CTC GTG GCT TTG G −3'; for IL-1β forward 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'; reverse 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'; for TNF-α forward 5'-CTC AAA TGG GCT TTC

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CGA ATT-3'; reverse 5'-TCC AGC CTC ATT CTG AGA CAG A-3'; for IL-6 forward 5'-AGA AGG AGT GGC TAA GGA CCA A-3'; reverse 5'-AAC GCA CTA GGT TTG CCG AGT A-3'; for GAPDH Forward 5'-TTC ACC ACC ATG GAG AAG GC-3'; Reverse: 5'-GGC ATG GAC TGT GGT CAT GA-3'. The amplified DNA fragments were quantified by densitometry using the MULTI-ANALYST program of Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

2.8. Measurement of ROS by flow cytometry

BV-2 cells were incubated with 10 mM CM-H_2DCFDA (Invitrogen), a fluorescence-based ROS indicator, at 37 °C for 15 min at the end of the different treatments. The DCF fluorescence intensities of 10,000 cells were analyzed by FACScan (BD FACSCalibur).

2.9. Statistical analysis

Data are expressed as mean ± SD. Differences between groups were tested for statistical significance using the Student's t test, and p values of < 0.05 were considered significant.

3. Results

3.1. Compound R6 inhibits NO production in BV2 microglial cells

To examine the inhibitory effects of the DMNQ derivatives on the NO production of BV2 microglia, the BV2 cells were pre-treated with 30 μM of the compounds for 30 min and were then treated with LPS (1 μg/ml) for 24 h. As shown in Fig. 2, the compounds R1, R2, R4, R5 and R6 significantly inhibited the production of NO, whereas compound R3 had no inhibitory effect. Moreover, compound R6 exhibited a greater inhibitory effect on the LPS-induced production of NO than the other compounds. Thus, our following research focused on the functional studies of compound R6. To investigate whether compound R6 could affect the cell viability, BV-2 microglial cells were treated with compound R6 without LPS stimulation, and the cell viability was measured by an MTT assay (Fig. 3). The results showed that compound R6 did not affect the cell viability.

3.2 Compound R6 inhibits LPS-induced NO production and iNOS expression in BV2 microglial cells

To examine the concentration dependence and time course of the inhibitory activity of compound R6 on NO production and the iNOS protein expression, the BV-2 microglial cells were pre-treated with compound R6 at various concentrations and then subsequently treated with LPS (1 μg/ml) for 24 h. As shown in Fig. 4 (A and

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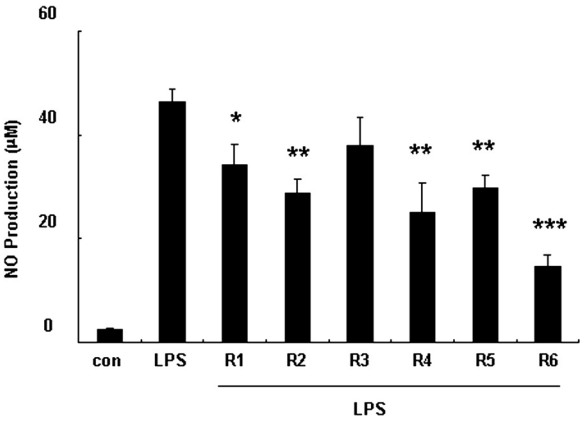

Fig. 2. New compounds inhibit the LPS-induced production of NO in BV2 microglial cells. BV2 microglial cells were pre-treated with the indicated compounds for 30 min followed by treatment with LPS (1 μg/mL) for 24 h. Then, the NO production was analyzed in the medium using Griess reagent. Three independent replicates were performed for all the experiments. The data are presented as the mean ± SD. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.001.

C), that compound R6 dose-dependently inhibited the LPS-induced NO production (approximately 35% at 30 μM) and iNOS expression (almost basal levels at 30 μM). Followed experiments were performed to understand of the inhibitory effect of compound R6 on NO secretion. The BV-2 microglial cells were pre-treated with compound R6 (30 μM), followed by LPS (1 μg/ml) stimulation for indicated times. As shown in Fig. 4 (B and D), that compound R6 time-dependently inhibited NO production and iNOS expression. To evaluate the inhibitory efficiency of compound R6 on the LPS-induced NO production, we compared its inhibitory

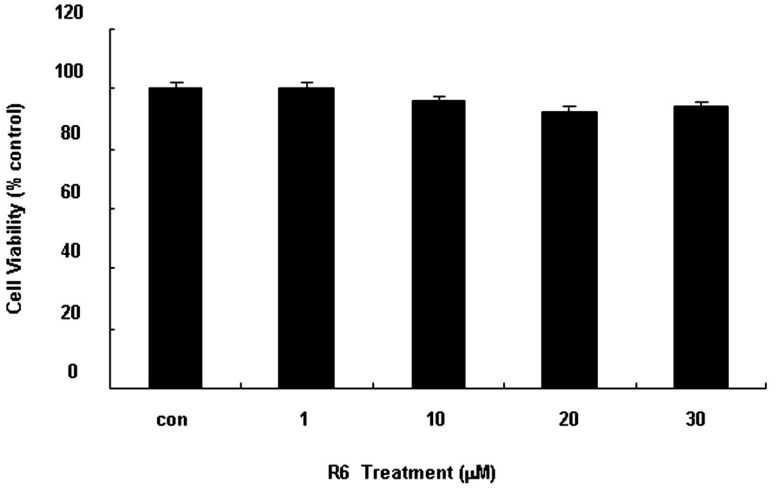

Fig. 3. Effect of compound R6 on cell viabilities. The BV2 microglial cells were treated with compound R6 at different concentrations for 24 h without LPS treatment. Cell viabilities were probed using an MTT assay. Three independent replicates were performed for all the experiments. The data are presented as the mean ± SD. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.001.

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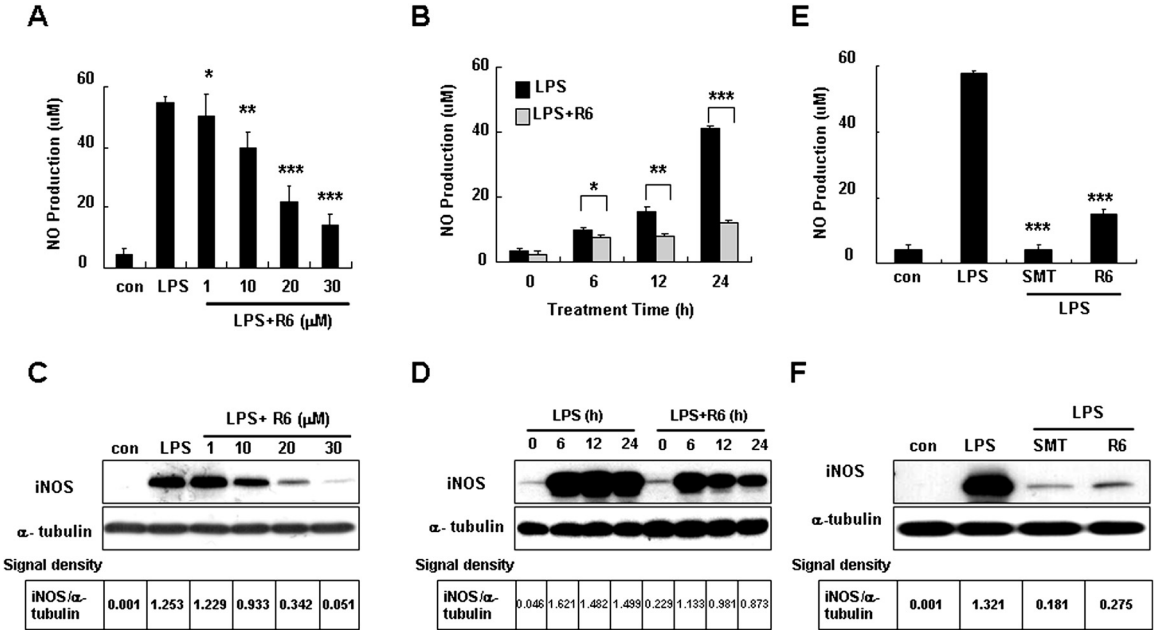
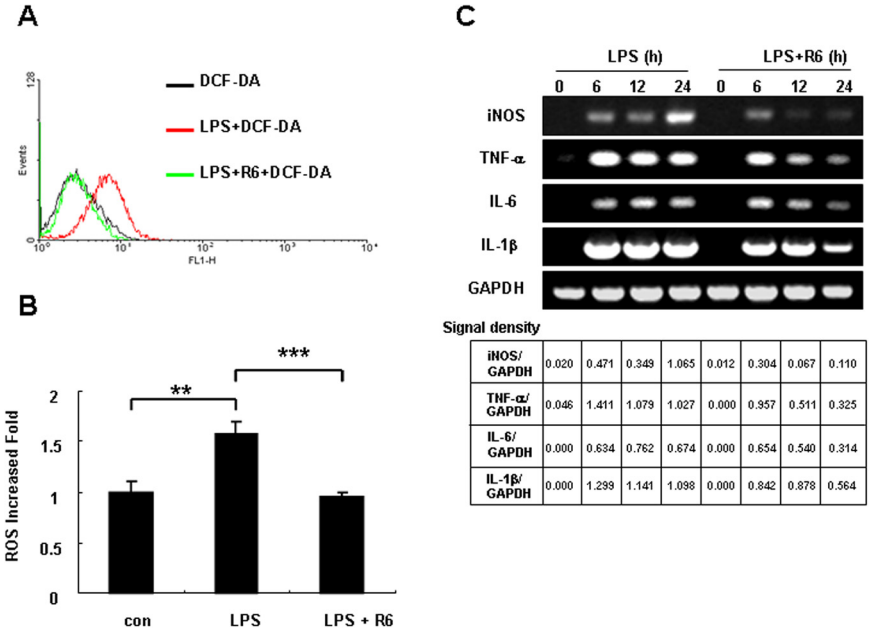


Fig. 4. Compound R6 inhibits the LPS-induced production of NO and expression of iNOS in BV2 microglial cells. BV-2 microglial cells were pre-treated with various concentrations of compound R6 (1 μM, 10 μM, 20 μM and 30 μM) for 30 min, followed by LPS (1 μg/mL) stimulation for 24 h, and the NO production (A) and iNOS expression (C) were detected using Griess reagent and Western blotting, respectively. BV-2 microglial cells were pre-treated with compound R6 (30 μM) for 30 min, followed by LPS (1 μg/mL) stimulation for the indicated times. NO production (B) was detected in the medium using Griess reagent, and the cellular iNOS expression was examined by Western blotting (D). BV-2 microglial cells were pre-treated with compound R6 (30 μM) and SMT (a selective inhibitor of iNOS, 1 mM) for 30 min, followed by LPS (1 μg/mL) stimulation for the indicated times. NO production (E) was detected in the medium using Griess reagent, and the cellular iNOS expression was examined by Western blotting (F). Three independent replicates were performed for all the experiments. The data are presented as the mean ± SD. *$p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$. Full, unmodified images of this figure are available as Supplementary Material.

activity with SMT, a selective inhibitor of iNOS (Figs. 4 E and F). The results showed that compound R6 inhibitory efficiency on LPS-induced microglial NO production and iNOS expression were similar to that of SMT, suggesting that compound R6 may be a good inhibitor candidate for NO production.

3.3 Compound R6 decreases LPS-induced cellular ROS levels and the expression of cytokines in BV2 microglial cells

LPS stimulation triggers the production of inflammatory mediators, the expression of cytokines and an increase in cellular ROS levels. To investigate the effect of compound R6 on the LPS-induced microglial ROS levels and the expression of pro-inflammatory cytokines, BV-2 microglial cells were pre-treated with compound R6 for 30 min, followed by LPS (1 μg/ml) stimulation for the indicated times. As shown in Fig. 5C, the LPS-induced mRNA expression of pro-inflammatory cytokines, such as TNF-α, iNOS, IL-6 and IL-1β, and the cellular ROS levels were significantly down-regulated by treatment with compound R6 (Figs. 5 A and B).



iNOS/GAPDH	0.020	0.471	0.349	1.065	0.012	0.304	0.067	0.110
TNF-α/GAPDH	0.046	1.411	1.079	1.027	0.000	0.957	0.511	0.325
IL-6/GAPDH	0.000	0.634	0.762	0.674	0.000	0.654	0.540	0.314
IL-1β/GAPDH	0.000	1.299	1.141	1.098	0.000	0.842	0.878	0.564

Fig. 5. Compound R6 decreases LPS-induced cellular ROS levels and the expression of cytokines in BV2 microglial cells. BV-2 microglial cells were pre-treated with compound R6 (30 μM) for 30 min, followed by LPS stimulation. Then, the intracellular ROS levels were analyzed by FACS. (A) The increased fold of ROS levels are presented by the mean ± SD (n = 3). (B) The mRNA expression of iNOS, TNF-α, IL-6 and IL-1β were examined by semi-quantitative RT-PCR (C). Three independent replicates were performed for all the experiments. **$p < 0.01$, ***$p < 0.001$. Full, unmodified images of this figure are available as Supplementary Material.

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3.4 Compound R6 down-regulates the LPS-induced phosphorylation of MAPKs and IκB-α degradation

To understand the mechanism of action for the inhibition of the LPS-induced NO production and the expression of cytokines by compound R6, we examined the effect of compound R6 on the phosphorylation of MAPKs (JNK, p38, ERK,) and IκB-α degradation stimulated by LPS. BV2 microglial cells were pre-treated with 30 μM of compound R6 for 30 min, followed by LPS (1 μg/ml) treatments for indicated times. As shown in Fig. 6, that compound R6 significantly down-regulated the LPS-induced phosphorylation of MAPKs (JNK, ERK and p38) (Figs. 6 A–C) as well as IκB-α degradation (Fig. 6 D).

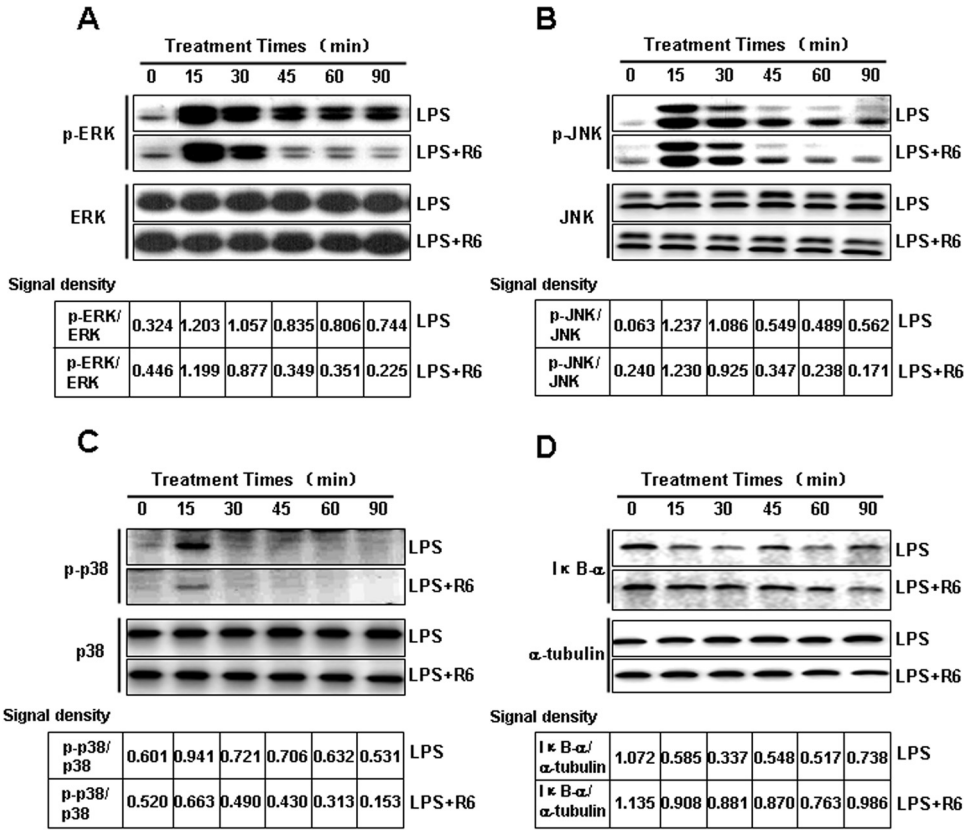

Fig. 6. Compound R6 down-regulates the phosphorylation of MAPKs and IκB-α degradation. BV2 microglial cells were pre-treated with compound R6 (30 μM) for 30 min, followed by LPS (1 μg/ml) treatment for the indicated times. The Western blot was performed to examine the phosphorylation levels for the MAPKs, including ERK (A), JNK (B) and p38 (C). The effect of compound R6 on the NF-κB pathway was also examined by detecting the degradation of IκB-α (D). Three independent replicates were performed for all the experiments. Full, unmodified images of this figure are available as Supplementary Material.

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4. Discussion

Microglia play a crucial roles in neurodegenerative disease by either single or chronic exposure to environmental toxins, neuronal damage, cytokines, and disease proteins [19]. Increasing evidence has shown that many inflammatory mediators, such as TNF-α, IL-1β, and IL-6, have been found in the striatum in human Parkinson's Disease (PD) and Alzheimer's Disease (AD) postmortem brains [20, 21, 22, 23], and an up-regulation of iNOS and cyclooxygenase 2 in amoeboid microglia are located in the substantia nigra of PD patients [24]. Thus, controlling the activation of the brain microglia may be an important approach to delay the progress of neuron degenerative diseases.

1,4-naphthoquinone derivatives have powerful pharmacological effects of antimicrobial and antitumor activities [25, 26], and these derivatives may be toxic to cells through a number of mechanisms, including intercalation, arylation, redox cycling, induction DNA strand breaks, and the generation of free radicals [27]. In this study, we examined the effect of six newly synthesized DMNQ derivatives on the LPS-induced production of NO in BV2 microglial cells. The results showed that among the six newly synthesized DMNQ derivatives, compound R6 exhibited the good inhibitory effect on the LPS-induced production of NO in BV2 microglial cells (Fig. 2) and did not affect the cell viability (Fig. 3). When examining the dose- and time-dependent inhibitory effect of compound R6 on the LPS-induced production of NO in BV2 microglial and the expression of iNOS protein (Figs. 4 A–D), it was found that compound R6 exhibited a significant inhibitory effect on NO production through decreasing the expression of the iNOS protein in BV2 microglial cells.

LPS stimulation triggers diverse microglial responses, including phagocytosis, ROS production, and inflammatory mediator production through MAPKs signaling cascades in microglial cells [28, 29]. ROS play a essential role in microglial activation and in the progression of neurodegenerative diseases, such as Alzhiemer's disease and Parkinson's disease [30]. Compound R6 significantly attenuated cellular ROS levels stimulated by LPS in BV2 microglial cells (Fig. 5 A and B). Furthermore, LPS stimulation could also increase the expression of cytokines in microglial cells [25, 26]. Thus, the effects of compound R6 on the LPS-induced gene expression of cytokines were also determined (Fig. 5C). The results showed that compound R6 significantly inhibited the mRNA expression of iNOS, TNF-α, IL-6 and IL-1β, suggesting that compound R6 has a potency to inhibit the LPS-induced activation of BV2 microglial cells. All these phenomena suggest that ROS-dependent signaling pathways are most likely responsible for the inhibitory effects described above.

MAPK family plays crucial roles in the LPS-induced pro-inflammatory products and neuroinflammation [31, 32, 33]. It was reported that DMNQ derivatives

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exhibit anti-tumor activity by inducing apoptosis via caspases and mitogen activated protein (MAP) kinase-dependent pathways [34, 35, 36]. Our previous study showed that JNK signaling plays a important role in the regulation of the LPS-stimulated production of NO and the expression of iNOS, and the regulatory effect of the JNK signaling pathway on NO production was dependent on cellular ROS [37]. Additionally, increasing evidence suggests that the ROS-dependent NF-κB signaling pathway also participates in the LPS-induced expression of cytokines and activation of microglial cells [38, 39, 40, 41, 42, 43]. Our results showed that treatment with compound R6 significantly down-regulated the LPS-induced phosphorylation of MAPKs (ERK, JNK and p38) and the degradation of IκB-α (NF-κB inhibitor) (Fig. 6) in an early time course, suggesting that compound R6 exhibits its inhibitory effect on the LPS-induced activation of BV2 microglial cells through blocking the primary signaling pathways that are ROS-dependent.

5. Conclusion

Taken together, our results showed that compound R6, synthesized from DMNQ, significantly inhibited NO production, the expression of cytokines and decreased the cellular ROS levels in LPS-stimulated BV-2 microglial cells by down-regulating the ROS-dependent MAPK/NF-κB signaling pathways. Our findings provide a new approach to therapeutic targets for glia-mediated neuroinflammatory diseases.

Declarations

Author contribution statement

Hu-Nan Sun: Performed the experiments; Wrote the paper.

Gui-Nan Shen, Yong-Zhe Jin, Yu Jin, Ying-Hao Han, Li Feng, Lei Liu, Mei-Hua Jin, Ying-Hua Luo, Tea-Ho Kwon, and Yu-Dong Cui: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Cheng-Hao Jin: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2016.e00132

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