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Design, synthesis and evaluation of a series of non-steroidal anti-inflammatory drug conjugates as novel neuroinflammatory inhibitors

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ABSTRACT

Neuroinflammation is involved in the process of several central nervous system (CNS) diseases such as Parkinson's disease, Alzheimer's disease, ischemia and multiple sclerosis. As the macrophages in the central nervous system, microglial cell function in the innate immunity of the brain and are largely responsible for the inflammation-mediated neurotoxicity. Prevention of microglia activation might alleviate neuronal damage and degeneration under the inflammatory conditions, and therefore, represents a possible therapeutic approach to the aforementioned CNS diseases. Here we report the synthesis of a number of non-steroidal anti-inflammatory drug (NSAID) conjugates, and the evaluation of their anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells and primary mouse microglial cells. Among the tested analogues, compounds **8** and **11** demonstrated potent inhibition of nitric oxide production with no or weak cell toxicity. Compound **8** also significantly suppressed the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, cyclooxygenase (COX)-2 as well as inducible nitric oxide synthase (iNOS) in LPS-stimulated BV-2 microglial cells. Further mechanistic studies indicated that compound **8** significantly suppressed phosphorylation of mitogen-activated protein kinases (MAPKs) and subsequent activation of activator of transcription 1 (AP-1). Furthermore, in a co-culture system, compound **8** inhibited the cytotoxicity generated by LPS-activated microglia toward HT-22 neuroblastoma cells. Collectively, these experimental results demonstrated that compound **8** possessed potent anti-neuroinflammatory activity via inhibition of microglia activation, and might serve as a potential lead for the therapeutic treatment of neuroinflammatory diseases.

1. Introduction

Neuroinflammation is a self-defense reaction in the central nervous system (CNS) to protect the human body from injurious stimuli and to initiate the healing process. However, chronic neuroinflammation could become the causative factor in the pathogenesis of a broad range of diseases such as Parkinson's disease, Alzheimer's disease, ischemia, and multiple sclerosis [1,2]. As the macrophages in the central nervous system, microglial cell function in the innate immunity of the brain and are largely responsible for the inflammation-mediated neurotoxicity [3,4]. Prevention of microglia activation might alleviate neuronal damage and degeneration under the causative inflammatory conditions, and therefore, represents a possible therapeutic approach to the aforementioned CNS diseases.

Non-steroidal anti-inflammatory drugs (NSAIDs, representatives, diclofenac and 5-ASA, are shown in Fig. 1) are the treatment of choice for peripheral acute and chronic inflammation and pain [5,6]. They have also been shown to reduce the risk of developing Parkinson's disease and Alzheimer's disease [7,8]. The beneficial effects have been attributed mainly to the anti-inflammatory functions elicited by these drugs. However, the application of NSAIDs in CNS was circumvented by their limited exposure in the brain [9], resulting in the need for higher doses, and therefore, higher risk of side effects. Many NSAID analogues were developed in the past decades, aiming at enhancing efficacy, reducing side effects, or both [10,11]. One example was the NSAID–ADT–OH conjugate (Fig. 1, ACS-15, compound **6**; ATB-429, compound **7**) [12,13]. ADT–OH (Fig. 1, compound **3**) is a slow-releasing hydrogen sulfide donor. Hydrogen sulfide has been demonstrated to show multiple physiological functions including neuronal protection and anti-inflammatory effects [14–16]. The conjugate ACS-15 indeed exhibited enhanced potency compared with diclofenac (compound **1**) in the inhibition of neuroinflammation induced by microglial and astrocytic activation [17].

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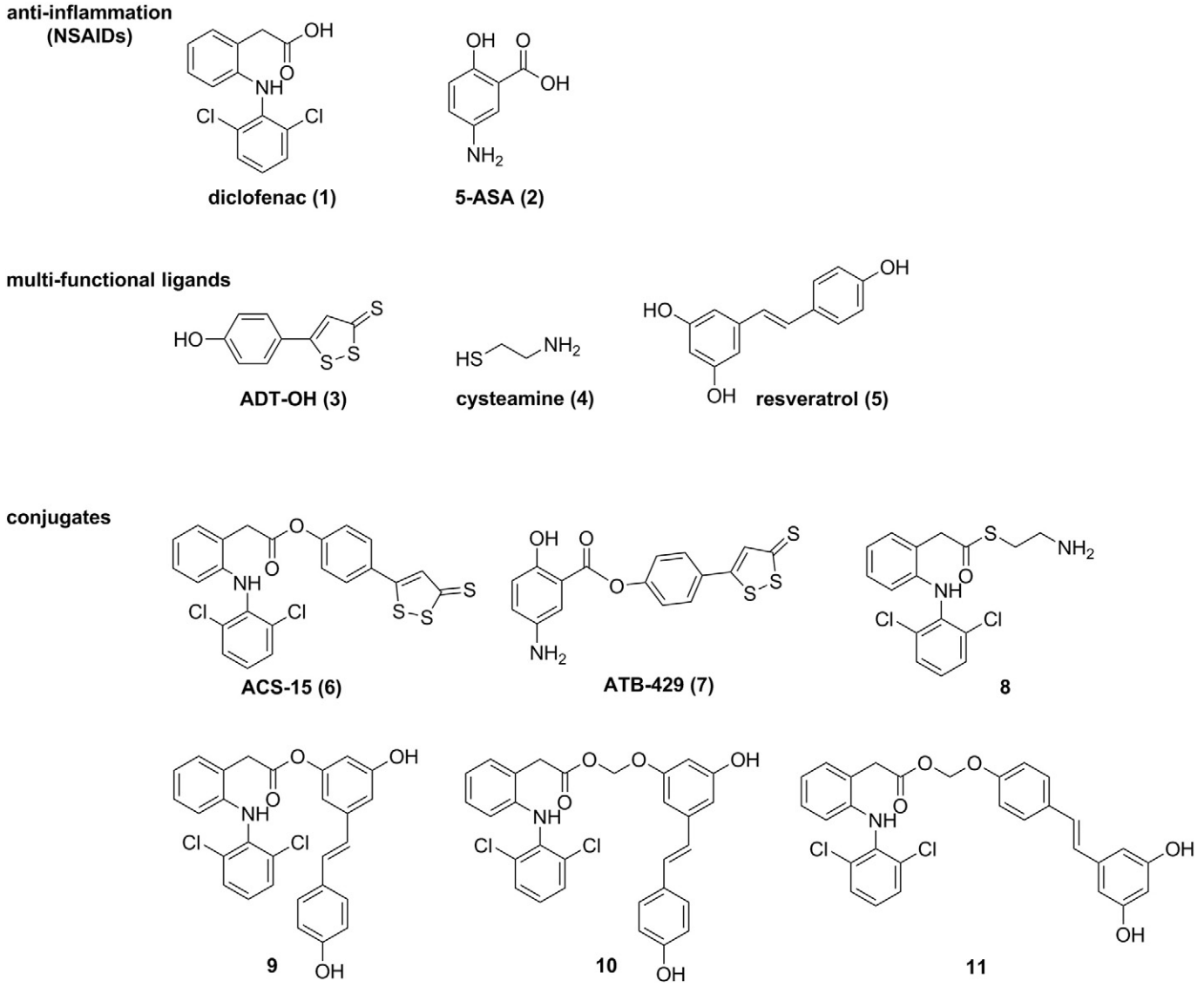

Fig. 1. Examples of NSAIDs, multi-functional ligands, known conjugates (**6**, **7**), and new conjugates (**8**–**11**).

Encouraged by these results, we decided to explore other NSAID conjugates as potential drug leads to attenuate neuroinflammation. Cysteamine (Fig. 1, compound **4**), an anti-oxidant aminothiol, was approved by FDA for the treatment of nephropathic cystinosis, a rare lysosomal storage disease [18]. Cysteamine exerts its biological functions through multiple mechanisms, including anti-oxidation, metal chelation, among others [19]. Oxidative neuronal damage and abnormal metal homeostasis are both involved in the development of Alzheimer's disease and Parkinson's disease [20–22], thus making cysteamine an attractive candidate to conjugate with NSAIDs. Because the conjugates are intended for CNS indications, and therefore need to cross the blood brain barrier, certain physical–chemical properties, such as low molecular weight, low polar surface area are especially important [23]. The fact that cysteamine has an extremely small molecular weight, along with an excellent safety profiles, further strengthens its candidacy as a NSAID conjugate; therefore, compound **8** was synthesized. An alternative way to conjugate cysteamine to diclofenac via an amide bond was developed [24]; however, the free thiol may suffer from spontaneous oxidation, which could complicate biological analysis.

Resveratrol, a natural stilbene which is a constituent of red wine, has been shown to prevent or slow a wide range of ailments such as cardiovascular disease, cancer and ischemic injuries [25,26]. Like cysteamine, resveratrol also exerts its biological functions through multiple mechanisms. Well studied molecular targets of resveratrol include quinone reductase 1 and 2 (anti-oxidation) [27], COX (anti-inflammation) [28], and SIRT1 (longevity) [29–31], a member of the sirtuin deacylase family. Despite overwhelmingly favorable results, however, the therapeutic application of resveratrol was hampered by its fast metabolism in circulation [32]. Major metabolites are sulfate and glucuronide on the hydroxyl groups of resveratrol. Here we propose using diclofenac to block the metabolic soft spots of resveratrol; therefore, compounds **9**–**11** were synthesized. The conjugates may bring the multifunctional effects from diclofenac and resveratrol into synergy.

In the primary screening experiment, NO release was used as a marker during LPS-stimulated BV-2 microglial cell activation. We have included compounds **1**–**7** as reference compounds to evaluate the anti-neuroinflammatory activities of compounds **8**–**11**.

2. Materials and methods

2.1. Chemistry

The synthetic approach to compounds **8**–**11** is outlined in Fig. 2. Treatment of diclofenac with *tert*-butyl 2-mercaptoethylcarbamate

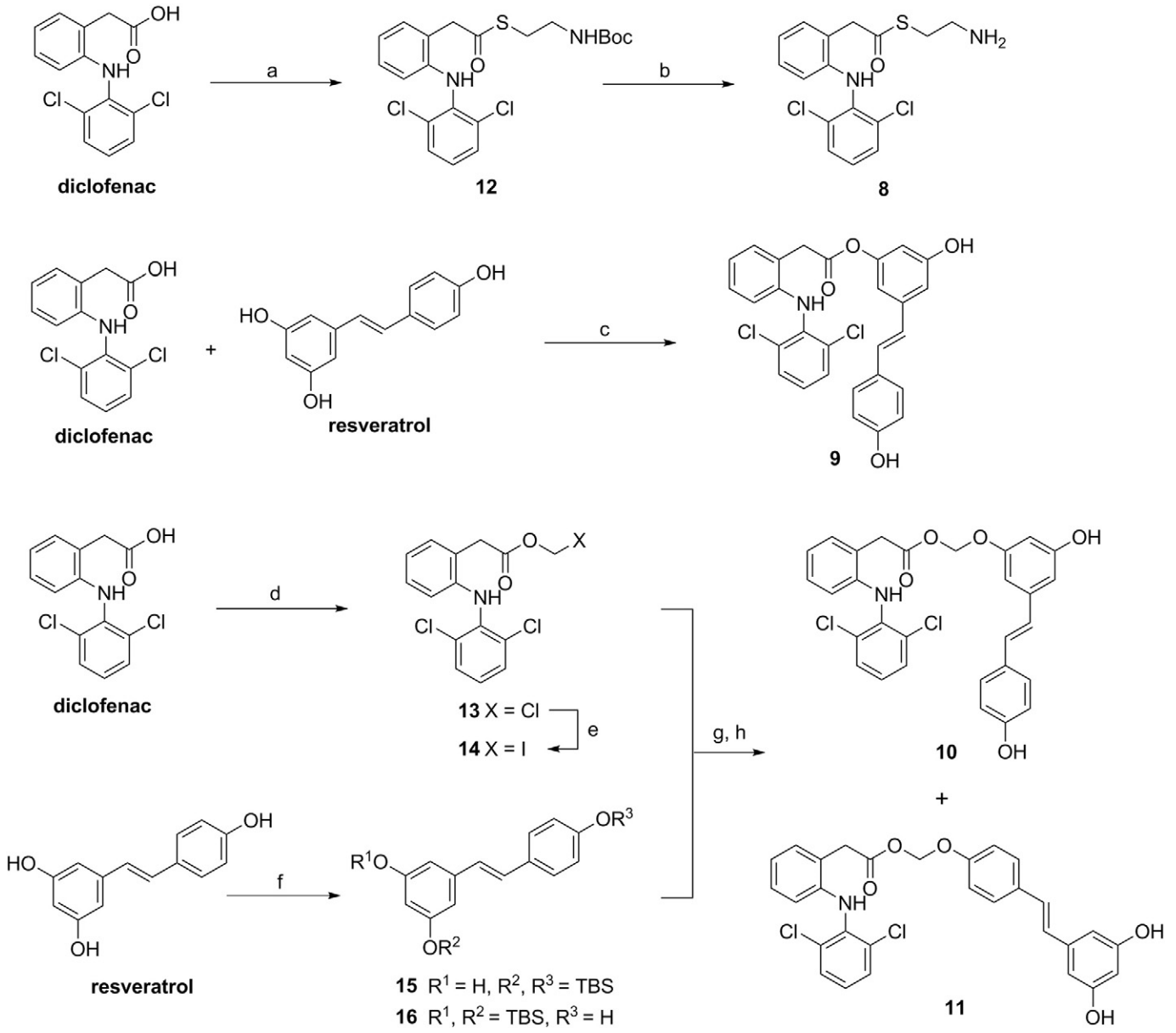

Fig. 2. Reagents and conditions: (a) *tert*-butyl 2-mercaptoethylcarbamate, DCC, DMAP, DCM, r. t., 16 h. (b) HCl/EtOAc, r. t., 8 h. (c) DCC, DMAP, DCM, dioxane, r. t., 24 h. (d) $ClCH_2OSO_2Cl$, Bu_4HSO_4, DCM, H_2O, r. t., 1 h. (e) NaI, acetone, r. t., 24 h. (f) TBSCl, imidazole, DMF, r. t., 24 h. (g) Ag_2CO_3, MeCN, r. t., 10 h. (h) TBAF, AcOH, r. t., 0.5 h.

and *N*,*N*-dicyclohexylcarbodiimide (DCC) provided intermediate **12**, which was deprotected with HCl/EtOH to give the desired compound **8** as an HCl salt. Compound **9** was obtained via direct coupling of diclofenac with resveratrol using standard DCC coupling conditions. To synthesize compounds **10** and **11**, chloromethyl ester **13** was prepared by alkylation of diclofenac using chloromethyl chlorosulfate, and then reacted with NaI in acetone to yield iodomethyl ester **14**. Resveratrol was treated with TBSCl and imidazole to give a mixture of compounds **15** and **16**, which were alkylated with iodomethyl ester **14** and subsequently deprotected with TBAF to afford compounds **10** and **11** (Fig. 2).

2.2. Biological assay

2.2.1. Reagents and cell culture

The reagents used in this study are following: Anti-α-tubulin, horseradish peroxidase-conjugated secondary antibodies and bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) were obtained from Sigma-Aldrich (St. Louis, MO). ELISA kits and recombinant mouse IFN-γ were obtained from R&D Systems (Minneapolis, MN). Anti-iNOS and anti-COX-2 were obtained from Abcam (Cambridge, MA). Anti-IκBα and anti-phospho-IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1/2, p38, JNK and c-jun were obtained from Cell Signaling Technology (Beverly, MA). DMEM, DMEM/F-12 and FBS were obtained from Invitrogen (Carlsbad, CA). BV-2 murine microglia cell line, RAW 264.7 macrophage cell and HT-22 mouse neuroblastoma cell lines were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin at 37 °C, 5% CO_2.

2.2.2. Primary culture of microglia and astrocytes

All of the animal studies were approved by the Institutional Review Board of Soochow University. The care and use of animals were performed in accordance with the guidelines of National Institute of Health. Primary culture of microglia and astrocytes were isolated from cerebral cortices of newborn of Institute of Cancer Research (ICR) mice as described previously [33]. In brief, stripped meninges were incubated in serum free DMEM/F12 containing 0.2% of trypsin for 10 min at

37 °C and then repeatedly suspended using different pore size of tips in DMEM/F12 containing 10% FBS. After centrifugation and resuspension, the cells were plated on poly-D-lysine-coated 75 mm flasks and cultured for 14 days at 37 °C, 5% CO_2. The microglia cells were separated from mixed glial cultures by shaking at 150 rpm for 2 h. The astrocyte cultures were obtained from mixed glial cell by shaking at 280 rpm for 12 h. The purity of cultured microglia or astrocytes (95%) were confirmed by immunostaining with CD11b or GFAP respectively (data not shown).

2.2.3. Cytotoxicity assay

The measurement of the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [34]. In brief, the cells were pretreated with different concentration of compound prior to LPS challenge for 30 min. After incubation with LPS for 24 h, the cell culture media were removed and 30 μL of MTT (0.5 mg/mL) solution was added to each well. After incubation for 4 h at 37 °C, the cell culture supernatants were aspirated and 100 μL of dimethyl sulfoxide (DMSO) was added into each well. The absorbance of solubilized formazan was measured by microplate reader at 540 nm of absorbance.

2.2.4. Nitrite quantification

The measurement of NO levels in condition medium was performed with Griess reagent as described previously [35]. In brief, after treatment of the cells with different concentration of compound or stimulating agents, the cell culture media were collected. 50 μL of the cell culture medium was added to 96 well plates containing 50 μL of Griess reagent [36] and incubated for 10 min at room temperature. The optical density was obtained by measuring absorbance at 550 nm with microplate reader. To calculate concentration of NO_2^-, serially diluted sodium nitrite solution was used as the standard reagent.

2.2.5. Measurement of level of TNF-α, IL-6 and PGE_2

The amounts of TNF-α, IL-6 and PGE_2 in the cell culture medium were measured by specific ELISA kits according to the instruction of the manufacturer.

2.2.6. Isolation of total RNA and quantitative real-time PCR

Cells were cultured in 6 well plates at a density of 2×10^5 cells/well. After incubation for overnight, the cells were pretreated with different concentration of compound or vehicle for 30 min prior to stimulation of LPS (0.1 μg/mL) for 6 h. The extraction of total RNA and preparation of cDNA were performed using TRIzol reagent (Invitrogen, CA) and reverse transcription kit (Takara, Dalian, China) respectively. The experiment was performed in accordance with the instruction of the manufacturer. The specific primers used for PCR are the following: iNOS forward, TAG GCA GAG ATT GGA GGC CTT G; iNOS reverse, GGG TTG CTG AAC TTC CAG TC; COX-2 forward, CAG GCT GAA CTT CGA AAC A; COX-2 reverse, GCT CAC GAG GCC ACT GAT ACC TA; TNF-α forward, CAG GAG GGA GAA CAG AAA CTC CA; TNF-α reverse, CCT GGT TGG CTG CTT GCT T; IL-6 forward, TCC AGG ATG AGG ACA TGA GCA C; IL-6 reverse, GAA CGT CAC ACA CCA GCA GGT TA; and GAPDH forward, TGT GTC CGT CGT GGA TCT GA, GAPDH reverse, TTGCTG TTG AAG TCG CAG GAG. Quantitative real-time PCR was conducted with CFX96 PCR instrument (BIORAD, USA) using specific primers and SYBR Premix II kit (Takara, Dalian, China). The CT values of each target gene were normalized to that of GAPDH. For calculation of relative quantification, the delta CT method was used [34].

2.2.7. Western blot analysis

The cells were pre-incubated with different concentration of compound or vehicle for 30 min prior to treatment of LPS (0.1 μg/mL). After LPS treatment, the cell lysates were obtained and the equal amounts of proteins were subjected to Western Blot analysis as described previously [36].

2.2.8. NF-κB and AP-1 reporter assay

The establishment of stable BV-2 cell line expressing NF-κB or AP-1 reporter construct was performed as previously described [37]. In brief, the BV-2 microglia cells stably expressing NF-κB or AP-1 constructs (Cignal™ Lenti Reporters, Qiagen) were seeded on 24 well plates (1×10^5 cells/well). The cells were pretreated with compounds or vehicle for 30 min prior to LPS (0.1 μg/mL) treatment. After 16 h of LPS stimulation, luciferase activity was measured using the luciferase assay kit (Promega) according to the instruction of the manufacturer and the level of promoter activity was expressed as relative units.

2.2.9. Microglia/neuron co-culture

For the co-culture of microglia/neuron, microglia condition media system was used as described previously [37]. In brief, BV-2 microglia cells were seeded in 6 well cell culture plates. The BV-2 microglia cells were pretreated with compound or vehicle for 30 min prior to LPS (0.1 μg/mL) treatment. After stimulation by LPS for 24 h, BV-2 cells condition medium (CM) was collected. The HT-22 neuroblastoma cells were incubated with CM from compound or vehicle treated BV-2 cells for 36 h, and then the cell viability was accessed by MTT assay.

2.2.10. Statistical analysis

For the statistical analysis, SPSS software (version 16) was used. The data are presented as the mean ± standard deviation of 3 independent experiments. For the multiple comparisons, the data were analyzed using One-way ANOVA followed by the Student Newman Keuls post hoc analysis. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Inhibition of NO production in activated microglial cells

Excessive activation of microglia is a signature of neuroinflammation and is related to the neurodegenerative progress by releasing various neurotoxic mediators [1]. The inhibition of microglial activation might be an effective therapeutic option for the treatment of neurodegenerative diseases. NO, a major pro-inflammatory factor, is a hallmark of microglial activation and plays an important role in the neuroinflammatory process and neuronal cell death [1]. Therefore, we evaluated the inhibitory effects of the novel NSAID conjugates on the NO generation in LPS activated microglia cells. In parallel, we evaluated cell viability by MTT assay to mitigate the possibility that the decrease of NO level was due to cell growth inhibition or cytotoxicity. The results were summarized in Table 1. The two NSAIDs, diclofenac and 5-ASA,

Table 1
Compound activity on inhibition of NO production in LPS-activated BV-2 microglia and cell viability.

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]	
		(10 μM)	(5 μM)
1	246.64 ± 2.39	107.35	102.79
2	59.87 ± 1.77	95.92	97.06
3	16.24 ± 1.21	96.08	97.62
4	124.25 ± 2.09	97.86	99.78
5	22.86 ± 1.35	103.37	106.81
6	16.00 ± 1.20	96.98	94.56
7	19.56 ± 1.29	98.96	100.37
8	4.80 ± 0.68	104.46	102.26
9	24.14 ± 1.38	103.14	96.67
10	18.95 ± 1.27	93.91	93.91
11	14.43 ± 1.15	93.83	93.98

[a] IC_{50}: the concentration that produces 50% inhibitory effect on NO production. Values obtained from three independent experiments and the mean inhibition of NO production relative to the LPS control for respective compound. The data are presented as mean ± standard deviation.

[b] Cell viability after treatment with (5–10 μM) of each compound was expressed as a percentage (%) of the value obtained from LPS alone group. The data are presented as mean ± standard deviation.

were virtually inactive (247 and 60 μM, respectively) under the current experimental conditions. Cysteamine was also inactive (124 μM). However, both ADT–OH and resveratrol demonstrated moderate inhibitory activities (16 and 23 μM, respectively), consistent with those of the previous reports [13,17,39]. ACS-15 and ATB-429 both showed improvement of the inhibitory potency (16 and 20 μM, respectively) compared with their corresponding NSAIDs, diclofenac, and 5-ASA, respectively. However, this improvement could be attributed to the inhibitory activity from the other half of the conjugate, ADT–OH (16 μM). The three resveratrol-diclofenac conjugates, compounds **9**, **10**, and **11**, all demonstrated moderate inhibitory effects (24, 19 and 14 μM, respectively) comparable to or better than that of resveratrol (23 μM). Among them, compound **11** exhibited a decent improvement compared with both parent molecules, indicating that a synergetic effect was indeed possible. This was validated in the case of cysteamine–diclofenac conjugate, as compound **8** (4.8 μM) was much more potent compared with diclofenac and cysteamine. All of the 11 compounds were well tolerated by the BV-2 microglial cells in the two concentrations (5 and 10 μM) tested as demonstrated by the cell viability assay (Table 1). Compounds **1**, **4**, **5**, **8** and **11** were selected for a detailed dose responses assessment (Fig. 3A–E). As shown in Fig. 3, compounds **8** and **11** significantly reduced NO production in a dose dependent manner in the LPS-stimulated BV-2 microglial cells. Consistent with the IC_{50} determination experiments, compound **8** is much more potent than the parent compounds **1** and **4** on inhibiting NO production in LPS-stimulated BV-2 microglial cells. Similarly, compound **11** was also more effective than the parent compounds **1** and **5**. Compound **8** represented the most potent compound that we evaluated in this assay. The generality of the inhibitory effect of compound **8** on NO production was confirmed in LPS or LPS/IFN-γ stimulated BV-2 microglial cells, primary microglia, RAW 264.7 murine macrophage cells and primary astrocytes (Fig. 4). In addition, cell viability assay demonstrated that compound **8** at the indicated concentrations (1.25–5 μM) did not decrease the viability of the aforementioned cells in the presence or absence of LPS or LPS/IFN-γ (data not shown).

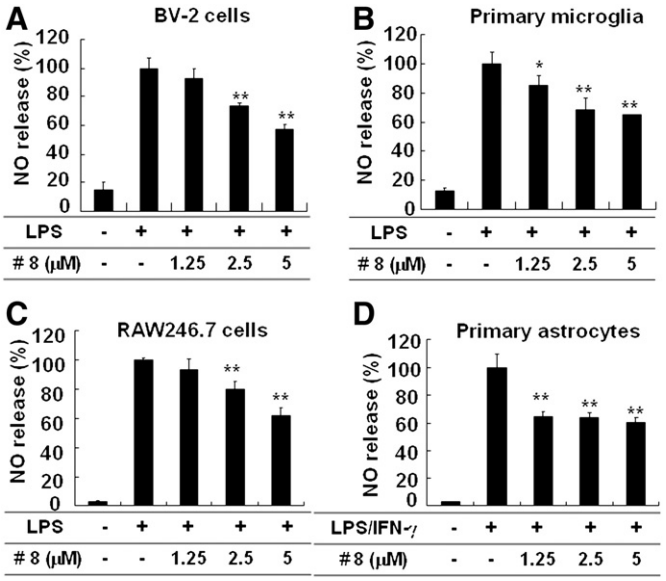

Fig. 4. Effect of compound **8** on NO production in LPS-stimulated BV-2 microglia, primary microglia, RAW 264.7 macrophage cells, and LPS/IFN-γ-stimulated primary astrocytes. The cells were pretreated with compound **8** (1.25–5 μM) for 30 min, followed by LPS (0.1 μg/mL) or LPS/IFN-γ (50 unit/mL) treatment for 24 h. The nitrite in the cell culture medium was quantified using Griess reaction (A, BV-2 cells; B, primary microglia; C, RAW 264.7 cells; D, primary astrocytes). The results shown were obtained from 3 independent experiments. Asterisk indicates significant difference from LPS or LPS/IFN-γ treatment alone group ($^{*}p < 0.05$, $^{**}p < 0.01$).

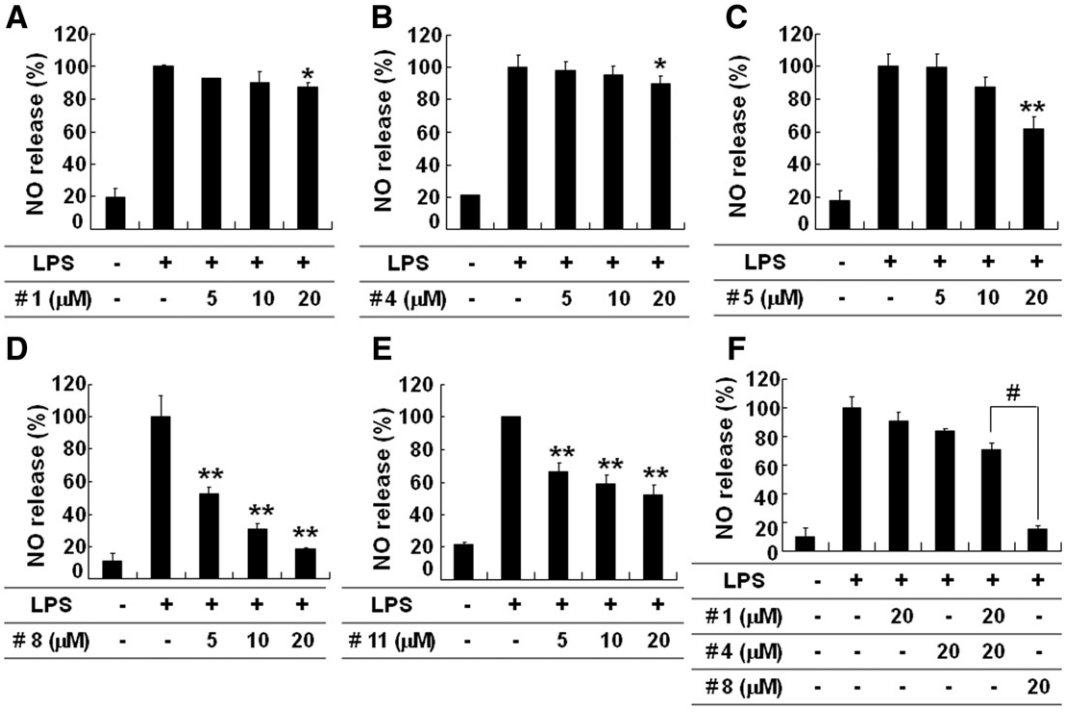

Fig. 3. Effect of compounds (A, **1**; B, **4**; C, **5**; D, **8**; E, **11**) on NO production in LPS-induced BV-2 microglial cells. BV-2 microglial cells were pretreated with indicated concentrations of compounds for 30 min, followed by LPS (0.1 μg/mL) treatment for 24 h. The contents of nitrite in the cell culture medium were quantified using Griess reaction. Comparison of the effect of compounds **1**, **4**, **8** and equal molar mixture of compounds **1** and **4** on NO production in LPS-induced BV-2 microglia cells (**F**). Data represent the mean ± standard deviation of 3 independent experiments carried out in triplicate. Asterisk indicates significant difference from LPS treatment alone group ($^{*}p < 0.05$, $^{**}p < 0.01$). $^{\#}p < 0.01$ indicates significant difference from compound **8** treatment group.

3.2. Compound 8 inhibited the expression of pro-inflammatory genes in LPS-stimulated BV-2 microglial cells

Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression are induced in activated microglia and responsible for synthesis of NO and PGE_2, respectively [40]. Likewise, pro-inflammatory cytokines such as TNF-α, IL-6 are over produced in activated microglia cells and actively contribute to neurodegenerative process [41]. The effect of compound **8** on iNOS, COX-2, TNF-α and IL-6 gene expression at mRNA levels was determined by quantitative real-time PCR. As shown in Fig. 5, compound **8** suppressed the gene expression of iNOS, COX-2, TNF-α and IL-6 in a dose dependent manner in LPS-activated BV-2 microglial cells. The inhibitory effect of compound **8** on the expression of iNOS, COX-2, TNF-α, IL-6 and PGE_2 at protein levels was further confirmed by immunoblotting analysis. As shown in Fig. 6, compound **8** significantly inhibited LPS-induced iNOS, COX-2, TNF-α, IL-6 and PGE_2 expression at protein level.

3.3. Compound 8 inhibited MAPK phosphorylation and AP-1 activation in LPS-activated BV-2 microglial cells

NF-κB is a key transcriptional factor that controls the expression of inflammatory cytokines and enzymes in activated microglia [42]. The inhibition of NF-κB activity has been implicated in the suppressive effect of numerous chemicals on the gene expression of iNOS, COX-2, TNF-α, and IL-6 in microglial cells [43,44]. We therefore investigated the effect of compound **8** on NF-κB activation in LPS-activated microglial cells. As shown in Fig. 7A and B, compound **8** did not suppress LPS-induced IκB phosphorylation, IκB degradation and NF-κB luciferase activity, suggesting that the NF-κB pathway was not involved in the anti-inflammatory mechanism of compound **8** in BV-2 cells. Previously, it was demonstrated that inhibition of NF-κB activity by S-diclofenac was dependant on H_2S releasing property [45]. It seemed that compound **8** elicited its anti-inflammatory effect through an alternative mechanism. AP-1 activation is also known to play a critical role in the signal transduction that controls pro-inflammatory gene expression in activated glial cells [46]. AP-1 activation in glial cells is mainly mediated by mitogen-activated protein kinases (MAPKs), including ERK, p38 and c-Jun *N*-terminal kinase (JNK) [47]. Therefore, the effect of compound **8** on the MAPK pathways was investigated by Western blot. The results revealed that compound **8** suppressed the phosphorylation of ERK and JNK in LPS-activated BV-2 microglial cells, whereas p38 phosphorylation was not affected (Fig. 7C). Since activated ERK and JNK directly bind and phosphorylate c-jun which is a major component of AP-1 transcriptional factor in LPS-activated microglia [47,48], the effect of compound **8** on c-jun phosphorylation was determined by Western blot. As shown in Fig. 7D, compound **8** markedly suppressed LPS induced phosphorylation of c-jun in LPS-activated BV-2 microglia cells. To confirm that compound **8** suppressed LPS-induced microglial activation through the inhibition of AP-1 activity, we next examined the effect of compound **8** on LPS-induced AP-1 luciferase activity. As shown in Fig. 7E, compound **8** significantly inhibited LPS induced AP-1 luciferase activity in BV-2 microglia cells. This is consistent with reports that diclofenac inhibited AP-1 activity in various cell lines [13,49]. These results suggest that MAPK/AP-1 pathway might be implicated in the inhibitory effects of compound **8** on the LPS triggered inflammatory responses in microglia cells.

3.4. Compound 8 reduced microglial neurotoxicity in microglia/neuron co-culture model

To investigate the potential neuroprotective effect of compound **8** in vitro, we took advantage of a microglia/neuron co-culture model. The neuronal toxicity of conditioned media (CM) of LPS-activated BV-2 microglial cells was evaluated with HT-22 hippocampal cells. A significant decrease of HT-22 cell viability was observed after the HT-22 cells were treated with CM harvested from LPS-activated BV-2 microglial cells. However, the viability of HT-22 cells being treated with CM harvested from compound **8** pre-incubated BV-2 cells was significantly improved (Fig. 8A). To investigate whether compound **8** could protect against oxidative stress-induced HT-22 cell death, we determined the viability of HT-22 cells after H_2O_2 treatment for 24 h in the presence or absence of compound **8**. The results showed that compound **8** also ameliorate H_2O_2 induced HT-22 cell death (Fig. 8B).

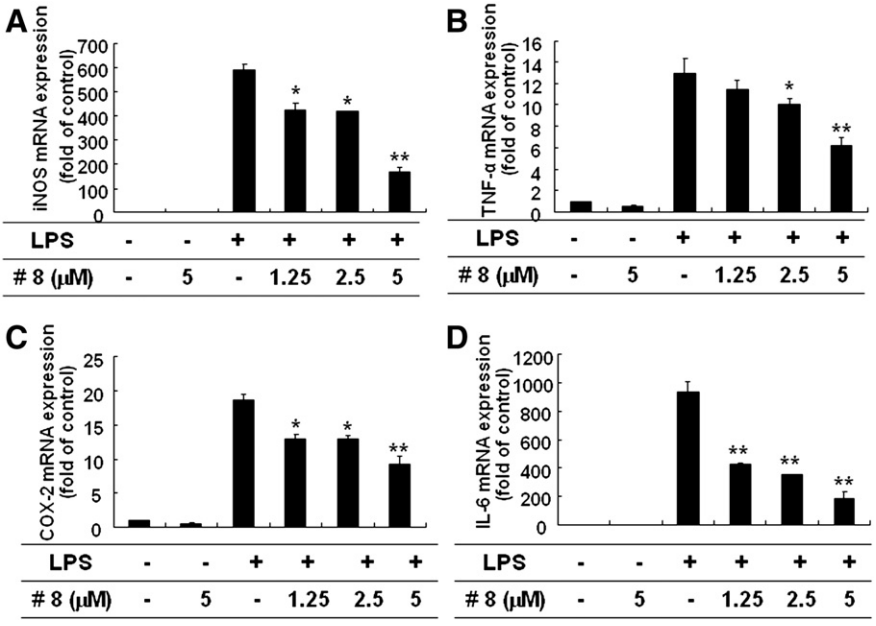

Fig. 5. Effect of compound **8** on iNOS, TNF-α, IL-6 and COX-2 expression at mRNA level in LPS-stimulated BV-2 microglial cells. The BV-2 microglial cells were pretreated with compound **8** (1.25–5 μM) for 30 min, followed by LPS treatment (0.1 μg/mL). The iNOS (A), TNF-α (B), COX-2 (C) and IL-6 (D) mRNA levels were determined by SYBR green quantitative-RT-PCR. Data represent the mean ± standard deviation of 3 independent experiments carried out in triplicate. Asterisk indicates significant difference from LPS or LPS/IFN-γ treatment alone group (*$p < 0.05$, **$p < 0.01$).

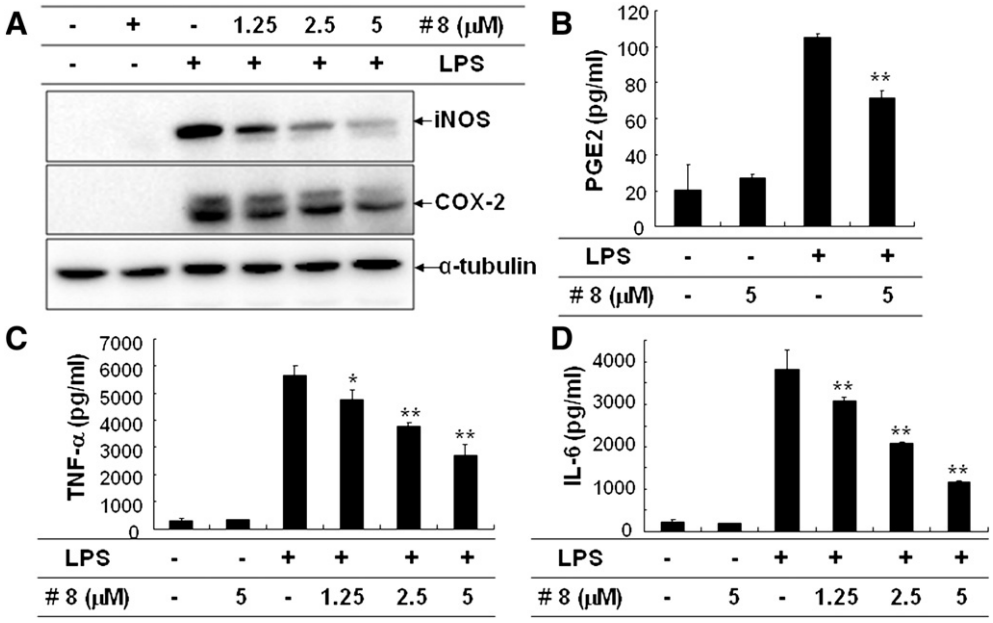

Fig. 6. Effect of compound **8** on iNOS, TNF-α, IL-6 and COX-2 expression at protein level in LPS-stimulated BV-2 microglial cells. The BV-2 microglial cells were pretreated with compound **8** (1.25–5 μM) for 30 min, followed by LPS treatment (0.1 μg/mL). The iNOS (A, upper), COX-2 (A, lower) levels were determined by Western blot. The amounts of PGE2 (B), TNF-α (C) and IL-6 (D) in the supernatants were measured using ELISA kit. Data represent the mean ± standard deviation of 3 independent experiments carried out in triplicate. Asterisk indicates significant difference from LPS or LPS/IFN-γ treatment alone group ($^{*}p < 0.05$, $^{**}p < 0.01$).

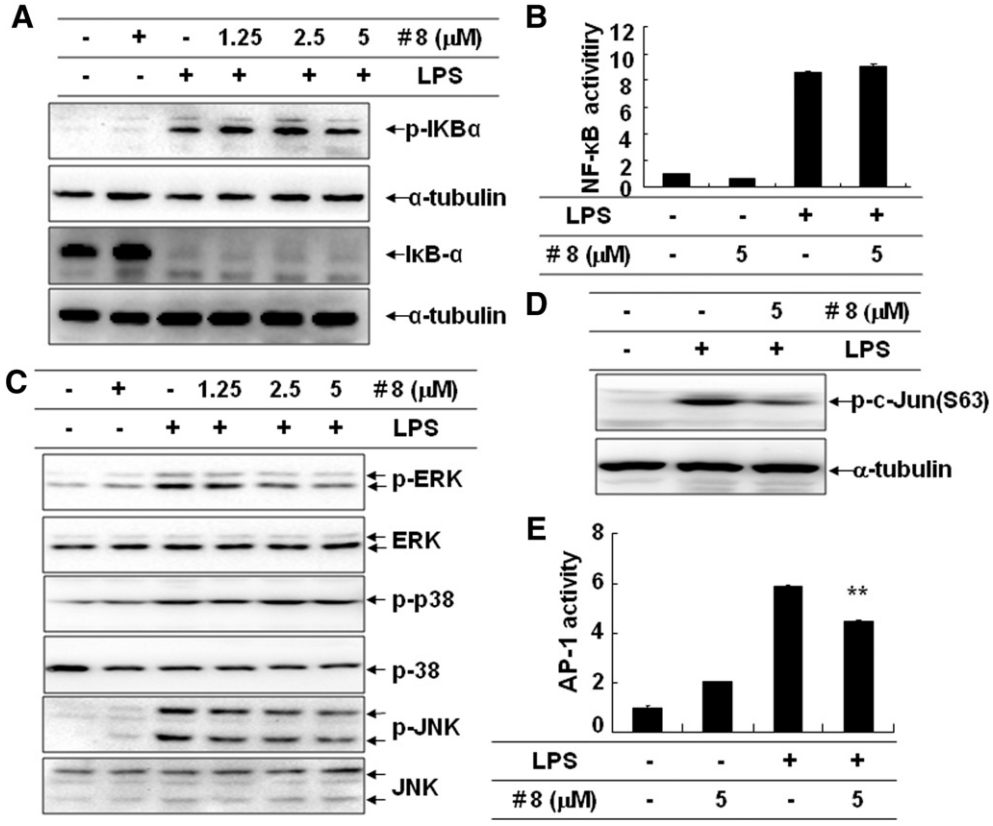

Fig. 7. Effect of compound **8** on NF-κB, MAPKs and AP-1 activation in LPS-activated BV-2 microglial cells. BV-2 microglial cells were pretreated with compound **8** (1.25–5 μM) for 30 min, followed by LPS treatment (0.1 μg/mL) for indicated time point. The phosphorylated–IκB-α (10 min) and IκB-α (20 min) levels were determined by Western blot using respective antibodies (A). BV-2 cells stably expressing NF-κB (B) or AP-1 (E) reporter construct was pretreated with compound **8** (5 μM) for 30 min, followed by LPS treatment (0.1 μg/mL) for 16 h. Luciferase activity was measured by luminometry. The luciferase activity is expressed as relative values and the values of control are set to a relative value of 1. Data represent the mean ± standard deviation of 3 independent experiments carried out in triplicate. Asterisk indicates significant difference from LPS or LPS/IFN-γ treatment alone group ($^{*}p < 0.05$, $^{**}p < 0.01$). The activation of MAPKs (ERK1/2, upper; p38, middle; JNK, lower) was determined by Western blot using respective antibodies (C). The phosphorylated-c-jun (1 h) level was determined by Western blot (D).

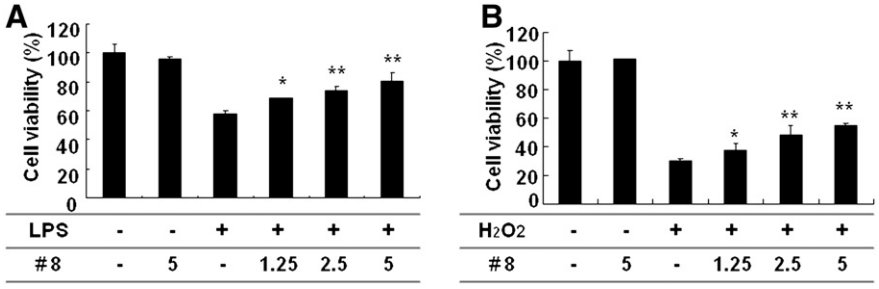

Fig. 8. Effect of compound **8** on microglial neurotoxicity. The BV-2 microglial cells were pretreated with compound **8** (1.25–5 μM) for 30 min, followed by LPS treatment (0.1 μg/mL). After treatment of LPS for 24 h, CM from control, compound **8** alone, LPS alone and LPS/compound **8** treated BV-2 microglial cells was added to HT-22 cells in 96 well plates. HT-22 cell viability was assessed by MTT assay after 36 h (A). HT-22 cells were pretreated with indicated concentration of compound **8** prior to 500 μM of H_2O_2 for 30 min. The HT-22 cell viability was measured by MTT assay (B). Data represent the mean ± standard deviation of 3 independent experiments carried out in triplicate. Asterisk indicates significant difference from LPS or LPS/IFN-γ treatment alone group (*$p < 0.05$, **$p < 0.01$).

3.5. Stability of compound 8, in vitro and in vivo transition

Since compound **8** is a thioester conjugate, we did preliminary stability test on it. Compound **8** as an HCl salt was stable at room temperature for days. Compound **8** was also evaluated in water and DMSO and remained unchanged after 24 h at room temperature. However, when we evaluated compound **8** in rat pharmacokinetics (P.K.) study, compound **8** demonstrated a large clearance (>100 mL/min/kg), indicating a high hurdle to translate the in vitro results into in vivo efficacy (data not shown).

4. Discussion

In the present study, we synthesized a number of non-steroidal anti-inflammatory drug (NSAID) conjugates and evaluated their anti-inflammatory effects in microglia cells. Among the tested analogues, compounds **8** (diclofenac–cysteamine conjugate) and **11** (diclofenac–resveratrol conjugate) exhibited potent inhibitory activities on nitric oxide production with no or weak cell toxicity. Compound **8** significantly suppressed the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, cyclooxygenase (COX)-2 as well as inducible nitric oxide synthase (iNOS) in LPS-stimulated BV-2 microglia cells. Further mechanistic studies indicated that compound **8** significantly suppressed phosphorylation of mitogen-activated protein kinases (MAPKs) and subsequent activation of activator of transcription 1 (AP-1). Furthermore, in a co-culture system, compound **8** inhibited the cytotoxicity generated by LPS-activated microglia toward HT-22 neuroblastoma cells.

Over activation of microglia is a signature of neuroinflammation and contributes to the neurodegenerative progress by releasing various neurotoxic mediators [1]. The inhibition of microglial activation represented effective potential therapeutic approach for the treatment of neurodegenerative diseases. NO, a major pro-inflammatory factor, is a hallmark of microglial activation and plays an important role in the neuroinflammatory process and neuronal cell death [1]. Therefore, we evaluated the inhibitory effects of the novel NSAID conjugates on the NO generation in LPS activated microglia cells. We found that (i) the parent drugs (diclofenac and cysteamine) alone showed weak inhibitory activity on NO production; (ii) co-administration of diclofenac and cysteamine with equimolar doses resulted in enhanced inhibition of NO production; (iii) diclofenac–cysteamine conjugate (compound **8**) demonstrated the most potent inhibitory effect on NO production, much more than that of the combined diclofenac and cysteamine (Table 1 and Fig. 3). Previously, it was reported that S-diclofenac displayed enhanced anti-inflammatory activity over the individual parent compound in an additive fashion because the conjugate broke down into diclofenac and H_2S [13]. On the other hand, it was demonstrated that co-administration of diclofenac and L-cysteine displayed less anti-inflammatory activity than individual parent compound, while diclofenac-L-cysteine conjugate exhibited enhanced anti-inflammatory activity in vivo [50]. In line with this, we observed that diclofenac–cysteamine conjugate inhibited NO production more effectively than that of the combination of diclofenac and cysteamine, suggesting that the synergistic effect of the conjugate was unlikely related to the hydrolysis of the thioester bond. Additional literature reports showed that amide prodrug of ibuprofen and naproxen were metabolized very slowly in vivo [51], and amide derivatives of indomethacin were not readily hydrolyzed into indomethacin in plasma. Taken together, we suspect that the synergistic effect of diclofenac–cysteamine conjugate on NO inhibition in LPS-activated microglia cells might involve different mechanisms: antioxidant cysteamine enhanced anti-inflammatory action of diclofenac, such as by increasing cellular glutathione levels; or compound **8** or its metabolites per se through alternative mechanisms. It is well known that iNOS and COX-2 catalyze the production of NO and PGE2 from L-arginine and arachidonic acid, respectively, and are shown to be induced by various inflammatory stimuli including pro-inflammatory cytokine and LPS. Although diclofenac is a selective COX inhibitor, many studies have demonstrated that diclofenac and its derivates exhibited inhibitory effects on expression of iNOS, COX-2 and pro-inflammatory cytokines including TNF-a, IL-6 [13,17]. In agreement with this study, we found that compound **8** markedly suppressed expression of iNOS, COX-2, TNF-a and IL-6 at mRNA or protein levels, suggesting that this compound may be a potential lead for the therapeutic treatment of microglial activation-associated neuroinflammatory diseases.

Transduction of LPS signaling within microglia was mainly mediated by Toll-like receptor 4 (TLR4). TLR4 activation leads to activation of many intracellular signaling pathways including IκB kinase (IKK) complex, MAPKs and PI3K/Akt [48]. These intracellular signal molecules ultimately lead to the activation of transcriptional factors NF-κB and AP-1, which control gene expression of many pro-inflammatory cytokines and enzymes. It is well established that LPS induced NF-κB activation is mediated by IκB phosphorylation, IκB degradation and nuclear translocation of NF-κB. In the nucleus, the NF-κB binds to a κB site in the promoter region of target genes, thereby regulating transcriptional gene expression [52]. In our work, it was observed that compound **8** inhibited neither the IκB degradation nor the NF-κB promoter activity in LPS-activated microglia cells. This was consistent with previous observation that diclofenac alone did not suppress the activation of NF-κB [13]. In addition to NF-κB, AP-1 is another main transcriptional factor that plays a critical role in the expression of pro-inflammatory genes such as iNOS, COX-2, IL-6 and ROS [48]. Transcriptional activation of AP-1 is mediated by the phosphorylation of MAPKs which has been implicated in the signal transduction pathways for inflammatory responses of microglia cells. It was demonstrated that diclofenac and its derivates significantly attenuated LPS triggered AP-1 DNA-binding activity [13]. In agreement with these results, we also found that diclofenac–cysteamine conjugate significantly inhibited LPS-induced phosphorylation of ERK1/2 and JNK, ultimately led to the blockage of transactivation of AP-1.

Previous studies indicated that ERK is an upstream signal molecule of NF-κB in peripheral cells [53]. On the contrary, it was suggested that NF-κB and ERK are two unrelated signal pathways in inflammatory responses [54]. We have shown that an ERK specific inhibitor, U0126, did not block NF-κB activation, suggesting that NF-κB activation is not induced by ERK phosphorylation [37]. We therefore suggest that diclofenac–cysteamine conjugate reduces the production of pro-inflammatory mediators though inhibition of the intracellular signal transduction pathway MAPKs/AP-1.

Pro-inflammatory cytokines and free radicals released by activated microglial cells are the major neurotoxic factors and are actively involved in the neuronal cell apoptosis and progression of neuronal degeneration associated diseases [38,55]. Thus, the inhibition of microglial activation may mitigate neurotoxicity [33]. In fact, a number of epidemiological studies have demonstrated that the use of NSAID is associated with lower risk of neurodegenerative diseases. In addition to inflammation, oxidative stress is also implicated in the pathogenesis of neurodegenerative diseases. Cysteamine is an organic compound generated in mammals as product of the coenzyme A metabolism [18]. Like cysteamine, cysteine can also be oxidized into taurine, which is one of the most abundant amino acids in the brain and possesses anti-oxidant activity [56]. Increasing evidence suggested that cysteamine and cysteine sustain neuroprotective and anti-oxidative properties in vitro as well as in vivo [57]. In the current study, we found that diclofenac–cysteamine not only reduce microglial cell activation induced neurotoxicity, but also inhibit H_2O_2 induced neuronal cells death. These result suggested that two potential mechanisms are involved in the neuroprotection of compound 8: i) diclofenac–cysteamine conjugate suppress microglial neurotoxicity by inhibiting microglial activation; ii) diclofenac–cysteamine could directly scavenge H_2O_2 induced free radicals in neurons. In fact, it was demonstrated that another NSAIDs–cysteamine conjugates significantly inhibit lipid peroxidation of rat hepatic microsomal lipids and directly interacted with free radical DPPH [50]. Although the condition media of LPS-stimulated microglia may not reflect microenvironments of neurodegenerative diseases, it partially mimics the neuroinflammatory conditions where secreted pro-inflammatory factors by microglia cells induce neuronal cell apoptosis. Ultimately, the neuroprotective effects of these compounds need to be validated in proper neuroinflammatory disease animal models. Collectively, these experimental results demonstrated that compound **8** possessed potent anti-neuroinflammatory activity via inhibition of microglia activation, and might serve as a potential lead for the therapeutic treatment of neuroinflammatory diseases.

Abbreviations

ACS-15 4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 2-(2-(2,6-dichlorophenylamino)phenyl)acetate;
ADT–OH 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione; AP-1, activator protein 1;
5-ASA 5-aminosalicylic acid;
ATB-429 4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 5-amino-2-hydroxybenzoate;
CM conditioned media;
CNS central nervous system;
COX cyclooxygenase;
DCC *N*,*N*-dicyclohexylcarbodiimide;
DMAP 4-dimethylaminopyridine;
DMEM Dulbecco's modified Eagle's medium;
DMSO dimethyl sulfoxide;
EDTA ethylenediaminetetraacetic acid;
ELISA enzyme-linked immunosorbent assay;
ERK extracellular signal-regulated kinases;
FBS fetal bovine serum;
GAPDH glyceraldehydes 3-phosphate dehydrogenase;
GFAP glial fibrillary acidic protein;
HBSS Hank's balanced salt solution;
ICR institute of cancer research;
IFN interferon;
IκB inhibitor of kappa B;
IL interleukin;
iNOS inducible nitric oxide synthase;
JNK c-Jun N-terminal kinase;
LPS lipopolysaccharide;
MAPKs mitogen-activated protein kinases;
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
NF-κB nuclear factor-kappa B;
NSAID non-steroidal anti-inflammatory drug;
PGE_2 prostaglandin E2;
RT-PCR real-time polymerase chain reaction;
S.D. standard deviation;
SDS sodium dodecyl sulfate;
Tris–HCl *tris*-(2-carboxyethyl)-phosphine hydrochloride.

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Appendix A. Supplementary data

Supplementary data (Experimental data and NMR spectra) associated with this article can be found, in the online version. Supplementary data to this article can be found online at doi: http://dx.doi.org/10.1016/j.intimp.2015.02.033

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