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NOTE

Curcuminoid analogs inhibit nitric oxide production from LPS-activated microglial cells

Jiraporn Tocharus · Sataporn Jamsuwan · Chainarong Tocharus · Chatchawan Changtam · Apichart Suksamrarn

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Abstract The chemically modified analogs, the demethylated analogs **4**–**6**, the tetrahydro analogs **7**–**9** and the hexahydro analogs **10**–**12**, of curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) were evaluated for their inhibitory activity on lipopolysaccharide activated nitric oxide (NO) production in HAPI microglial cells. Di-*O*-demethylcurcumin (**5**) and *O*-demethyldemethoxycurcumin (**6**) are the two most potent compounds that inhibited NO production. The analogs **5** and **6** were twofold and almost twofold more active than the parent curcuminoids **1** and **2**, respectively. Moreover, the mRNA expression level of inducible NO synthase was inhibited by these two compounds. The strong neuroprotective activity of analogs **5** and **6** provide potential alternative compounds to be developed as therapeutics for neurological disorders associated with activated microglia.

Keywords Curcuminoid analog · HAPI microglia · Inducible nitric oxide synthase · Di-*O*-demethylcurcumin · *O*-Demethyldemethoxycurcumin

J. Tocharus (✉)
Department of Physiology, Faculty of Medicine,
Chiang Mai University, Chiang Mai 50200, Thailand
e-mail: sukbunteung@hotmail.com

S. Jamsuwan
Department of Anatomy, Faculty of Medical Science,
Naresuan University, Phitsanulok 65000, Thailand

C. Tocharus
Department of Anatomy, Faculty of Medicine,
Chiang Mai University, Chiang Mai 50200, Thailand

C. Changtam · A. Suksamrarn
Department of Chemistry, Faculty of Science,
Ramkhamhaeng University,
Bangkapi, Bangkok 10240, Thailand

Introduction

Microglia are the principal immune cells of the central nervous system (CNS) [1]. They are activated in response to brain injury from inflammation, damage, or disease, and then release neurotoxic factors, including pro-inflammatory cytokines such as tumor necrosis factor α (TNF α), interleukin (IL)-1β, IL-6, as well as nitric oxide (NO) [2, 3]. Among these mediators, NO is the product of the inducible isoforms of the inducible NO synthase (iNOS) enzyme that is induced in response to proinflammatory cytokine and bacterial lipopolysaccharide (LPS) [4, 5]. NO released from microglia is known to induce neurotoxicity. iNOS expression and NO generation by activated microglia have been described in several brain pathologies including multiple sclerosis, cerebral ischemia and Alzheimer's disease [6, 7], while inhibition of NO production provides significant neuroprotection [8].

Curcumin (**1**) (Fig. 1) is a major chemical component of curcuminoids, which in turn are isolated from turmeric (*Curcuma longa* L.). Another two minor curcuminoids, demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**), are natural analogs of curcumin. Curcumin is metabolized into dihydrocurcumin (DHC) and tetrahydrocurcumin (THC), which are converted to monoglucuronide conjugates including curcumin glucuronide. Previous studies have reported that curcumin and its natural analogs are promising agents for the prevention and treatment of neurodegenerative diseases via their inhibiting effect on microglia activation [9–11]. Curcumin and its natural analogs have a significantly inhibitory effect on NO production by rat primary microglia induced by LPS through inhibiting expression of iNOS at both the protein and mRNA level [10, 12, 13]. Previous studies have reported that analogs of curcumin exhibited higher physiological

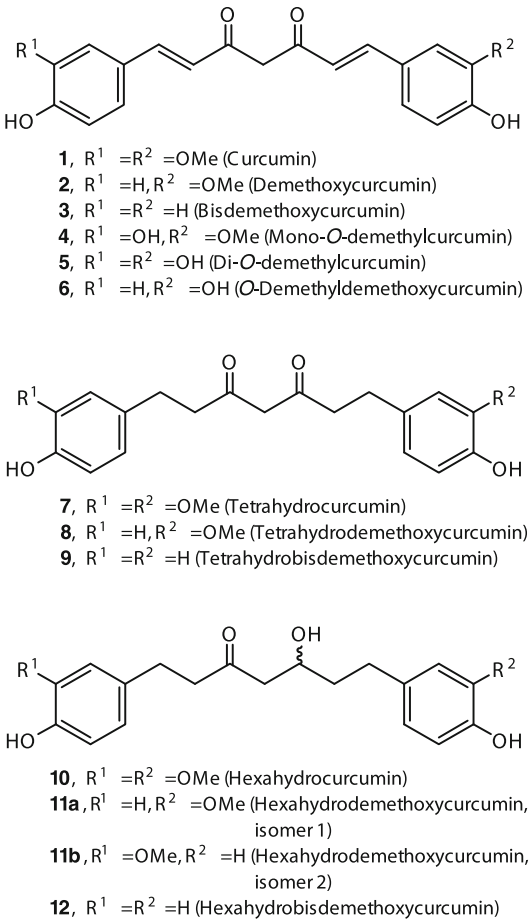

Fig. 1 Chemical structures of parent curcuminoids **1**–**3** and modified analogs **4**–**12**

activities and pharmacological activities than the parent curcumin itself by inhibiting NO and proinflammatory cytokines [10–13]. It is therefore of interest to investigate whether chemical modification of curcuminoids would improve their neuroprotective properties. This study compared the inhibitory effect on NO production from LPS-activated microglia of synthetic curcuminoid analogs—the demethylated analogs **4**–**6**, the tetrahydro analogs **7**–**9**, and the hexahydro analogs **10**–**12** (Fig. 1).

Results and discussion

The cytotoxic effect of the curcuminoids **1**–**12** on HAPI microglial cells was determined by MTT reduction assay. The curcuminoids **1**–**3** and their analogs **4**–**12** at concentrations of 20–40 μM decreased cell viability significantly in a concentration dependent manner. On the other hand, cells treated with curcuminoids **1**–**3** and their analogs **4**–**12** at concentrations of 10 μM exhibited viability similar to the control (untreated control). A concentration of <10 μM of compounds **4**–**12** was therefore selected for further experiments (data not shown).

The curcuminoids **1**–**3** and their analogs **4**–**12** were evaluated for their inhibitory effect on NO production in LPS-activated HAPI microglial cells. The results from previous studies indicated that the parent curcuminoids curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) have both NO scavenging and inhibitory NO production activity [14, 15]. Curcumin (**1**) showed strong NO inhibitory potential in primary microglia cells in LPS-mediated NO production via inhibition of the activation of JNK, p38 and NF-κB [16]. In addition, demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**)—minor components of curcuminoids—also exhibited strong inhibition of NO activity in LPS-activated microglial cells [10, 17, 18]. These results suggest that curcuminoid analogs also exhibit an inhibitory effect on NO production in LPS-activated microglia cells similar to that of the parent compounds and *S*-methylisothiourea (S-MT), a selective iNOS inhibitor used as a positive control in the evaluation of the inhibitory effects on the NO production with IC_{50} values varying from 5.14 to 10.94 μM (Table 1). Among these compounds, the demethylated analogs, di-*O*-demethylcurcumin (**5**) and *O*-demethyldemethoxycurcumin (**6**) were more effective than their parent compounds, with IC_{50} value of 5.14 and 6.12 μM, respectively. Analogs **5** and **6** were twofold and almost twofold more active than the respective parent curcuminoids **1** and **2**. At a concentration of 2–8 μM, compounds **5** and **6** showed an NO inhibitory effect in a dose-dependent manner (Fig. 2). The significant inhibitory activity of the demethylated analogs **5** and **6** could be the result of the presence of two and one extra phenolic hydroxyl groups on the phenyl rings. The enhanced inhibitory activity of these compounds might also be due to the presence of the 1,2-dihydroxy phenolic (catechol) system in the molecule, since such a system has been reported to exhibit high anti-oxidant activity [19].

Curcuminoid analogs **5** and **6** further downregulated the expression of iNOS mRNA, indicating that the action of these compounds occurred at the transcriptional level. A similar result was also observed in cells treated with the parent compounds (Fig. 3). The results showed that pretreated S-MT inhibited the expression of iNOS mRNA caused by LPS in a concentration-dependent manner (Fig. 3). The results further supported the notion that LPS induces NO overexpression in microglia. Higher concentrations of S-MT inhibited the basal level of iNOS mRNA expression.

The impairment of NO generation that led to decreased nitrite production in this study might be due to both direct NO scavenging activity of these two analogs and indirect

Table 1 Inhibition of nitric oxide (NO) production by curcuminoids **1–3** and their synthetic analogs **4–12** in lipopolysaccharide (LPS)-activated HAPI microglial cells

Compound	Inhibition of NO production: IC_{50} (μM)
Curcumin (**1**)	10.94 ± 1.11
Demethoxycurcumin (**2**)	10.32 ± 1.52
Bisdemethoxycurcumin (**3**)	9.46 ± 1.3
Mono-*O*-demethylcurcumin (**4**)	10.80 ± 1.06
Di-*O*-demethylcurcumin (**5**)	5.14 ± 0.41
O-demethyldemethoxycurcumin (**6**)	6.12 ± 1.46
Tetrahydrocurcumin (**7**)	9.63 ± 1.59
Tetrahydrodemethoxycurcumin (**8**)	8.90 ± 0.05
Tetrahydrobisdemethoxycurcumin (**9**)	10.01 ± 0.56
Hexahydrocurcumin (**10**)	7.99 ± 0.12
Hexahydrodemethoxycurcumin (**11**)	8.70 ± 0.46
Hexahydrobisdemethoxycurcumin (**12**)	9.72 ± 0.68

Values represent the mean ± SEM of three separate determinations

blockade effects of NO-producing pathways by suppression of iNOS at the transcriptional and translational levels.

In conclusion, nine curcuminoid analogs were synthesized and evaluated for their inhibitory NO production in microglial cell activated by LPS in vitro. Among them, the curcuminoid analogs **5** and **6** are more potent than the parent curcuminoids. These two compounds may have therapeutic potential in the treatment of neurodegenerative diseases accompanied by microglial activation.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD). Nitrite, phosphoric acid, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS (*E. coli* serotype 055:B5), and S-MT were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

The immortalized rat microglial cell line HAPI was generously provided by J.R. Connor (Hershey Medical Center, Hershey, PA). Microglial cells were maintained in DMEM supplemented with FBS (10%, v/v), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

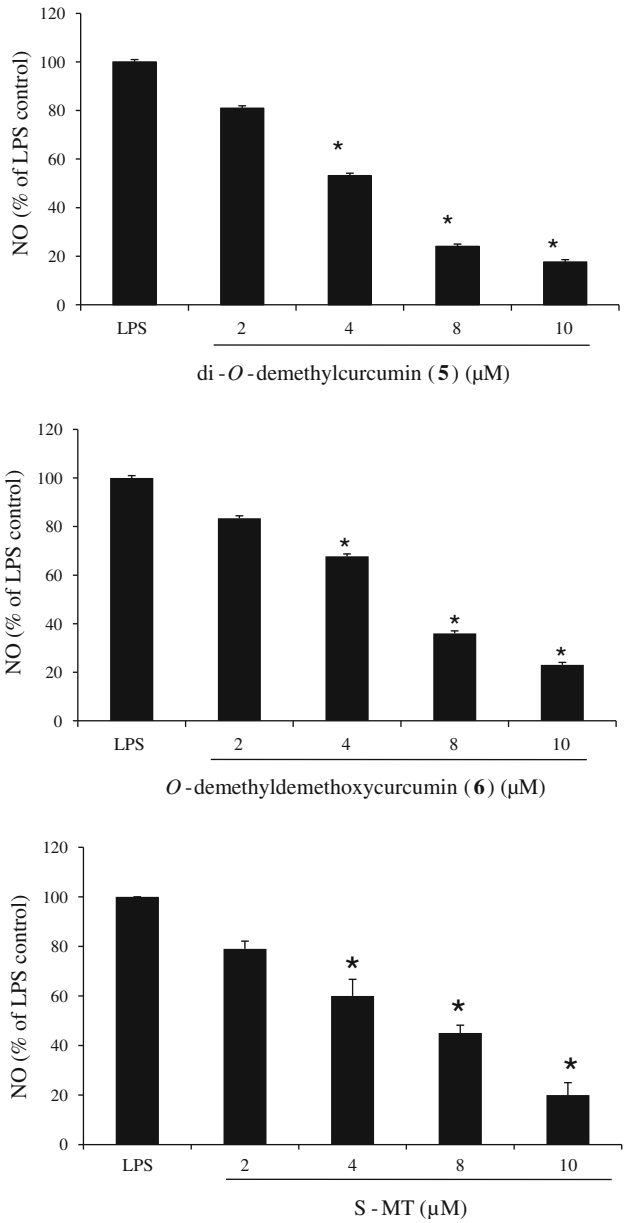

Fig. 2 Effect of di-*O*-demethylcurcumin (**5**), *O*-demethyldemethoxycurcumin (**6**) and *S*-methylisothiourea (S-MT) on lipopolysaccharide (LPS)-induced NO production in HAPI microglia cells. Cells were treated with the analogs **5**, **6** or S-MT (2–10 μM) in the presence or absence of LPS (1 μg/ml) for 24 h. The culture supernatants were collected and analyzed for nitrite production using Griess reagent. The results were expressed as percentage values taking the LPS treatment group as 100%. Data were represented as mean ± SEM of three separate experiments. Significance compared with LPS-only treatment group: $*P < 0.05$, $***P < 0.001$

Preparation of curcuminoid analogs

The parent curcuminoids **1–3** were isolated from the rhizome of *Curcuma longa* L. The curcuminoids were modified chemically to the corresponding demethylated analogs **4–6**, tetrahydro analogs **7–9**, and hexahydro analogs **10–12**.

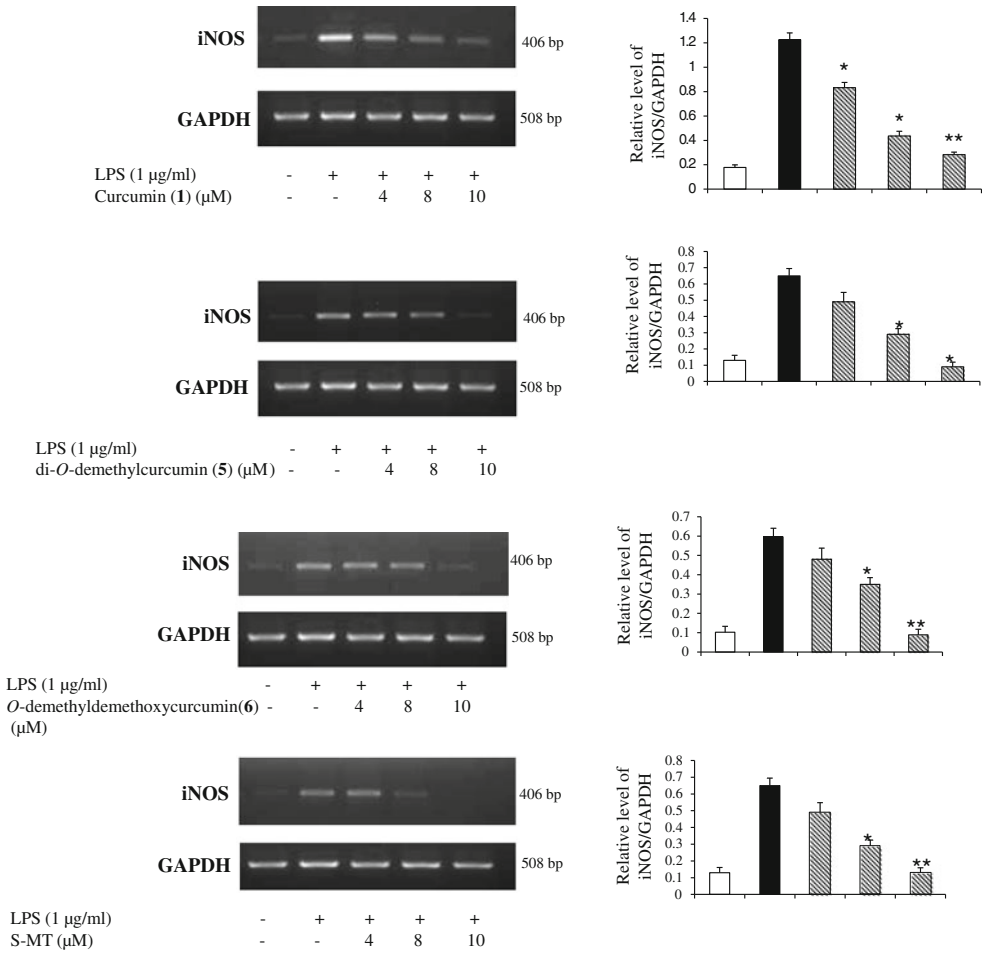

Fig. 3 Effect of di-*O*-demethylcurcumin (**5**) *O*-demethyldemethoxycurcumin (**6**) and S-MT on iNOS expression in LPS-activated HAPI microglia cells. Cells (1.5×10^5 cells/well) were pretreated with the analogs **5**, **6** or S-MT (2–10 μM) for 2 h before stimulating with 1 μg/ml LPS for 6 h. Total RNA was isolated and analyzed for GAPDH and iNOS mRNA by semi-quantitative RT-PCR. GAPDH is used as a reference to control for equal loading. The level of iNOS was quantified as the relative expression normalized against GAPDH in the same sample. Data were derived from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the LPS-only group

The chemical structures of compounds **4**–**12** are shown in Fig. 1. Thus, starting from curcumin, demethylation to the corresponding mono-*O*-demethyl analog **4** and di-*O*-demethyl analog **5** were accomplished in yields of 42% and 33%, respectively, by treatment of curcumin with boron tribromide in dry dichloromethane. Demethylation of the demethoxycurcumin was similarly achieved to the corresponding *O*-demethyl analog **6** in 64% yield. The spectroscopic (IR, ^{1}H NMR and mass spectra) data of **4**, **5** and **6** were consistent with reported values [19]. A number of non-conjugated analogs of the parent curcuminoids **1**–**3** were also prepared for biological evaluation. Catalytic hydrogenation of curcumin, with palladium on charcoal as a catalyst, furnished THC (**7**) and hexahydrocurcumin (**10**) in 68% and 18% yields, respectively. The spectroscopic data of compounds **7** and **10** were consistent with reported values [20, 21]. From the demethoxycurcumin, tetrahydro analog **8** and hexahydro **11** were similarly prepared in 62% and 15% yields, respectively. The spectroscopic data of compound **8** were consistent with reported values, and those of compound **11** revealed the existence of a 1:1 mixture of **11a** and **11b** [22]. From bisdemethoxycurcumin, the tetrahydro analog **9** and hexahydro analog **12** were similarly prepared at 65% and 12% yields, respectively. The spectroscopic data of compounds **11** and **12** were consistent with reported values [22]. The purity of all synthesized compounds was verified by thin layer chromatography (TLC) using Merck's precoated silica gel 60 F_{254} plates, with dichloromethane–methanol and *n*-hexane–ethyl acetate as two developing solvent systems. The spots on TLC were detected under UV light and by spraying with anisaldehyde–sulfuric acid reagent followed by heating. The purity of the compounds was further confirmed by NMR spectroscopy.

Cell viability assay

Cell viability was measured by quantitative colorimetric assay with MTT. HAPI microglial cells were cultured at a density of 1.5×10^4 cells/well in 96-well plate overnight. Various concentrations of curcuminoids **1**–**3** and their synthetic curcuminoid analogs **4**–**12** were added to HAPI microglial cells. After 24 h, 10 mg/ml of MTT were added to each well and further incubated for 4 h. Formazan was

solubilized with dimethyl sulfoxide (DMSO) and was determined by measuring the absorbance at 540 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT).

Measurement of nitrite

HAPI microglial cells were plated onto a 96-well plate at a density of 10^5 cells/well. Cells were pretreated with curcuminoids **1**–**3** and their synthetic curcuminoid analogs **4**–**12** for 2 h followed by LPS for 24 h. The supernatant was mixed with an equal volume of Griess reagent [a mixture of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid], and the absorbance was measured in a microplate reader at 540 nm optical density, using sodium nitrite ($NaNO_2$) at known concentration as a standard curve.

Reverse transcription polymerase chain reaction

For semiquantitative reverse transcription polymerase chain reaction (RT-PCR), microglial cells in a 96-well plate were incubated with LPS (1 μg/ml) or test compounds for 6 h. Total RNA was isolated using TRIzol according to the manufacturer's protocol. Then, random-primed cDNA species were prepared from total RNA using SuperScript III RNase H-reverse transcriptase. Specific DNA sequences were amplified with a PCR mixture. Each PCR primer used in this study was as follows: iNOS (accession no. 010927), 5′ TCACTGGGACAGCACAGA AT (sense) and 5′ TGTGTCTGCAGATGTGCTGA (antisense) and GAPDH (accession no. 001473623), 5′ TCCCT CAAGATTGTGAGCAA (sense) and 5′ AGATCCACAA CGGATACATT (antisense). Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. The level of transcript for the constitutive housekeeping gene product, GAPDH, was measured quantitatively for each sample to control differences in RNA concentration.

Statistical analysis

All data are shown as mean ± standard error of mean (SEM). Statistical comparison between different treatments results was analyzed by one-way ANOVA followed by the Dunnett test. Differences with P value <0.05 were considered statistically significant.

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