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A synthetic curcuminoid derivative inhibits nitric oxide and proinflammatory cytokine synthesis

Chau Ling Tham [a], Choi Yi Liew [a], Kok Wai Lam [b], Azam-Shah Mohamad [a], Min Kyu Kim [a], Yoke Kqueen Cheah [a], Zainul-Amirudin Zakaria [a], Mohd-Roslan Sulaiman [a], Nordin H. Lajis [b], Daud A. Israf [a,*]

[a] Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[b] Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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ABSTRACT

Curcumin is a highly pleiotropic molecule with significant regulatory effects upon inflammation and inflammatory related diseases. However curcumin has one major important limitation in which it has poor bioavailability. Design of synthetic structural derivatives of curcumin is but one approach that has been used to overcome its poor bioavailability while retaining, or further enhancing, its drug-like effects. We have synthesized a series of curcumin analogues and describe the effects of 2,6-bis-4-(hydroxyl-3-methoxy-benzylidine)-cyclohexanone or BHMC upon nitric oxide and cytokine synthesis in cellular models of inflammation. BHMC showed a significant dose–response inhibitory action upon the synthesis of NO and we have shown that this effect was due to suppression of both iNOS gene and enzyme expression without any effects upon scavenging of nitrite. We also demonstrated that BHMC has a very minimal effect upon iNOS activity with no effect at all upon the secretion of PGE_2 but has a strong inhibitory effect upon MCP-1 and IL-10 secretion and gene expression. Secretion and gene expression of TNF-α and IL-6 were moderately inhibited whereas IL-8 and IL-1β were not altered. We conclude that BHMC selectively inhibits the synthesis of several inflammatory mediators. BHMC should be considered a promising drug lead for preclinical and further pharmacological studies.

1. Introduction

Curcumin (diferuloylmethane) or 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione], a member of the curcuminoid family, is the major active component of turmeric, a yellow compound originally isolated from the plant *Curcuma longa* L. Over the last two decades many studies have shown that curcumin has a number of biological and pharmacological activities such as anti-carcinogen (Aggarwal et al., 2004; Cen et al., 2009; Yoysungnoen et al., 2008), anti-malarial (Mishra et al., 2008), antioxidant, anti-mutagenic, anti-bacterial (Parvathy et al., 2009), anti-angiogenic (Yoysungnoen et al., 2008), immune-modulatory (Allam, 2009), chemo-preventive (Chan et al., 1998), and anti-inflammatory (Chan et al., 1998; Liang et al., 2009, in press).

Curcumin is a highly pleiotropic molecule with significant regulatory effects upon inflammatory, proliferative and oxidative tissue damage. It is not surprising that preclinical studies have shown that curcumin can prevent neurodegenerative, respiratory, cardiovascular, neoplastic, metabolic and autoimmune diseases (Aggarwal and Harikumar, 2009). Curcumin has been shown to regulate a diverse array of cellular signaling pathways, gene expression and also inducible enzyme activity (Aggarwal and Sung, 2009). Of the host of inflammatory related mediators, enzymes, and transcription factors, curcumin has shown strong inhibitory effects on proinflammatory cytokines such as tumor necrosis factor (TNF)-α, Interleukin (IL)-1, IL-6; prostaglandin, inducible enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), protein kinases, transcription factors such as NF-κB, STAT-3 and AP-1 to name a few (Aggarwal and Sung, 2009).

Curcumin has entered many clinical trials and proven to be safe and effective in the prevention and treatment of mainly enteric diseases. Despite the effective modulatory activity of curcumin upon many cellular targets linked to various diseases, one of the most important limitations with curcumin is its bioavailability (Anand et al., 2007). Pharmacokinetic studies have shown that orally administered curcumin undergoes hepatic conjugation leading to the formation of glucoronides and sulphates (Anand et al., 2007; Garcea et al., 2005). Systemic administration of curcumin causes it to undergo reduction. In any case, the products following biotransformation are clearly biologically inactive (Sandur et al., 2007). Numerous approaches have been adopted in an effort to increase the bioavailability of this promising natural molecule. These approaches attempt to either interfere with glucuronidation, protect curcumin with liposomal formulations and nanoparticles, or design synthetic structural derivatives of curcumin (Aggarwal and Sung, 2009).

* Corresponding author. Tel./fax: +603 8947 2337.
E-mail address: daud@medic.upm.edu.my (D.A. Israf).

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Our group has adopted the chemical synthesis of curcumin analogues as an approach to overcome the limitation of bioavailability. The presence of the β-diketone moiety renders curcumin to be rapidly metabolized by aldo-keto reductase in the liver, therefore limiting the potential therapeutic or prophylactic effect of curcumin on many types of disease. The phenolic OH has been shown to be essential for its antioxidant activity (Parvathy et al., 2009). Taking into account these structural functionalities, a synthetic diarylpentanoid, BHMC was synthesized based on the chemical structure of curcumin, by eliminating the unstable β-diketone moiety and modifying it into conjugated double bonds while preserving the phenolic OH group. Preliminary screening of a panel of synthetic derivatives of curcumin for nitric oxide inhibitory activity has enabled us to select BHMC for further *in vitro* studies. We report here the effect of BHMC upon both nitric oxide and proinflammatory cytokine synthesis in cellular models of inflammation.

2. Materials and methods

2.1. Materials

Antibiotics (5000 U/ml penicillin and 5000 μg/ml streptomycin), Amphotericin B (0.25 μg/ml), Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and Roswell Park Memorial Institute medium (RPMI)-1640 were purchased from Hyclone (Utah, USA). TNF-α, IL-1β, IL-6, IL-8, IL-10 and monocyte chemotactic protein (MCP)-1 assay kits were purchased from BD Pharmingen (San Diego, CA, USA). Prostaglandin E_2 EIA monoclonal kit and rabbit anti-mouse iNOS polyclonal antibody were purchased from Cayman Chemicals, USA. HRP-conjugated donkey anti-rabbit Ig-G and HRP-conjugated mouse anti-mouse β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other reagents of Western blot analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Amresco (Solon, Ohio). The polyvinylidene fluoride (PVDF) membrane was purchased from Milipore (Bredford, MA, USA). Super Signal West Femto Maximum Sensitivity Substrate and Bicinchoninic acid (BCA) protein determination kit (Cat No. 23225) were purchased from Pierce (Rockford, IL, USA). Phorbol-12–Myristate-13–Acetate (PMA), NS-398, *E. coli* lipopolysaccharide (LPS) 055:B5, sulphanilamide, naphthylenediamine, sodium nitroprusside (SNP), 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), and Nω-nitro-L-arginine-methyl ester hydrochloride (L-NAME) were purchased from Sigma Chemical co. (St. Louis, MO, USA). One-step RT-PCR kit and RNeasy Extraction kit were purchased from Qiagen (Valencia, CA, USA). Benzonase nuclease and 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were purchased from Calbiochem (San Diego, CA, USA). Gene and protein ladder were purchased from Fermentas (Glen Burnie, MD, USA).

2.2. Compound synthesis

BHMC or 2,6-bis-4-(hydroxyl-3-methoxy-benzylidine)-cyclohexanone was chemically synthesized at the Institute of Bioscience, Universiti Putra Malaysia. Briefly, a mixture of vanillin (20 mmol, 2 equiv) and cyclohexanone (10 mmol, 1 equiv) was dissolved in 20 ml of absolute ethanol. Concentrated HCl (2.0 ml) was added drop wise over 5 min to the stirred mixture. The mixture was further refluxed for 1 h. One hundred milliliters of distilled water was then poured into the dark viscous solution and extracted with ethyl acetate. The ethyl acetate layer was collected and subjected to further purification using column chromatography. The structure was determined by nuclear magnetic resonance and mass spectrometry (Fig. 1A). BHMC is 99.9% pure as determined by HPLC. Our preliminary screening of several synthetic derivatives involved the testing of several batches with the Griess assay. We observed consistent results between different batches with variation not exceeding 3%. BHMC was dissolved in 100% DMSO as a stock at 100 mmol/l and diluted to appropriate concentrations for assays. The final concentration of DMSO in all assays was kept constant at 0.1%.

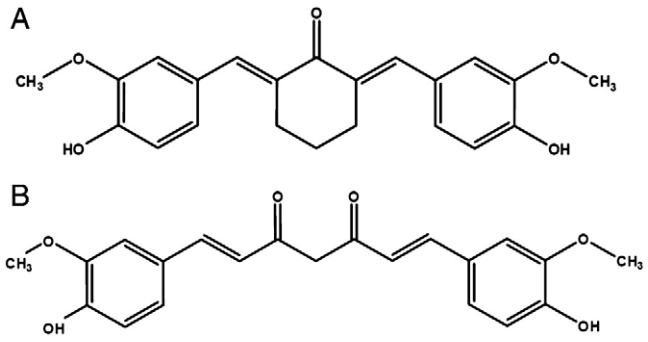

Fig. 1. Chemical structure of (A) BHMC and (B) curcumin.

2.3. Cell culture

U937 and RAW 264.7 were purchased from the American Type Culture Collection (ATCC). U937 were cultured in RPMI-1640 supplemented with 10% FBS, 4.5 g/l glucose, sodium pyruvate (1 mmol/l), L-glutamine (2 mmol/l), amphotericin B, streptomycin (50 μg/ml) and penicillin (50 U/ml). RAW 264.7 were cultured in DMEM supplemented with 10% FBS, 4.5 g/L glucose, sodium pyruvate (1 mmol/l), L-glutamine (2 mmol/l), amphotericin B, streptomycin (50 μg/ml) and penicillin (50 U/ml). Cells were split when cell confluency reached 80–90%.

2.4. Differentiation of U937 monocytes into macrophages

The human macrophage U937 cells at logarithmic phase of growth were harvested, adjusted to 2×10^6 cells/ml in the presence of 10 nM PMA, and incubated at 37 °C in 5% CO_2 incubator for 24 h to differentiate into adherent macrophages. After 24 h incubation, cells were washed twice with phosphate buffered saline (PBS) to remove non-adherent cells. Cell counting was carried out each time before and after the cell differentiation process to make sure that there was consistency in differentiation. The variation in the differentiation process was minimal with more than 90% of differentiated cells remaining attached and viable. Remaining adherent cells were quiesced in fresh RPMI medium in the absence of PMA for 24 h before stimulation with 10 μg/ml of LPS.

2.5. Induction of RAW 264.7 and U937 cells

Cells at a confluency of 80–90% were centrifuged at 120 g at 4 °C for 7 min. The concentration was adjusted to 2×10^6 cells/ml and cell viability was always more than 90%, as determined by trypan blue dye exclusion. A total of 50 μl of cell suspension was dispensed into wells of a tissue-culture-grade 96-well plate (10×10^4 cells/well) and incubated for 2 h at 37 °C, 5% CO_2 to attach the cells. Unattached cells were discarded gently after 2 h. The attached cells were induced with 10 μg/ml of *Escherichia coli* LPS (Strain 055:B5) in the presence or absence of sample at a final volume of 100 μl/well. The sample stock was serially diluted in DMSO to decreasing concentrations ranging from 12.5 to 0.4 μmol/l, where the final concentration of DMSO in media was maintained at 0.1%. Untreated cells and drug controls were stimulated with LPS and also had the same amount of DMSO in culture medium. Cells were then incubated for 24 h at 37 °C in 5% CO_2.

2.6. Cell viability assay

Cell viability was assessed by the MTT method after the removal of spent media. In the MTT assay a total of 100 µl of DMEM or RPMI-1640 containing 5% FBS was added to each well followed by 20 µl of MTT (5 mg/ml). The formazan crystals were dissolved with 100 µl of 100% DMSO per well after 4 h. The absorbance was measured at λ 570 nm with a microplate reader (UVM 340, ASYS Hitech GmbH, Austria, Europe) by using a reference wavelength of 650 nm. Cell viability was determined as the percentage of untreated stimulated cells.

2.7. Measurement of nitric oxide production

To evaluate the inhibitory activity of test materials on nitric oxide (NO) production, culture media was assayed using Griess reaction. RAW 264.7 macrophages were incubated with LPS treatment with the presence or without compound simultaneously. A concentration of 10 µg/ml of *E. coli* LPS were treated in RAW 264.7 cells which were seeded in a 96-well plate for 24 h. Briefly, cell culture supernatants were mixed with an equal volume of Griess reagent [1%sulfanilamide/0.1%N-(1-naphtyl)-ethylene diamide dihydrochloride in 2.5% H_3PO_4], and absorbance was read at 550 nm with microplate reader (Asys HiTech UVM 340, USA). The amount of nitrite in the sample was calculated from a sodium nitrite standard curve.

2.8. Nitrite-scavenging activity

In an attempt to determine whether BHMC was scavenging secreted nitrite in culture media, we subjected the compound to a chemical scavenging assay. Briefly, 50 µl of serially-diluted BHMC was pipetted into a 96-well flat-bottomed plate. Next, 50 µl of 20 mM sodium nitroprusside dissolved in PBS (SNP, 10 mM final concentration) was added into each well and the plate was then incubated at room temperature for 150 min. A control experiment was conducted to determine NO scavenging effect of 0.1% DMSO in media. PTIO, a nitrite scavenger, was used as a positive control. After incubation, an equal volume of Griess reagent was added into each well in order to measure the nitrite content. The absorbance of these solutions was measured at 550 nm against the corresponding blank solutions. Fresh culture medium was used as the blank in all the experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve (0–100 µM) freshly prepared in deionized water.

2.9. Indirect determination of iNOS activity

Induction of cells into an inflammatory state prior to treatment with BHMC enables the synthesis of intracellular iNOS and accumulation of high levels with corresponding enhanced synthesis and secretion of NO. Since the induction with LPS will induce iNOS synthesis, it is suggested that variations in the secretion of NO following treatment with BHMC point to an effect upon iNOS activity rather than gene or enzyme expression. RAW 264.7 cells were placed in a 96-well plate and treated with LPS for 12 h. The cells were washed three times with PBS and incubated in the presence of serial dilutions of BHMC without LPS in medium for a further 12 h. L-NAME was used as a specific inhibitor of iNOS enzyme activity (positive control) and 0.1% DMSO as a solvent control. The supernatants were removed and assayed for nitrite using the Griess assay as described above.

2.10. Prostaglandin E_2 (PGE_2) immunoassay

In order to determine the effect of BHMC on prostaglandin production, U937 cells were differentiated and stimulated as described earlier. Following 24 h incubation with varying concentrations of BHMC spent media was collected for prostaglandin immunoassay. Supernatants of spent cell culture media were stored at −80 °C prior to assay. PGE_2 concentrations were determined with an enzyme immunoassay kit (Cayman Chemicals, USA, Cat No. 514010) according to the manufacturer's instructions. NS-398 was used at 50 µM as a drug control.

2.11. Cytokine immunoassay

For evaluation of secreted cytokines level, U937 cells were differentiated and stimulated as described above. Following 18 h incubation with varying concentrations of BHMC spent media was collected for cytokine immunoassay. Supernatants of spent cell culture media were stored at −80 °C prior to assay. Spent media was analyzed for TNF-α, IL-1β, IL-6, IL-8, IL-10 and MCP-1 by enzyme-linked immunosorbent assay (ELISA) using commercial kits (BD Pharmingen, USA, Cat No. 555212, 557953, 555220, 555244, 555157, 555179 BD OptEIA) according to the manufacturer's instructions.

2.12. Reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cell monolayers was isolated using Qiagen RNeasy Mini Extraction kit (Qiagen, USA, Cat No. 74106) as suggested by the manufacturer. RNA concentration was determined and 100 ng of RNA was used for Qiagen One-Step RT-PCR kit (Qiagen, USA, Cat No. 210212) according to the manufacturer's instructions. RNA integrity was examined by formaldehyde agarose gel electrophoresis and concentrations were determined by UV spectrophotometry (DU 530 Life Science UV/Visible Spectrophotometer, Fullerton, CA). The master mix was performed in an Eppendorf thermal cycler for reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min, final extension at 72 °C for 10 min. The reaction products from PCR were examined by 1.8% agarose gel electrophoresis and stained with ethidium bromide. Band intensities were quantified by Image J Java-based image processing program (NIH, USA) and normalized by comparison to the RT-PCR products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. PCR product for each gene was run in parallel as a molecular size marker (providing bands at 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp and 100 bp) except for iNOS gene, which was run in parallel with another molecular size marker (providing bands at 766 bp, 500 bp, 350 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp, 75 bp, 50 bp and 25 bp). Primers used in this study were adapted from published gene sequences (Table 1).

2.13. Western blot analysis

RAW 264.7 cells were stimulated with 10 µg/ml of LPS as described earlier and treated with BHMC for 18 h for iNOS protein detection. Whole cell protein extract was used to analyze iNOS protein. Protein sample was extracted by using an equal volume of sample buffer (125 mM, 4% SDS, 20% glycerol, 0.004% bromophenol blue) with benzonase nuclease (25 U/ml) was added in to digest nucleic acid. After that, sample was boiled for 10 min to denature protein. Equal amounts of protein (50 µg) were electrophoresed on a 7% SDS-polyacrylamide gel and blotted on a 0.45 µm PVDF membrane. The membrane was blocked with 5% BSA in Tris buffered saline (TBS)–Tween 20 (0.05%) for 1 h and incubated overnight at 4 °C with rabbit anti-mouse polyclonal iNOS antibody (1:1000) in TBS–Tween containing 5% BSA. After washing three times with TBS–Tween, the membrane was hybridized with HRP-conjugated donkey anti-rabbit secondary antibody (1:5000) for 2 h and washed three times with TBS–Tween. The same membrane was stripped and re-probed with HRP-conjugated mouse anti-mouse β-actin (1:10,000). The membrane was incubated with ECL reagent for 2 min and viewed by chemiluminescence. Band intensities were quantified by Image J Java-based image processing program and normalized by comparison to β-actin.

Table 1
Primer sequences, operating conditions, and PCR product sizes.

Gene name	Oligonucleotide sequences (5′–3′)	Denaturing temperature and time (°C, s)	Annealing temperature and time (°C, s)	Elongation temperature and time (°C, s)	Cycle number	Product size (bp)
hTNF-α	F-GAGCACTGAAAGCATGATCCGGGAC R-TTGGTCTGGTAGGAGACGGCGATGC	94 (60)	67 (45)	72 (60)	25	495
hIL-1β	F-GCATCCAGCTACGAATCTCC R-CCACATTCAGCACAGGACTC	94 (60)	62 (45)	72 (60)	25	700
hIL-6	F-CACCGGGAACGAAAGAGAAG R-AGCAGGCTGGCATTTGTGGT	94 (60)	66 (45)	72 (60)	23	450
hIL-10	F-CATCAGGGTGGCGACTCTAT R-CCCAAGCCCAGAGACAAGAT	94 (60)	62 (45)	72 (60)	25	430
hIL-8	F-ACCGGAAGGAACCATCTCACT R-GCATCTGGCAACCCTACAACA	94 (60)	69 (45)	72 (60)	25	444
hMCP-1	F-GCTCATAGCAGCCACCTTCATTC R-TGCAGATTCTTGGGTTGTGGAG	94 (60)	69 (45)	72 (60)	24	297
miNOS	F-GAATCTTGGAGCGAGTTGTGG R-AGGAAGTAGGTGAGGGCTTGG	94 (60)	66 (45)	72 (60)	26	139
mGAPDH	F-CCCTGTTGCTGTAGCCGTAT R-TGTTCCTACCCCCAATGTGT	94 (60)	Depends on target gene			
hGAPDH	F-TGAAGGTCGGAGTCAACGGATTTGGT R-CATGTGGGCCATGAGGTCCACCAC	94 (60)	Depends on target gene			

hTNF-α: human tumor necrosis factor-alpha; mGAPDH: murine glyceraldehyde-3-phosphate dehydrogenase.

2.14. Statistical analysis

Data was presented as means ± S.E.M of three separate experiments performed in triplicate. The IC_{50} values were calculated using one parameter model [$y = 100/(1 + (a/x)$] using GraphPad Prism software. SPSS version 15.0 was used to determine the differences between groups by using one-way analysis of variance (ANOVA) followed by post hoc comparison using Dunnett test. *P*-value of less than 0.05 was considered to be significant.

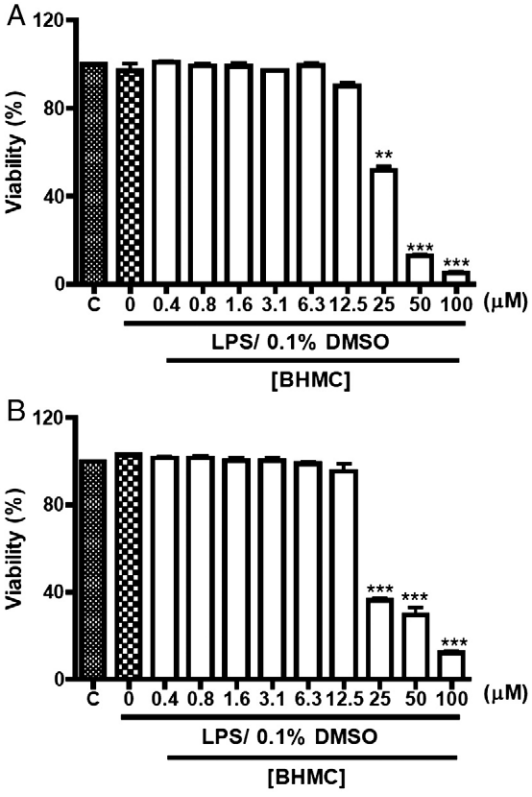

Fig. 2. Effect of BHMC on RAW 264.7 and U937 cell viability using MTT assay. (A) RAW 264.7 macrophages (2×10^6 cells/ml) were treated with indicated concentrations of BHMC for 24 h. (B) Differentiated U937 monocytes (2×10^6 cells/ml) were treated with indicated concentrations of BHMC for 24 h. All values are the mean ± S.E.M. of three independent experiments. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$, significantly different from the control group.

3. Results

3.1. Cytotoxicity

Fig. 2 shows the effect of BHMC upon both RAW 264.7 and U937 cell viability. Treatment with BHMC caused a reduction in cell viability at 25 μM and above in both cell lines. Therefore BHMC was used at 12.5 μM and below for further assays.

3.2. BHMC inhibits NO secretion in a dose-dependent manner with minimal inhibition of iNOS activity

Fig. 3A shows the dose–response of BHMC towards the secretion of NO. LPS-induced RAW 264.7 cells produced large amounts of nitrite. Significant inhibition of nitrite was demonstrated at almost all concentrations tested with an IC50 value of 2.93 ± 0.15 μM. To further investigate whether the inhibition of NO production by BHMC was mediated through the inhibition of iNOS enzyme activity, we assessed the effects of BHMC on iNOS activity by induction of enzyme expression followed by treatment with BHMC. Fig. 3B shows a slight reduction in nitrite synthesis following prior induction of iNOS before treatment and therefore we conclude that BHMC has a minimal effect upon iNOS activity.

3.3. BHMC inhibits iNOS gene and protein expression

Fig. 4A and B shows the effect of various doses of BHMC upon iNOS gene and protein expression respectively. Both gene and protein expression were reduced in a dose-dependent manner.

3.4. BHMC does not scavenge nitrite and does not inhibit PGE_2 secretion

All of the tested concentrations of BHMC did not reveal any level of nitrite-scavenging activity (data not shown). Therefore the inhibitory effect of BHMC on NO secretion is not caused by its scavenging activity. BHMC did not inhibit the secretion of PGE_2 at all concentrations tested (data not shown).

3.5. BHMC variably inhibits cytokine/chemokine secretion and gene expression

Table 2 and Fig. 5 shows the IC50 values of BHMC inhibitory effects upon cytokine secretion and dose–response effects upon cytokine gene expression respectively.

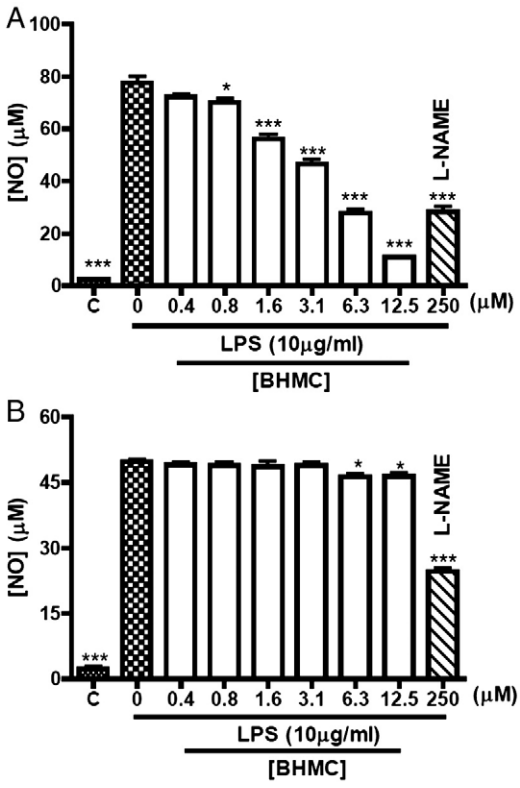

Fig. 3. Effect of BHMC upon nitric oxide production and iNOS activity by RAW 264.7 macrophages. (A) RAW 264.7 macrophages (2×10^6 cells/ml) were treated with 10 μg/ml *E. coli* LPS and indicated concentrations of BHMC. (B) RAW 264.7 macrophages (2×10^6 cells/ml) were treated with 10 μg/ml *E. coli* LPS for 12 h prior to treatment with indicated concentrations of BHMC. Nitrite levels were determined by the Griess reaction after 24 h treatments. All values are the mean ± S.E.M. of three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.005$, significantly different from the LPS-induced control group.

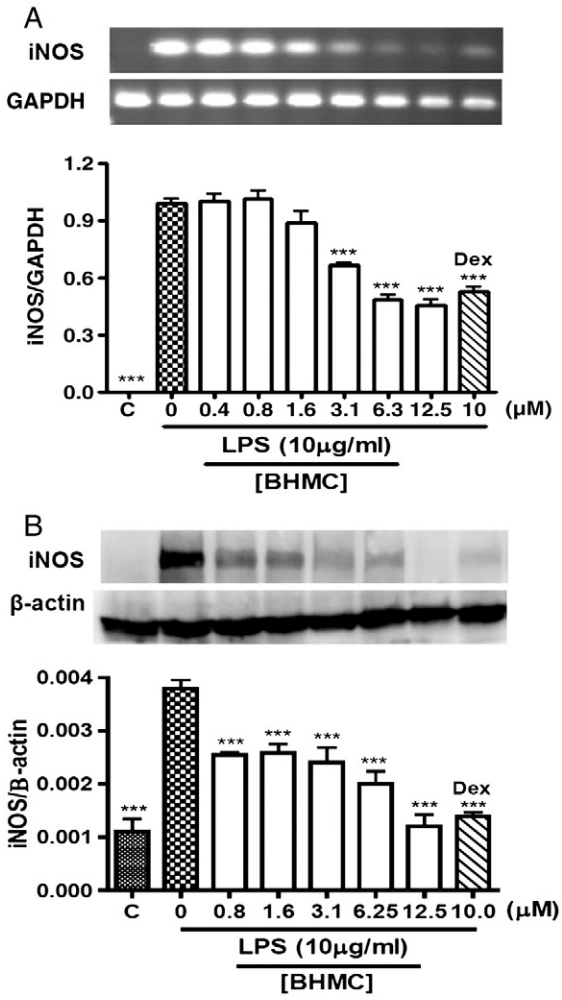

Fig. 4. Effect of BHMC upon iNOS gene and protein expression in LPS-stimulated RAW 264.7 macrophages. (A) Cells were induced with 10 μg/ml *E. coli* LPS and treated with increasing concentration of BHMC for 4 h. RNA was extracted and analyzed by Reverse-Transcriptase-PCR. (B) Cells were induced with 10 μg/ml *E. coli* LPS and treated with increasing concentration of BHMC for 18 h. Protein was extracted and probed against rabbit anti-mouse polyclonal iNOS antibody. All values are the mean ± S.E.M. of three independent experiments. *P0.05, **$P<0.01$ and ***$P<0.005$, significantly different from the LPS-induced control group. Cur: Curcumin; Dex: Dexamethasone.

BHMC showed a strong inhibition of MCP-1 and IL-10 secretion. TNF-α and IL-6 were also inhibited in a dose-dependent fashion albeit at a moderate level and minimal effects were noted upon the secretion of IL-1β and IL-8. Results from RT-PCR (Fig. 5) also showed that inhibition of MCP-1 and IL-10 mRNA expression was strong. In fact MCP-1 gene expression was totally abolished at the concentration of 3.1 μM and above. Effects upon TNF-α and IL-6 gene expression were moderate and the expression of IL-8 and IL-1β genes were not affected at all and reflect the lack of effect upon cytokine synthesis and secretion.

4. Discussion

This study shows that our modification of curcumin into BHMC preserved several inhibitory characteristics upon proinflammatory mediators, while enabling the compound to be more specific since it lost its inhibitory effect upon PGE_2 secretion and had minimal inhibitory effect upon IL-1β and IL-8. BHMC showed a strong inhibitory effect upon NO synthesis, which we have shown to be due to suppression of both iNOS gene and protein expression rather than an effect upon iNOS activity or the simple scavenging of nitrite in the media. An interesting observation is the particularly strong inhibitory effect upon MCP-1 synthesis.

Although BHMC possessed a prominent suppressive effect on MCP-1 synthesis, its effect upon IL-8 secretion was minimal with no effect upon IL-8 gene expression. This finding suggests that the inhibitory effect of BHMC was geared towards chronic rather than acute inflammatory networks (Mukaida et al., 1998). In contrast, curcumin affects chemokines non-specifically whereby both IL-8 and MCP-1 are inhibited (Hidaka et al., 2002; Jain et al., 2009). The inhibition of curcumin on the production of IL-8, macrophage inflammatory protein (MIP)-1α, MCP-1, IL-1β and TNF-α from monocytes and alveolar macrophages is achieved via several mechanisms. The promoter gene encoding these cytokines contains sequences for binding several nuclear transcriptional factors including AP-1 and NF-κB (Siebenlist et al., 1994; Baeuerle and Henkel, 1994). Furthermore curcumin has been shown to inhibit the activation of c-Jun/AP-1 in osteoblastic cells (Hanazawa et al., 1993) and mouse fibroblasts

Table 2
The IC_{50} of BHMC upon TNF-α, IL-1β, IL-6, IL-8, IL-10 and MCP-1 secretions by LPS-induced U937 cells. Concentrations of cytokines were determined by immunoassay.

Cytokine/chemokine	IC_{50} (μM)
TNF-α	5.537 ± 0.477
IL-1β	n.d.[a]
IL-6	6.542 ± 0.613
IL-8	n.d.[a]
IL-10	2.448 ± 0.177
MCP-1	0.981 ± 0.241

[a] n.d.: not determined (none of the doses tested caused 50% of inhibition).

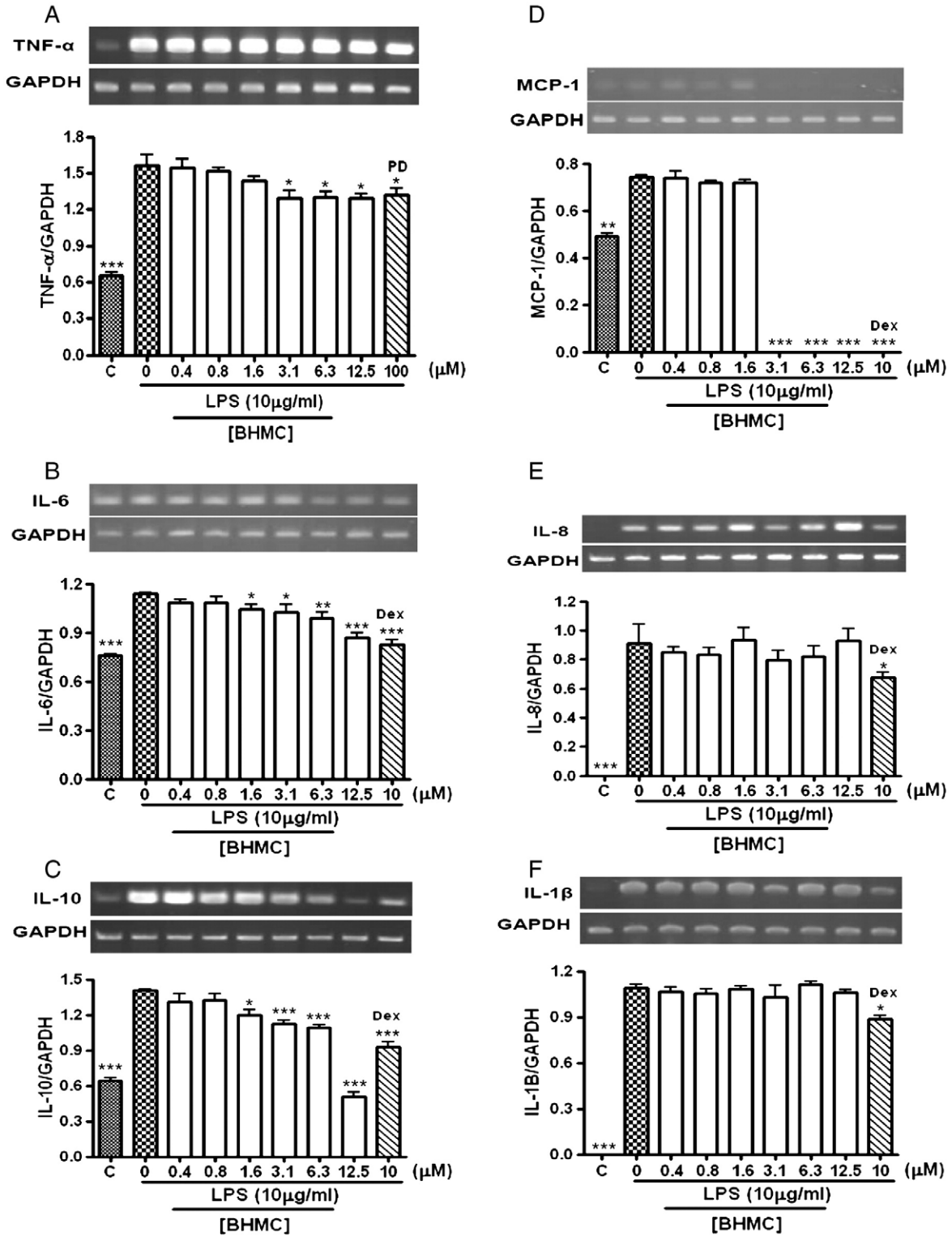

Fig. 5. Effect of BHMC on cytokine and chemokine gene expression in LPS-induced U937 cells by using One Step Reverse-Transcriptase-PCR. (A) TNF-α. (B) IL-6. (C) IL-10. (D) MCP-1. (E) IL-8. (F) IL-1β. All values are the mean ± S.E.M. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$, significantly different from the LPS-treated control group. PD: PD 98059; Dex: Dexamethasone.

(Huang et al., 1991), and of NF-κB in a human monocytic macrophage cell line (Chan, 1995; Singh and Aggarwal, 1995). Inhibition of MCP-1 gene expression has also been demonstrated in closely related analogues in which this effect was associated with inhibition of NF-κB gene expression (Liang et al., in press). Strong inhibition of NO, PGE_2, and proinflammatory cytokine synthesis by curcumin (Jin et al., 2007) has been ascribed to down-regulation of NF-κB-dependent transcription (Chen et al., 2008). Closely related analogues of BHMC have also been shown to inhibit the secretion of proinflammatory cytokines, iNOS and COX 2 through inhibition of NF-κB gene expression (Liang et al., in press). In view of the differences in the inhibitory nature of BHMC upon cytokine synthesis compared to closely related analogues and

curcumin, further work is warranted to determine whether BHMC affects the NF-κB signaling pathway and other proinflammatory signaling pathways. Of particular interest is the finding that the modifications of curcumin that lead to the synthesis of BHMC have abolished inhibition PGE_2 and IL-1β, mediators of which seem to be a target of other closely related compounds.

Curcumin has been reported to inhibit the induction of iNOS in RAW 264.7 cells. The activity and protein expression of iNOS are decreased by curcumin via reduction of iNOS mRNA expression (Cen et al., 2009). Furthermore, the question of whether curcumin is a potent scavenger of nitrite remains controversial (Sreejayan and Rao, 1997; Zhang et al., 2008). In this study we show that BHMC has a strong inhibitory activity upon NO synthesis of which we demonstrate this effect to be due to inhibition of iNOS synthesis and not an effect upon enzymatic activity or scavenging of released cellular nitrite. The use of the RAW 264.7 cell line in our studies of NO inhibition was based on the fact that U937 cells do not produce measurable amounts of nitrite following induction. The question of whether human macrophages possess the complete machinery necessary for NO synthesis remains controversial. It has been reported that human monocytic U937 cells contain all the cofactors necessary for NO synthesis, except BH4 (Bertholet et al., 1999). Further work will consider the use of induced cells from whole blood of donor subjects and efficacy tests for BHMC in mouse models of diseases that are related to excessive iNOS activation.

In our present study, BHMC strongly inhibited both the secretion and gene expression of IL-10, this is in contrast with curcumin, which was shown to enhance the level of Interferon (IFN)-β and IFN-α-induced IL-10 production in T cells (Fahey et al., 2007). The suppression of IL-10 production and gene expression by glucocorticoids and indomethacin in LPS-induced human peripheral blood mononuclear cells (PBMCs) is suggested to be a major mechanism by which these drugs suppress allergic inflammation (Cremer et al., 2001) and asthmatic allergy (Robinson et al., 1996). The role of IL-10, an anti-inflammatory cytokine, in the pathogenesis of inflammatory disease and its pharmacological modulation remains complicated. However, studies using chalcone compounds have shown that the inhibition of IL-10 is due to inhibitory effects upon mitogen activated protein kinases (MAPKs) (Liu et al., 2006). Indeed curcumin and its derivatives have been shown to inhibit MAPK pathways (Aggarwal and Shishodia, 2006) albeit with stimulation of IL-10 synthesis (Fahey et al., 2007). It is indeed likely that BHMC may affect MAPK pathways too, an interesting problem for further work.

In conclusion, BHMC effectively suppressed the production of various inflammatory mediators. The main inhibitory effect is upon the synthesis of NO, MCP-1 and IL-10. At present, we have shown that BHMC acquires this effect by inhibiting both gene and protein expression of iNOS and several cytokines. Whether BHMC may also act upon transcription factors and related cell signaling pathways remains to be proven. However we are inclined to believe that differences in structural and functional characteristics of BHMC compared to curcumin may also be reflected in a different, more selective effect upon proinflammatory signaling pathways. Further dissection of the mechanism may provide more accurate information regarding the precise target molecule involved.

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