

MAPKs and NF- κ B pathway inhibitory effect of bisdemethoxycurcumin on phorbol-12-myristate-13-acetate and A23187-induced inflammation in human mast cells

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Abstract. Inflammation-associated damage may occur in any tissue following infection, exposure to toxins, following ischemia, and in allergic and auto-immune reactions. Inflammation may also result from mast cell degranulation induced by the intracellular calcium concentration. The inflammatory process may be inhibited by compounds that affect mast cells. Bisdemethoxycurcumin [1,7-bis(4-hydroxyphenyl) hepta-1,6-diene-3,5-dione, BDCM] is the active component of turmeric. It has anticancer, antioxidant and antibacterial properties. To investigate the molecular mechanism associated with the anti-inflammatory activity of BDCM, human mast cell line 1 (HMC-1) cells were treated with phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187 (A23187) to induce the inflammatory process. Various HMC-1 cells were pretreated with BDCM prior to stimulation of inflammation. BDCM inhibited the inflammation-triggered production of cytokines including interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α . BDCM inhibition extended to the gene level. In activated HMC-1 cells, phosphorylation levels of extracellular signal-regulated kinase, c-jun N-terminal kinase and p38 mitogen-activated protein kinase were decreased by treatment with BDCM. BDCM also inhibited nuclear factor-(NF)- κ B activation and I κ B degradation. In conclusion, BDCM suppresses the expression of TNF- α , IL-8, and IL-6 by inhibiting the NF- κ B and mitogen activated protein kinase signaling pathways.

Introduction

Inflammation-related damage can occur in any tissue following infection, exposure to toxins, after ischemia, and in allergic and auto-immune reactions. Inflammatory processes are systematized by inflammatory cells, such as mast cells (1). Mast cells are widely present in the connective tissues of mammals and other vertebrates and are physically in close proximity to blood vessels (2). The cells are significant effector cells in inflammation as well as allergic reactions, because they secrete various cytokines (3). Regulation of cytokine secretion from mast cells could be a useful therapeutic strategy for allergic inflammatory diseases. The signaling pathway inducing mast cell degranulation has been characterized (4). Mast cell activation induces phosphorylation of tyrosine kinase and migration of calcium ion (Ca^{2+}) in the body (5). Tyrosine phosphorylation is an important event in intracellular signal transduction induced activation of protein kinase C and secretion through Ca^{2+} influx (6). Calcium acts as a second messenger in mast cell activation, with activation in response to increased intracellular Ca^{2+} (7). The release of intracellular Ca^{2+} is essential for the activation of mitogen-activated protein kinase (MAPK) (8,9). MAPK participates in the mast cell regulation of cytokine production in response to particular extracellular stimuli, which subsequently begins the biological responses that drive cell differentiation, proliferation, and apoptosis. These include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) (10). Phosphorylation-mediated ERK activation regulates cytoplasmic and nuclear targets, and various cell responses including proliferation, migration, differentiation and death (11). JNK is involved in a signaling pathway concerned with the inflammatory response. Activated JNKs affect the formation of the activator protein 1 (AP-1) transcription factor complex that participates in the expression of many inflammatory factors and controls the synthesis of many inflammatory cytokines (12). P38 is involved in the production of pro-inflammatory cytokines by regulating the expression of nuclear factor- κ -light-chain-enhancer of

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activated B cells (NF- κ B) (13). In addition, ERKs, JNKs, and p38 are involved in control of interleukin (IL)-6 mRNA or protein production in response to Fc ϵ RI aggregation (14). Activated NF- κ B regulates the expression of cytokines, chemokines, and cell adhesion molecules (CAM). The activity of NF- κ B occurs when I κ B is phosphorylated and disassociated by I κ B kinase (IKK) catalysis (15).

Curcuma longa L. is a perennial herb in family Zingiberaceae. It is a traditional medicine used to treat disorders, anorexia, diabetic wounds, hepatic disorders, rheumatism, and sinusitis in China and India (16). The three main ingredients of *Curcuma longa* L. are curcumin (diferuloylmethane), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC), with curcumin being most abundant (17). Curcumin has physiological activities including antioxidant, anti-inflammation, and anticancer activities (18-20). However, curcumin is unstable and easily degrades *in vivo*, so more stable curcuminoids are needed to replace it (21). BDCM is more comparatively stable *in vivo*, is more readily taken up into the cell nucleus (22,23), and possesses anticancer, antioxidant and antibacterial activities (24-26). BDCM has anti-inflammatory activity in lipopolysaccharide-induced RAW 264.7 macrophages, with the inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NF- κ B. BDCM also suppresses carrageenan-induced paw edema in mice (27,28).

The anti-inflammatory effect of BDCM in human mast cells is unknown. The present study investigated the influence of BDCM on cytokine, MAPK, and NF- κ B activity in HMC-1 induced with phorbol-12-myristate-13-acetate (PMA) and A23187.

Materials and methods

Reagents and antibodies. Bisdemethoxycurcumin (Fig. 1) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PMA and A23187 (calcymycin; C29H37N3O6) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Iscove's modified Dulbecco's medium (IMDM) was obtained from Welgene (Daegu, Korea). Anti-human tumor necrosis factor- α (TNF- α ; 555212), anti-IL-6 (555220), anti-IL-8 (555244), biotinylated anti-human TNF- α (51-26372E), anti-IL-6 (51-26452E), IL-8 (51-26542E), and anti-recombinant human TNF- α (51-26376E), anti-IL-6 (51-26456E), and anti-IL-8 (51-26546E) antibodies were obtained from BD Pharmingen (San Diego, CA, USA). The reverse transcription kit was purchased from Qiagen (Valencia, CA, USA). Nuclear and cytoplasmic extraction reagents were purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Anti-phosphorylated (p)-ERK1/2 (sc-7383), anti-p-JNK1/2 (sc-6254), anti-p-p38 (sc-7973), anti-ERK1/2 (sc-93), anti-JNK1/2 (sc-571) anti-p38 (sc-535), anti- β -actin (sc-47,778), anti-NF- κ B (sc-8008), anti-mouse, and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and cell viability. The human mast cell line 1 (HMC-1) was cultured in IMDM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 50 μ g/ml streptomycin, and 1.2 mM α -thioglycerol at 37°C in an incubator with an atmosphere of 5% CO₂. Cell viability was

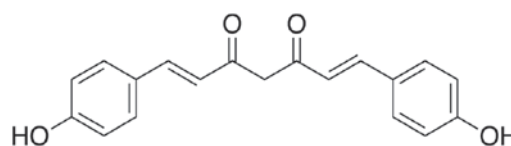


Figure 1. Chemical structure of bisdemethoxycurcumin.

evaluated by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA) assay following incubation in the presence of 25 or 50 μ M BDCM for 24 h at the aforementioned temperature and CO₂ conditions using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA) at an optical density of 490 nm.

Analysis of cytokine production. The OptEIA™ human enzyme-linked immunosorbent assay (ELISA; BD Bioscience, San Jose, CA, USA) was used to assay culture supernatants to measure TNF- α , IL-6, and IL-8 secretion. HMC-1 cells were seeded at 5x10⁵ cells per well in 24-well plates and treated with 25 or 50 μ M BDCM for 30 min. The cells were then stimulated for 8 h with 50 nM PMA and 1 μ M A23187 (Sigma-Aldrich). Cytokines in the supernatant were measured using ELISA. Each well of the 96-well microplate was coated with capture antibody diluted in coating buffer (0.1 M carbonate, pH 9.5). Each plate was sealed and incubated overnight at 4°C. After washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20, non-specific binding sites were blocked with PBS containing 10% FBS (pH 7.0) for 1 h. A total of 100 μ l of each sample, or TNF- α , IL-6, and IL-8 standards were added to wells and incubated for 2 h at room temperature. A total of 100 μ l of detection antibody conjugated with and avidin-horseradish peroxidase (HRP) diluted in assay buffer was applied for 1 h. A total of 100 μ l of substrate solution (tetramethylbenzidine, TMB) was added to each wells and incubated for 30 min at room temperature in the dark. A total of 50 μ l of stop solution (2 M H₂SO₄) was added and the absorbance was determined at 450 nm.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from HMC-1 cells using easy BLUE™ Total RNA Extraction kit (iNtRon, Seongnam, Korea). The total RNA was dissolved in DEPC-treated distilled water. A spectrophotometer (Biotek) was used to evaluate RNA purity by measuring the ratio of the absorbance at 260 and 280 nm. Complementary deoxyribonucleic acid (cDNA) was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, Seoul, Korea) according to the manufacturer's instructions. RT-qPCR was performed in triplicate using power SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in a StepOne Plus Real-Time-PCR system (Applied Biosystems). The amplification was 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by 1 cycle 95°C for 15 sec and 60°C for 1 min. The expression levels of the target genes relative to the endogenous reference gene, β -actin, were calculated using the $\Delta\Delta$ Cq method using StepOne software v2.3 (Applied Biosystems). The primer sequences are listed in Table I.

Table I. Sequences of oligonucleotide primers designed for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
hTNF- α	
Forward	GACAAGCCTGTAGCCCATGTTGTA
Reverse	CAGCCTTGGCCCTTGAAGA
hIL-6	
Forward	AAGCCAGACTGTGCAGATGACTA
Reverse	TGTCCTGCAGCCACTGGTTC
hIL-8	
Forward	ACACTGCGCCAACACAGAAATTA
Reverse	TTTGCTTGAAGTTTCACTGGCATC
β -actin	
Forward	ATTGCCGACAGGATGCAGAAC
Reverse	ATGGAGCCACCGATCCACA

TNF, tumor necrosis factor; IL, interleukin.

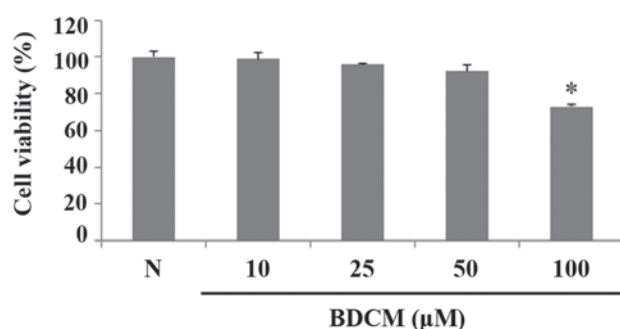


Figure 2. Effect of bisdemethoxycurcumin on viability of HMC-1 cells. Cell viability was evaluated using MTS assay. Data are expressed as means \pm standard error of the mean of duplicate determinations from three separate experiments. * $P < 0.05$ vs. normal cells. BDCM, bisdemethoxycurcumin; HMC, human mast cell line.

Western blot analysis. BDCM-pretreated and 1-h PMA and A23187 stimulated cells were collected and lysed with ice-cold lysis buffer (iNtRon). Following centrifugation at 13,000 rpm for 20 min, the supernatant was transferred to a 1.5 ml microtube. A total of 20–30 μ l of denatured protein lysate was separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis and the resolved proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-human-phospho-ERK antibody, anti-human-phospho-JNK antibody, or anti-human-phospho-p38 antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C. HRP-conjugated antibody against mouse IgG (Santa Cruz Biotechnology, Inc.) diluted 1:2,500 in 3% BSA was used as the secondary antibody. The proteins were detected using EzWestLumi plus luminal substrate (ATTO Co., Tokyo, Japan). After stripping, the membranes were reprobed with anti-human ERK antibody, anti-human JNK antibody, or anti-human p38 antibody (Santa Cruz Biotechnology, Inc.) as respective loading controls.

Cytoplasmic and nuclear protein extraction. Cytoplasmic and nuclear proteins were extracted from BDCM-pretreated and 2-h PMA and A23187 stimulated HMC-1 cells. Nuclear extraction was performed according to the manufacturer's instructions using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.). Cell volume of 20 μ l corresponded to a volume ratio of CER I:CER II:NER (200:11:100 μ l). A tube containing CER I was first vortexed vigorously at the highest speed setting for 1 sec to fully suspend the cell pellet. The tube was then incubated on ice for 10 min. Ice-cold CER II was then added to the tube followed by vortexing for 5 sec at the highest speed setting. The tube was then incubated on ice for 1 min before being vortexed for 5 sec at the highest speed setting. The tube was then centrifuged at 13,000 rpm for 5 min in a microcentrifuge. The supernatant (cytoplasmic extract) was then immediately transferred to a clean and pre-chilled tube and stored until use. Ice-cold NER was added to the pellet and the tube was vortexed for 15 sec at the highest speed setting. The sample was placed on ice and vortexed for 15 sec every 10 min for a total of 40 min. The tube was then centrifuged at 13,000 rpm for 5 min in a microcentrifuge. The supernatant (nuclear extract) was then immediately transferred to a clean and pre-chilled tube. The extracted cytoplasmic and nuclear protein was detected by western blot analysis.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Student's t-test for multiple comparisons was performed using SPSS Ver. 23 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability measurement. MTS assay was performed to measure cell viability of HMC-1 treated with BDCM. BDCM did not affect the viability of HMC-1 cells at concentrations of 10, 25, and 50 μ M. The cytotoxicity of BDCM was observed at concentrations of 100 μ M (Fig. 2).

Effect of BDCM on production of pro-inflammatory cytokines. ELISA was used to evaluate the effect of BDCM on the production of IL-6, IL-8, and TNF- α . The production of all three cytokines in HMC-1 cells considerably increased after stimulation with PMA and A23187 (Fig. 1). However, the productions of these cytokines were decreased by pretreatment with either concentration of BDCM (Fig. 3).

Effect of BDCM on pro-inflammatory cytokine gene expression. RT-qPCR was used to determine the expression levels of the genes encoding IL-6, IL-8, and TNF- α . Expression of the three genes in HMC-1 cells were significantly increased after stimulation with PMA and A23187, but were suppressed by pretreatment with either concentration of BDCM (Fig. 4).

Effect of BDCM on activation of MAPKs. Western blots were used to investigate the effect of BDCM on activation of MAPKs (ERK, JNK, and p38). Phosphorylation of the three MAPKs was increased by stimulation of PMA and A23187 in HMC-1 cells. Both concentrations of BDCM

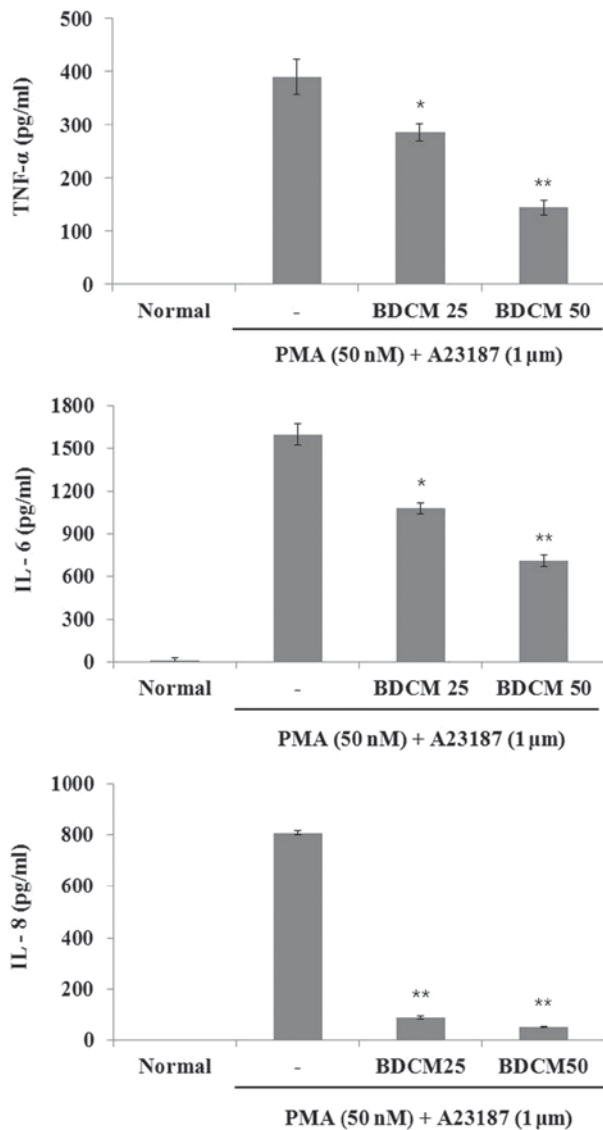


Figure 3. Effect of bisdemethoxycurcumin on production of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α . Human mast cell line 1 (HMC-1) cells were treated with bisdemethoxycurcumin (BDCM; 25 and 50 μ M) before being stimulated with phorbol-12-myristate-13-acetate (PMA; 50 nM) and calcium ionophore A23187 (A23187, 1 μ M) for 8 h. Values are expressed as the means \pm standard error (n=3 experiments). *P<0.05 and **P<0.005 vs. PMA and A23187-stimulated HMC-1 cells.

reduced JNK phosphorylation induced by PMA and A23187. Phosphorylation of ERK and p38 was reduced by BDCM at 50 μ M (Fig. 5).

Effects of BDCM on NF- κ B activation, I κ B α phosphorylation, and degradation. The expression of NF- κ B signaling molecules and NF- κ B transcriptional activity was investigated at the protein level. NF- κ B and p-I κ B α expressions in HMC-1 cells were increased by stimulation with PMA and A23187. Expressions of NF- κ B and p-I κ B α were decreased in BDCM-pretreated cells and I κ B α degradation was inhibited (Fig. 6).

Discussion

Mast cells are influential effector cells in the immune system. The importance of mast cells in allergic diseases, anaphylaxis,

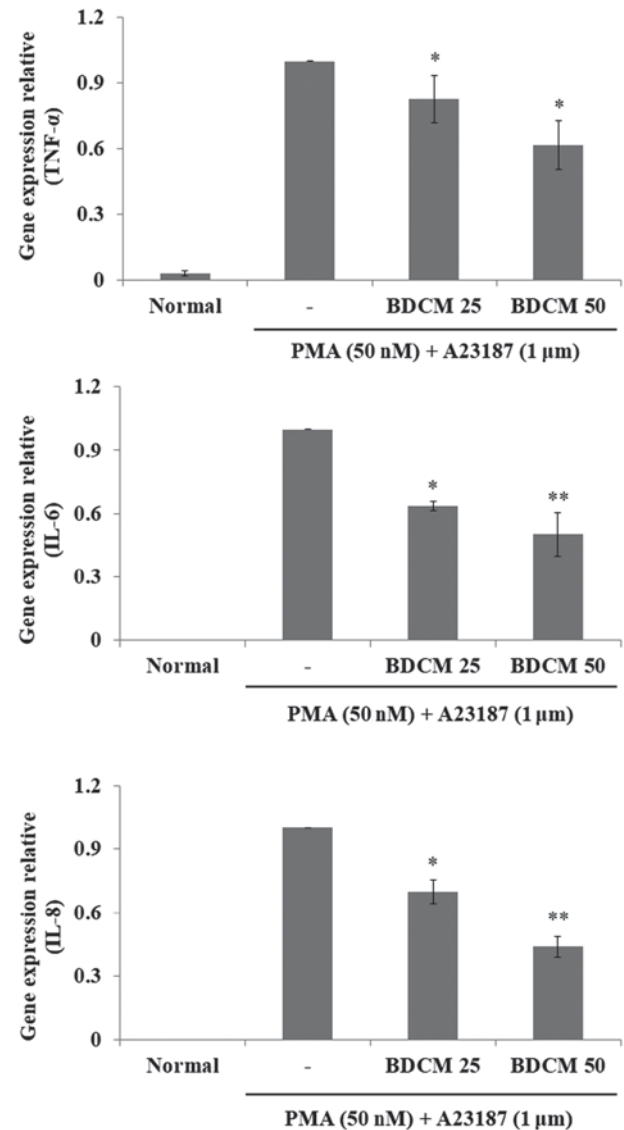


Figure 4. Effect of bisdemethoxycurcumin on the mRNA expression of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α as analyzed by RT-qPCR. Human mast cell line 1 (HMC-1) cells were treated with bisdemethoxycurcumin (BDCM; 25 and 50 μ M) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA; 50 nM) and A23187 (1 μ M) for 8 h. Values are expressed as the means \pm standard error (n=3 experiments). *P<0.05 and **P<0.005 vs. PMA and A23187-stimulated HMC-1 cells.

and autoimmunity is established. Mast cell-related diseases result from increased mast cell number and/or activity.

Activity of genes encoding several cytokines and their protein production were increased in HMC-1 cells stimulated with by PMA and A23187, as was phosphorylation of MAPKs and activity NF- κ B. These observations were consistent with the degranulation of HMC-1 cells in response to increased intracellular Ca^{2+} . The inflammatory reaction caused by the mast cell degranulation was alleviated if HMC-1 cells were first pretreated with BDCM. Phosphorylation of JNK, ERK, and p38 was also inhibited by BDCM as was NF- κ B and p-I κ B α production.

p38 regulates cell proliferation, apoptosis, environmental stress, and neuropathic pain. It is activated by various stress conditions and inflammatory cytokines, such as ultraviolet irradiation, osmotic and oxidative stress, heat shock, IL-1 β ,

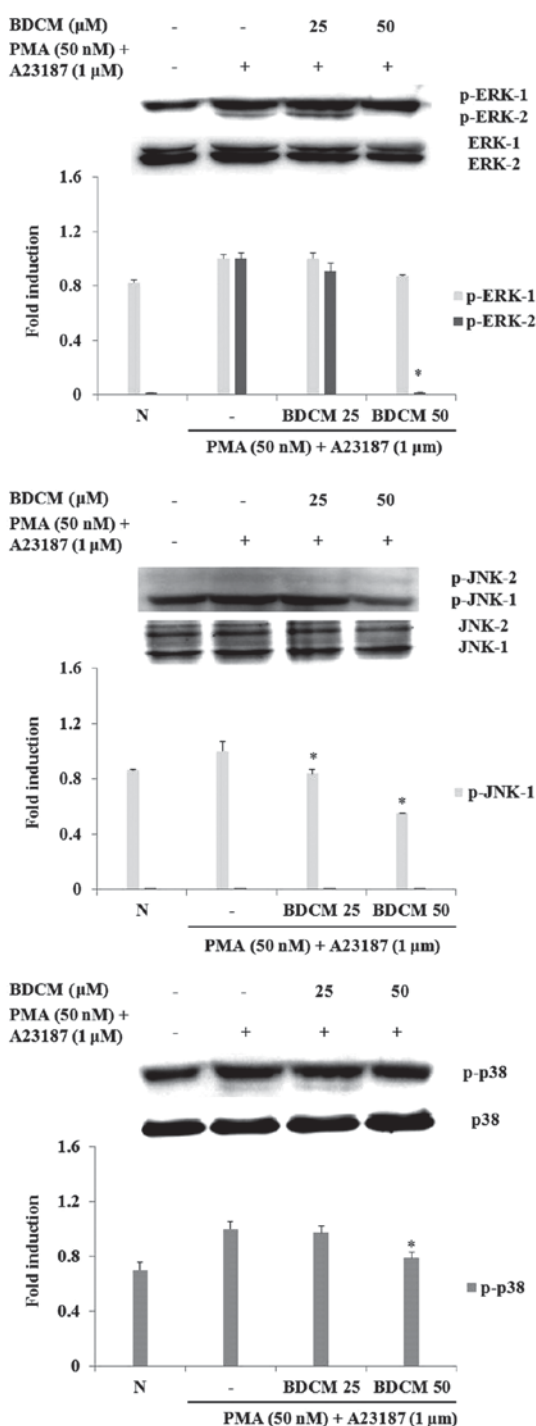


Figure 5. Effect of bisdemethoxycurcumin on the activation of mitogen-activated protein kinases (MAPKs) was analyzed by western blot analysis. Human mast cell line 1 (HMC-1) cells were treated with bisdemethoxycurcumin (BDCM; 25 and 50 μ M) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA; 50 nM) and A23187 (1 μ M) for 1 h. Values are expressed as the means \pm standard error (n=3 experiments). *P<0.05 vs. PMA and A23187-stimulated HMC-1 cells.

TNF- α , and transforming growth factor- β . The activated p38 transfers from the cytosol into the nucleus or to the other regions of the cell, activates downstream kinases, and regulates inflammatory processes, such as those involving iNOS, TNF- α , IL-1 β , and COX-2 (29).

Many recent studies have described the interaction of *Curcuma longa* L. and NF- κ B. Because this transcription

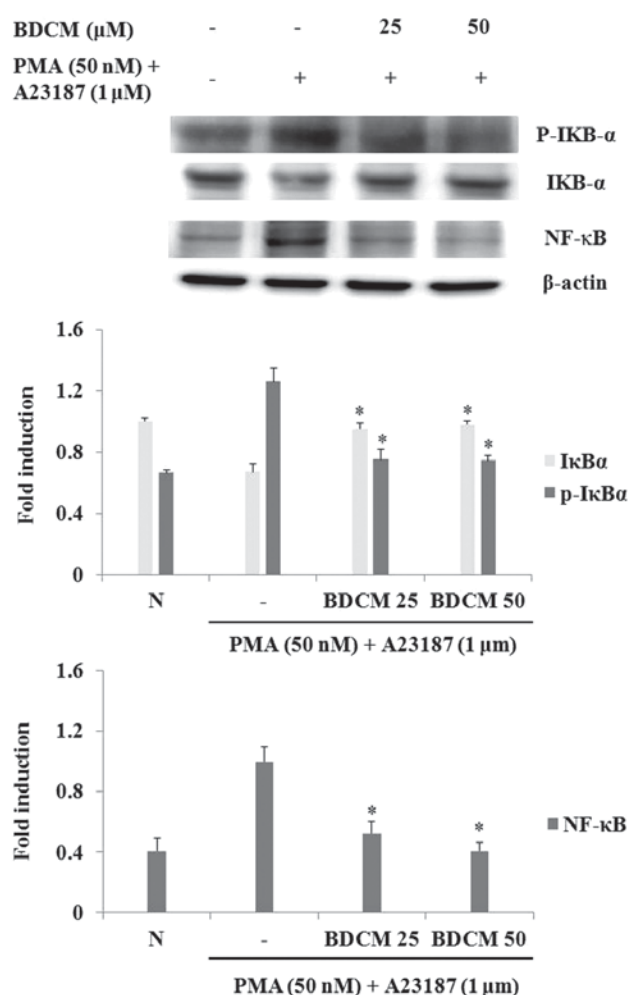


Figure 6. Effect of bisdemethoxycurcumin on the expression of nuclear factor- κ B (NF- κ B), p-IκB α and IκB α was analyzed by western blot analysis. Human mast cell line 1 (HMC-1) cells were treated with bisdemethoxycurcumin (BDCM; 25 and 50 μ M) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA; 50 nM) plus A23187 (1 μ M) for 2 h. Values are expressed as the means \pm standard error (n=3 experiments). *P<0.05 vs. PMA and A23187-stimulated HMC-1 cells.

factor is closely related to inflammatory and immune responses, *Curcuma longa* L. mediates its effects, at least in part, through the inhibition of NF- κ B activation. NF- κ B activation is regulated by MAPK via several mechanisms, but accumulated evidence shows that inhibitory proteins called I- κ B regulate NF- κ B activation by MAPKs that induce specific phosphorylation. In addition, previous studies have demonstrated a role for NF- κ B activation and inflammatory cytokine production regulation in inflammatory responses (30). The expression of TNF- α , IL-6, and IL-8 genes is dependent on the activation of transcription factor NF- κ B in mast cells.

This means that BDCM, one of the major components of *Curcuma longa* L., can inhibit the expression of inflammatory mediators by inhibiting NF- κ B activation and I- κ B depression in HMC-1. Therefore, BDCM suppressed the nuclear translocation of NF- κ B, phosphorylation of I- κ B, and degradation of I- κ B. These results indicate that the molecular mechanism by which BDCM suppresses the expression of pro-inflammatory cytokines and inflammatory mediators is through NF- κ B

inactivation. Specifically, I- κ B degradation and translocation are blocked. Inhibition of NF- κ B and MAPK affects the expression of cytokines and cytokine-associated genes.

In summary, BDCM regulates the expression of IL-6, IL-8, and TNF- α in PMA and A23187-induced HMC-1 cells, and inhibits the ERK, JNK, p38 MAPK, and NF- κ B pathways. These results implicate BDCM as a valuable compound to suppress the NF- κ B signaling pathway in mast cell-mediated inflammatory diseases.

Acknowledgements

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