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European Journal of Pharmacology 521 (2005) 1–8

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# Suppressive effect of novel aromatic diamine compound on nuclear factor-κB-dependent expression of inducible nitric oxide synthase in macrophages

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Received 31 March 2005; received in revised form 12 July 2005; accepted 19 July 2005
Available online 23 September 2005

**Abstract**

$N^1$-Benzyl-4-methylbenzene-1,2-diamine (BMD) is a novel synthetic compound. In the present study, BMD compound was discovered to inhibit nitric oxide (NO) production in macrophages RAW 264.7. BMD compound attenuated lipopolysaccharide (LPS)-induced synthesis of both mRNA and protein of inducible nitric oxide synthase (iNOS), and inhibited LPS-induced iNOS promoter activity, indicating that the aromatic diamine compound could down-regulate iNOS expression at the transcription level. As a mechanism of the anti-inflammatory action, suppression of BMD compound on nuclear factor (NF)-κB activation has been documented. BMD compound exhibited dose-dependent inhibitory effect on LPS-mediated NF-κB transcriptional activity in the macrophages. Further, the compound inhibited LPS-mediated nuclear translocation of NF-κB p65 and DNA binding activity of NF-κB complex, in parallel, but did not affect LPS-mediated degradation of inhibitory κBα protein (IκBα). These results indicate that BMD compound could inhibit nuclear localization step of NF-κB p65 without affecting IκBα degradation. Finally, BMD compound could provide an invaluable tool to investigate NF-κB-dependent iNOS expression, in addition to its therapeutic potential in NO-associated inflammatory diseases.
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*Keywords:* Aromatic diamine compound; Inducible nitric oxide synthase; Nuclear factor-κB; Anti-inflammation; Macrophages

## 1. Introduction

Nitric oxide (NO) is a short-lived free radical that can be produced from L-arginine by catalytic reaction of NO synthase (NOS) (Nathan, 1992). NOS-1 and -3 are expressed constitutively and located principally in endothelial cells and neurons, respectively (Griffith and Stuehr, 1995; Marletta, 1994). Endothelial NOS-1-derived NO is involved in physiological regulation of blood pressure by acting as a vasodilator, whereas NO produced by neuronal NOS-3 is associated with neurotransmission (Arnal et al., 1999; Wang et al., 1999). In contrast, expression of NOS-2, which commonly called as inducible NOS (iNOS), generally requires a cascade of cellular signaling events leading to transcriptional activation (Aktan, 2004). Macrophages have been shown to induce iNOS in response to bacterial lipopolysaccharide (LPS) and inflammatory cytokines (Bogdan et al., 2000; MacMicking et al., 1997). However, it is also clear that iNOS induction can be happened in a variety of other cell types including vascular smooth muscle and neuron cells, but also that high-output NO by iNOS may provoke deleterious consequences such as septic shock, neurotoxicity, and inflammatory diseases (Dalkara et al., 1998; Sessler et al., 2004; Zamora et al., 2000). Indeed, NO production in macrophages is related to the level of iNOS

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doi:10.1016/j.ejphar.2005.07.013

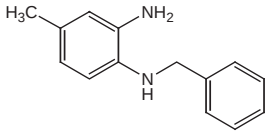


Fig. 1. Chemical structure of $N^1$-benzyl-4-methylbenzene-1,2-diamine (BMD).

expression, which is dependent upon several transcription factors including nuclear factor (NF)-κB (Lowenstein et al., 1993; Kim et al., 1997; Xie et al., 1994).

NF-κB transcription factor is functional as homo- or hetero-dimeric forms of Rel family proteins such as RelA (p65), RelB, cRel, p50 and p52 which are sequestered as an inactive complex in the cytoplasm, binding to inhibitory κB (IκB) proteins such as IκBα, IκBβ, IκBε, p105 and p100 (Baeuerle and Baltimore, 1996; Beg et al., 1992). LPS, a major component of the outer membranes of Gram-negative bacteria, can trigger a variety of inflammatory reactions after binding to Toll-like receptor 4 (Jones et al., 2001). Downstream signaling components of the receptor include myeloid differentiation protein MyD88, interleukin-1 receptor-associated kinase, and tumor necrosis factor receptor-associated factor 6, which can activate IκB kinase (IKK) complex (Akira, 2001; O'Neill and Dinarello, 2000; Wesche et al., 1997). Activation of IKK complex results in phosphorylation of IκB, which marks for ubiquitination followed by proteasome-mediated degradation (Lee et al., 1998). IκB degradation unmasks the nuclear localization signal motif of NF-κB, allowing the transcription factor to move into the nucleus, and the NF-κB binds to the promoter regions of immune and inflammatory genes including iNOS for transcriptional regulation (Guha and Machman, 2001; Tian and Brasier, 2003).

In our random screening study to discover anti-inflammatory agents, chemically synthetic $N^1$-benzyl-4-methylbenzene-1,2-diamine (BMD) (Fig. 1) was found to inhibit NO production in LPS-stimulated macrophages RAW 264.7. The BMD compound down-regulated LPS-induced iNOS expression at the transcription level. As a mechanism of the anti-inflammatory action shown by BMD compound, suppression of LPS-mediated NF-κB activation, specifically to nuclear localization step of NF-κB p65 without affecting IκB degradation, has been documented.

## 2. Materials and methods

### 2.1. Materials

LPS (*Escherichia coli* 055:B5) and interferon (IFN)-γ were purchased from Sigma-Aldrich (St. Louis, USA), and fetal bovine serum (FBS) from Invitrogen (Carlsbad, USA). Antibodies against iNOS, NF-κB p65, IκBα, extracellular signal-regulated kinase-1/2 (ERK-1/2), c-Jun N-terminal kinase (JNK) or p38 kinase were obtained from Santa Cruz Biotech (Santa Cruz, USA). An iNOS-luciferase reporter construct (Lowenstein et al., 1993), encoding the promoter region (−1592/+183) of murine iNOS gene fused to luciferase gene as a reporter, was kindly supplied by Dr. C.J. Lowenstein.

### 2.2. Chemical preparation of BMD compound

Hydrochloric acid (36%, 0.5 ml) was slowly added to a mixture (20 ml) of *N*-benzyl-4-methyl-2-nitroaniline (2.0 g, 8.3 mmol) and Fe powder (4.6 g) in 20% ethanol at room temperature. The mixture was refluxed for 1 h and then filtered without cooling. The filtrate was evaporated under vacuum, and subjected to flash column chromatography to isolate the BMD compound (purity, >98%), with a yield of 51.4%. Spectral data of BMD compound were described elsewhere (Lee, 2003).

### 2.3. Cell culture

Macrophages RAW 264.7 were obtained from ATCC (Manassas, USA). The cells were cultured in DMEM (13.4 mg/ml Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, pH 7.1) containing 10% FBS and maintained at 37 °C with 5% $CO_2$. The RAW 264.7 cells harboring pNF-κB-secretory alkaline phosphatase (SEAP)-NPT reporter construct (Moon et al., 2001) were cultured in the same conditions except supplement of 500 μg/ml geneticin to the media.

### 2.4. NO determination

Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with either LPS (1 μg/ml) for indicated times or LPS (100 ng/ml) plus interferon (IFN)-γ (3 ng/ml) for 24 h. Amounts of nitrite, a stable metabolite of NO, were measured using Griess reagent (Archer, 1993). Briefly, cell-free culture media (100 μl) was reacted with 1 : 1 mixture (100 μl) of 1% sulfanilamide in 5% $H_3PO_4$ and 0.1% *N*-(1-naphthyl)ethylenediamine in distilled water, and then absorbance at 540 nm was measured.

### 2.5. Western immunoblot analysis

Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 5 min (phospho-IκBα), 20 min (phospho-ERK-1/2), 20–100 min (IκBα), 1 h (NF-κB p65) or 18 h (iNOS). Cytoplasmic or nuclear extracts were resolved on SDS-acrylamide gel by electrophoresis, and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, USA). Membranes were blocked in Tris-buffered saline with Tween-20 (TBST) solution (25 mM Tris–HCl, 150 mM NaCl, 0.5% Tween 20, pH 7.5) containing 5% nonfat dried milk for 1 h, and then incubated overnight at room temperature with anti-

iNOS antibody (1 : 1500), anti-NF-κB p65 antibody (1 : 300), anti-IκBα antibody (1 : 300), anti-phospho-IκBα antibody (1 : 500) or anti-phospho-ERK-1/2 antibody (1 : 250). After washing with TBST solution, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1 : 2500) for 3 h at room temperature. After washing with TBST solution, the blots were treated with ECL Western blotting reagents (Amersham-Pharmacia, San Francisco, USA) and then exposed to X-ray film.

### 2.6. Immunoprecipitation experiment

Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 20 min. Lysates (200 μg protein) of the cells were pre-cleared using 25 μl of protein A/G-Sepharose, and then mixed with 2 μg of anti-JNK antibody or anti-p38 kinase antibody. After rotation for at least 4 h at 4 °C, the immunoprecipitates were resolved on SDS acrylamide gel by electrophoresis and then subjected to Western immunoblot analysis with anti-phospho-JNK antibody or anti-phospho-p38 kinase antibody.

### 2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 4 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR using an RNA PCR kit (Bioneer, Daejeon, Korea). Primers used for quantification of iNOS transcript were constructed as described previously (Kim et al., 2004). Briefly, total cellular RNA was reverse transcribed into cDNA by incubation at 42 °C for 1 h. The resulting cDNA samples were heated at 94 °C for 5 min, subjected to 30 cycles of PCR with 30-s denaturation at 94 °C, 30-s annealing at 56 °C and 90-s extension at 72 °C, and then followed by an additional 5-min extension at 72°. The RT-PCR products were resolved on 1.5% agarose gel by electrophoresis and then stained with ethidium bromide.

### 2.8. Transient transfection and luciferase assay

Macrophages RAW 264.7 were transiently transfected with both iNOS-luciferase reporter plasmid (Lowenstein et al., 1993) and pSV-β-galactosidase control vector (Promega, Madison, USA) using LipofectAMINE (Invitrogen, Carlsbad, USA). The transfected RAW 264.7 cells were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Lysates of the cells were subjected to luciferase assay using Luciferase Reporter Assay System (Promega, Madison, USA) and to β-galactosidase assay using β-Galactosidase Enzyme Assay System (Promega, Madison, USA).

### 2.9. Measurement of NF-κB transcriptional activity

Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct (Moon et al., 2001), encoding four copies of κB sequence and SEAP gene as a reporter, were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Aliquots of the cell-free culture media were heated at 65 °C for 5 min, and reacted with an assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate) in the dark at room temperature for 1 h. SEAP activity was measured as relative fluorescence units (RFU) with emission 449 nm and excitation 360 nm.

### 2.10. Electrophoretic mobility shift assay (EMSA)

Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Nuclear extracts were reacted with NF-κB specific [$^{32}$P]-labeled oligonucleotide (Promega, Madison, USA) in a binding buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 μg/ml poly(dI-dC), 4% glycerol, pH 7.5) on ice for 10 min. The complexes between oligonucleotide and nuclear protein were resolved on non-denaturing acrylamide gel by electrophoresis. The gels were dried and then exposed to X-ray film.

### 2.11. Statistical analysis

Results are expressed as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnet test. Values of $P<0.01$ were considered significant.

## 3. Results

### 3.1. BMD compound inhibits NO production in macrophages RAW 264.7

Macrophages RAW 264.7 in resting state released basal amounts of nitrite, a stable metabolite of NO, between 6.0±1.1 μM and 9.5±3.1 μM during incubation for 6–24 h (Fig. 2A). However, the cells markedly increased NO production, 19.6±1.6 μM of nitrite at 9 h, 34.1±1.1 μM at 12 h and 61.2±3.2 μM at 24 h after stimulation with LPS alone (Fig. 2A). BMD compound inhibited the nitrite production at 9–24 h after LPS stimulation in a dose-dependent manner (Fig. 2A). At 24 h treatment, BMD compound exhibited dose-dependent inhibitory effects, corresponding to 24.5±3.7% inhibition at 3 μM, 53.5±4.9% at 10 μM and 91.5±3.5% at 30 μM, on LPS-induced NO production (Fig. 2A and B). As a positive control, parthenolide also inhibited LPS-induced NO production in a dose-dependent manner with an $IC_{50}$ value of 3.5 μM (Fig. 2B). Upon co-stimulation with LPS and IFN-γ, the RAW 264.7 cells released 78.1±

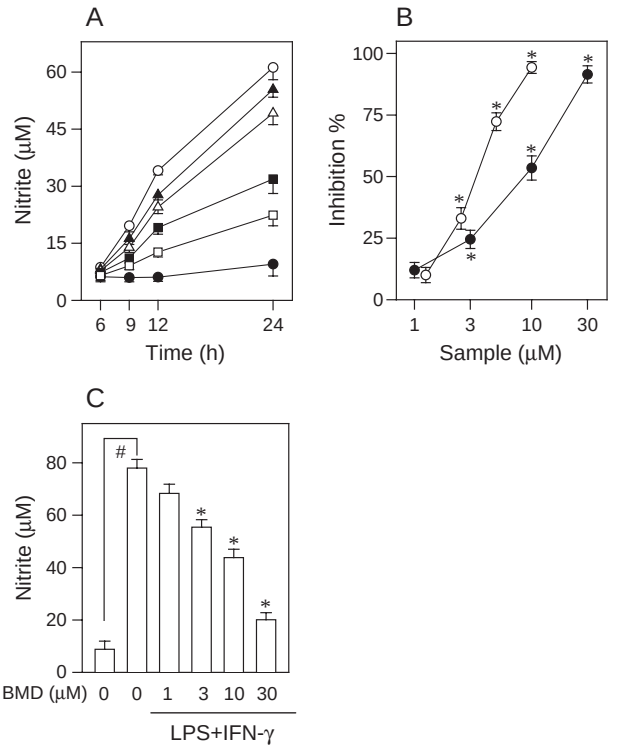


Fig. 2. Inhibition of NO production by BMD compound. Macrophages RAW 264.7 were treated with LPS (1 μg/ml) alone (○), LPS (1 μg/ml) plus BMD compound with a concentration of 1 μM (▲), 3 μM (△), 10 μM (■) or 30 μM (□), and media alone (●) for indicated times. Amounts of nitrite, a stable metabolite of NO, were measured with the cell-free culture media (A). Effects of BMD compound (●) or parthenolide (○) on NO production at 24 h after LPS stimulation are represented as inhibition % (B). The cells were pre-treated with BMD compound for 2 h, stimulated with LPS (100 ng/ml) plus IFN-γ (3 ng/ml) for 24 h, and then amounts of nitrite were measured (C). Values are mean±S.E.M. ($N$=5). *$P$<0.01 vs. LPS alone-treated group. $^{\#}P$<0.01 vs. media alone-treated group.

3.3 μM of nitrite over the basal amounts of 8.8±3.1 μM (Fig. 2C). BMD compound inhibited LPS plus IFN-γ-induced nitrite production in a dose-dependent manner, corresponding to 32.6±3.8% inhibition at 3 μM, 49.4±4.1% at 10 μM and 83.7±3.4% at 30 μM (Fig. 2C). Neither BMD compound nor parthenolide at the effective concentrations showed any cytotoxic effects to the RAW 264.7 cells (data not shown).

### 3.2. BMD compound attenuates LPS-induced iNOS synthesis

To examine whether inhibitory effect of BMD compound on NO production was attributable to its influence on iNOS synthesis, Western immunoblot analysis was carried out. iNOS protein was hardly detectable in resting macrophages RAW 264.7, but pronounced amounts of iNOS protein were induced upon exposure to LPS alone (Fig. 3A). BMD compound decreased LPS-induced synthesis of iNOS protein in a dose-dependent manner, corresponding to 24.8±5.1% inhibition at 3 μM, 77.6±4.2% at 10 μM and 87.3±5.4% at 30 μM (Fig. 3A). However, synthesis of housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was not affected by treatment of LPS and/or BMD compound (Fig. 3A). Semi-quantitative RT-PCR was also carried out to understand whether BMD compound could influence LPS-induced synthesis of iNOS transcript. The iNOS transcript was hardly detectable in resting macrophages RAW 264.7, but markedly increased upon exposure to LPS alone (Fig. 3B). BMD compound inhibited LPS-induced synthesis of iNOS transcript in a dose-dependent manner, corresponding to 34.7±3.4% inhibition at 3 μM, 48.2±4.8% at 10 μM and 90.1±7.6% at 30 μM (Fig. 3B). However,

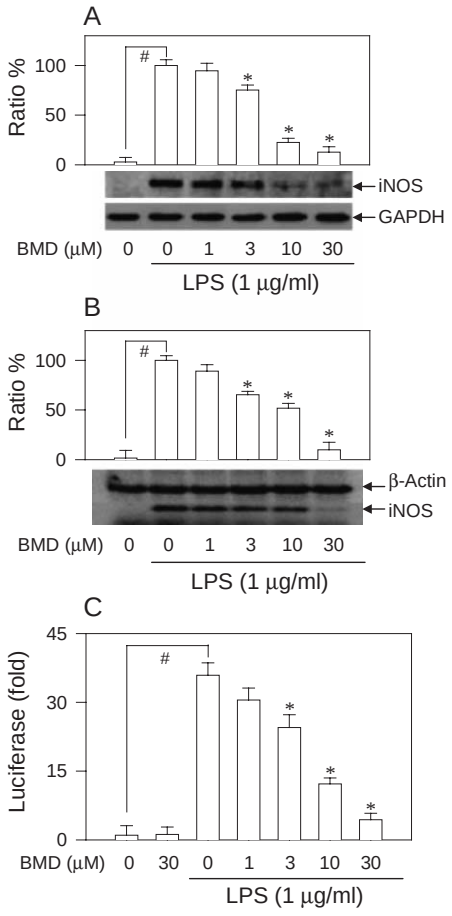


Fig. 3. Inhibition of iNOS expression by BMD compound. Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS for 18 h. Lysates of the cells were subjected to Western blot analysis with anti-iNOS antibody. One of similar results is represented and relative ratio % is also shown, where iNOS signal was normalized to GAPDH signal (A). The cells were pre-treated with BMD compound for 2 h and stimulated with LPS for 4 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar results is represented and relative ratio % is also shown, where iNOS signal was normalized to β-actin signal (B). The cells transfected transiently with both iNOS-luciferase reporter plasmid and pSV-β-galactosidase control vector were pre-treated with BMD compound for 2 h and stimulated with LPS for 16 h. Luciferase and β-galactosidase activities were measured with lysates of the cells. Luciferase expression as a reporter of iNOS promoter activity is represented as relative fold, where luciferase activity was normalized to β-galactosidase activity (C). Values are mean±S.E.M. ($N$=3). $^{\#}P$<0.01 vs. media alone-treated group. *$P$<0.01 vs. LPS alone-treated group.

synthesis of housekeeping β-actin transcript was not affected by treatment of LPS and/or BMD compound (Fig. 3B).

### 3.3. BMD compound down-regulates LPS-induced iNOS expression at the transcription level

Transcriptional control of iNOS expression by BMD compound was documented using a promoter activity, which was analyzed in macrophages RAW 264.7 transfected transiently with iNOS-luciferase construct containing murine iNOS promoter (−1592/+183) fused to luciferase gene as a reporter (Lowenstein et al., 1993). Upon exposure to LPS alone, luciferase expression was increased to 36-fold over the basal level (Fig. 3C). No significant difference in the luciferase expression was found between resting RAW 264.7 cells and the cells treated with BMD compound (30 μM) alone (Fig. 3C). BMD compound inhibited LPS-induced luciferase expression in a dose-dependent manner, corresponding to 33.1±4.2% inhibition at 3 μM, 68.2±2.8% at 10 μM and 90.4±2.9% at 30 μM (Fig. 3C).

### 3.4. BMD compound inhibits LPS-mediated NF-κB transcriptional activity

NF-κB activation has been evidenced to play a key mechanism in LPS-induced iNOS expression in macrophages (Lowenstein et al., 1993; Kim et al., 1997, 1993; Xie et al., 1994). NF-κB transcriptional activity was monitored using macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct that contains four copies of κB sequence fused to SEAP gene as a reporter (Moon et al., 2001). Upon exposure to LPS alone, SEAP expression was increased to about 3- to 4-fold over the basal level, indicating that cellular NF-κB is transcriptionally functional (Fig. 4A). No significant difference in the SEAP expression was found between resting RAW 264.7 cells and the cells treated with BMD compound (30 μM) alone (Fig. 4A). BMD compound inhibited LPS-mediated SEAP expression in a dose-dependent manner, corresponding to 28.5±2.5% inhibition at 3 μM, 53.3±5.2% at 10 μM and 92.3±6.6% at 30 μM (Fig. 4A and B). Parthenolide also inhibited LPS-mediated SEAP expression with an $IC_{50}$ value of 3.6 μM (Fig. 4B).

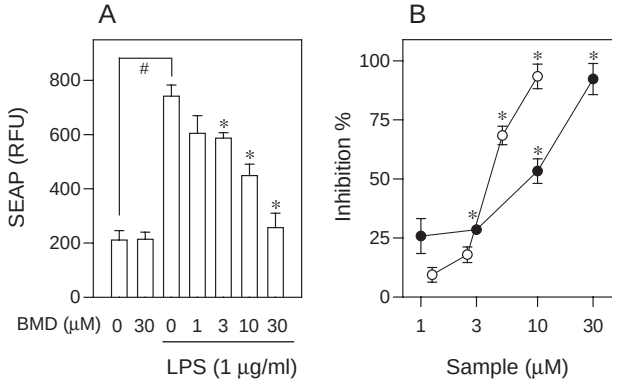


Fig. 4. Inhibition of NF-κB transcriptional activity by BMD compound. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT reporter construct were pre-treated with BMD compound for 2 h and stimulated with LPS for 16 h. SEAP expression as a reporter of NF-κB transcriptional activity was measured with the cell-free culture media, and is represented as relative fluorescence units (RFU) (A). Effects of BMD compound (●) or parthenolide (○) on LPS-induced NF-κB transcriptional activity are represented as inhibition % (B). Values are mean±S.E.M. ($N$=5). $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

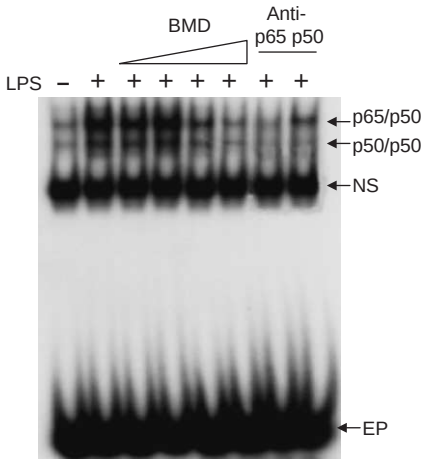


Fig. 5. Inhibition of DNA binding activity of NF-κB by BMD compound. Macrophages RAW 264.7 were pre-treated with BMD compound (1–30 μM) for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Nuclear extracts of the cells were reacted with [$^{32}$P]-labeled oligonucleotide specific to NF-κB. Neutralization experiments were also carried out by incubation with 1 μg of anti-NF-κB p65 or p50 antibody followed by centrifugation before the DNA binding reaction. An EMSA result is represented, where NF-κB complex of p65/p50 or p50/p50, nonspecific signal (NS) and excess probe (EP) are indicated by an arrow.

### 3.5. BMD compound prevents not only LPS-mediated DNA binding activity of NF-κB complex but also nuclear translocation of NF-κB p65

To elucidate inhibitory mechanism on NF-κB activation, we next determined whether BMD compound could affect DNA binding activity of NF-κB in LPS-stimulated macrophages RAW 264.7, which was analyzed using EMSA with a [$^{32}$P]-labeled oligonucleotide corresponding to the κB sequence. Upon exposure to LPS alone, DNA binding activity of NF-κB complex, p65/50 and p50/p50, was markedly increased within 1 h (Fig. 5). BMD compound (1–30 μM) decreased LPS-mediated DNA binding activity of NF-κB complex in a dose-dependent manner (Fig. 5). To further investigate whether BMD compound could affect nuclear translocation of NF-κB, Western immunoblot analysis for NF-κB p65 was carried out with nuclear extracts of LPS-stimulated macrophages RAW 264.7. Amounts of NF-κB p65 in the nucleus were markedly increased upon exposure to LPS alone for 1 h (Fig. 6). BMD compound inhibited LPS-mediated nuclear

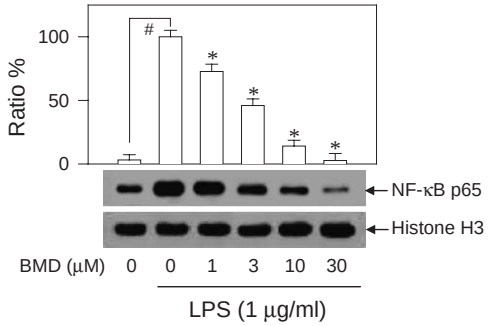


Fig. 6. Inhibition of nuclear translocation of NF-κB p65 by BMD compound. Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS for 1 h. Nuclear extracts of the cells were subjected to Western blot analysis with anti-NF-κB p65 antibody. One of similar results is represented and relative ratio % is also shown, where NF-κB p65 signal was normalized to histone H3 signal. Values are mean±S.E.M. ($N=3$). $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

translocation of NF-κB p65 in a dose-dependent manner, corresponding to 27.3±5.7% inhibition at 1 μM, 54.1±5.2% at 3 μM, 85.9±4.5% at 10 μM and 97.3±5.5% at 30 μM (Fig. 6).

### 3.6. BMD compound prevents neither LPS-mediated degradation nor phosphorylation of IκBα

Another immunoblot analysis was carried out with cytoplasmic extracts of LPS-stimulated macrophages RAW 264.7 to understand whether BMD compound could affect IκBα degradation. IκBα degradation was dramatically caused within 20–40 min upon exposure to LPS alone, and amounts of IκBα at the cytoplasm were recovered to the normal level at 80 min after LPS stimulation (Fig. 7A). BMD compound (30 μM) did not show significant inhibitory effect on LPS-mediated IκBα degradation, but also IκBα recovery in the time course study (Fig. 7A). However, parthenolide (10 μM) as a positive control exhibited inhibitory effect on IκBα degradation (Fig. 7A). IκBα can be phosphorylated on its Ser-32 and -36 residues by IKK complex, which marks for ubiquitin-dependent IκBα degradation (Lee et al., 1998). IκBα phosphorylation was also documented by Western immunoblot analysis with cytoplasmic extracts of LPS-stimulated macrophages RAW 264.7. Upon exposure to LPS alone for 5 min, IκBα phosphorylation was markedly taken place but IκBα degradation was not started yet (Fig. 7B). However, phosphorylated forms of IκBα were hardly detectable in resting macrophages RAW 264.7 (Fig. 7B). At the 5-min point after LPS stimulation, BMD compound (1–30 μM) did not inhibit LPS-mediated IκBα phosphorylation, at all (Fig. 7B).

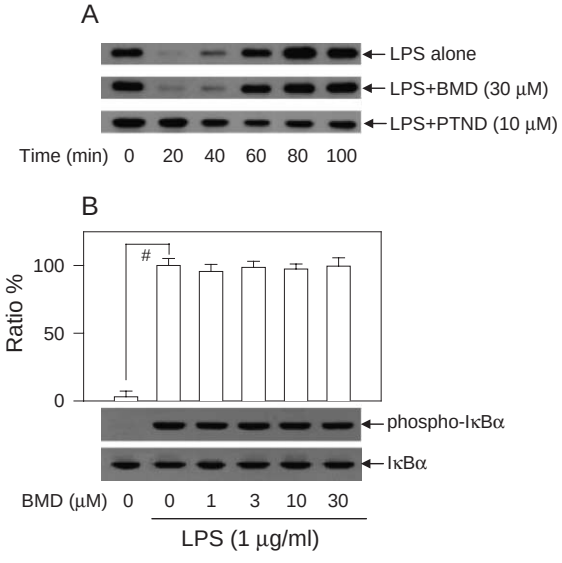


Fig. 7. Effect of BMD compound on IκB degradation and phosphorylation. Macrophages RAW 264.7 were pre-treated with BMD compound or parthenolide (PTND) for 2 h and stimulated with LPS (1 μg/ml) for indicated times. Cytoplasmic extracts of the cells were subjected to Western blot analysis with anti-IκBα antibody (A). The cells were treated with BMD compound for 2 h and stimulated with LPS for 5 min. Cytoplasmic extracts of the cells were subjected to Western blot analysis with anti-phospho-IκBα antibody. One of similar results ($N=3$) is represented and relative ratio % is also shown, where phospho-IκBα signal was normalized to total IκBα signal (B). $^{\#}P<0.01$ vs. media alone-treated group.

### 3.7. BMD compound does not inhibit LPS-induced activation of mitogen-activated protein (MAP) kinase

MAP kinase pathway is also involved in LPS induction of iNOS (Guha and Mackman, 2001). To understand whether BMD compound could inhibit LPS-induced activation of MAP kinases such as ERK-1/2, JNK and p38 kinase, Western immunoblot analysis and immunoprecipitation were carried out. Upon exposure to LPS alone, phosphorylation of ERK-1/2, JNK or p38 kinase was markedly taken place, whereas phosphorylated forms of the MAP kinases were hardly detectable in resting macrophages RAW 264.7 (Fig. 8). BMD compound (3–30 μM) did not inhibit LPS-induced phosphorylation of ERK-1/2, JNK or p38 kinase (Fig. 8).

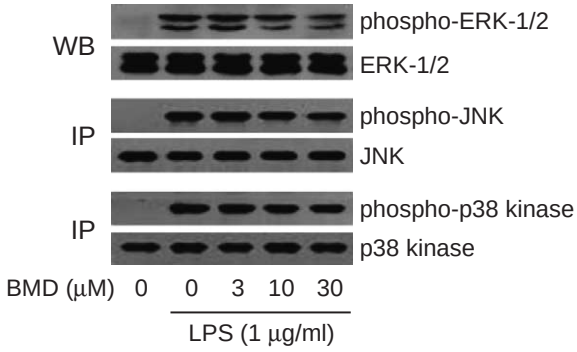


Fig. 8. Effect of BMD compound on LPS-induced activation of MAP kinase. Macrophages RAW 264.7 were pre-treated with BMD compound for 2 h and stimulated with LPS for 20 min. Lysates of the cells were analyzed by either Western immunoblot analysis (WB) for phospho-ERK-1/2 or immunoprecipitation (IP) for phospho-JNK and phospho-p38 kinase.

## 4. Discussion

In the present study, chemically synthetic BMD compound (Fig. 1) was discovered to inhibit NO production in macrophages RAW 264.7 stimulated with LPS alone or LPS plus IFN-γ (Fig. 2). BMD compound attenuated LPS-induced synthesis of both mRNA and protein of iNOS, in parallel, and inhibited LPS-induced iNOS promoter activity (Fig. 3), indicating that the aromatic diamine compound could down-regulate iNOS expression at the transcription level.

NF-κB activation has been evidenced as a major mechanism for LPS-induced iNOS expression in macrophages (Lowenstein et al., 1993; Kim et al., 1997; Xie et al., 1994). BMD compound inhibited LPS-mediated NF-κB transcriptional activity in a dose-dependent manner (Fig. 4). Furthermore, BMD compound inhibited LPS-mediated DNA binding activity of NF-κB complex as well as nuclear translocation of NF-κB p65, in parallel (Figs. 5 and 6). However, BMD compound could not influence LPS-mediated degradation and phosphorylation of IκBα (Fig. 7). These results indicate that BMD compound could inhibit LPS-mediated nuclear translocation of NF-κB p65 without affecting IκBα degradation, which is a rare mechanism for control of NF-κB activation. However, BMD compound did not inhibit LPS-induced activation of ERK-1/2, JNK and p38 kinase (Fig. 8), where MAP kinase pathways are also involved in transcriptional activation of LPS-inducible iNOS (Guha and Mackman, 2001).

In the nuclear import machinery, nuclear localization signal motif of NF-κB binds to karyopherin α, which are docked to karyopherin β at cytoplasmic face of the nuclear pore (Cunningham et al., 2003). Once docking has occurred, the complex is subsequently translocated through the pore into the nucleus by an energy-dependent process involving GTPase Ran/TC4 and the Ran interacting factor NTF2/p10 (Chook and Blobel, 2001). Synthetic peptide SN50, containing a hydrophobic membrane-translocating region and the nuclear localization signal motif of NF-κB p50, was reported to inhibit nuclear translocation of NF-κB p50 in response to LPS or TNF-α (Kolenko et al., 1999). Target of synthetic peptide SN50 is the nuclear localization signal motif on NF-κB p50, which is recognized by karyopherin α. Even though molecular target of BMD compound in the nuclear import machinery remains to be determined, this study demonstrated that a non-peptide aromatic diamine compound could prevent nuclear localization step of NF-κB p65.

Parthenolide, as a positive control, inhibited not only LPS-induced NO production (Fig. 2B) but also LPS-mediated NF-κB transcriptional activity (Fig. 4B). Further, parthenolide exhibited inhibitory effect on LPS-induced IκBα degradation (Fig. 7A). Parthenolide is a sesquiterpene lactone abundant in many medicinal plants used for inflammatory diseases as the traditional remedy. As a mechanism of NF-κB inhibitory action, parthenolide has been reported to prevent IκB degradation by influence on the IKK complex, specifically to Cys-179 residue of IKKβ (Hehner et al., 1999) and is able to alkylate Cys-38 residue of NF-κB p65, resulting in suppression of DNA binding activity of the transcription factor (Garcia-Pineres et al., 2001).

In conclusion, BMD compound inhibited LPS-induced NO production in macrophages RAW 264.7 by its down-regulatory action on iNOS expression at the transcription level. As a mechanism of the anti-inflammatory action shown by BMD compound, suppression of LPS-mediated NF-κB activation, specifically to nuclear translocation step of NF-κB p65, has been demonstrated. Finally, BMD compound could provide an invaluable tool to investigate NF-κB-dependent iNOS expression, in addition to its therapeutic potential in NO-associated inflammatory diseases.

## Acknowledgements

Dr. Alavala M. Reddy is appreciated for his help in preparing this manuscript. This work was financially supported by a grant (02-PJ2-PG3-21605-0004) from Korea Ministry of Health and Welfare, and a research fund of RCBH, RRC.

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