DBM1285 Suppresses Tumor Necrosis Factor α Production by Blocking p38 Mitogen-Activated Protein Kinase/Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 Signaling Pathway

Jong Soon Kang, Hwan Mook Kim, In Young Choi, Sang-Bae Han, Yeo Dae Yoon, Hyunju Lee, Ki Hwan Park, Ig Jun Cho, Chang Woo Lee, Kiho Lee, Ki Hoon Lee, and Song-Kyu Park

Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Cheongwon, Chungbuk, Republic of Korea (J.S.K., H.M.K., Y.D.Y., H.L., K.H.P., I.J.C., C.W.L., K.L., K.H.L., S.-K.P.); Dongbu HiTek Co., Ltd., Daeduck Science Town, Daejeon, Republic of Korea (I.Y.C.); and College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea (S.-B.H.)

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ABSTRACT

Tumor necrosis factor α (TNF-α) is a major inflammatory cytokine that plays an important role in the development of various inflammatory diseases. TNF-α has been considered as a potential therapeutic target for the treatment of chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease. In this study, we report that cyclopropyl-{4-[4-(4-fluorophenyl)-2-piperidin-4-yl-thiazol-5-yl]pyrimidin-2-yl}amine (DBM1285) is a novel inhibitor of TNF-α production. DBM1285 concentration-dependently inhibited lipopolysaccharide (LPS)-induced TNF-α secretion in various cells of macrophage/monocyte lineage, including mouse bone marrow macrophages, THP-1 cells, and RAW 264.7 cells. However, LPS-induced mRNA expression of TNF-α was not affected by DBM1285 in these cells. Further studies demonstrated that the inhibitory effect of DBM1285 on TNF-α production might be mediated by post-transcriptional regulation through the modulation of the p38 mitogen-activated protein kinase (MAPK)/MAPK-activated protein kinase 2 (MK2) signaling pathway. We also confirmed that DBM1285 directly inhibits p38 MAPK enzymatic activity. In vivo administration of DBM1285 inhibited LPS-induced increase in the plasma level of TNF-α in mice. Whole-blood in vivo target inhibition assay also revealed that DBM1285 attenuates p38 MAPK activity after oral administration in mice. Moreover, DBM1285 suppressed zymosan-induced inflammation and adjuvant-induced arthritis in murine models. Collectively, these results suggest that DBM1285 inhibits TNF-α production, at least in part, by blocking the p38 MAPK/MK2 pathway. Furthermore, in vivo results suggest that DBM1285 might be a possible therapeutic candidate for the treatment of TNF-α-related chronic inflammatory diseases.

Tumor necrosis factor α (TNF-α) is a multifunctional proinflammatory cytokine produced mainly by cells of the monocyte/macrophage lineage, which plays an important role in chronic inflammatory diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease, by amplifying an inflammatory signal in multiple pathways (Montecucco and Mach, 2009). Human TNF-α is expressed as a 26-kDa protein on the plasma membrane, cleaved by TNF-α-converting enzyme (TACE), and released as a mature 17-kDa protein (Paladino et al., 2003). The expression of the TNF-α gene is controlled at both the transcriptional and post-transcriptional levels, and a variety of signaling pathways are involved in these processes. Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases involved in a variety of cellular processes, such as inflammation, cell growth/differentiation, and cell survival/death (Chang and Karin, 2001). Three kinds of MAPKs, including extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK, have been identified so far. Among these, p38 MAPK

ABBREVIATIONS: TNF-α, tumor necrosis factor α; DBM1285, cyclopropyl-{4-[4-(4-fluorophenyl)-2-piperidin-4-yl-thiazol-5-yl]pyrimidin-2-yl}amine; BIRB796, 1-{5-tert-butyl-2-p-toly-2H-pyrazol-3-yl}-3-{4-[2-morpholin-4-yl-ethoxy]naphthalene-1-yl}urea; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MK2, mitogen-activated protein kinase-activated protein kinase 2; RA, rheumatoid arthritis; NSAID, nonsteroidal anti-inflammatory drug; DMARD, disease-modifying antirheumatic drug; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; DMSO, dimethyl sulfoxide; TACE, TNF-α-converting enzyme; BM, bone marrow; ARE, AU-rich element.
is a key molecule involved in the induction of inflammatory genes such as TNF-α, inducible nitric oxide synthase, cyclooxygenase-2, and various adhesion molecules (Saklatvala, 2004; Schindler et al., 2007). The p38 MAPK is comprised of four different isoforms (p38α, p38β, p38γ, and p38δ); p38α is the main isoform involved in the regulation of TNF-α production (Schindler et al., 2007).

Among TNF-α-related diseases, RA is one of the most prevalent systemic autoimmune disorders (Smolen and Steiner, 2003). It affects approximately 1% of the population and is more frequent in women than men (Smolen and Steiner, 2003; Brennan and McInnes, 2008). Therapeutic approaches for RA are divided into two main categories: treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs). NSAIDs affect only a small portion of the inflammatory cascade, such as prostaglandin generation by inhibition of cyclooxygenases, and have not been a major treatment option for RA patients. Recently, it has been suggested that aggressive treatment with DMARDs, rather than NSAIDs, is required for better long-term life quality for RA patients by attenuating disease progression and retaining joint function. Small-molecule DMARDs, such as methotrexate and sulfasalazine, have been used for a long time clinically. Although DMARDs remain the most common therapy for RA, they provide only partial clinical benefits and cause significant toxicity (Brennan and McInnes, 2008). However, the recent development of biological DMARDs targeting TNF-α and interleukin-1β has revolutionized RA treatment (Segal et al., 2008).

Although biological DMARDs have proven to be clinically successful, they have innate limitations that include the requirement for injection and reduced efficacy after repeated treatment caused by antibody formation against the applied drug (Radstake et al., 2009). Therefore, the development of new orally available small-molecule DMARDs with enhanced efficacy and reduced toxicity is continuously required, and numerous small-molecule DMARDs targeted for RA treatment are currently under development (Pettus and Wurz, 2008; Stanczyk et al., 2008). To develop a small-molecule inhibitor of TNF-α production we screened chemical libraries developed by Dongbu HiTek Co., Ltd. Among the chemicals examined, DBM1285 [cyclopropyl-{4-[4-(4-fluorophenyl)-2-piperidin-4-yl-thiazol-5-yl]pyrimidin-2-yl}amine; Fig. 1] displayed the most potent inhibition of TNF-α production in vitro and in vivo. In this study, we examined the effect of DBM1285 on lipopolysaccharide (LPS)-induced TNF-α production in vitro and in vivo and arthritis progression in an animal model of RA. We also elucidated the mechanism responsible for DBM1285-mediated inhibition of TNF-α production. Our results indicate DBM1285 is a potential therapeutic candidate for the treatment of RA.

Materials and Methods

Chemicals, Cells, and Animals. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. DBM1285 and 1-5-tert-butyl-2-p-toly1-2H-pyrazol-3-yl]-3-[4-(2-morpholin-4-yl-ethoxy)naphthalene-1-yl]urea (BIRB796) were synthesized and supplied by Dongbu HiTek Co., Ltd. DBM1285 is characterized as a small-lipophilic compound with molecular weight of 395.5 and cLogP of 3.68 (calculated by ChemBioDraw Ultra 12.0; CambridgeSoft Corporation, Cambridge, MA). DBM1285 and BIRB796 were dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in culture medium for in vitro experiments (final DMSO concentration of 0.1%). For in vivo experiments, DBM1285 was dissolved in phosphate-buffered saline supplemented with 5% Tween 80. Six- to 8-week-old female C57BL/6 mice and Sprague-Dawley rats were purchased from Koatech (Pyungtaek, Gyeonggi, Korea) and cared for as described previously (Kang et al., 2009). All animals were allowed to acclimate to the local environment for 1 week. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Korea Research Institute of Bioscience and Biotechnology.

THP-1 cells (TIB-202; American Type Culture Collection, Manassas, VA) and RAW 264.7 cells (TIB-71; American Type Culture Collection) were grown in RPMI medium (Invitrogen, Carlsbad, CA) and Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Bone marrow (BM) macrophages were generated from BM cells obtained from female C57BL/6 mice. In brief, BM cells were flushed out from femurs and tibias. After lysing red blood cells, whole BM cells (2 × 10^6 cells/ml) were cultured in 100-mm^2 culture dishes in 10 ml/dish of RPMI complete medium containing 10 ng/ml macrophage colony-stimulating factor. On culture day 3, another 10 ml of fresh RPMI complete medium containing 10 ng/ml macrophage colony-stimulating factor was added, and half of the medium was changed on day 6. On day 8, cells were harvested and used as BM macrophages. In general, >90% of cells recovered from these cultures were CD11b<sup>+</sup>, and >95% of cells were F4/80<sup>+</sup>. Cell viability was determined with Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. In brief, the labeling mixture was prepared by mixing 50 volumes of 1 mg/ml sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate with 1 volume of 0.383 mg/ml N-methylbenzoxazinyl methyl sulfate. This mixture was added to the cultures and incubated for 2 h at 37°C. Absorbance was measured at 490 nm with a reference wavelength at 650 nm.

Enzyme-Linked Immunosorbent Assay. BM macrophages, THP-1 cells, and RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 6 h in the presence or absence of 0.001, 0.01, 0.1 or 1 μg/ml DBM1285 or BIRB796. Culture supernatants were recovered, and the concentration of TNF-α was determined by using the mouse TNF-α DuoSet (for BM macrophages and RAW 264.7 cells; R&D Systems, Minneapolis, MN) or the human TNF-α DuoSet (for THP-1 cells; R&D Systems) according to the manufacturer’s instructions.

Real-Time Reverse Transcription-Polymerase Chain Reaction. The expression of mRNA transcripts was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) as described previously (Han et al., 2007). Total RNAs were isolated by using TRIzol reagent (Invitrogen) as described previously (Chomczynski and Mackey, 1995). Equal amounts of RNA were reverse-transcribed into cDNA by using oligo(dT)₁₅ primers. The primer sequences used were: mouse TNF-α, sense 5′-CCCTGTC GTA GC-3′, antisense 5′-TTG ACC TCA GCG CTC AGT TG-3′; human TNF-α, sense 5′-GAG TGA TAA CCA GCC TGT AGC CCA TGT

![Fig. 1. Chemical structure of DBM1285.](image-url)
TGT AGC A-3', antisense 5'-GGC AAT GAT GAT CCC AAA GTA GAC CTG CCC AGA CT-3'; mouse β-actin, sense 5'-TGG AAT CCT GTG GCA TTC ATG AAA C-3', antisense 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; and human β-actin, sense 5'-GGG TCA GAA GGA TTC CTA TG-3', antisense 5'-GGT CTC AAA CAT GAT CGT CGG-3'.

**TACE Assay.** Enzyme assay was performed by using recombinant human TACE and substrate (MCA-Pro-Leu-Ala-Gln-Ala-Val-DPA-Arg-Ser-Ser-Ser-Arg-NH2) purchased from R&D Systems. Recombinant human TACE (0.01 µg) was loaded in a F16 Black Maxisorp Plate (Nunc, Roskilde, Denmark), and DBM1285 (0.001, 0.01, 0.1, or 1 µM) was added to each well. Enzyme reaction was started by adding substrate (10 µM), and fluorescence was measured at excitation and emission wavelengths of 320 and 405 nm, respectively, in the kinetic mode. Specific enzyme activity was measured according to the manufacturer’s instructions and expressed as percentage of the vehicle-treated group.

**Western Immunoblot Analysis.** Twenty micrograms of whole-cell lysates was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Each membrane was preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.5, containing 0.05% Tween 20 and 5% nonfat milk. Each nitrocellulose membrane was incubated with specific antibodies against phosho-p38 MAPK, nonphosho-p38 MAPK, phosho-MK2, and nonphosho-MK2 (Cell Signaling Technology, Danvers, MA). Immunoreactive bands were then detected by incubating with secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase and visualizing with enhanced chemiluminescence reagents (GE Healthcare).

**Kinase Assay.** Recombinant human p38α MAPK (active), recombinant human MK2 (active), and MK2 substrate peptide were purchased from Millipore Corporation (Billerica, MA). Kinase assay was performed according to the manufacturer’s instructions by serially adding substrate, recombinant enzymes, DBM1285 (0.001, 0.01, 0.1, or 1 µM), and [γ-32P]ATP and incubating it for 10 min at 30°C. Twenty microliters of reaction product was transferred to 2 X 2-cm P81 paper (Millipore Corporation) and washed three times with 0.75% phosphoric acid. The paper was washed once with acetone and subjected to scintillation counting.

**In Vivo TNF-α Assay.** Mice were fasted for 24 h before the start of the experiment. DBM1285 (0.1, 1, or 10 mg/kg) was given by oral administration. After 15 min, LPS (2 mg/kg) was intraperitoneally injected into each mouse. After 90 min, blood samples were collected from retro-orbital puncture, and plasma were prepared. The amount of TNF-α in plasma was measured by enzyme-linked immunosorbent assay (ELISA) as described earlier.

**In Vivo Target Inhibition Assay.** The in vivo validation of inhibitory effect of DBM1285 on p38 MAPK activity was performed as described previously (Zhao et al., 2008). In brief, mice were pre-treated with vehicle or 10 mg/kg DBM1285. After 15 min, whole blood was collected in heparin tubes (BD Biosciences, San Jose, CA) by retro-orbital puncture. Fluorescein isothiocyanate-conjugated rat anti-mouse Ly-6G antibody (BD Biosciences), aliphosphocyanin-conjugated rat anti-mouse CD11b antibody (BD Biosciences), and LPS (1 µg/ml) were added to the blood and incubated at 37°C for 15 min. Twenty volumes of BD Phosflow Lyse/Fix buffer (BD Biosciences) was added to lyse red blood cells and fix white blood cells. Cells were collected by centrifugation and resuspended in BD Phosflow Perm buffer III (BD Biosciences) with anti-phospho-MK2 antibody (Thr384; Cell Signaling Technology) for intracellular staining of phospho-MK2. Cells were collected by centrifugation and finally stained with anti-rabbit immunoglobulin-phycoerythrin (BD Biosciences) and incubated at room temperature for 30 min. Stained cells were collected and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**Zymosan-Induced Inflammation.** Inflammation was induced by subcutaneous injection of 50 µl of 3 mg/ml zymosan A isolated from *Saccharomyces cerevisiae* (Sigma-Aldrich) into the hind footpads of C57BL/6 mice. DBM1285 was orally administered from days 0 to 6. On day 7, popliteal lymph nodes were isolated and weighed as described previously (Frasnelli et al., 2005).

**Adjuvant-Induced Arthritis.** The adjuvant-induced arthritis model used has been described previously (Ishikawa et al., 2005). Paw volume was measured 1 day before the start of the experiment. Arthritis was induced by subcutaneous injection of 0.5 mg of *Mycobacterium tuberculosis* (Difco, Detroit, MI) in 50 µl of mineral oil into the right hind footpads of Sprague-Dawley rats on day 0. DBM1285 (1, 3, or 10 mg/kg) was administrated orally daily from days 0 to 25. Left hind paw volumes were measured by the water displacement method using a model MK-550 plethysmometer (Muramachi Kikai, Tokyo, Japan). Changes in paw volume were expressed as percentage change of initial paw volume of each rat.

**Statistical Analysis.** Results are expressed as mean ± S.D. One-way analysis of variance and Dunnett’s t test were used for multiple comparisons by using Prism (GraphPad Software Inc., La Jolla, CA). The criterion for statistical significance was set at p < 0.05.

**Results**

**Effect of DBM1285 on LPS-Induced TNF-α Secretion in Cells of Macrophage/Monocyte Lineage.** We first examined the effect of DBM1285 on LPS-induced TNF-α secretion in various cells, including mouse BM macrophages, THP-1 human monocyte cells, and RAW 264.7 mouse macrophage-like cells. As shown in Fig. 2A, DBM1285 concentration-dependently inhibited LPS-induced TNF-α secretion in BM macrophages. Treatment of BM macrophages with 0.01, 0.1, and 1 µM of DBM1285 caused 28, 84, and 100% inhibition of TNF-α secretion, respectively. In THP-1 cells and RAW 264.7 cells, DBM1285 also suppressed LPS-induced TNF-α secretion in a dose-dependent manner (Fig. 2, B and C). Treatment of cells with 0.01, 0.1, and 1 µM DBM1285 inhibited TNF-α secretion in THP-1 cells by 50, 62, and 84%, respectively, and inhibited TNF-α secretion in RAW 264.7 cells by 18, 55, and 91%, respectively. IC₅₀ for TNF-α secretion was 0.023, 0.01, and 0.073 µM in BM macrophages, THP-1 cells, and RAW 264.7 cells, respectively. The concentration and duration of DBM1285 treatment used in this study had no significant effect on the viability of BM macrophages, THP-1 cells, and RAW 264.7 cells (data not shown). In addition, concanavalin A-induced T cell proliferation and LPS-induced B cell proliferation were not affected even at 10 µM DBM1285 (Supplemental Fig. 1).

**Effect of DBM1285 on LPS-Induced mRNA Expression of TNF-α in BM Macrophages and THP-1 Cells.** To investigate whether the DBM1285-mediated inhibition of TNF-α secretion might be mediated by transcriptional regulation of cognate genes, we examined the effect of DBM1285 on the mRNA expression of TNF-α by real-time RT-PCR. LPS-induced TNF-α mRNA expression was not affected by DBM1285 treatment in BM macrophages (Fig. 3A), THP-1 cells (Fig. 3B), and RAW 264.7 cells (Supplemental Fig. 2).

**Effect of DBM1285 on TACE Activity.** To investigate whether TACE might be involved in the inhibitory effect of
TNF-α secretion by DBM1285, we examined the effect of DBM1285 on TACE activity by performing an enzyme assay. DBM1285 had no effect on the enzymatic activity of TACE (Fig. 4).

**Effect of DBM1285 on p38 MAPK Activity.** It is well known that the p38 MAPK/MK2 signaling pathway is involved in post-transcriptional regulation of TNF-α biosynthesis by modulation of the activity of AU-binding proteins in AU-rich regions of the 3’ untranslated region of TNF-α mRNA (Kotlyarov and Gaestel, 2002). Therefore, we examined the effect of DBM1285 on LPS-induced phosphorylation of p38 MAPK in BM macrophages and THP-1 cells to characterize whether the p38 MAPK pathway could be involved in the DBM1285-mediated inhibition of TNF-α production. DBM1285 blocked the LPS-induced phosphorylation of p38 MAPK in BM macrophages (Fig. 5A) and THP-1 cells (Fig. 5B), although the degree of inhibition did not correspond to that of TNF-α secretion. In addition, LPS-induced phosphorylation of p38 MAPK was not affected by DBM1285 in RAW 264.7 cells (Supplemental Fig. 2). However, phosphorylation

Fig. 2. Effect of DBM1285 on LPS-induced TNF-α secretion. Mouse BM macrophages (A), THP-1 cells (B), and RAW264.7 cells (C) were pretreated with vehicle (VH; DMSO) or the indicated concentrations of DBM1285 (DBM) for 1 h before being incubated with LPS (1 µg/ml) for 6 h. The culture supernatants were subsequently isolated and analyzed for TNF-α secretion by ELISA. UN, untreated. Each column shows the mean ± S.D. of triplicate determinations. Statistical significance was determined by using Dunnett’s t test versus the vehicle-treated group (*, p < 0.05).

Fig. 3. Effect of DBM1285 on LPS-induced TNF-α mRNA expression. Mouse BM macrophages (A) and THP-1 cells (B) were pretreated with vehicle (VH; DMSO) or the indicated concentrations of DBM1285 (DBM) for 1 h before being incubated with LPS (1 µg/ml) for 6 h. Total RNAs were isolated, and the mRNA expression of TNF-α and β-actin was determined by real-time RT-PCR. The amount of TNF-α mRNA was normalized by that of β-actin and expressed as fold induction versus untreated group. UN, untreated. Each column shows the mean ± S.D. of triplicate determinations. Statistical significance was determined by using Dunnett’s t test versus the vehicle-treated group (*, p < 0.05).

Fig. 4. Effect of DBM1285 on TACE activity. Recombinant human TACE was treated with vehicle (VH; DMSO) or the indicated concentrations of DBM1285 (DBM). TACE activity was measured as described under *Materials and Methods* by measuring fluorescence signal. Each column shows the mean ± S.D. of triplicate determinations.
of MK2, a downstream target of p38 MAPK and a regulator of TNF-α biosynthesis, was dramatically suppressed by DBM1285 treatment in LPS-stimulated BM macrophages (Fig. 5C). To further characterize the molecular target of DBM1285, we examined the effect of DBM1285 on kinase activity of MK2 (Fig. 6C). To further characterize the molecular target of DBM1285 in LPS-stimulated BM macrophages (A and C) and THP-1 cells (B) were pretreated with vehicle (VH: DMSO) or the indicated concentrations of DBM1285 (DBM) for 1 h before being incubated with LPS (1 µg/ml) for 30 min. Cells were lysed and phosphorylated, and nonphosphorylated forms of p38 MAPK (A and B) and MK2 (C) were detected by Western immunoblot analysis. UN, untreated.

Inhibition of LPS-Induced TNF-α Production and Validation of p38 MAPK Inhibition by Oral Administration of DBM1285 in Mice. To investigate whether DBM1285 could also exert its effect in vivo, we first examined the effect of DBM1285 on LPS-induced TNF-α production in mice. As shown in Fig. 8A, oral administration of DBM1285 suppressed LPS-induced increases in the serum level of TNF-α in a dose-related manner. To further confirm the inhibition of p38 MAPK in vivo by DBM1285 treatment, we treated mice with DBM1285 for 15 min and analyzed p38 MAPK activity by measuring intracellular MK2 phosphorylation in a monocyte population of whole blood cells. As shown in Fig. 8B, MK2 phosphorylation was detected in LPS-treated samples. However, DBM1285 treatment markedly suppressed phosphorylation of MK2 by LPS treatment (Fig. 8B).

Effect of DBM1285 on Zymosan-Induced Inflammation and Adjuvant-Induced Arthritis in Murine Models. We investigated the effect of DBM1285 in a zymosan-induced inflammation model. Popliteal lymph node weight was substantially increased in vehicle-treated mice and sup-
pressed by oral administration of DBM1285 in a concentration-dependent manner (Fig. 9A). In addition, we demonstrated that DBM1285 potently suppressed the plasma level of TNF-α in zymosan-treated mice (Fig. 9B). Moreover, we examined the effect of DBM1285 on RA development in an adjuvant-induced arthritis model. Rats were injected subcutaneously with adjuvant in their right rear feet on day 0, and disease progression was monitored in their left rear feet for 26 days. Vehicle-treated rats showed severe inflammation in their left rear feet; paw volume was increased to 280.4% of initial volume at day 18 and gradually decreased thereafter (Fig. 9C). However, daily oral administration of 10 mg/kg DBM1285 caused 49% inhibition at day 18 and 41% inhibition at day 26 (Fig. 8C). Body weights of DBM1285-treated rats were not significantly decreased compared with those of vehicle-treated rats during experiment, and LD50 was estimated to be over 300 mg/kg according to single and 14-day repeated-dose oral toxicity experiments (data not shown).

Discussion

The results of the present study support the potential of DBM1285 as an inhibitor of TNF-α production and a therapeutic candidate for TNF-α-related diseases. Here, we have shown that DBM1285 significantly inhibits LPS-induced secretion of TNF-α in mouse and human cells of macrophage/monocyte lineage, including mouse BM macrophages, THP-1 cells, and RAW 264.7 cells. Our results also demonstrate that, although submicromolar concentrations of DBM1285 can markedly suppress TNF-α secretion in LPS-stimulated cells of macrophage/monocyte lineage, treatment with 10 μM DBM1285 has no effect on concanavalin A-induced T cell proliferation and LPS-induced B cell proliferation, suggesting that the effect of DBM1285 might be cell type-specific. In general, high specificity is associated with reduced adverse effect. Therefore, it is assumed that DBM1285 might be a good therapeutic candidate for TNF-α-related diseases. It is
noteworthy that DBM1285 was active in animals after oral administration. In vivo administration of DBM1285 resulted in a decrease in LPS-induced serum levels of TNF-α in mice. Moreover, daily oral administration of DBM1285 also suppressed RA progression in an adjuvant-induced arthritis model. Collectively, our results suggest that DBM1285 is an orally available therapeutic candidate for RA and the anti-arthritic activity of DBM1285 might be mediated by blocking TNF-α secretion by cells of macrophage/monocyte lineage.

To investigate the mechanism responsible for the inhibitory effect of DBM1285 on TNF-α secretion, we first analyzed the effect of DBM1285 on LPS-induced mRNA expression of the TNF-α gene. In contrast to the results of TNF-α secretion, DBM1285 did not affect TNF-α mRNA expression in LPS-stimulated BM macrophages, THP-1 cells, and RAW 264.7 cells, suggesting that the inhibitory effect of DBM1285 on TNF-α secretion is not mediated at the transcriptional level. It is well known that the expression of TNF-α is controlled at both the translational and post-transcriptional levels (Kumar et al., 2003). Because DBM1285 does not affect the transcriptional regulation of TNF-α, we searched targets involved in post-transcriptional regulation of TNF-α. TACE is one of the most well known molecular targets that regulate TNF-α secretion post-transcriptionally (DasGupta et al., 2009). It mediates proteolytic cleavage of the membrane-bound form of TNF-α, resulting in the secretion of the active soluble form of TNF-α (Black, 2002). Therefore, we assessed the possibility of whether DBM1285 exerts its inhibitory effect on TNF-α secretion by blocking TACE activity. Our observation that DBM1285 had no effect on TACE activity suggests that TACE is not a target of DBM1285.

p38 MAPK plays an important role in cellular responses to external stress signals (Schett et al., 2008) and is implicated in the expression of inflammatory mediators; inhibitors of p38 MAPK exert anti-inflammatory effect in various preclinical animal models (Kumar et al., 2003). Although a number of p38 MAPK inhibitors have failed in clinical trials so far, several inhibitors of p38 MAPK are still in clinical trials for RA treatment. MK2 is a downstream target of p38 MAPK that is essential for LPS-induced TNF-α biosynthesis (Kotlyarov et al., 1999). Furthermore, the major post-transcriptional effects of p38 MAPK are mediated by MK2 (Kotlyarov and Gaestel, 2002). MK2 regulates stability and translational efficacy of AU-rich element (ARE)-containing genes in their 3’ untranslated region by direct phosphorylation of ARE-binding proteins (Kumar et al., 2003). In fact, it has been reported that MK2 regulates biosynthesis of TNF-α through ARE-dependent modulation of translational efficiency (Tietz et al., 2006). Therefore, we hypothesized that the p38 MAPK/MK2 signaling pathway might be involved in the inhibitory effect of DBM1285 on TNF-α secretion. To test this hypothesis, we first examined the effect of DBM1285 on the phosphorylation of p38 MAPK. Our results showed that DBM1285 slightly blocked LPS-induced phosphorylation of p38 MAPK in BM macrophages and THP-1 cells. Although DBM1285 suppressed the phosphorylation of p38 MAPK, the potency of DBM1285 in p38 MAPK phosphorylation did not correspond to that in TNF-α secretion. Therefore, we examined the effect of DBM1285 on MK-2 phosphorylation in LPS-stimulated BM macrophages and demonstrated that DBM1285 potently inhibited LPS-induced MK2 phosphorylation. These results suggest that DBM1285 blocks the activity of p38 MAPK, although its effect on p38 MAPK phosphorylation is minimal. We further confirmed by kinase assay that DBM1285 suppresses enzymatic activity of p38 MAPK and p38 MAPK-mediated phosphorylation of MK2, but not MK2 activity itself. We also confirmed the inhibition of p38 MAPK activity by DBM1285 treatment in vivo by whole-blood in vivo target inhibition assay. Collectively, these results suggest that DBM1285 directly binds to p38 MAPK, resulting in the blockade of downstream signaling cascade both in vitro and in vivo.
BIRB796 is a well known inhibitor of p38 MAPK (Kumar et al., 2003). To compare the potency and mode of action of DBM1285 with those of BIRB796, we examined the effect of BIRB796 on LPS-induced TNF-α expression and p38 MAPK phosphorylation. Our results showed that BIRB796 dose-dependently suppressed LPS-induced secretion of TNF-α. It was also demonstrated that the potency of DBM1285 in the inhibition of TNF-α secretion was comparable with or better than that of BIRB796. As in the case of DBM1285, BIRB796 did not affect LPS-induced mRNA expression of TNF-α, suggesting that BIRB796 might also regulate TNF-α secretion at the post-transcriptional level. However, analysis of the effect of BIRB796 on p38 MAPK phosphorylation revealed a dramatic inhibition of LPS-induced phosphorylation of p38 MAPK. This result is different from that obtained with DBM1285, which showed slight inhibition of p38 MAPK phosphorylation and suggests that these two compounds do not share the same mechanism to regulate the activity of p38 MAPK. A previous report described that BIRB796 inhibited p38 MAPK activity through direct binding (Regan et al., 2003). From these results, we can speculate that both DBM1285 and BIRB796 bind directly to p38 MAPK to exert their inhibitory effect, but their binding sites and exact inhibitory mechanisms are different (Fig. 10). Further studies are required to fully address this question.

In summary, the results presented in this article demonstrate that DBM1285 inhibits TNF-α production in vitro and in vivo and exerts an antiarthritic effect in an animal model. We also show that the inhibitory effect of DBM1285 on TNF-α production is mediated by the inhibition of p38 MAPK activity and the downstream signaling cascade. Our results indicate DBM1285 is a promising drug candidate for RA treatment.

References


Address correspondence to: Dr. Song-Kyu Park, Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Cheongwon, Chungbuk, Republic of Korea. E-mail: spark123@kribb.re.kr