

Original Article

8-(Tosylamino)quinoline inhibits macrophage-mediated inflammation by suppressing NF- κ B signaling

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Aim: The macrophage-mediated inflammatory response may contribute to the development of cancer, diabetes, atherosclerosis and septic shock. This study was to characterize several new compounds to suppress macrophage-mediated inflammation.

Methods: Peritoneal macrophages from C57BL/6 male mice and RAW264.7 cells were examined. Anti-inflammatory activity was evaluated in the cells exposed to lipopolysaccharide (LPS). The mechanisms of the anti-inflammatory activity were investigated via measuring transcription factor activation in response to specific signals and via assaying the activities of the target kinases.

Results: Of 7 candidate compounds tested, 8-(tosylamino)quinoline (8-TQ, compound 7) exhibited the strongest activities in suppressing the production of NO, TNF- α , and PGE₂ in LPS-activated RAW264.7 cells and peritoneal macrophages (the IC₅₀ values=1–5 μ mol/L). This compound (1.25–20 μ mol/L) dose-dependently suppressed the expression of the pro-inflammatory genes for iNOS, COX-2, TNF- α , and the cytokines IL-1 β and IL-6 at the level of transcription in LPS-activated RAW264.7 cells. 8-TQ (20 μ mol/L) significantly suppressed the activation of NF- κ B and its upstream signaling elements, including inhibitor of κ B (I κ B α), I κ B α kinase (IKK) and Akt in LPS-activated RAW264.7 cells. In *in vivo* experiments, oral administration of 20 and 40 mg/kg 8-TQ for 3 d significantly alleviated the signs of LPS-induced hepatitis and HCl/EtOH-induced gastritis, respectively, in ICR mice.

Conclusion: 8-TQ (compound 7) exerts significant anti-inflammatory activity through the inhibition of the Akt/NF- κ B pathway, thus may be developed as a novel anti-inflammatory drug.

Keywords: 8-(tosylamino)quinoline; anti-inflammatory effect; lipopolysaccharide; macrophage; RAW264.7 cell; hepatitis; gastritis; NF- κ B; Akt

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Introduction

Macrophages, the terminally differentiated progeny of monocytes, are characterized by cell-surface markers of the inflammatory response, including toll-like receptors (TLRs), the Fc receptor, and the complement receptor. Although macrophages function primarily as phagocytes and antigen-presenting cells, they may also activate other immuno-regulatory cells, including neutrophils and T- and B-lymphocytes^[1, 2], through the production of pro-inflammatory cytokines, such as interleukins (ILs) and tumor necrosis factor (TNF)- α ;

chemokines; and inflammatory mediators such as nitric oxide (NO) and prostaglandin E₂ (PGE₂). The activation and chemotactic migration of inflammatory and immune cells into tissues play essential roles in the defense against viral, bacterial, and fungal infections. Uncoupled from normal controls, however, components of the immune response may contribute to a variety of acute and chronic disorders, including cancer, diabetes, septic shock, autoimmune diseases, and atherosclerosis^[3–5]. In these disorders, tissue-associated macrophages predominate among the cells that directly injure tissues. These considerations led us to seek to develop a drug that might suppress macrophage-mediated inflammation. A variety of *in vitro* and *in vivo* models of inflammatory disease have been used in drug-screening studies. Macrophages in these systems may be activated by treatment with ligands such as lipopolysaccha-

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ride (LPS), peptidoglycan, and poly(I:C)^[6].

Recent approaches to anti-inflammatory drug development have focused on key signaling proteins as targets and have tested compounds for activity against them. Previously targeted proteins include the transcription factors nuclear factor (NF)- κ B and activator protein (AP)-1 and their upstream activating enzymes, including inhibitor of κ B (I κ B α), I κ B α kinase (IKK), Akt, phosphoinositide-dependent kinase-1 (PDK1), phosphoinositide 3-kinase (PI3K), the tyrosine kinases Syk and Src, and enzymes in the mitogen-activated protein kinase (MAPK) cascade [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38]. These proteins play critical roles in regulating pro-inflammatory gene expression.

BAY11-7082 is a representative IKK inhibitor that actively suppresses various inflammatory cytokines^[7], the induction of heme oxygenase-1^[8] and ICAM-1 expression^[9] and may potentiate neutrophil apoptosis^[10]. This compound may prove beneficial in the treatment of inflammatory conditions such as arthritis^[11]. Because we did not initially identify this compound, however, we face restrictions in developing it further. We believe we can overcome such restrictions by using derivatives of the original compound. For this study, we selected seven commercially available compounds (1 through 7) based on structural similarity to BAY 11-7082. We evaluated the anti-inflammatory activities of these seven analogs and investigated their molecular mechanisms.

Materials and methods

Materials

Test compounds 1 through 7 were purchased from Sigma-Aldrich Co (St Louis, MO, USA) at greater than 95% purity. Sodium carboxymethylcellulose (NaCMC), polyethylene glycol 400, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), GM-CSF, and LPS (*E coli* 0111:B4) were also obtained from Sigma. LY294002 (LY), BAY11-7082 (BAY), U0126, and wortmannin were from Calbiochem (La Jolla, CA, USA). Luciferase constructs containing binding promoters for NF- κ B and AP-1 were used as reported previously^[12, 13]. Enzyme immunoassay (EIA) kits and enzyme-linked immunosorbent assay (ELISA) kits for PGE₂ and TNF- α were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum and RPMI-1640 medium were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of Sigma reagent grade. Phospho-specific or total antibodies to transcription factors (p65, p50, c-Jun, STAT-1, and c-Fos), ERK (extracellular signal-related kinase), p38, JNK (c-Jun N-terminal kinase), I κ B α , IKK β , Akt, p85/PI3K, γ -tubulin, β -actin, and non-receptor tyrosine kinases (Src and Syk) were obtained from Cell Signaling Technology Inc (Beverly, MA, USA).

Animals

C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from Dae Han Bio Link Co Ltd, Chungbuk, Korea, and maintained in plastic cages under conventional conditions. Water

and pellet diets (Samyang Corp, Daejeon, Korea) were available *ad libitum*. Studies were performed in accordance with the guidelines established by the Kangwon University Institutional Animal Care and Use Committee.

Preparation of peritoneal and bone marrow-derived macrophages

Peritoneal exudates were obtained from C57BL/6 male mice (7–8 weeks old, 17–21 g) by lavage 4 d after the intraperitoneal injection of 1 mL of sterile 4% thioglycolate broth (Difco Laboratories, Detroit, MI, USA) as reported previously^[14, 15]. After washing with RPMI-1640 medium containing 2% FBS, peritoneal macrophages (1×10^6 cells/mL) were plated in 100-mm tissue culture dishes for 4 h at 37°C in a 5% CO₂ humidified atmosphere. To prepare bone marrow-derived macrophages, femurs and tibias were isolated from mice, and the muscle was removed. Bones were cut with scissors at both ends and flushed with 5 mL of RPMI-1640 with a 25-gauge needle. Cells were seeded at a density of 2×10^5 nucleated bone marrow cells/cm² in RPMI-1640 containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 ng/mL GM-CSF in 6-well CellBIND plates (Corning Life Sciences, Lowell, MA, USA) containing 3 mL per well. After a 24-h incubation, the cells were rinsed three times with 3 mL of RPMI-1640 to remove non-adherent cells and cultured further with 3 mL of RPMI-1640 containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 ng/mL GM-CSF, hereafter referred to as “complete medium.” The cell culture medium was replaced every 3 d with fresh complete medium. After 3 weeks in culture, experiments were performed in serum-free RPMI-1640 containing 50 ng/mL GM-CSF and additions as indicated.

Cell culture

Peritoneal macrophages and cell lines (RAW264.7 and HEK293 cells) were cultured with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), glutamine, and antibiotics (penicillin and streptomycin) at 37°C under 5% CO₂. For each experiment, cells were detached with a cell scraper. At our experimental cell density (2×10^6 cells/mL), the proportion of dead cells was less than 1% according to trypan blue dye exclusion tests.

NO, PGE₂, and TNF- α production

After the preincubation of RAW264.7 cells or peritoneal macrophages (1×10^6 cells/mL) for 18 h, the cells were pre-treated with test compounds 1 through 7 for 30 min and were then incubated with LPS (1 μ g/mL) for 24 h. The inhibitory effects of the test compounds on NO, PGE₂, and TNF- α production were determined by analyzing the NO, PGE₂, and TNF- α levels with the Griess reagent and enzyme-linked immunosorbent assay (ELISA) kits, as described previously^[16–18].

Cell viability test

RAW264.7 cells (1×10^6 cells/mL) were preincubated for 18 h and were then incubated for 24 h following the addition of

test compounds 1 through 7 to the cells. The cytotoxic effects were evaluated by the MTT assay^[19]. At 3 h prior to culture termination, 10 μ L of an MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added, and the cells were returned to culture until the end of the experiment. Incubation was halted by the addition of 15% sodium dodecyl sulfate to each well to solubilize the formazan^[20]. The absorbance at 570 nm (OD_{570-630 nm}) was measured using a SpectraMax 250 microplate reader.

mRNA analysis by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR)

To determine the cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells with TRIzol reagent (Gibco BRL), according to the manufacturer's instructions. Total RNA was stored at -70°C until use. Analysis of mRNA was also performed using semiquantitative RT-PCR according to the manufacturer's instructions (Bioneer, Daejeon, Korea) as reported previously^[21]. The results were expressed as the ratio of the optical density at 280 nm to the GAPDH mRNA concentration. The primers (Bioneer) used are listed in Table 1.

Luciferase reporter gene activity assay

HEK293 cells (1 \times 10⁶ cells/ml) were transfected with 1 μ g of NF- κ B-Luc, CREB-Luc, or AP-1-Luc plasmid, in addition to β -galactosidase plasmid, in the presence or absence of an Akt construct using the polyethyleneimine method in a 12-well plate according to the manufacturer's protocol^[22]. The cells were used for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega) as previously described^[23, 24].

Preparation of total lysates and nuclear fractions, immunoblotting, and immunoprecipitation

RAW264.7 cells (5 \times 10⁶ cells/mL) or livers were washed 3

times in cold PBS with 1 mmol/L sodium orthovanadate and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 2 mmol/L ethyleneglycotetraacetic acid, 50 mmol/L β -glycerophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μ g/mL aprotinin, 10 μ g/mL pepstatin, 1 mmol/L benzimidazole, and 2 mmol/L PMSF) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16000 \times g for 10 min at 4°C and stored at -20°C until needed.

Nuclear lysates were prepared in a three-step procedure^[25]. After treatment, cells were collected with a rubber policeman, washed with 1 \times PBS, and lysed in 500 μ L of lysis buffer on ice for 4 min. The cell lysates were then centrifuged at 19326 \times g for 1 min in a microcentrifuge. In the second step, the pellet (the nuclear fraction) was washed once in wash buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mmol/L KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at -80°C, thawed on ice and centrifuged at 19326 \times g for 5 min. The supernatant was collected as the nuclear extract.

For immunoprecipitation, cell lysates containing equal amounts of protein (500 μ g) from RAW264.7 cells cultured at 1 \times 10⁷ cells/mL and treated or not treated with LPS (1 μ g/mL) for 2.5 min were pre-cleared with 10 μ L of protein A-coupled Sepharose beads (50% *v/v*) (Amersham, UK) for 1 h at 4°C. Pre-cleared samples were incubated with 5 μ L of anti-Akt antibody overnight at 4°C. Immune complexes were mixed with 10 μ L of protein A-coupled Sepharose beads (50% *vv*) and stirred by rotation for 3 h at 4°C.

Soluble cell lysates or boiled immunoprecipitated beads were analyzed on Western blots, and the phosphorylated or total transcription factors (p65, c-Jun, and c-Fos), MAPK proteins (ERK, p38, and JNK), I κ B α , IKK α / β , Akt, p85/PI3K, PDK1, γ -tubulin, β -actin, and non-receptor tyrosine kinases (Src and Syk) were visualized as previously reported^[26].

Akt and PI3K kinase assays

To evaluate the Akt- and PI3K kinase-inhibitory activity in the extracts using purified enzymes, a kinase profiler service from Millipore (http://www.millipore.com/life_sciences/flx4/1d_kinases) was used. In a final reaction volume of 25 μ L, Akt (1, 2, and 3) or PI3K (α , β , and γ ; human; 1-5 mU) protein was incubated with the reaction buffer. The reaction was initiated by the addition of MgATP. After incubation for 40 min at room temperature, the reaction was stopped by adding 5 mL of a 3% phosphoric acid solution. Ten microliters of the reaction product was then spotted onto a GF/P30 filtermat (PerkinElmer, Inc), which was then washed three times for 5 min in 75 mmol/L phosphoric acid and once in methanol prior to drying and scintillation counting.

EtOH/HCl-induced gastritis, LPS-induced hepatitis, and acute toxicity tests

Inflammation of the stomach was induced with EtOH/HCl

Table 1. Primers for genes investigated using RT-PCR analysis.

Gene		Primer sequences
TNF- α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCACGTCGTAGC-3'
IL-1 β	F	5'-CAGGATGAGGACATGAGCACC-3'
	R	5'-CTCTGCAGACTCAAACCTCCAC-3'
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'
	R	5'-TGCTGGTGACAACCCAGGCC-3'
IL-12 p40	F	5'-CAGAAGCTAACCATCTCCTGGTTG-3'
	R	5'-TCCGGAGTAATTTGGTGCTTCACAC-3'
iNOS	F	5'-CCCTCCGAAGTTCTGGCAGCAGC-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F	5'-CACTACATCCTGACCCACTT-3'
	R	5'-ATGCTCCTGCTTGTAGTATGT-3'
GAPDH	F	5'-CACTCACGGCAAATCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

F, forward; R, reverse.

according to a published method^[27]. Fasted ICR mice were orally treated with compound 7 (10 to 40 mg/kg) or ranitidine (40 mg/kg) suspended in 5% NaCMC. Thirty minutes later, 400 μ L of 60% ethanol in 150 mmol/L HCl was administered orally. Each animal was euthanized with an overdose of urethane 1 h after the administration of the necrotizing agents. The stomach was excised and gently rinsed under running tap water. After opening the stomach along the greater curvature and spreading it out on a board, the area (mm^2) of the mucosal erosive lesions was measured using a pixel-counter. Inflammation of the liver was induced by the injection of LPS according to a published method^[28]. Fasted C57BL/6 mice were orally treated with compound 7 (20 mg/kg) once per day for 6 d. One hour after the final administration, LPS (10 mg/kg) was intraperitoneally administered. Each animal was anesthetized with an overdose of urethane 1 h after the administration of hepatitis inducers, and blood was drawn from the portal vein. The livers were then excised and gently rinsed under running tap water. Serum was obtained by centrifugation of the blood at 3000 r/min for 15 min. The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with a Roche Modular spectrophotometric autoanalyser. In the acute toxicity test, compound 7 was dissolved in 20% PEG 400 diluted in 5% BSA in H_2O or suspended in 5% NaCMC and administered orally or by intraperitoneal injection. Lethality and body weight changes were determined after 7 d.

Statistical analysis

Data represent the mean \pm standard deviations (SD) from at least three independent experiments, each performed in triplicate, or are representative of three different experiments with similar results. For statistical comparisons, the results were analyzed using analysis of variance with Scheffe's *post-hoc* test and the Kruskal-Wallis/Mann-Whitney U-test. A *P* value <0.05 was considered to represent a significant difference. All statistical tests were carried out using SPSS (Statistical Package for the Social Sciences, SPSS Inc, Chicago, IL, USA).

Results and Discussion

In a search for novel anti-inflammatory drugs, we selected compounds (Figure 1A) similar in structure to BAY 11-7082, an IKK inhibitor, and tested them for inhibitory effects on the inflammatory mediators NO, TNF- α , and PGE₂. Compound 7 [8-(tosylamino)quinoline] suppressed NO production in a dose-dependent manner without affecting cell viability (Figure 2), whereas the other compounds tested showed no inhibitory effect (Figure 1B). Compound 7 suppressed the release of NO, PGE₂, and TNF- α by RAW264.7 cells (Figure 2A), peritoneal macrophages (Figure 2B), and bone-marrow derived macrophages (Figure 2C) during LPS exposure, with IC₅₀ values of 1 to 5 μ mol/L (Table 2). The inhibitory activity of compound 7 was comparable to that of BAY11-7082 (Figure 1C) when BAY11-7082 was tested as a treatment for arthritis^[11]. These findings support the further development of compound 7 as an anti-inflammatory drug.

Table 2. The half-maximal inhibitory concentration (IC₅₀) of compound 7 for the inhibition of the production of NO, TNF- α , and PGE₂ in RAW264.7 cells and peritoneal macrophages.

Cells	IC ₅₀ value (μ mol/L)		
	NO	PGE ₂	TNF- α
RAW264.7 cells	4.3	3.2	6.1
Peritoneal macrophages	7.9	3.9	4.1

To explore the inhibitory mechanism of compound 7, we analyzed the effects of this compound on the transcription of genes encoding inflammatory mediators. Compound 7 dose-dependently reduced the levels of TNF- α , IL-1 β , IL-6, IL-12, COX-2, and iNOS mRNAs, implying that this drug inhibits inflammatory mediator production at the level of transcription (Figure 3). To identify the transcription factors targeted by compound 7, we measured transcription factor levels in nuclear fractions in cells exposed to LPS as an inflammatory stimulus. In this setting, compound 7 suppressed p65 upregulation after 15 min and 2 h without exerting inhibitory effects on c-Jun and c-Fos (Figure 4A left panel). We found a similar pattern of inhibition in LPS-treated peritoneal macrophages (Figure 4A, right panel). In the reporter gene assay performed with constructs containing NF- κ B, CREB or AP-1 binding promoters, similar inhibitory patterns for the activation of transcription factors were observed. Thus, compound 7 suppressed NF- κ B-mediated luciferase activities that were stimulated by PMA or cotransfection with other adaptor molecules such as TRIF and MyD88 (Figure 4B), but this compound did not suppress AP-1 or CREB activities (Figure 4C). These results indicate that compound 7 modulates NF- κ B signaling at an early stage.

Because NF- κ B activation is linked to a cascade of kinase activation, we sought to identify the exact target of compound 7 by determining the levels of phosphorylated I κ B α , IKK, Akt, PDK1, PI3K, and Src or Syk after LPS stimulation. At 5 and 60 min, compound 7 strongly suppressed I κ B α phosphorylation (Figure 5A). Because of the effect at 5 min, we turned our attention to early signaling events to investigate the target of compound 7. Interestingly, I κ B α and its upstream kinase IKK α/β were phosphorylated at 1 min, and Akt phosphorylation had not diminished at this time. In agreement with this result, compound 7 did not suppress the phosphorylation of Syk or Src, tyrosine kinases that contribute to I κ B α phosphorylation at 5 min^[29]. These data imply that compound 7 targets molecules upstream of IKK, such as Akt kinase, either directly or indirectly. To confirm these possibilities, a direct kinase assay was performed with purified PI3K and Akt. Contrary to expectations, there was no inhibition of any type of PI3K and Akt (Figure 5C). Nonetheless, the molecular association between Akt and IKK observed after LPS treatment was clearly reduced by treatment with compound 7 (Figure 5D), suggesting that this compound seems to target to the binding event between Akt and its substrate protein IKK. Meanwhile,

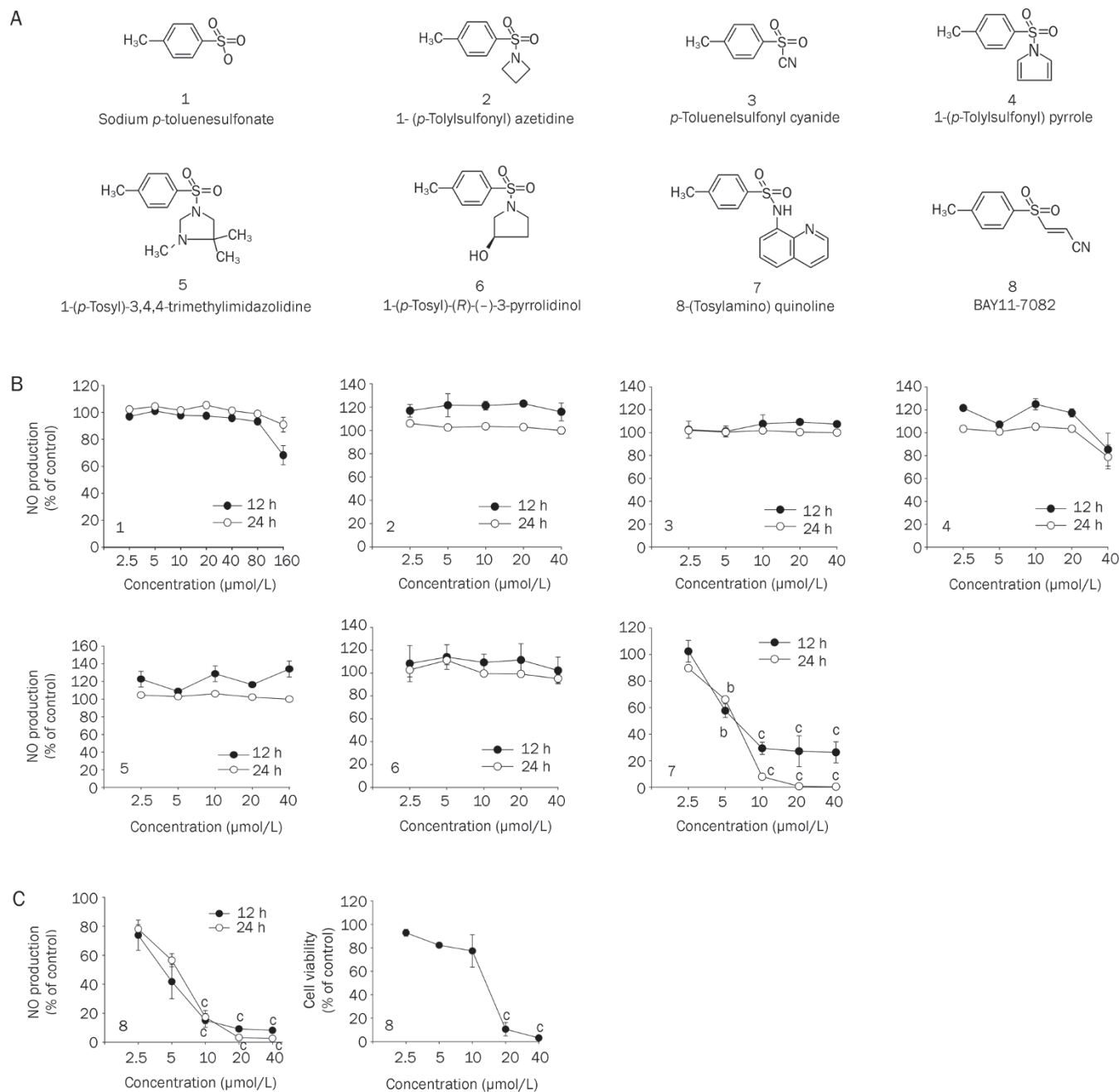


Figure 1. Effects of compounds 1 through 7 and BAY11-7082 on NO production. (A) Chemical structures of compounds 1 through 7. (B and C left panel) The Griess assay was used to determine the NO levels in culture supernatants of RAW264.7 cells or peritoneal macrophages treated with the test compounds and LPS (1 $\mu\text{g}/\text{mL}$) for 12 or 24 h. (C right panel) The viability of RAW264.7 cells was determined by the MTT assay. Mean \pm SD. $n=4$. ^b $P<0.05$, ^c $P<0.01$ vs the control.

to confirm that compound 7 does not inhibit AP-1, we evaluated the levels of phosphorylated MAPK-related kinases (ERK, JNK, and p38). As expected, compound 7 did not suppress the phosphorylation of these enzymes (Figure 5E).

The Akt pathway, considered to be one of the major targets of compound 7, was initially recognized as a significant cell survival signal and subsequently as a potential target for cancer therapy. Based on recent evidence, however, attention

has shifted to the role of Akt signaling in LPS-induced inflammatory responses^[30]. It is noteworthy that anti-inflammatory herbal extracts from *Eleutherococcus senticosus*, *Dichroa febrifuga*, and *Cymbopogon citratus*^[31-33] and ethnopharmacological agents such as curcumin, resveratrol, and quercetin^[34-36] may suppress the Akt pathway. The Akt inhibitors LY294002 and wortmannin displayed anti-inflammatory activity, reflected by the suppression of NO and PGE₂ (Figure 6A). Furthermore,

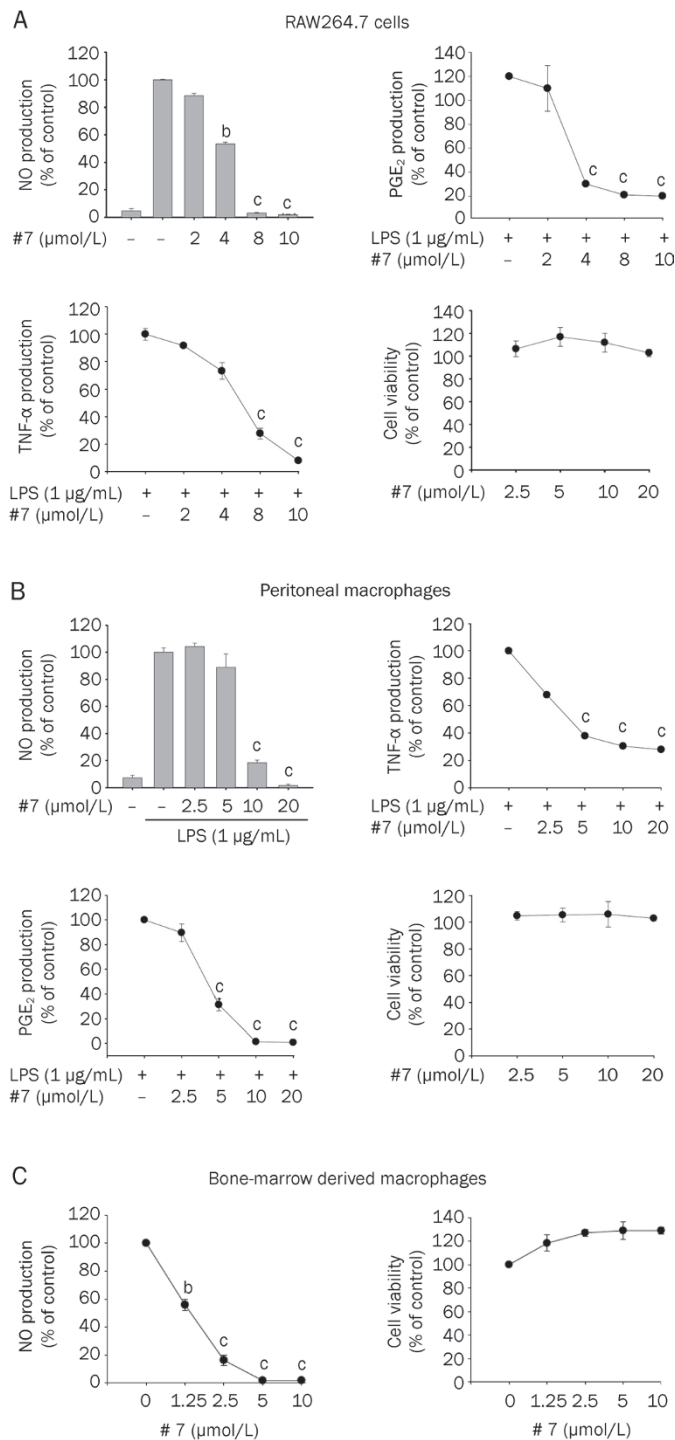


Figure 2. Effect of compound 7 on the production of inflammatory mediators. (A, B and C) The levels of NO, PGE₂, and TNF- α were determined by the Griess assay, EIA, and ELISA in culture supernatants of RAW264.7 cells, peritoneal macrophages or bone marrow-derived macrophages treated with compound 7 and LPS (1 μ g/mL) for 6 or 24 h. The viability of RAW264.7 cells and peritoneal macrophages was determined using the MTT assay. ^b $P < 0.05$, ^c $P < 0.01$ vs the control.

the overexpression of Akt induced NF- κ B activation up to 2.5 fold as assessed by measuring NF- κ B-mediated luciferase

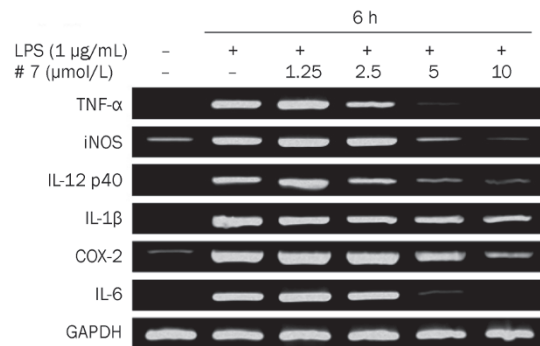


Figure 3. Effect of compound 7 on the expression of pro-inflammatory genes. The levels of iNOS, COX-2, TNF- α , IL-1 β , IL-6, and IL-12 p40 mRNAs were determined by semiquantitative RT-PCR.

activity (Figure 6B). These findings suggest that Akt pathway inhibition may be a determining event in compound 7-mediated anti-inflammatory action. We will investigate how this compound mediates Akt-IKK binding inhibition without affecting Akt kinase activity in future experiments in our lab. In these experiments, the mode of binding between Akt and IKK and the inhibition of this binding by compound 7 will be analyzed using mutant constructs of these proteins and immunoprecipitation.

To determine whether compound 7 is active by the oral route, we tested its effect against EtOH/HCl-stimulated gastritis, an *in vivo* model of inflammatory disease that is widely used in drug development. At a single dose of 40 mg/kg, compound 7 significantly reduced gastric tissue injury following EtOH/HCl administration, as did ranitidine (40 mg/kg), the positive control (Figure 7A). In addition, this compound also suppressed LPS-induced hepatitis symptoms as assessed by measuring the serum levels of enzymes (ALT and AST) indicative of liver damage, implying that *in vivo* TLR4-mediated inflammatory symptoms are also ameliorated by this compound. The acute administration of compound 7 to mice at 500 mg/kg for 1 week by the oral or intraperitoneal route induced no perturbation in body weight or change in mortality (Figure 7C). These results support the further testing of compound 7 as an orally available, well-tolerated anti-inflammatory drug^[37].

In summary, we have shown that compound 7 may suppress the production of NO, TNF- α , and PGE₂ in LPS-treated macrophages and may attenuate HCl/EtOH-induced gastritis. In exploring the anti-inflammatory mechanism of compound 7, we found that this compound can block NF- κ B activation by suppressing upstream signaling by I κ B α , IKK, and Akt (Figure 8). We propose that compound 7 might be used as a novel anti-inflammatory drug. To investigate this application, we will test compound 7 for efficacy *in vivo* using models of acute disorders (septic shock and carrageenan-induced arthritis) and chronic disorders (collagen- or adjuvant-induced arthritis) in a pre-clinical study.

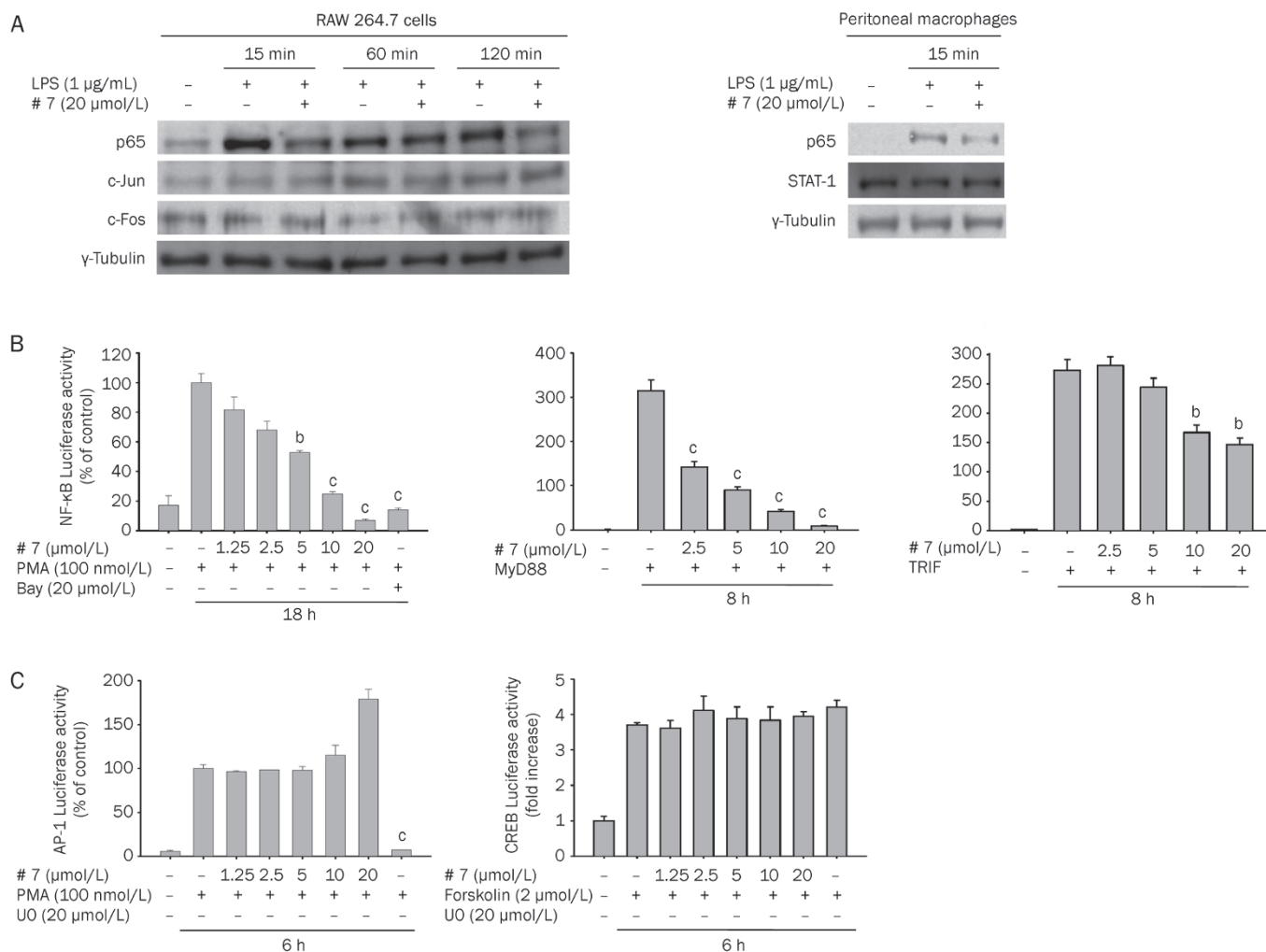


Figure 4. Effect of compound 7 on the activation of transcription factors (NF- κ B and AP-1). (A) The levels of NF- κ B (p65), STAT-1, and AP-1 (c-Jun/c-Fos) in nuclear fractions from LPS-treated RAW264.7 cells and peritoneal macrophages were determined by immunoblotting analysis with antibodies against the total proteins. (B and C) HEK293 cells cotransfected with plasmid constructs for NF- κ B-Luc, CREB-Luc or AP-1-Luc; adaptor molecules (MyD88 and TRIF) (each at 1 µg/mL); and β -gal (as a transfection control) were treated with compound 7, BAY (BAY11-7082) or UO (UO126) in the presence or absence of PMA (100 nmol/L). The luciferase activity was measured using a luminometer. ^a $P < 0.05$, ^c $P < 0.01$ vs the control.

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Author contribution

Yongwoo JUNG, Sungyoul HONG, and Jae Youl CHO designed the research; Yongwoo JUNG, Se Eun BYEON, Dae Sung YOO, Tao YU, Yanyan YANG, Ji Hye KIM, Eunji Kim, Yong Gyu LEE, and Deok JEONG performed the research; Yongwoo JUNG, Man Hee RHEE, Eui Su CHOUNG, Sungyoul HONG, and Jae Youl CHO analyzed the data; and Jae Youl CHO wrote the paper.

Abbreviations

ERK, extracellular signal-related kinase; TLR, Toll-like recep-

tors; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; AP-1, activator protein-1; JNK, c-Jun N-terminal kinase; Akt, protein kinase B; ATF2, activating transcription factor 2; CREB, cAMP response element-binding; IKK, I κ Ba kinase; IRAK1, interleukin-1 receptor-associated kinase 1; MKK, MAP kinase kinase; MyD88, myeloid differentiation primary-response protein-88; TRAF6, tumor necrosis factor-receptor-associated factor-6; TAK1, TGF- β -activated kinase-1; PDK1, phosphoinositide-dependent protein kinase-1; Syk, spleen tyrosine kinase; TRIF, TIR-domain-containing adapter-inducing interferon- β ; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphoinositide 3-kinase; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase polymerase chain reaction; ALT, serum alanine aminotransferase; AST, aspartate aminotransferase

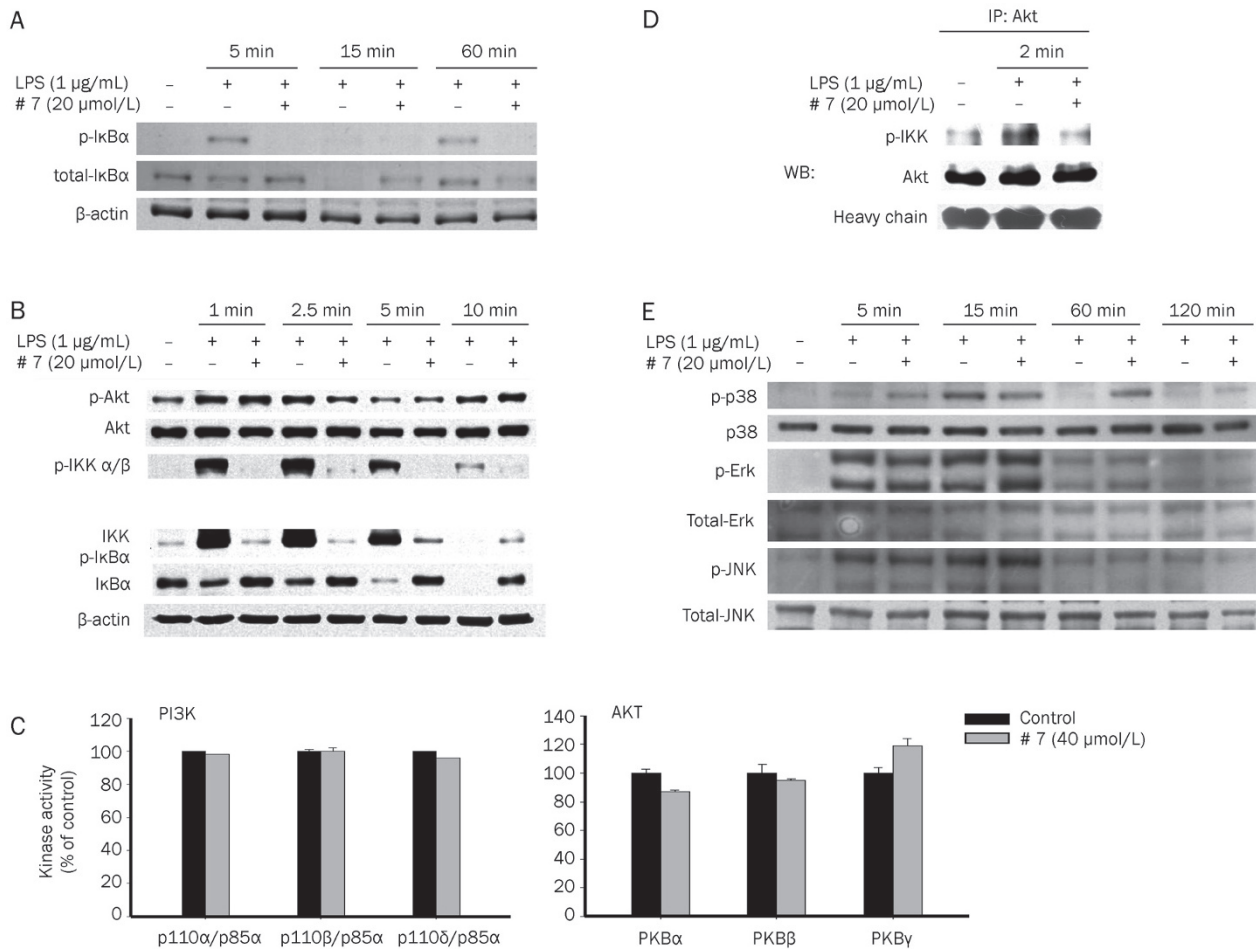


Figure 5. Effects of compound 7 on the activation of signaling enzymes upstream of NF- κ B translocation. (A, B, and E) The levels of phospho- and total proteins of I κ B α , IKK α / β , Akt, ERK, p38, JNK, and β -actin in cell lysates were determined using phospho-specific and total-protein antibodies, respectively. (C) The kinase activities of Akt and PI3K were determined by a direct kinase assay using purified enzymes. The control activity of each enzyme (Akt or PI3K) was set to 100%. (D) The effects of compound 7 on the formation of the signaling complex composed of Akt and phospho-IKK in the total lysates from LPS-treated RAW264.7 cells (5×10^6 cells/mL) were determined by immunoprecipitation with an anti-Akt antibody and immunoblotting with antibodies to p-IKK.

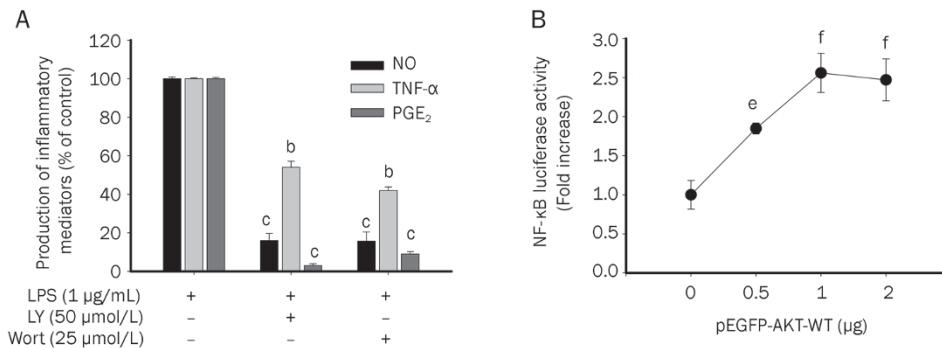


Figure 6. Functional role of Akt in the induction of inflammatory responses. (A) Culture supernatants prepared from LPS-treated RAW264.7 cells pre-treated with standard PI3K/Akt inhibitors [LY294002 (LY) and wortmannin (Wort)] were assayed for NO, TNF- α , and PGE $_2$. (B) HEK293 cells were cotransfected with plasmid constructs for either NF- κ B-Luc or Akt (1 μ g/mL each) and β -gal (as a transfection control). The luciferase activity was measured with a luminometer. ^c $P < 0.05$, ^e $P < 0.01$ vs the control. ^b $P < 0.05$, ^f $P < 0.01$ vs the normal.

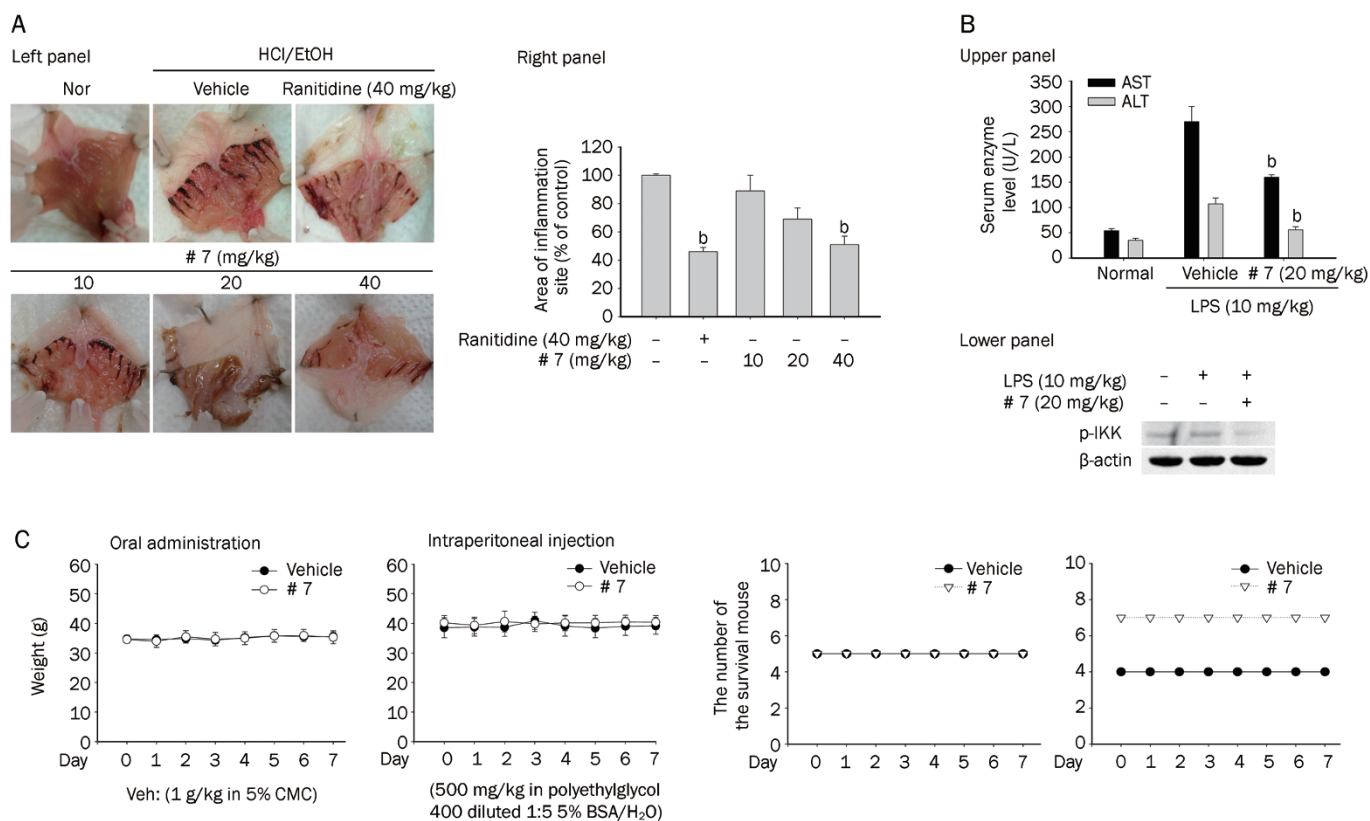


Figure 7. Effects of compound 7 on gastric inflammation in mice induced by HCl/EtOH treatment and on acute toxicity. (A) Mice were treated orally with compound 7 or ranitidine for 3 d and then treated orally with HCl/EtOH. After 24 h, the gastric lesions in the stomach were measured by pixel counting (right panel) and photographed (left panel). The area of gastric lesions present after treatment with the inducer alone is represented by 100%. (B) Mice orally treated with compound 7 for 6 d were intraperitoneally treated with LPS. After 1 h, serum was prepared to measure the biochemical parameters (AST and ALT) (upper panel), and the levels of p-IKK and β -actin were analyzed by Western blotting of the liver lysate (lower panel). (C) Lethality and body weight changes were followed for 7 d after the oral administration (1 g/kg) or intraperitoneal injection of compound 7 (500 mg/kg). ^b*P*<0.05 vs the control.

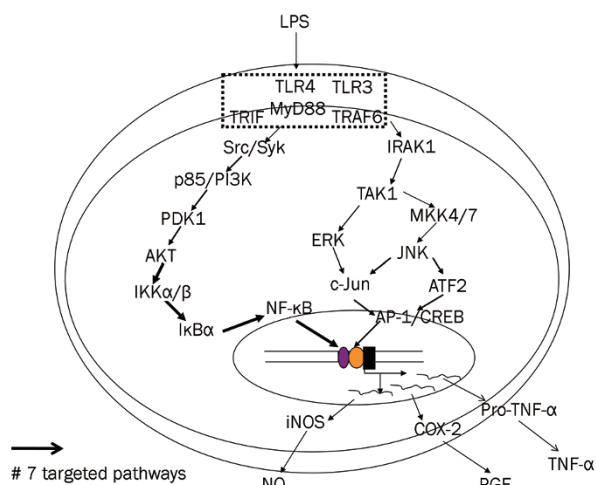


Figure 8. Putative pathway of the inhibition of LPS-activated inflammatory signaling by compound 7.

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