

Diallyl trisulfide exerts anti-inflammatory effects in lipopolysaccharide-stimulated RAW 264.7 macrophages by suppressing the Toll-like receptor 4/nuclear factor- κ B pathway

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Abstract. Diallyl trisulfide (DATS; di-2-propen-1-yl trisulfide) is an organic polysulfide compound found in garlic and other allium vegetables. Although certain studies have demonstrated that DATS possesses strong anti-inflammatory activity, the underlying molecular mechanisms remain largely unresolved. In the present study, the anti-inflammatory potential of DATS was investigated using the murine macrophage RAW 264.7 cell model. At non-toxic concentrations, DATS inhibited the production of nitric oxide (NO) and prostaglandin E₂ by inhibiting inducible NO synthase and cyclooxygenase-2 expression at the transcriptional level in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. DATS attenuated the release of the pro-inflammatory cytokines, tumor necrosis factor- α and interleukin-1 β , by inhibiting mRNA expression, respectively. DATS also suppressed LPS-induced DNA-binding activity of nuclear factor- κ B (NF- κ B), as well as the nuclear translocation of the NF- κ B p65, which correlated with the inhibitory effects of DATS on inhibitor κ B (I κ B) degradation. In addition, DATS was observed to significantly suppress LPS-induced Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 expression and the binding of LPS to macrophages, indicating

the antagonistic effect of DATS against TLR4. Furthermore, blocking TLR4 signaling with the specific TLR4 signaling inhibitor, CLI-095, increased the anti-inflammatory potential of DATS in LPS-stimulated RAW 264.7 macrophages. These data demonstrate that DATS may attenuate the initiation of LPS-mediated intracellular signaling cascades by suppressing activation of NF- κ B and by inhibiting binding of LPS to TLR4 on macrophages.

Introduction

Inflammation is the attempt of the body to remove harmful stimuli and initiate the healing process. However, chronic inflammatory processes are involved in the pathogenesis of common inflammation-associated diseases (1,2). Various infectious agents, such as bacteria and viruses, cause inflammation and macrophages play a critical role in initiating and propagating inflammatory responses by releasing pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E₂ (PGE₂) and cytokines to promote the inflammatory response (3,4).

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, activates a number of major inflammatory cellular effects by inducing several interconnecting mechanisms through specific surface molecules known as pathogen-associated molecular patterns, which bind to Toll-like receptors (TLRs), particularly TLR4, leading to the release of a wide variety of inflammatory inducers (5,6). The LPS-initiated signaling cascade occurs by binding of LPS to its receptor complex, and this leads to activation of the nuclear factor- κ B (NF- κ B) pathway (7,8). In normal cells, NF- κ B is constitutively localized in the cytosol as a homodimer or heterodimer, which is associated with the NF- κ B inhibitor, inhibitory- κ B α (I κ B). Upon stimulation with LPS, I κ B proteins are phosphorylated, ubiquitinated and rapidly degraded by the ubiquitin-proteasome pathway,

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and the resulting free NF- κ B migrates into the nucleus and activates transcription of NF- κ B-dependent inflammatory enzymes and cytokines (5,9,10). These observations indicate that antagonistic agents of TLR4 can inactivate pro-inflammatory downstream signaling pathways by suppressing the differential target gene expression and cellular responses.

Several epidemiological studies have shown that consuming allium vegetables is associated with a decreased risk of various diseases (11,12). Among them, garlic (*Allium sativum* L.) has long been used for culinary and medicinal purposes in numerous cultures. Accumulating studies have demonstrated that garlic has a wide range of biological activities against a number of chronic diseases, including cardiovascular problems, diabetes, infections and even cancer (13-15). Garlic is a particularly rich source of organo-sulfur compounds (OSCs), such as diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), which are believed to be responsible for its flavor and aroma, as well as its potential health benefits (16-18). In particular, studies have shown that the antioxidant potential is in the order DATS > DADS > DAS (19-21). Na *et al* (22) found the same order for the anticancer potential, suggesting that the number of sulfur atoms plays a vital role in the biological activities of OSCs. Although DATS has been suggested recently as the strongest inhibitor of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) among OSCs (21,23), the molecular mechanisms underlying the anti-inflammatory effects of DATS have not been extensively explored.

Therefore, the present study was carried out to further investigate the potential anti-inflammatory effect and underlying molecular mechanism of DATS using a murine RAW 264.7 macrophage model, which can be stimulated with LPS to mimic a status of inflammation. The results suggest that DATS effectively suppresses LPS-induced inflammatory signaling by inactivating the NF- κ B pathway and inhibiting TLR4.

Materials and methods

Cell culture. The murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine, at 37°C in a 5% CO₂ humidified air environment.

DATS treatment and MTT assay. DATS was obtained from LKT Laboratories (St. Paul, MN, USA) and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as a 100 mM stock solution and was stored in aliquots at -20°C. RAW 264.7 cells were incubated with different concentrations of DATS or 100 ng/ml LPS (Sigma-Aldrich) alone, or pretreated with DATS for 1 h before LPS for the cell viability assay. After 24 h, the medium was removed and the cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution for 2 h. The supernatant was discarded and the formazan blue, which was formed in the cells, was dissolved in DMSO. Optical density was measured at 540 nm with a microplate

reader (Dynatech Laboratories, Chantilly, VA, USA) and growth inhibition was assessed as the percent viability, in which vehicle-treated cells were considered as 100% viable.

Measurement of NO production. Concentrations of NO in the culture supernatants were determined by measuring nitrite, a stable oxidation product of nitric oxide, using the Griess reagent (Sigma-Aldrich). Briefly, cells (5x10⁵ cells/ml) were stimulated in 24-well plates with DATS and/or LPS for 24 h. Subsequently, 100 μ l of each culture supernatant was mixed with an equal volume of Griess reagent. After 10 min incubation at room temperature, absorbance was measured at 540 nm and nitrite production was determined with a NaNO₂ standard curve (24).

Determination of PGE₂, tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β production. The cells were incubated with DATS in either the presence or absence of LPS (100 ng/ml) for 24 h to measure the quantity of PGE₂. A 100 μ l aliquot of culture medium supernatant was collected and the concentration (pg/ml) of PGE₂ in the cell culture medium was calculated based on the concentrations of the standard solution using a PGE₂ enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA). The levels of TNF- α and IL-1 β were also measured with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions (25).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) to produce cDNAs. RT-generated cDNAs encoding *iNOS*, *COX-2*, *TNF- α* and *IL-1 β* genes were amplified by PCR using selective primers, which were purchased from Bioneer (Seoul, Korea). The PCR primers were as follows: mouse *iNOS* (5'-ATG TCC GAA GCA AAC ATC AC-3' and 5'-TAA TGT CCA GGA AGT AGG TG-3'), *COX-2* (5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3'), *TNF- α* (5'-ATG AGC ACA GAA AGC ATG ATC-3' and 5'-TAC AGG CTT GTC ACT CGA ATT-3') and *IL-1 β* (5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' and 5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'). Following amplification, the PCR reactants were electrophoresed in 1% agarose gels and visualized by ethidium bromide (Sigma-Aldrich) staining. In a parallel experiment, glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Protein extraction and western blotting. The cells were harvested and lysed with lysis buffer [20 mM sucrose, 1 mM EDTA, 20 μ M Tris-Cl (pH 7.2), 1 mM dithiothreitol, 10 mM KCl, 1.5 mM MgCl₂ and 5 μ g/ml aprotinin] for 1 h. In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. For western blot analysis, equivalent amounts of proteins were separated by

electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). After blocking with 5% skimmed milk, the membranes were incubated with protein-specific antibodies for 1 h, subsequently incubated with appropriate enzyme-linked secondary antibodies [mouse IgG, HRP-linked whole antibody (NA931) and rabbit IgG, HRP-linked whole antibody (NA934)] (Amersham Corp., Arlington Heights, IL, USA) and visualized by enhanced chemiluminescence (Amersham Corp.) according to the manufacturer's instructions. Anti-iNOS (#610330) and anti-COX-2 (#160106) antibodies were purchased from BD Biosciences (San Jose, CA, USA) and Cayman Chemical Co., respectively. Anti-IL-1 β (SC7884), anti-TLR4 (SC10741), anti-MyD88 (SC11356), anti-NF- κ B p65 (SC109), anti-actin (SC1616) and anti-GAPDH (SC32233) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-TNF- α (#3737) and anti-I κ B (#4812) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-nucleolin (AB22758) antibody was obtained from Abcam (Cambridge, MA, USA).

Electrophoretic mobility assay (EMSA). EMSA was performed with the nuclear extract. Synthetic complementary NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') binding oligonucleotides (Santa Cruz Biotechnology, Inc.) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA before being transferred onto a positively charged nylon membrane (HybondTM-N⁺) in 0.5X Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm². Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

Immunofluorescence staining. The RAW 264.7 cells were cultured on glass coverslips in 6-well plates for 24 h, stimulated with LPS in the presence or absence of DATS, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and permeabilized with 100% MeOH for 10 min at 20°C. The anti-NF- κ B p65 antibody was applied for 1 h followed by a 1 h incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Subsequent to washing with PBS, nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich), and fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). RAW 264.7 cells were stimulated with Alexa Fluor 488-conjugated LPS (100 ng/ml, AF-LPS; Invitrogen) for 30 min in the presence or absence of DATS for the LPS/TLR4 complex formation assay. The cells were fixed, stained with anti-TLR4 antibody for 90 min at 4°C and then incubated with secondary antibodies conjugated with Alexa Fluor 594 (1:200; Invitrogen) for 1 h. The stained cells were observed under a fluorescence microscope.

Measurement of TLR4 expression on cell surface. RAW 264.7 cells were incubated with AF-LPS in the presence or absence of

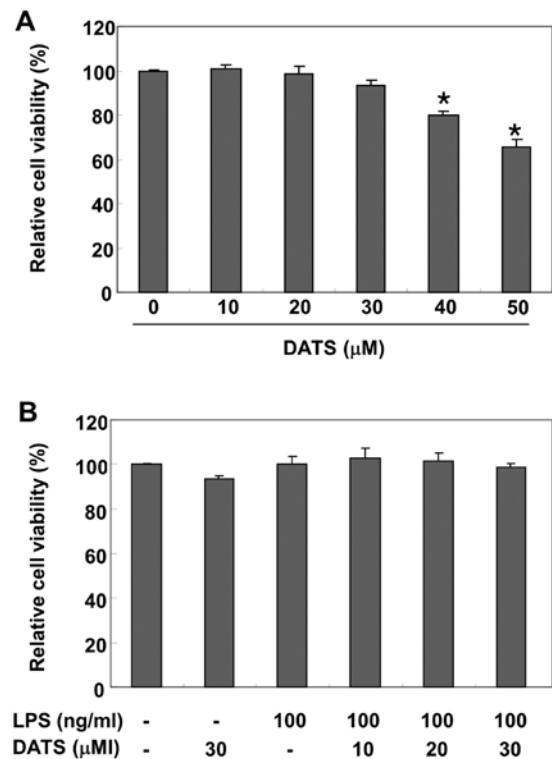


Figure 1. Effects of diallyl trisulfide (DATS) and lipopolysaccharide (LPS) on the cell viability of RAW 264.7 macrophages. (A) Cells were treated with the indicated concentrations of DATS alone, or (B) pretreated with DATS for 1 h before LPS treatment. After 24 h, cell viability was assessed using MTT reduction assays. Data are expressed as mean \pm standard deviation of three independent experiments. Significance was determined by Student's t-test ($P < 0.05$ vs. untreated control).

DATS for 1 h. The cells were washed twice with PBS, harvested with 0.005% EDTA and analyzed by flow cytometry. Alexa 488 was excited using a 488 argon-ion laser line and detected on a channel FL1 using a 530 nm emission filter. The fluorescence emission of samples was recorded by a flow cytometer (26).

Statistical analysis. Data are presented as mean \pm standard deviation. Statistical significance was determined using an analysis of variance followed by Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of DATS and LPS on RAW 264.7 macrophage viability.

To examine whether DATS is cytotoxic to RAW 264.7 macrophages, the cells were exposed to DATS for 24 h in the presence or absence of LPS, and cell viability was measured by the MTT assay. The results showed no effect of DATS on cell viability at concentrations of 10, 20 or 30 μ M (Fig. 1A); however, at higher concentrations the MTT assay suggested an adverse influence of DATS on cell viability. Furthermore, no significant cytotoxic effects were observed at ≤ 30 μ M DATS in the presence of LPS (Fig. 1B).

DATS reduces LPS-induced production of pro-inflammatory mediators and cytokines. Pro-inflammatory mediators including NO and PGE₂, and pro-inflammatory cytokines,

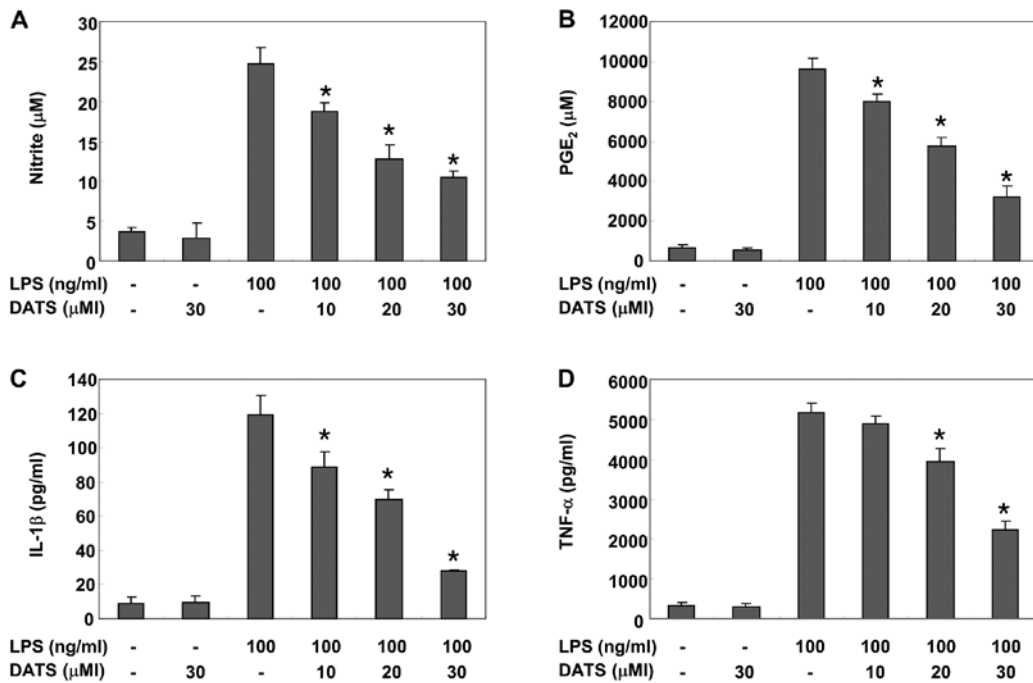


Figure 2. Effects of diallyl trisulfide (DATS) on the production of pro-inflammatory mediators and cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Cells were pretreated with the different concentrations of DATS for 1 h before incubation with LPS (100 ng/ml). (A) Following a 24 h treatment, the supernatants were prepared, and the nitrite content was measured using the Griess reaction. The amounts of (B) prostaglandin E₂ (PGE₂), (C) interleukin-1β (IL-1β), and (D) tumor necrosis factor-α (TNF-α) were measured in the culture media using commercial enzyme-linked immunosorbent assay kits. Each value represents the mean ± standard deviation of triplicate cultures (*P<0.05 vs. LPS-treated group).

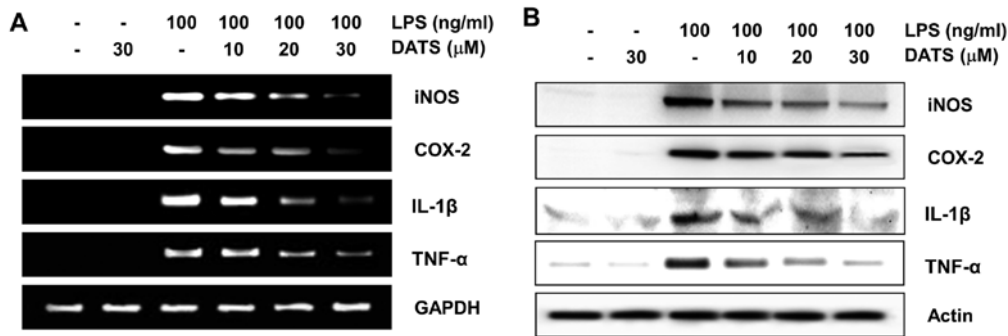


Figure 3. Effects of diallyl trisulfide (DATS) on the expression of pro-inflammatory mediators and cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Cells were pretreated with the different concentrations of DATS for 1 h before incubation with LPS (100 ng/ml). (A) Following a 6 h treatment, total RNA was isolated, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the indicated primers. (B) Cells were lysed after 24 h treatment culture and cellular proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin were used as internal controls for the RT-PCR and western blot assays, respectively.

such as IL-1β and TNF-α, that were released into the culture medium were measured using the Griess reagent and ELISA to examine the inhibitory effect of DATS on LPS-induced inflammatory responses. According to the NO detection assay, LPS alone markedly induced NO production compared to that generated by the control. However, pretreatment with DATS significantly repressed the levels of NO production in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner ≤30 μM (Fig. 2A). The levels of PGE₂ and tested cytokines also increased significantly in the culture media of LPS-stimulated RAW 264.7 cells, but the increases were significantly attenuated in a concentration-dependent manner by DATS pretreatment (Fig. 2B-D).

DATS inhibits LPS-induced expression of pro-inflammatory enzymes and cytokines. The effects of DATS on the levels of iNOS, COX-2, TNF-α and IL-1β mRNA and protein expression was measured by RT-PCR and western blot analyses to elucidate the mechanism involved in the inhibition of pro-inflammatory mediators and cytokines generated by DATS in LPS-stimulated RAW 264.7 cells. mRNA and protein expression in unstimulated RAW 264.7 cells was undetectable or extremely low. However, expression increased markedly in response to LPS, which was significantly inhibited by pretreatment with DATS (Fig. 3). These results indicate that the reduced expression of pro-inflammatory enzymes and cytokines at the transcriptional levels contributed to the

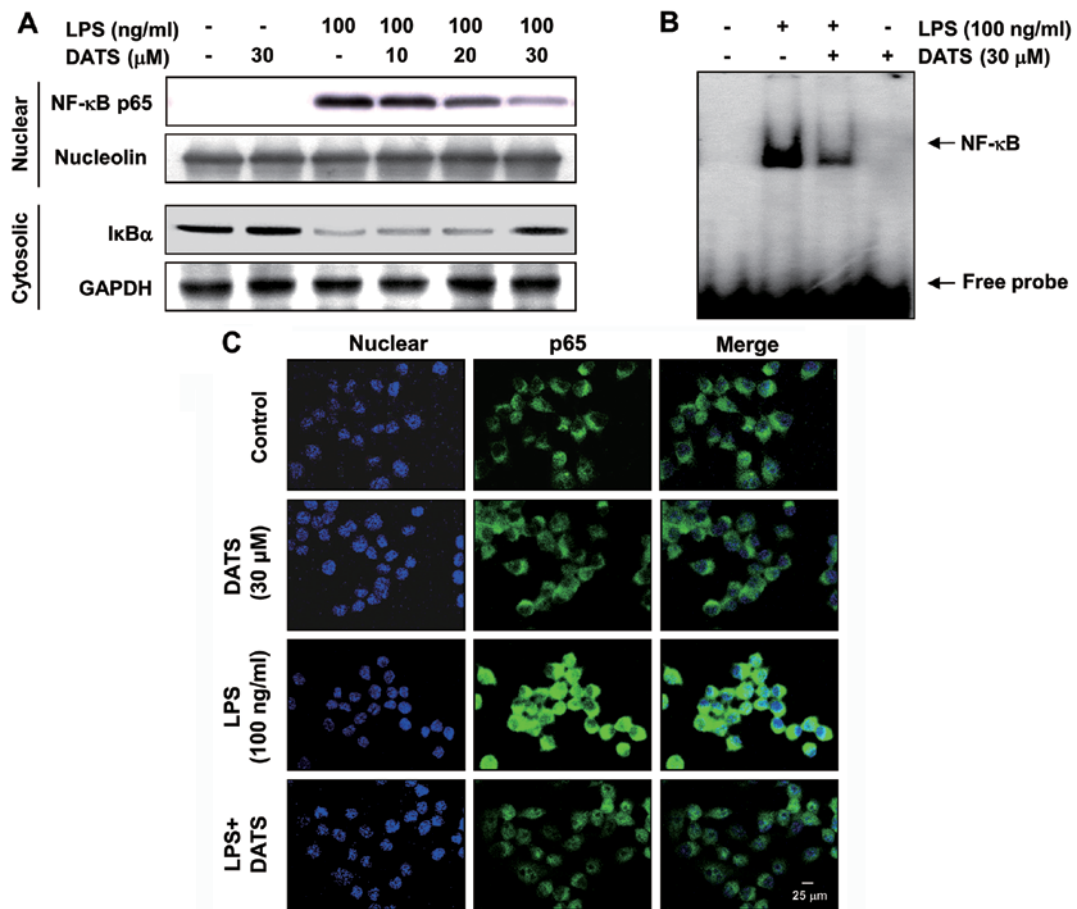


Figure 4. Effects of diallyl trisulfide (DATS) on lipopolysaccharide (LPS)-induced nuclear translocation of the nuclear factor- κ B (NF- κ B) and degradation of inhibitor κ B α (I κ B α) in RAW 264.7 macrophages. (A) Cells were treated with the indicated concentrations of DATS for 1 h before LPS treatment (100 ng/ml) for 30 min. Nuclear and cytosolic proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels followed by western blotting using anti-NF- κ B p65 and anti-I κ B α antibodies. Nucleolin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for the nuclear and cytosolic fractions, respectively. (B) Cells were pre-incubated with DATS 1 h before stimulation with LPS for 30 min. Nuclear extracts were subsequently assayed for NF- κ B activity by electrophoretic mobility assay (EMSA). (C) Cells were pretreated with 30 μ M DATS for 1 h prior to stimulation with LPS (500 ng/ml) for 30 min. Localization of NF- κ B p65 was visualized with a fluorescence microscope following immunofluorescence staining with anti-NF- κ B p65 antibody and an fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (IgG) antibody (green). Nuclei of the corresponding cells were visualized with DAPI (blue). The cells were visualized using a fluorescence microscope.

inhibitory effect of DATS on LPS-induced NO, PGE₂, IL-1 β and TNF- α production.

DATS blocks LPS-induced nuclear translocation of NF- κ B p65 and degradation of I κ B α . As previous studies suggested that NF- κ B is an important transcription factor regulating the expression of pro-inflammatory enzymes and cytokines (5,9,10), whether DATS blocked the NF- κ B signaling pathway was explored. The western blot analysis results showed that the amount of NF- κ B p65 in the nucleus was markedly increased within 30 min of exposure to LPS alone, concomitant with degradation of I κ B α in the cytosol. However, LPS-induced NF- κ B p65 levels in the nuclear fractions decreased markedly by DATS pretreatment, and LPS-induced I κ B α degradation was clearly blocked in a concentration-dependent manner by pretreatment with DATS (Fig. 4A). EMSA also showed that treatment with LPS causes an increase in NF- κ B DNA-binding activity at 30 min, while pretreatment of the cells with DATS for 1 h resulted in a significant reduction in the DNA-binding activity of NF- κ B (Fig. 4B). In parallel with immunoblotting data, the immunofluorescence images revealed that the

nuclear accumulation of NF- κ B p65 was not induced in the cells following treatment with DATS alone in the absence of LPS stimulation; however, it was strongly induced following LPS stimulation, and the shift of NF- κ B to the nucleus was completely abolished subsequent to pretreating the cells with DATS (Fig. 4C). Taken together, these results suggest that DATS treatment inhibits LPS-induced NF- κ B translocation by attenuating the I κ B α degradation.

DATS inhibits LPS-induced TLR4 and myeloid differentiation factor 88 (MyD88) expression, and the interaction between LPS and TLR4. The effects of DATS was assessed on the expression of LPS-induced TLR4 and MyD88 (a TLR4-associated molecule) expression to determine the involvement of the TLR4 signaling pathway in the DATS-mediated anti-inflammatory potential. The increased levels of TLR4 and MyD88 proteins in LPS-treated RAW 264.7 cells were completely blocked in a concentration-dependent fashion by pretreatment with DATS (Fig. 5A). Furthermore, when cells were treated with AF-LPS alone, fluorescence of LPS and TLR4 were observed outside the cell membrane by the immunofluorescence

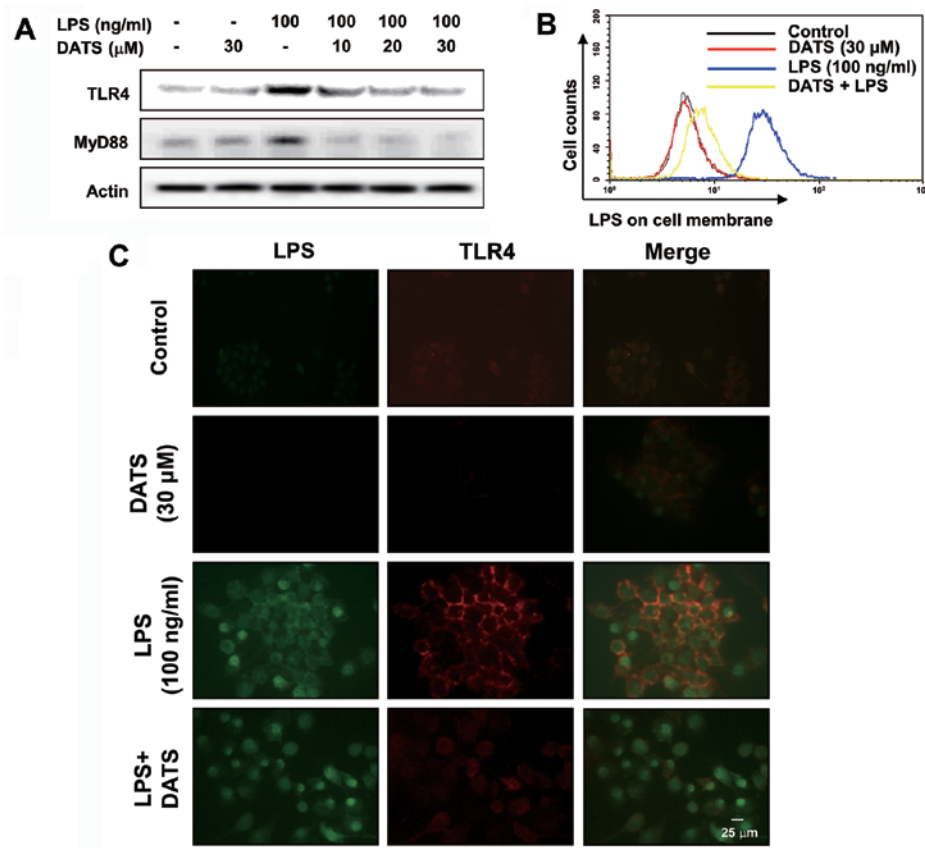


Figure 5. Inhibition of lipopolysaccharide (LPS)-induced Toll-like receptor 4 (TLR4) and MyD88 expression, and interaction between LPS and TLR4 by diallyl trisulfide (DATS) in LPS-stimulated RAW 264.7 macrophages. (A) RAW 264.7 cells were pretreated with 30 μ M DATS for 1 h prior to LPS treatment, and the total proteins were isolated at 6 h after LPS treatment. The levels of TLR4 and MyD88 protein were assessed by western blot analysis using the anti-TLR4 and anti-MyD88 antibodies, and an enhanced chemiluminescence (ECL) detection system. Actin was used as an internal control. (B) RAW 264.7 cells were incubated with AF-LPS for 1 h in absence or presence of DATS, and the LPS binding in the surface of BV2 cells was measured by flow cytometry. (C) Cells were incubated with 100 ng/ml AF-LPS for 30 min in the absence or presence of DATS (30 μ M), and subsequently the interaction between AF-LPS and TLR4 was detected by a fluorescence microscope.

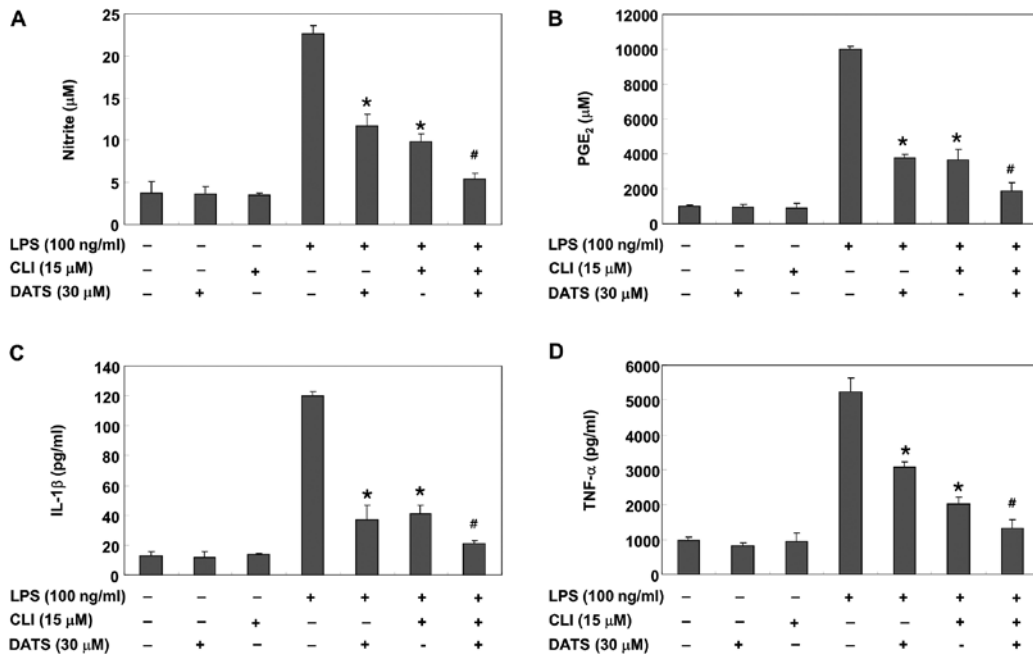


Figure 6. Toll-like receptor 4 (TLR4) blocking with CLI-095 increases the inhibitory potential of pro-inflammatory mediators and cytokine production by diallyl trisulfide (DATS) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Cells were treated with 30 μ M DATS alone or in combination with 15 μ M CLI-095 (InvivoGen, San Diego, CA, USA) for 1 h before LPS treatment. Following a 24 h treatment, the amounts of (A) nitric oxide (NO), (B) prostaglandin E₂ (PGE₂), (C) interleukin-1 β (IL-1 β) and (D) tumor necrosis factor- α (TNF- α) were measured in supernatants. Data are means \pm standard deviations of three independent experiments (*P<0.05 vs. LPS treated cells; #P<0.05 vs. LPS + DATS treated cells).

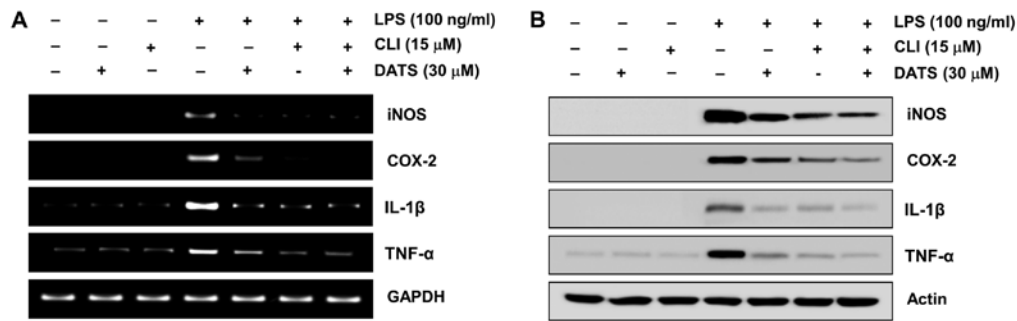


Figure 7. Toll-like receptor 4 (TLR4) blocking with CLI-095 increases the inhibitory potential of pro-inflammatory mediators and cytokine production by diallyl trisulfide (DATS) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Cells were treated with 30 μ M DATS alone or in combination with 15 μ M CLI-095 (InvivoGen, San Diego, CA, USA) for 1 h before LPS treatment. The levels of (A) inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) mRNA and (B) protein were assessed by reverse transcription-polymerase chain reaction and western blot assays after 6 and 24 h treatment, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin were used as internal controls, respectively.

assay (Fig. 5B). However, the fluorescence intensity of TLR4 was markedly attenuated in the presence of DATS, suggesting that DATS may inhibit the interaction between LPS and TLR4 on the RAW 264.7 cell surface. In addition, treatment with LPS in the presence of DATS significantly prevented the binding of LPS to the RAW 264.7 cell surface (Fig. 5C), indicating that DATS may interfere with the clustering of TLR4 with MyD88.

CLI-095, a TLR4 inhibitor, increases the anti-inflammatory potential of DATS. The effects of CLI-095, which blocks TLR4-mediated signaling, were evaluated to further confirm the involvement of the TLR4 signaling pathway in the DATS-mediated anti-inflammatory potential. CLI-095 reduced the LPS-induced production of pro-inflammatory mediators and cytokines by suppressing the transcriptional levels of their corresponding genes (Fig. 6). Furthermore, DATS and CLI-095 co-treatment synergistically inhibited LPS-induced synthesis of NO and PGE₂, as well as expression of iNOS and COX-2. DATS and CLI-095 co-treatment synergistically inhibited LPS-induced release and expression of IL-1 β and TNF- α (Fig. 7). These results verify that the inhibitory effects of DATS on the LPS-induced inflammatory responses may result from suppression of the TLR4 signaling pathway.

Discussion

In the present study, DATS was shown to be suitable for reducing LPS-induced NO and PGE₂ synthesis in RAW 264.7 macrophages. This suppression was possibly due to inhibiting the upregulation of iNOS and COX-2 at the transcriptional level during macrophage activation by LPS. Parallel inhibitory effects on synthesis and expression of TNF- α and IL-1 β were also observed in LPS-stimulated RAW 264.7 cells.

During the inflammatory process, LPS induces the overproduction of pro-inflammatory mediators, such as NO and PGE₂, which are generated by the inducible enzymes iNOS and COX-2, respectively (3,4). NO is controlled by NO synthase and iNOS is markedly upregulated in inflammatory disorders. Another enzyme that plays a pivotal role in mediating inflammation is COX-2, which catalyzes the rate-limiting step in the synthesis of PGE₂ from arachidonic acid. In addition to

NO and PGE₂, numerous inflammatory cytokines have been recently demonstrated to be initiators and mediators of the inflammatory response. Among these, TNF- α and IL-1 β are the major pro-inflammatory cytokines produced by activated macrophages, and their excessive production has been linked to the development of chronic inflammatory diseases (27,28). Additionally, TNF- α and IL-1 β production is critical for the synergistic induction of NO and PGE₂ production in LPS-stimulated macrophage cells (27,29). Therefore, overproduction of these inflammatory factors is a histopathological characteristic of various inflammation-mediated diseases, and the selective inhibition of their production and function may be effectively therapeutic in the control of inflammatory disorders.

Consistent with the findings of the present study (Figs. 2 and 3), DATS inhibited LPS-induced NO and iNOS expression, as well as intracellular reactive oxygen species (ROS) generation, and the inhibitory effect of DATS on LPS-induced iNOS expression is possibly attributed to its antioxidant potential to inhibit NF- κ B activation in RAW 264.7 macrophages (21). DATS has been reported to reduce the levels of LPS-induced PGE₂ synthesis and several cytokines, including TNF- α , by inactivating mitogen-activated protein kinases and NF- κ B signaling pathways in the same cell line (23). This study also revealed that DATS reduces LPS-induced ROS and activates transcription factor NF-E₂p45-related factor 2-mediated expression of heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1, demonstrating that cross-talk between antioxidant and anti-inflammation in the inhibition of LPS-induced inflammation by DATS. In addition, DATS inhibits TNF- α production by inhibiting its transcriptional activity and downregulating NF- κ B activation in inflamed mucosa of ulcerative colitis (30). Additionally, DATS ameliorates dextran sulfate sodium-induced mouse colitis presumably by blocking inflammatory signaling mediated by NF- κ B and signal transducer and activator of transcription 3 transcription factors, as well as expression of their target proteins, including COX-2 and iNOS (31). A previous study has also suggested that DATS suppresses high glucose-induced cardiomyocyte apoptosis by inhibiting NADPH oxidase-related ROS and downstream c-Jun N-terminal kinase/NF- κ B signaling (32). A previous study showed that DATS has an anti-cancer effect

by inhibiting the levels of certain critical genes involved in cancer cell growth and metastasis by suppressing NF- κ B activation (33). In agreement with these previous observations, the present data also revealed that the anti-inflammatory effects of DATS appeared to involve inhibiting NF- κ B activation by blocking LPS-stimulated I κ B α degradation and nuclear translocation of the NF- κ B p65 protein (Fig. 4). Based on the present study findings and previous studies, DATS may exert its anti-inflammatory effect by suppressing the production of these pro-inflammatory mediators and cytokines by modulating the NF- κ B signaling pathway.

By contrast, increasing evidence suggests that signaling pathways downstream of TLR4, a pattern recognition receptor that generates innate immune responses to pathogens such as LPS, play an important role in the pathogenesis of inflammation (6). TLR4 has a common TIR motif in the cytoplasmic domain and shares different adaptor proteins, including MyD88 for NF- κ B activation (34). During infection, LPS directly binds with cellular TLR4 and induces a cascade of inflammatory events by triggering overproduction of inflammatory inducers through receptor dimerization and the recruitment of adapter molecules (35,36). Several studies have reported that certain anti-inflammatory agents compete with LPS binding to TLR4, resulting in the suppression of downstream signaling pathways (37,38). In addition, TLR4 and MyD88 are upregulated in various inflammatory processes induced by LPS (39,40). Consequently, the pharmacological reduction of LPS-inducible inflammatory regulators through inhibiting the association between LPS and TLR4 is regarded as an attractive therapeutic strategy for numerous acute and chronic inflammatory diseases. In the present study, increased expression of TLR4 and MyD88 proteins was concentration-dependently reduced in the presence of DATS (Fig. 5A). The data also indicate that DATS has inhibitory effects on the binding of LPS with TLR4 (Fig. 5B and C), suggesting a possible antagonistic effect of DATS against TLR4. In addition, combined pretreatment of DATS with CLI-095, a specific TLR4 signaling inhibitor (41,42), synergistically attenuated the release of NO and PGE₂, as well as expression of *iNOS* and *COX-2* mRNA and protein (Fig. 6). In accordance with these results, co-treatment also reduced the production and expression of TNF- α and IL-1 β compared to those in the DATS or CLI-095 alone treated groups in LPS-treated RAW 264.7 macrophages (Fig. 7). These observations suggest that DATS may inhibit the initiation of intracellular inflammatory signaling cascades by attenuating the binding of LPS to TLR4 on macrophages, which is a pivotal upstream signal for NF- κ B activation. Therefore, the antagonistic function of DATS against TLR4 may be responsible for the anti-inflammatory effects of DATS in LPS-stimulated RAW 264.7 macrophages.

In conclusion, the present data reveal that DATS inhibited LPS-induced *iNOS*, *COX-2*, TNF- α and IL-1 β expression, which was accompanied by reducing the corresponding transcriptional gene expression through interfering with the clustering of LPS with TLR4. This resulted in blocking the activation of NF- κ B signaling pathway in RAW 264.7 macrophages. These findings provide a further partial molecular description of the mechanism that underlies the anti-inflammatory properties of DATS.

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References

- Lind L: Circulating markers of inflammation and atherosclerosis. *Atherosclerosis* 169: 203-214, 2003.
- Bertolini A, Ottani A and Sandrini M: Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacol Res* 44: 437-450, 2001.
- Kanno S, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, Obara Y, Nakahata N and Ishikawa M: Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. *Life Sci* 78: 673-681, 2006.
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B and Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-2088, 1998.
- Takeda K and Akira S: Microbial recognition by Toll-like receptors. *J Dermatol Sci* 34: 73-82, 2004.
- Shuto T, Kato K, Mori Y, Viriyakosol S, Oba M, Furuta T, Okiyonedo T, Arima H, Suico MA and Kai H: Membrane-anchored CD14 is required for LPS-induced TLR4 endocytosis in TLR4/MD-2/CD14 overexpressing CHO cells. *Biochem Biophys Res Commun* 338: 1402-1409, 2005.
- Chan ED and Riches DW: IFN- γ + LPS induction of *iNOS* is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol Cell Physiol* 280: C441-C450, 2001.
- Hattori Y, Hattori S and Kasai K: Lipopolysaccharide activates Akt in vascular smooth muscle cells resulting in induction of inducible nitric oxide synthase through nuclear factor-kappa B activation. *Eur J Pharmacol* 481: 153-158, 2003.
- Anwar MA, Basith S and Choi S: Negative regulatory approaches to the attenuation of Toll-like receptor signaling. *Exp Mol Med* 45: e11, 2013.
- Datla P, Kalluri MD, Basha K, Bellary A, Kshirsagar R, Kanekar Y, Upadhyay S, Singh S and Rajagopal V: 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol, from *Eulophia ochreatea*, inhibits inflammatory signalling mediated by Toll-like receptors. *Br J Pharmacol* 160: 1158-1170, 2010.
- Tattelman E: Health effects of garlic. *Am Fam Physician* 72: 103-106, 2005.
- Moyad MA: Bladder cancer recurrence: Part II. What do I tell my patients about lifestyle changes and dietary supplements? *Curr Opin Urol* 13: 379-383, 2003.
- Yi L and Su Q: Molecular mechanisms for the anti-cancer effects of diallyl disulfide. *Food Chem Toxicol* 57: 362-370, 2013.
- Padiya R and Banerjee SK: Garlic as an anti-diabetic agent: recent progress and patent reviews. *Recent Pat Food Nutr Agric* 5: 105-127, 2013.
- Capasso A: Antioxidant action and therapeutic efficacy of *Allium sativum* L. *Molecules* 18: 690-700, 2013.
- Iciek M, Kwiecień I and Włodek L: Biological properties of garlic and garlic-derived organosulfur compounds. *Environ Mol Mutagen* 50: 247-265, 2009.
- Lea MA: Organosulfur compounds and cancer. *Adv Exp Med Biol* 401: 147-154, 1996.
- Amagase H, Petesch BL, Matsuura H, Kasuga S and Itakura Y: Intake of garlic and its bioactive components. *J Nutr* 131: 955S-962S, 2001.
- Chen C, Pung D, Leong V, Hebbar V, Shen G, Nair S, Li W and Kong AN: Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Radic Biol Med* 37: 1578-1590, 2004.
- Fukao T, Hosono T, Misawa S, Seki T and Ariga T: The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride. *Food Chem Toxicol* 42: 743-749, 2004.

21. Liu KL, Chen HW, Wang RY, Lei YP, Sheen LY and Lii CK: DATS reduces LPS-induced iNOS expression, NO production, oxidative stress, and NF-kappaB activation in RAW 264.7 macrophages. *J Agric Food Chem* 54: 3472-3478, 2006.
22. Na HK, Kim EH, Choi MA, Park JM, Kim DH and Surh YJ: Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. *Biochem Pharmacol* 84: 1241-1250, 2012.
23. You S, Nakanishi E, Kuwata H, Chen J, Nakasone Y, He X, Liu X, Zhang S, Zhang B and Hou DX: Inhibitory effects and molecular mechanisms of garlic organosulfur compounds on the production of inflammatory mediators. *Mol Nutr Food Res* 57: 2049-2060, 2013.
24. Kyung J, Kim D, Park D, Yang YH, Choi EK, Lee SP, Kim TS, Lee YB and Kim YB: Synergistic anti-inflammatory effects of *Laminaria japonica* fucoidan and *Cistanche tubulosa* extract. *Lab Anim Res* 28: 91-97, 2012.
25. Lee SH, Kim DW, Eom SA, Jun SY, Park M, Kim DS, Kwon HJ, Kwon HY, Han KH, Park J, Hwang HS, Eum WS and Choi SY: Suppression of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice by transduced Tat-Annexin protein. *BMB Rep* 45: 354-359, 2012.
26. Joh EH and Kim DH: Lancemaside A inhibits lipopolysaccharide-induced inflammation by targeting LPS/TLR4 complex. *J Cell Biochem* 111: 865-871, 2010.
27. Aggarwal BB and Natarajan K: Tumor necrosis factors: developments during the last decade. *Eur Cytokine Netw* 7: 93-124, 1996.
28. Brennan FM and McInnes IB: Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 118: 3537-3545, 2008.
29. Blatteis CM, Li S, Li Z, Perlik V and Feleder C: Signaling the brain in systemic inflammation: the role of complement. *Front Biosci* 9: 915-931, 2004.
30. Bai AP, Ouyang Q and Hu RW: Diallyl trisulfide inhibits tumor necrosis factor-alpha expression in inflamed mucosa of ulcerative colitis. *Dig Dis Sci* 50: 1426-1431, 2005.
31. Lee HJ, Lee HG, Choi KS, Surh YJ and Na HK: Diallyl trisulfide suppresses dextran sodium sulfate-induced mouse colitis: NF-kB and STAT3 as potential targets. *Biochem Biophys Res Commun* 437: 267-273, 2013.
32. Kuo WW, Wang WJ, Tsai CY, Way CL, Hsu HH and Chen LM: Diallyl trisulfide (DATS) suppresses high glucose-induced cardiomyocyte apoptosis by inhibiting JNK/NFkB signaling via attenuating ROS generation. *Int J Cardiol* 168: 270-280, 2013.
33. Lai KC, Hsu SC, Kuo CL, Yang JS, Ma CY, Lu HF, Tang NY, Hsia TC, Ho HC and Chung JG: Diallyl sulfide, diallyl disulfide, and diallyl trisulfide inhibit migration and invasion in human colon cancer colo 205 cells through the inhibition of matrix metalloproteinase-2, -7, and -9 expressions. *Environ Toxicol* 28: 479-488, 2013.
34. Akira S and Takeda K: Toll-like receptor signalling. *Nat Rev Immunol* 4: 499-511, 2004.
35. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA and O'Neill LA: Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413: 78-83, 2001.
36. Jawan B, Kao YH, Goto S, Pan MC, Lin YC, Hsu LW, Nakano T, Lai CY, Sun CK, Cheng YF, Tai MH, Eng HL, Wang CS, Huang CJ, Lin CR and Chen CL: Propofol pretreatment attenuates LPS-induced granulocyte-macrophage colony-stimulating factor production in cultured hepatocytes by suppressing MAPK/ERK activity and NF-kappaB translocation. *Toxicol Appl Pharmacol* 229: 362-373, 2008.
37. Jung JS, Shin KO, Lee YM, Shin JA, Park EM, Jeong J, Kim DH, Choi JW and Kim HS: Anti-inflammatory mechanism of exogenous C2 ceramide in lipopolysaccharide-stimulated microglia. *Biochim Biophys Acta* 1831: 1016-1026, 2013.
38. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N and Hwang DH: Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J Biol Chem* 278: 37041-37051, 2003.
39. Islam MA, Cinar MU, Uddin MJ, Tholen E, Tesfaye D, Looft C and Schellander K: Expression of Toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages. *Vet Immunol Immunopathol* 146: 62-73, 2012.
40. Cario E and Podolsky DK: Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 68: 7010-7017, 2000.
41. Ii M, Matsunaga N, Hazeki K, Nakamura K, Takashima K, Seya T, Hazeki O, Kitazaki T and Iizawa Y: A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol Pharmacol* 69: 1288-1295, 2006.
42. Takashima K, Matsunaga N, Yoshimatsu M, Hazeki K, Kaisho T, Uekata M, Hazeki O, Akira S, Iizawa Y and Ii M: Analysis of binding site for the novel small-molecule TLR4 signal transduction inhibitor TAK-242 and its therapeutic effect on mouse sepsis model. *Br J Pharmacol* 157: 1250-1262, 2009.