Dysifragilone A inhibits LPS-induced RAW264.7 macrophage activation by blocking the p38 MAPK signaling pathway

HUIXIANG LI[1*], QIAN ZHANG[1*], XIN JIN[1], XIAOWEN ZOU[1], YUEXUAN WANG[1], DEXIANG HAO[1], FENGHUA FU[1], WEIHUA JIAO[2], CUIXIA ZHANG[3], HOUWEN LIN[2], KEIICHI MATSUZAKI[4] and FENG ZHAO[1]

[1]Key Laboratory of Molecular Pharmacology and Drug Evaluation, Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, School of Pharmacy, Yantai University, Yantai, Shandong 264005; [2]Research Center for Marine Drugs, State Key Laboratory of Oncogenes and Related Genes, Department of Pharmacy, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127; [3]Pharmaceutical Preparation Section, Xing Fu Hospital of Zhi Fu, Yantai, Shandong 264000, P.R. China; [4]School of Pharmacy, Nihon University, Narashinodai, Funabashi, Chiba 274-8555, Japan

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Abstract. Dysifragilone A, a sesquiterpene aminoquinone based on a rearranged avarone skeleton, has been previously isolated and identified from the South China Sea sponge *Dysidea fragilis*. In the present study, anti-inflammatory activity and the underlying molecular mechanism of dysifragilone A were studied using the classical inflammation model of lipopolysaccharide (LPS)-activated RAW264.7 macrophage cells and an MTT assay, Griess method, ELISA and western blotting were used. The results revealed that dysifragilone A significantly reduced the release of inflammatory mediators and inflammatory cytokines in activated RAW264.7 cells, including nitric oxide (NO), prostaglandin E_2,(PGE_2) and interleukin-6 (IL-6). The protein expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and the enzymatic activity of iNOS and COX-2 were also inhibited by dysifragilone A in a dose dependent manner. Further mechanistic investigations suggested that the anti-inflammatory activity of dysifragilone A results from the suppression of p38 mitogen-activated protein kinase (MAPK) activation in LPS-activated macrophages; however, this was not associated with inhibition of the extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) signaling pathways. Therefore, dysifragilone A and similar compounds may be anti-inflammatories that have potential to be used in the clinic.

Correspondence to: Professor Feng Zhao, Key Laboratory of Molecular Pharmacology and Drug Evaluation, Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, School of Pharmacy, Yantai University, 30 Qingquan Road of Laishan, Yantai, Shandong 264005, P.R. China
E-mail: ytuzhaofeng@163.com

Professor Houwen Lin, Research Center for Marine Drugs, State Key Laboratory of Oncogenes and Related Genes, Department of Pharmacy, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai 200127, P.R. China
E-mail: franklin67@126.com

*Contributed equally

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Introduction

Marine sponge (Porifera) is the oldest animal phylum in existence, and is also the simplest of animals with the most primitive multicellular structures and partially differentiated tissues. Sponge is a remarkable component of the marine benthos throughout the tropical and polar habitats, and lives in areas with strong currents or wave action (1). Sponges account for one fifteenth of all marine species on the Earth, and the species found in China accounts for approximately half of these.

The marine sponge surface forms a number of streamline textures and may avoid destruction by waves and currents and are able to defend against environmental factors such as hunting, overgrowth by fouling the shells of abalone and oyster or conquering spaces (2). Due to the splinter-like spicules and toxic chemicals produced by the sponge, the majority of carnivorous animals elude them (3). The disparate structural framework makes the marine sponge source rich and display a battery of cogent biological activities that are of potential interest to humans (4).

Novel constituents have been identified from *Dysidea fragilis*, such as azacyclopropene derivatives (5), diketopiperazines (6,7), polyhydroxylated sterols (8), sesquiterpenoid (9) and brominated diphenyl ethers (10). The diversity of the chemical structures implies the potential of various bioactivities, such as antibacterial, anti-inflammatory, antiviral and anti-cardiovascular activity. Previous studies on marine sponges of *Dysidea* genus from the Xisha Islands

in the South China Sea identified seven novel sesquiterpene quinones (dysidavarones A-D and dysideanones A-C) (11,12) from *Dysidea avara*, and 13 novel sesquiterpene aminoquinones (dysidaminones A-M) (13) and three other novel sesquiterpene aminoquinones based on a rearranged avarone skeleton (dysifragilones A-C) (14) from *Dysidea fragilis*. These newly isolated agents have been assessed for their inhibitory activities on lipopolysaccharide (LPS)-stimulated production of nitric oxide (NO) in RAW264.7 cells, and the results have demonstrated that dysifragilone A exhibits potent inhibitory activity. In the present research, the anti-inflammatory activities *in vitro* and the regulatory effect on the inflammatory signal transduction pathway of dysifragilone A were further investigated.

Materials and methods

Animal material. Samples of *Dysidea fragilis* were collected along the coast of Yongxing Island in the South China Sea on 11th April 2011, and the extraction, isolation and identification of dysifragilone A (15.9 mg; Fig. 1A) were performed by Shanghai Jiao Tong University (Shanghai, China) (14). Subsequently, the purity was determined by standardization of the peak area by high performance liquid chromatography, which was found to be 98.7% (via a UV detector). The data of ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR of dysifragilone A are displayed in Table I.

Chemicals and reagents. *E. coli* LPS and MTT were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hydrocortisone succinate (catalog no. 080-05581; lot CTE6574) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The NO concentration determination kit, mouse tumor necrosis factor-α (TNF-α) ELISA kit (catalog no. SEM024), mouse interleukin-6 (IL-6) ELISA kit (catalog no. SEM008) and the bicinchoninic acid protein assay kit were obtained from Yantai Science and Biotechnology Co., Ltd. (Shandong, China). The mouse prostaglandin E_2 (PGE_2) ELISA kit (catalog no. KGE004B) was obtained from R&D Systems, Inc. (USA). The NO synthase assay kit (fluorimetric method) was purchased from Beyotime Biotechnology (Haimen, China). The cyclooxygenase (COX) colorimetric inhibitor screening assay kit (catalog no. 701050) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The mouse anti-rabbit inducible nitric oxide synthase (iNOS) polyclonal antibody (catalog no. 160862) and mouse anti-rabbit COX-2 polyclonal antibody (catalog no. 160106) were purchased from Cayman Chemical Company. Goat anti-rabbit phosphorylated (p)-extracellular signal-regulated kinase (ERK) 1/2 polyclonal antibody (catalog no. AF1015), goat anti-rabbit p-c-Jun N-terminal kinase (JNK) polyclonal antibody (catalog no. AF3318), goat anti-rabbit p-p38 polyclonal antibody (catalog no. AF3455), and horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L; catalog no. S0001) were products of Affinity Biosciences (Cincinnati, OH, USA). Goat anti-rabbit β-actin polyclonal antibody (catalog no. sc-1616) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Dysifragilone A was dissolved in cell culture grade dimethyl sulfoxide (DMSO) (purity >99.9%) at 50 mM and stored at -20˚C, and then diluted to the concentration required.

Cell culture of RAW264.7 cells. RAW264.7 mouse monocyte-macrophage cells (TIB-71; American Type Culture Collection, Manassas, VA, USA), were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS at 37˚C in a cell incubator with 5% CO_2. The media was routinely replaced every 2 days. RAW264.7 cells were passaged until they achieved 80% of the petri dish area.

MTT assay for cytotoxicity. MTT assay was used to detect cell viability and cytotoxicity. Succinate dehydrogenase of mitochondria in living cells reduced the exogenous MTT reagent to formazan, which is deposited in the cells, and the number of living cells is proportional to the formazan crystals (15). RAW264.7 cells were seeded in a 96-well plate at a density of $1x10^6$ cells/ml. After 1 h incubation, the cells were treated with dysifragilone A at final concentrations of 12.5, 25, 50, 100 μM and the control group received an equal amount of 0.2% DMSO in the culture medium. After 24 h incubation, MTT solution (5 mg/ml) was then added to the 96-well plate, and the cells were incubated for another 4 h at 37˚C in cell incubator. After removal of the cell supernatant, 150 μl DMSO was added to dissolve the formazan. The absorbance was measured at 570 nm (reference, 630 nm) by using a microplate reader. The untreated cells were regarded as having 100% viability. Results are expressed as a percentage of viable cells compared with the control group.

Determination of nitrite concentration. RAW264.7 cells were seeded in a 96-well plate at the density of $1x10^6$ cells/ml. After 1 h incubation, the cells were treated with LPS (1 μg/ml), various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml), hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h. A total of 100 μl cell culture supernatant was removed to detect the NO concentration. The cell culture supernatant was added to 100 μl Griess reagent (an equal mixture of reagent A and reagent B) and then incubated for 10 min at room temperature. The absorbance at 540 nm was measured by using a microplate reader (16), and the nitrite concentrations were calculated by interpolation of a standard curve.

Determination of PGE_2 concentration. PGE_2, a pro-inflammatory mediator, is produced by COX-2. RAW264.7 cells were seeded in a 96-well plate at a density of $5x10^5$ cells/ml. After 1 h incubation, the cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and treated with hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. A total of 100 μl of the cell culture supernatant was removed to detect the levels of PGE_2 by using a commercial mouse PGE_2 ELISA kit in accordance with the manufacturer's protocol. The ELISA data represent the mean ± standard deviation. Samples were tested in duplicate in more than three independent experiments (17).

Determination of TNF-α and IL-6. RAW264.7 cells were seeded in a 96-well plate at a density of $5x10^5$ cells/ml. After

1 h incubation, the cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and treated with hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 6 h at 37˚C in a humidified atmosphere containing 5% CO_2. A total of 100 μl of the cell culture supernatant was taken out to detect the levels of TNF-α or IL-6 by using the respective ELISA kit in accordance with the manufacturer's protocol (18). The ELISA data represent the mean ± standard deviation. Samples were tested in duplicate in more than three independent experiments.

iNOS enzymatic activity determination. The determination of iNOS enzymatic activity has been previously reported (19). Briefly, RAW264.7 cells at a density of $5x10^5$ cells/ml were treated with LPS (1 μg/ml), treated with various concentration (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and treated with hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2, and the cell supernatant was removed from the 96-well plate. After adding 100 μl iNOS assay buffer (2X NOS assay buffer was mixed with an equal volume of Milli-Q water), 100 μl of NOS assay reaction solution (50% NOS assay buffer, 39.8% Milli-Q water, 5% L-arginine solution, 5% 0.1 mM nicotinamide adenine dinucleotide phosphate, 0.2% 4'-amino-5-methylamino-2',7'-difluorofluorescein diacetate) was added and incubated for 2 h at 37˚C in an incubator. The fluorescence was measured at excitation wavelength of 495 nm and emission wavelength of 515 nm by using a fluorescence microplate reader.

COX-2 enzymatic activity determination. The enzymatic activity of COX-2 was assayed by using a COX colorimetric inhibitor screening assay kit in a cell-free system in accordance with the manufacturer's protocol (19). Briefly, 160 μl assay buffer, 10 μl heme and 10 μl DMSO were added to three background wells. 150 μl assay buffer, 10 μl heme, 10 μl COX-2 enzyme and 10 μl DMSO were added to three 100% initial activity wells. 150 μl assay buffer, 10 μl heme, 10 μl COX-2 enzyme and 10 μl dysifragilone A (final concentration 1 mM) were added to three inhibitor wells. After carefully shaking the plate for a few sec, the plate was incubated for 5 min at 25˚C. After adding 20 μl colorimetric substrate solution to all wells, 20 μl arachidonic acid was quickly added to all wells. The plate was carefully shaken for a few sec and incubated for 2 min at 25˚C. The absorbance was measured at 590 nm using a microplate reader, and the suppression ratio of COX-2 enzymatic activity was calculated in accordance with the manufacturer's protocol.

Western blot analysis. RAW264.7 cells were treated with various concentration (12.5, 25, 50 and 100 μM) of dysifragilone A with or without LPS (1 μg/ml), and then washed with cold PBS (1X) and lysed in ultrasonic cell disruptor with cold PBS buffer after 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. The cell debris was removed from the samples by centrifugation (1,1749 x g, 4˚C, 6 min). After determination of the protein concentration for each sample by the bicinchoninic acid method, 60 μl sample was boiled in SDS-PAGE loading buffer (15 μl) for 5 min. A total of 30 μg protein was added to gels (8% gels for proteins of iNOS and COX-2, 10% gels for proteins of p-ERK, p-JNK and p-p38), which were subjected to electrophoresis prior to transfer onto nitrocellulose membranes. Membranes were then incubated with blocking buffer [5% nonfat-dried milk in Tris-buffered saline-Tween (TBS-T)] for 2 h at room temperature. After being washed three times in TBS-T, the membranes were incubated with anti-rabbit polyclonal primary antibodies diluted at 1:1,000 (anti-iNOS, anti-COX-2, anti-phospho-ERK 1/2, anti-phospho-JNK and anti-phospho-p38) overnight at 4˚C and anti-β-actin antibody. The membranes were washed three times in TBS-T and incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (1:1,000) for 1 h at room temperature. The membranes were then washed three times in TBS-T prior to visualization of proteins by using an Enhanced Chemiluminescence reagent (BeyoECL plus A and the same volume of BeyoECL plus B; Beyotime Biotechnology) and exposed to photographic films (Kodak, Rochester, NY, USA). Images of iNOS, COX-2, p-ERK 1/2, p-JNK, p-p38, and β-actin proteins were collected and quantified by densitometric analysis using the DigDoc100 program (Alpha Ease FC 2008 software; Alpha Innotech Corporation, San Leandro, CA, USA). Expression of iNOS, COX-2, p-ERK 1/2, p-JNK, and p-p38 were normalized to the expression levels of β-actin.

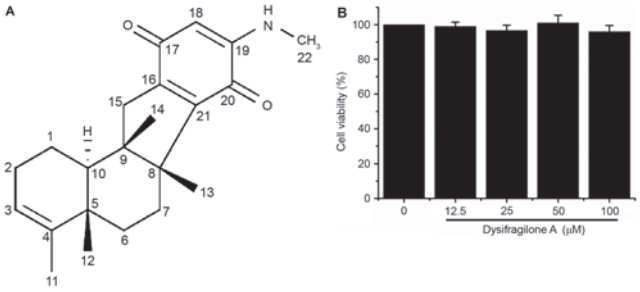

Figure 1. (A) Chemical structure of dysifragilone A. (B) Dysifragilone A induced no cytotoxicity on the RAW264.7 cells. RAW264.7 cells were exposed to dysifragilone A (12.5, 25, 50 and 100 μM) for 24 h. Cell viability was measured via an MTT assay.

Statistical analysis. All experimental results are presented as mean ± standard deviation. Statistical significance of differences between groups was determined by a one-way analysis of variance followed by the Least Significant Difference test using SPSS software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Dysifragilone A is not cytotoxic to RAW264.7 cells. RAW264.7 cells were treated with the indicated concentration of dysifragilone A for 24 h, and then the cell viability was determined by MTT assay. Dysifragilone A (up to 100 μM) did not cause cytotoxicity against RAW264.7 cells (Fig. 1B). The highest dose for treatment of cells in future experiments was 100 μM dysifragilone A, and was used to determine the effect of dysifragilone A on anti-inflammatory activities and the production of pro-inflammatory factors, inflammatory mediators or protein expression levels. As Dysifragilone A

Table I. ^{1}H-NMR and ^{13}C-NMR spectroscopic data of dysifragilone A ($CDCl_3$, δ).

Position	δ_H	δ_C	Position	δ_H	δ_C
1	1.55 (m)	21.1	11	1.52 (s, br)	17.9
1	1.56 (m)		12	1.01 (s)	18.9
2	1.92 (m)	27.2	13	1.04 (s)	23.4
2	2.05 (m)		14	1.03 (s)	16.3
3	5.15 (s, br)	120.9	15	2.82 (d, *J*=18.6 Hz)	42.2
4		143.5	15	2.25 (d, *J*=18.6 Hz)	
5		37.6	16		153.7
6	0.96 (td, *J*=13.8 3.0 Hz)	33.7	17		184.9
6	1.64 (dt, *J*=12.6 3.0 Hz)		18	5.29 (s)	96.9
7	1.50 (m)	26.6	19		149.0
7	2.56 (dt, *J*=15.0 2.4 Hz)		20		181.8
8		52.6	21		147.0
9		47.5	22	2.83 (d, *J*=5.4 Hz)	29.3
10	1.28 (m)	46.2	NH	5.68 (s, br)	

^{1}H-NMR and ^{13}C-NMR were obtained at 600 and 150 MHz, respectively. NMR, nuclear magnetic resonance; s, single peak; m, multiple peaks; br, broad peaks.

did not cause any significant cytotoxicity, this implied that the observed inhibition activities were not a result of cell death.

Effect of dysifragilone A on NO and PGE_2 production. RAW264.7 cells were exposed to LPS (1 μg/ml), various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h. The concentration of nitrite (NO_2^-) was detected by Griess assay as an indicator of NO production, and the levels of PGE_2 were determined by ELISA. Hydrocortisone succinate was used as a positive control. As shown in Fig. 2A, dysifragilone A caused a significant reduction in the production of NO stimulated by LPS, which was nearly equivalent to treatment with hydrocortisone succinate when 100 μM dysifragilone A was used. The production of PGE_2 was also strongly suppressed by dysifragilone A in a dose-dependent manner, compared with LPS treatment (Fig. 2B).

Effect of dysifragilone A on TNF-α and IL-6 release. RAW264.7 cells were exposed to LPS (1 μg/ml), various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 6 h. The levels of pro-inflammatory cytokines TNF-α and IL-6 were measured by using corresponding ELISA kits. As shown in Fig. 2C, hydrocortisone succinate significantly inhibited the release of TNF-α stimulated by LPS. However, dysifragilone A did not cause inhibition of the release of TNF-α induced by LPS. As displayed in Fig. 2D, hydrocortisone succinate significantly inhibited the release of IL-6 stimulated by LPS, while dysifragilone A only showed slight inhibitory activity on the release of IL-6 at concentrations >12.5 μM.

Effect of dysifragilone A on the expression levels of iNOS and COX-2 proteins. NO and PGE_2 are synthesized by iNOS and COX-2 in the inflammatory reaction. High NO and PGE_2 levels are associated with high expression levels of iNOS and COX-2 proteins (20). The present study determined protein expression levels of iNOS and COX-2 by western blotting. As shown in Fig. 3A, RAW264.7 cells were stimulated by LPS for 24 h and the protein expression levels of iNOS and COX-2 were enhanced compared with untreated cells. Dysifragilone A downregulated the expression of iNOS protein in a dose-dependent manner, which may account for the reduced production of NO caused by dysifragilone A (Fig. 2). In addition, dysifragilone A also significantly inhibited the expression of the COX-2 protein in a dose-dependent manner. The density of the iNOS and COX-2 proteins were normalized to β-actin and the data are presented in Fig. 3B and C, respectively.

Effect of dysifragilone A on the iNOS and COX-2 enzymatic activity. RAW264.7 cells were exposed to LPS (1 μg/ml), various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h. The fluorimetric method was used to detect the iNOS enzymatic activity. As shown in Fig. 4A, a 6-fold increase in iNOS enzymatic activity was observed by LPS treatment within 120 min compared with untreated cells. Dysifragilone A significantly suppressed iNOS enzymatic activity in RAW264.7 cells at concentrations of 50 and 100 μM. The cell-free colorimetric method was used to detect the inhibitory effect of COX-2 enzymatic activity induced by dysifragilone A. As demonstrated in Fig. 4B, dysifragilone A also inhibited the COX-2 enzymatic activity at concentrations of 50 and 100 μM.

Effect of dysifragilone A on the activation of the mitogen-activated protein kinase (MAPK) signaling pathway. MAPKs are involved in regulating the inflammatory reaction stimulated by LPS (15 min for JNK, 1 h for ERK and

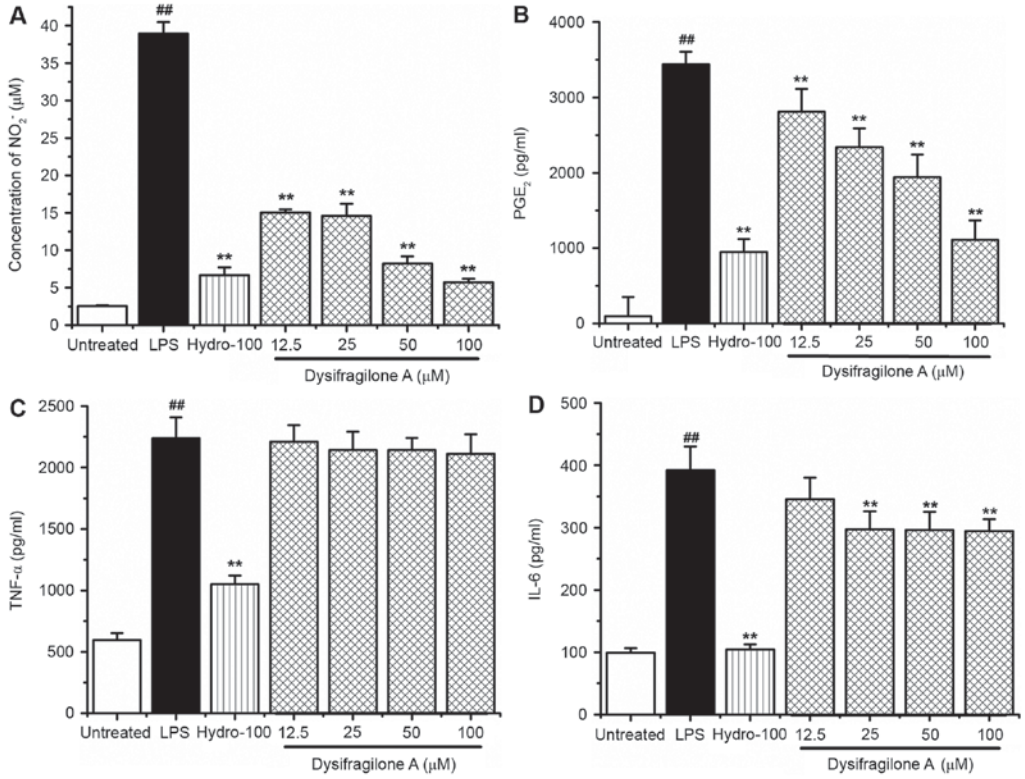

Figure 2. Effect of dysifragilone A on the production of NO, PGE_2, TNF-α and IL-6 induced by LPS. (A) RAW264.7 cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h. The concentration of nitrite in the cells culture supernatant was measured in triplicate, and the data represent the mean ± standard deviation from three independent experiments. (B) The levels of PGE_2 in the cells culture supernatant was measured in triplicate, and the data represent the mean ± standard deviation from three independent experiments. RAW264.7 cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 6 h. A total of 100 μl cell culture supernatant was removed to detect the levels of TNF-α or IL-6 by using respective ELISA kits. The experiment was performed in triplicate, and the results represent the mean ± standard deviation of (C) TNF-α or (D) IL-6 levels. $^{\#\#}$P<0.01 vs. untreated group; **P<0.01 vs. LPS treatment group. NO, nitric oxide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; LPS, lipopolysaccharide; Hydro-100, 100 μM hydrocortisone succinate; PGE_2, prostaglandin E_2.

10 min for p38), and may also be treatment targets for inflammation (21,22). In addition, the MAPK signaling pathway, including ERK 1/2, JNK and p38 MAPK, was activated by LPS (23). Therefore, in order to research whether ERK 1/2, JNK and p38 MAPKs have inhibitory activities on the inflammatory reaction by dysifragilone A, western blot analysis was used to detect these three MAPKs. As shown in Fig. 5A, LPS treatment significantly induced high levels of the p-ERK 1/2, p-JNK and p-p38 proteins compared with untreated cells. Dysifragilone A blocked LPS-induced p-p38 protein in a dose-dependent manner, whereas p-JNK and p-ERK 1/2 were not inhibited by dysifragilone A. The results suggested that dysifragilone A may exhibit an anti-inflammatory potential via suppression of the p38/MAPK signaling pathway following treatment with dysifragilone A for 24 h and treated with LPS for 10 min. However, total p38 was not detected, and this was a potential limitation of the present study. Densitometric data of p-ERK 1/2, p-JNK and p-p38 proteins are displayed in Fig. 5B-D, respectively.

Discussion

Inflammation is a stress reaction of the body to resist environmental stimuli including pathogens, damage or other foreign invasion (24). At inflammatory sites, a clinical symptom is painful heat. Excessive and continuous inflammatory responses may result in diseases associated with inflammation, such as rheumatoid arthritis (25), cancer (26,27), atherosclerosis (28), coronary artery disease (29), chronic bronchitis (30), pneumonia (31), and many other diseases (32). There are two types of medicines used in the clinic to treat inflammation, including non-steroidal anti-inflammatory drug (NSAID) and steroidal agents. Long-term use of NSAID may produce a gastrointestinal reaction, liver damage, other secondary effects on the nervous system, and effects on the urinary, blood and cardiovascular systems (33). The disadvantages of the steroidal agents are retention of water and sodium, risk of infection, rarefaction of bone and obesity. It is therefore essential to identify an effective anti-inflammatory drug with low toxicity.

Macrophages are acquired from monocytes of bone marrow precursors, and regulate inflammation by generation of pro-inflammatory cytokines (including TNF-α, IL-6 and IL-1β), chemokines (monocyte chemoattractant protein-1) and inflammatory mediators (iNOS, COX-2 and PGE_2) at the site of inflammation (34). In the present study, RAW264.7 cells were chosen to investigate the anti-inflammatory activity of dysifragilone A. LPS, the primary component of the outer membranes of gram-negative bacteria, is the primary start factor of the inflammatory response and is recognized by toll-like receptor 4 on the surface of host cells (35).

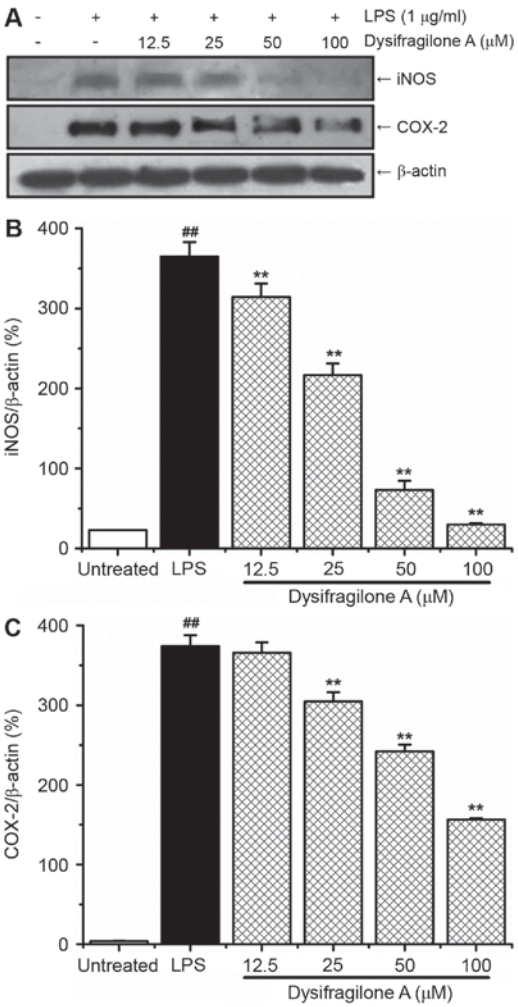

Figure 3. Effect of dysifragilone A on the expression levels of iNOS and COX-2 proteins induced by LPS. RAW264.7 cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h, and the expression of (A) iNOS and COX-2 proteins determined by western blot analysis. β-actin was internal reference to confirm the equal loading of proteins. The mean of three independent experiments used to represent the densitometric analysis of proteins of (B) iNOS and (C) COX-2. $^{\#\#}$P<0.01 vs. untreated group; **P<0.01 vs. LPS treatment group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide.

The bioactivities of sponges and marine bacteria associated with sponges have been extensively studied, such as antibacterial (36), antitumor (37), antifungal (38), antiviral (39), as well as cardiovascular disease (39) resistance activity. However, limited research regarding the anti-inflammatory molecular mechanism of dysifragilone A from *Dysidea fragilis* or related derivatives, has been available until now. In the current study, the anti-inflammatory activities of dysifragilone A in LPS-stimulated RAW264.7 cells were investigated, perhaps for the first time, and the underlying molecular mechanism was explored.

Previous experiments have verified that NO participates in the inflammatory reaction through recruiting leucocytes to the effected tissue (40). PGE_2 is one of the prostaglandins with the highest bio-distribution and mediates a variety of physiological and pathological processes, such as the symptoms of redness, swelling, heat and pain in inflammation (41). In addition, a study confirmed the pathological action of PGE_2 in most inflammatory diseases (42). Therefore, inhibiting the synthesis and release of NO and PGE_2 may be important for anti-inflammation. The results of this study revealed that dysifragilone A suppressed the overproduction of NO. The pro-inflammatory mediator PGE_2 also exhibited similar inhibitory activities to hydrocortisone succinate. Based on above analysis, further research of anti-inflammatory activity is essential.

NO and PGE_2 are overproduced by the enzymes iNOS and COX-2, respectively (43). Thus, inhibiting the expression of enzymes of iNOS and COX-2 may reduce the synthesis and release of NO and PGE_2. Consistent with the above analysis, dysifragilone A also downregulated the enzymatic activity of iNOS and COX-2 in a dose-dependent fashion.

During inflammation, activated RAW264.7 cells produce a large number of pro-inflammatory factors, which expand inflammatory responses (44). TNF-α and IL-6 are the primary cytokines that mediate inflammation. TNF-α is produced by activated mononuclear macrophages and activated by nuclear factor-κB (NF-κB) and MAPK pathways, which exert regulatory effects. IL-6 is produced by multiple cells, promotes liver synthetic plasma protein and serves a crucial role in acute responses and chronic inflammation. Thus, cytokines and chemokines may be measured to evaluate anti-inflammatory activities. The results demonstrated that dysifragilone A exhibited weakly inhibitory activities on cytokine IL-6 induced by LPS. However, dysifragilone A did not reduce the release of TNF-α stimulated by LPS.

Western blotting was used to determine the possible underlying molecular mechanism of anti-inflammation. Nuclear transcription factor NF-κB serves an important role in the mediating the expression levels of iNOS, COX-2 and pro-inflammatory cytokines (45). NF-κB is a dimer formed by the polypeptide chain p50 and p65 subunits, whereas in the quiescent condition, NF-κB is located in the cytoplasm combined with an inhibitory protein IκB-α. Stimulated by LPS, phosphorylation of IκB-α dissociates from NF-κB and releases p65 with p50, resulting in translocation of activated NF-κB into the nucleus, which then regulates the transcription of target genes, including iNOS, COX-2 and other pro-inflammatory cytokines (46). Further western blotting studies revealed that high concentrations of dysifragilone A downregulated the expression levels of iNOS and COX-2 proteins.

MAPK signaling pathways are known to influence the modulation of the inflammatory response and cellular processes (47). Three MAPK family members have been identified in mammalian cells, including ERK1/2, JNK and p38/MAPK (48). Signal transduction pathways of JNK, ERK1/2 and p38/MAPK and other pathways in cells are regulated by each other, and determine the final biological effect of cells by external stimuli. An indicator of activation of the three pathways is an increase in the expression of phosphorylated protein involved in the pathways. These three parallel MAPK signaling pathways promote the production and release of pro-inflammatory cytokines (49), iNOS and COX-2 expression and development of inflammation stimulated by LPS (50). Therefore, MAPK signaling pathways have a profound involvement in inflammation, and are considered as targets for screening compounds that have anti-inflammatory activity

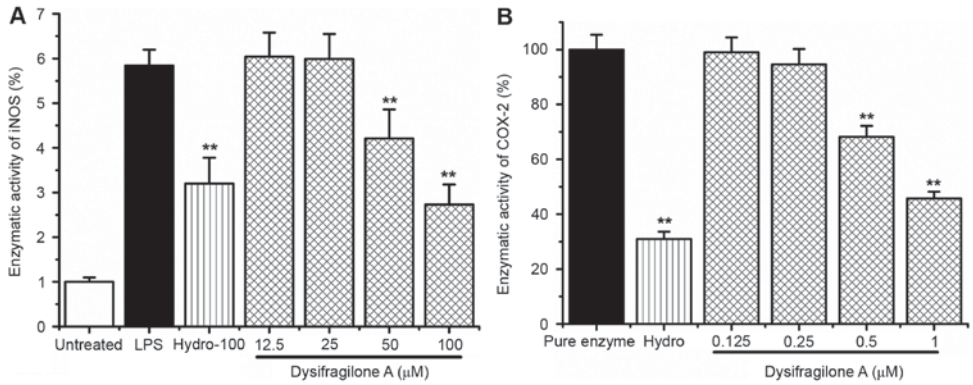

Figure 4. Effect of dysifragilone A on iNOS and COX-2 enzymatic activity. (A) RAW264.7 cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for 24 h. iNOS enzymatic activity was plotted as relative fluorescence units. The experiment was repeated twice. **P<0.01 vs. LPS group. (B) A colorimetric method was used to determine the inhibitory effect of dysifragilone A (0.125, 0.25, 0.5 and 1 mM) together with 1 mM hydrocortisone succinate on COX-2 enzymatic activity. COX-2 enzymatic activity was plotted as relative units compared with the pure enzyme group. The experiment was repeated twice. **P<0.01 vs. pure enzyme group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; Hydro-100, 100 μM hydrocortisone succinate.

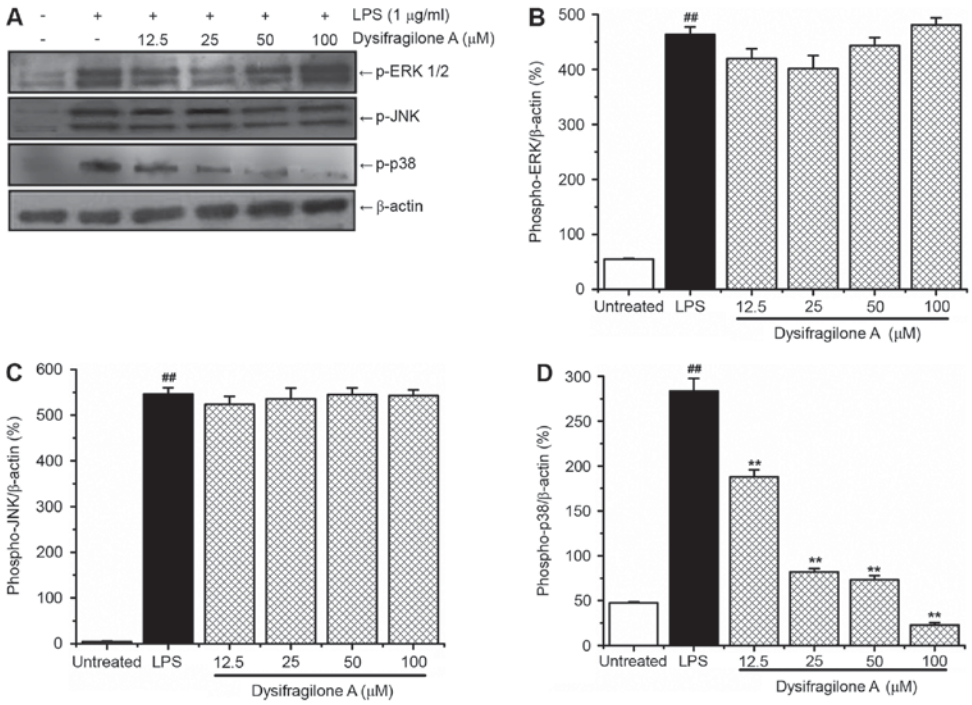

Figure 5. Effect of dysifragilone A on p-ERK 1/2, p-JNK and p-p38 proteins induced by LPS. RAW264.7 cells were treated with LPS (1 μg/ml), treated with various concentrations (12.5, 25, 50 and 100 μM) of dysifragilone A with LPS (1 μg/ml) and hydrocortisone succinate (100 μM) with LPS (1 μg/ml) for different times (15 min for JNK, 1 h for ERK and 10 min for p38), and the expression of (A) p-ERK 1/2, p-JNK and p-p38 proteins determined by western blot analysis. β-actin was internal reference to confirm the equal loading of proteins. Densitometric analysis represents the mean of three independent experiments of (B) p-ERK 1/2 protein, (C) p-JNK protein and (D) p-p38 protein. $^{\#\#}$P<0.01 vs. untreated group; **P<0.01 vs. LPS treatment group. LPS, lipopolysaccharide; p-ERK 1/2, phosphorylated-extracellular signal-regulated kinase 1/2; p-JNK, phosphorylated-c-Jun N-terminal kinase; p-p38, phosphorylated-p38.

at the molecular level. In the present study, dysifragilone A prevented the activation of p38/MAPK signaling pathway in LPS-induced RAW264.7 cells. However, dysifragilone A did not exhibit inhibitory activities on p-ERK 1/2 and p-JNK. Based on the above experimental results, it was speculated that dysifragilone A is likely to selective in the p38/MAPK signaling pathway.

In conclusion, the results of the present study demonstrated that dysifragilone A may inhibit the enzymatic activity of iNOS and COX-2 and the release of NO, PGE_2 and IL-6 in LPS-stimulated RAW264.7 cells. Further investigations demonstrated that dysifragilone A may also downregulate the expression levels of iNOS and COX-2 proteins and block the p38/MAPK signal pathway in RAW264.7 cells induced by LPS. These observations suggested that dysifragilone A was a valuable candidate compound to treat inflammatory diseases. However, animal experiments are essential in revealing the potential of anti-inflammatories in a more comprehensive way, and a limitation of the present study was a lack of experiments *in vivo*. To support the findings of the

present study, samples of dysifragilone A are required for animal experiments.

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References

1. Bell JJ: The functional roles of marine sponges. Estuar Coast Shelf S 79: 341-353, 2008.
2. Braekman JC and Daloze D: Chemical and biological aspects of sponge secondary metabolites. Phytochem Rev 3: 275-283, 2004.
3. Hooper JNA and Van Soest RWM: Systema porifera: A guide to the classification of sponges. New York: Kluwer Plenum: pp1-7, 2002.
4. Gerwick WH and Moore BS: Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. Chem Biol 19: 85-98, 2012.
5. Salomon CE, Williams DH and Faulkner DJ: New azacyclopropene derivatives from Dysidea fragilis collected in Pohnpei. J Nat Prod 58: 1463-1466, 1995.
6. Su JY, Zhong YL, Zeng LM, Wei S, Wang QW, Mak TCW and Zhou ZY: Three new diketopiperazines from a marine sponge Dysidea fragilis. J Nat Prod 56: 637-642, 1993.
7. Fu X, Zeng LM, Su JY and Pais M: A new diketopiperazine derivative from the South China Sea sponge Dysidea fragilis. J Nat Prod 60: 695-696, 1997.
8. Milkova TS, Mikhova BP, Nikolov NM, Popov SS and Andreev SN: Two new polyhydroxylated sterols from the sponge Dysidea fragilis. J Nat Prod 55: 974-978, 1992.
9. Guella G, Guerriero A and Pietra F: Sesquiterpenoids of the sponge Dysidea fragilis of the North-Brittany Sea. Helv Chim Acta 68: 39-48, 1985.
10. Utkina NK, Kazantseva MV and Denisenko V: Brominated diphenyl ethers from the marine sponge Dysidea fragilis. Chem Nat Compd 23: 508-509, 1987.
11. Jiao WH, Huang XJ, Yang JS, Yang F, Piao SJ, Gao H, Li J, Ye WC, Yao XS, Chen WS and Lin HW: Dysidavarones A-D, new sesquiterpene quinones from the marine sponge Dysidea avara. Org Lett 14: 202-205, 2012.
12. Jiao WH, Xu TT, Yu HB, Chen GD, Huang XJ, Yang F, Li YS, Han BN, Liu XY and Lin HW: Dysideanones A-C, unusual sesquiterpene quinones from the South China Sea sponge Dysidea avara. J Nat Prod 77: 346-350, 2014.
13. Jiao WH, Xu TT, Yu HB, Mu FR, Li J, Li YS, Yang F, Han BN and Lin HW: Dysidaminones A-M, cytotoxic and NF-κB inhibitory sesquiterpene aminoquinones from the South China Sea sponge Dysidea fragilis. RSC Adv 4: 9236-9246, 2014.
14. Jiao WH, Xu TT, Zhao F, Gao H, Shi GH, Wang J, Hong LL, Yu HB, Li YS, Yang F and Lin HW: Dysifragilones A-C, unusual sesquiterpene aminoquinones and inhibitors of NO production from the South China sea sponge Dysidea fragilis. Eur J Org Chem 2015: 960-966, 2015.
15. Denizot F and Lang R: Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89: 271-277, 1986.
16. Ishihara T, Kohno K, Ushio S, Iwaki K, Ikeda M and Kurimoto M: Tryptanthrin inhibits nitric oxide and prostaglandin E(2) synthesis by murine macrophages. Eur J Pharmacol 407: 197-204, 2000.
17. Wohlmuth H, Deseo MA, Brushett DJ, Thompson DR, Macfarlane G, Stevenson LM and Leach DN: Diarylheptanoid from Pleuranthodium racemigerum with in vitro prostaglandin E(2) inhibitory and cytotoxic activity. J Nat Prod 73: 743-746, 2010.
18. Zhao F, Xu H, He EQ, Jiang YT and Liu K: Inhibitory effects of sesquiterpenes from Saussurea lappa on the overproduction of nitric oxide and TNF-alpha release in LPS-activated macrophages. J Asian Nat Prod Res 10: 1045-1053, 2008.
19. Zhao F, Gao Z, Jiao W, Chen L, Chen L and Yao X: In vitro anti-inflammatory effects of bata-carboline alkaloids, isolated from Picrasma quassioiders, through inhibition of the iNOS pathway. Planta Med 78: 1906-1911, 2012.
20. Akram M, Kim KA, Kim ES, Shin YJ, Noh D, Kim E, Kim JH, Majid A, Chang SY, Kim JK and Bae ON: Selective inhibition of JAK2/STAT1 signaling and iNOS expression mediates the anti-inflammatory effects of coniferyl aldehyde. Chem Biol Interact 256: 102-110, 2016.
21. Kim YS, Ahn CB and Je JY: Anti-inflammatory action of high molecular weight Mytilus edulis hydrolysates fraction in LPS-induced RAW264.7 macrophage via NF-κB and MAPK pathways. Food Chem 202: 9-14, 2016.
22. Kim SH, Kim J and Sharma RP: Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. Pharmacol Res 49: 433-439, 2004.
23. Kaminska B: MAPK signaling pathways as molecular targets for anti-inflammatory therapy-from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 1754: 253-262, 2005.
24. Rao KM: MAP kinase activation in macrophages. J Leukoc Biol 69: 3-10, 2001.
25. Valledor AF, Comalada M, Santamaría-Babi LF, Lloberas J and Celada A: Macrophage proinflammatory activation and deactivation: A question of balance. Adv Immunol 108: 1-20, 2010.
26. Mantovani A, Allavena P, Sica A and Balkwill F: Cancer-related inflammation. Nature 454: 436-444, 2008.
27. Coussens LM and Werb Z: Inflammation and cancer. Nature 420: 860-867, 2002.
28. Libby P, Ridker PM and Maseri A: Inflammation and atherosclerosis. Circulation 105: 1135-1143, 2002.
29. Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352: 1685-1695, 2005.
30. Ignatova GL, Volchegorskii IA, Volkova EG and Kolesnikov OL: Intensity of lipid peroxidation: Indicator of inflammation severity in chronic bronchitis. B Exp Biol Med 124: 800-801, 1997.
31. Hoogendijk AJ, Roelofs J, van Lieshout M, Blok DC, Poll TVD and Wieland CW: Cyclin-dependent kinase inhibitor r-roscovitine reduces lipoteichoic acid lung inflammation and improves the resolution of antibiotic-treated Streptococcus Pneuumoniae pneumonia. Crit Care 13 (Suppl 4): S39, 2009.
32. Wellen KE and Hotamisligil GS: Inflammation, stress and diabetes. J Clin Invest 115: 1111-1119, 2005.
33. Liu J, Tang J, Zuo Y, Yu Y, Luo P, Yao X, Dong Y, Wang P, Liu L and Zhou H: Stauntoside B inhibits macrophage activation by inhibiting NF-κB and ERK MAPK signaling. Pharmacol Res 111: 303-315, 2016.
34. Butterfield TA, Best TM and Merrick MA: The dual roles of neutrophils and macrophages in inflammation: A critical balance between tissue damage and repair. J Athl Train 41: 457-465, 2006.
35. Fitzpatrick JK and Downer EJ: Toll-like receptor signaling as a cannabinoid target in Multiple Sclerosis. Neuropharmacology 113: 618-626, 2017.
36. Melander RJ, Liu HB, Stephens MD, Bewley CA and Melander C: Marine sponge alkaloids as a source of anti-bacterial adjuvants. Bioorg Med Chem Lett 26: 5863-5866, 2016.
37. Nishimura S, Matsunaga S, Yoshida M, Hirota H, Yokoyama S and Fusetani N: 13-Deoxytedanolide, a marine sponge-derived antitumor macrolide, binds to the 60S large ribosomal subunit. Bioorg Med Chem 13: 449-454, 2005.
38. EI-Hossary EM, Cheng C, Hamed MM, El-Sayed Hamed AN, Ohisen K, Hentschel U and Abdelmohsen UR: Antifungal potential of marine natural products. Eur J Med Chem 126: 631-651, 2017.
39. Mayer AM and Hamann MT: Marine pharmacology in 2000: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. Mar Biotechnol (NY) 6: 37-52, 2004.
40. Laroux FS, Pavlick KP, Hines IN, Kawachi S, Harada H, Bharwani S, Hoffman JM and Grisham MB: Role of nitric oxide in inflammation. Acta Physiol Scand 173: 113-118, 2001.

41. Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP and Versteeg HH: Prostanoids and prostanoid receptors in signal transduction. Int J Biochem Cell Biol 36: 1187-1205, 2004.
42. Kang CH, Han SH, Koo JR and So JS: Chrysanthemum zawadskii var. latilobum extract inhibits the production of nitric oxide and PGE_2 through inducible nitric oxide synthase (iNOS) and cyclooxygenase-2(COX-2) in RAW 264.7 cells. Biotechnol Bioproc Eng 18: 501-506, 2013.
43. Lee JK, Sayer BC, Chun KS, Lao HC, Shipley-phillips JK, Bonner JC and Langenbach R: Multi-walled carbon nanotubes induce COX-2 and iNOS expression via MAP kinase-dependent and -independent mechanisms in mouse RAW264.7 macrophages. Part Fibre Toxicol 9: 14, 2012.
44. Fujiwara N and Kobayashi K: Macrophages in inflammation. Curr Drug Targets Inflamm Allergy 4: 281-286, 2005.
45. Karin M and Lin A: NF-kappaB at the crossroads of life and death. Nat Immunol 3: 221-227, 2002.
46. Ham YM, Ko YJ, Song SM, Kim J, Kim KN, Yun JH, Cho JH, Ahn G and Yoon WJ: Anti-inflammatory effect of litsenolide B2 isolated from Litsea japonica fruit via suppressing NF-κB and MAPK pathways in LPS-induced RAW264.7 cells. J Funct Foods 13: 80-88, 2015.
47. Wu J, Xue X, Wu Z, Zhao HL, Cao HM, Sun DQ, Wang RM, Sun J, Liu Y and Guo RC: Anti-tumor effect of paeonol via regulating NF-κB, AKT and MAPKs activation: A quick review. Biomed Pre Nutr 4: 9-14, 2014.
48. Liu Y, Shepherd EG and Nelin LD: MAPK phosphatases-regulating the immune response. Nat Rev Immunol 7: 202-212, 2007.
49. Liu SJ, Shi Y, Liu C, Zhang M, Zuo ZC, Zeng CJ, Zhou GB, Xian H and Song TZ: The upregulation of pro-inflammatory cytokines in the rabbit uterus under the lipopolysaccaride-induced reversible immunoresponse state. Anim Reprod Sci 176: 70-77, 2017.
50. Arthur JS and Ley SC: Mitogen-activated protein kinases in innate immunity. Nat Rev Immunol 13: 679-692, 2013.