J. Microbiol. Biotechnol. (2014), 24(10), 1346–1353
http://dx.doi.org/10.4014/jmb.1405.05035

jmb

Anti-Inflammatory Activity of Questinol Isolated from Marine-Derived Fungus *Eurotium amstelodami* in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

Xiudong Yang[1,2], Min-Cheol Kang[2], Yong Li[3], Eun-A Kim[2], Sung-Myung Kang[4], and You-Jin Jeon[2,5]*

[1]*Changchun Center of Mass Spectrometry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China*
[2]*Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea*
[3]*College of Pharmacy Science, Changchun University of Traditional Chinese Medicine, Changchun 130017, P. R. China*
[4]*Pediatric Oncology Experimental Therapeutics Investigators Consortium (POETIC) Laboratory for Pre-Clinical and Drug Discovery Studies, University of Calgary, Calgary, AB T3B 6A8, Canada*
[5]*Marine and Environmental Research Institute, Jeju National University, Jeju 695-814, Republic of Korea*

Received: May 16, 2014
Revised: June 25, 2014
Accepted: June 27, 2014

First published online
July 2, 2014

**Corresponding author*
Phone: +82-64-754-3475;
Fax: +82-64-756-3493;
E-mail: youjinj@jejunu.ac.kr

pISSN 1017-7825, eISSN 1738-8872

In the present study, an anthraquinone derivative, questinol was successfully isolated from the broth extract of the marine-derived fungus *Eurotium amstelodami* for the first time. The structure of questinol was determined based on the analysis of the MS and NMR spectral data as well as comparison of those data with the published data. Moreover, the anti-inflammatory effect of questinol in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells was investigated. The results showed that questinol did not exhibit cytotoxicity in LPS-stimulated RAW 264.7 cells up to 200 μM. Questinol could significantly inhibit NO and PGE_2 production at indicated concentrations. Questinol was also found to inhibit the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Furthermore, the western blot analysis showed that questinol suppressed the expression level of iNOS in a dose-dependent manner. However, questinol could slightly inhibit the expression of COX-2 at the concentration of 200 μM. Therefore, our study suggests that questinol might be selected as a promising agent for the prevention and therapy of inflammatory disease.

Keywords: Anti-inflammation, *Eurotium amstelodami*, questinol, NO production, macrophages

Introduction

Inflammation is the result of the host response to pathogenic challenges or tissue injuries, and ultimately leads to the restoration of a normal tissue structure and function. Normal inflammatory responses are self-limited by a process that involves the down-regulation of pro-inflammatory proteins and the up-regulation of anti-inflammatory proteins [10, 12]. However, unbalanced or prolonged inflammation leads to progressive tissue damage and has been implicated in the development of many chronic diseases such as cancer and neurodegenerative disorders as well as diabetes and cardiovascular disease [28]. Lipopolysaccharide (LPS)-stimulated macrophages can generate a variety of inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin 1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and matrix metalloprotease-9 (MMP-9) [8, 23, 27].

Nitric oxide has been shown to be an important regulatory molecule in diverse physiological functions, including vasodilation, neural communication, and host defense [16, 17]. NO produced by activated macrophages *via* iNOS was initially considered a component of innate immunity in the fight against infections. Alternatively, NO is a free oxygen radical ($NO^{\bullet}$) and can act as a cytotoxic agent in pathological processes, particularly in inflammatory

disorders [2, 3]. Prostaglandins (PGs) also function as mediators of the inflammatory response to induce pain, fever, and other symptoms [8]. The rate-limiting enzyme in the synthesis of prostaglandins is cyclooxygenase (COX). Two isoforms of COX have been found: COX-1 and COX-2. COX-2 is induced by several stimuli, and is responsible for the production of large amounts of pro-inflammatory prostaglandins at the inflammatory site [13, 29]. PGE_2 is a major COX product at inflammatory sites, where it contributes to local blood flow increases, edema formation, and pain sensitization [7]. The pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 are known to be important mediators involved in the progress of several inflammatory diseases. Therefore, inhibition of NO, PGE_2, and cytokines (TNF-α, IL-1β, and IL-6) may have potential therapeutic value when related to inflammation.

The macrophage cell line (RAW 264.7) used in experiments has been established as a suitable model to investigate compounds interfering with LPS-inducible inflammatory cascades *in vitro* [22, 24]. Therefore, in the present work, we isolated questinol from the broth extract of a marine-derived fungus, *Eurotium amstelodami*, and evaluated its anti-inflammatory effect in LPS-stimulated RAW 264.7 cells.

Materials and Methods

General Materials

Column chromatographies were carried out on Silica Gel 60 (230–400 mesh; Merck, Germany), ODS (12 nm; YMC, Japan), Sephadex LH-20 (Sigma, St. Louis, MO, USA). Thin-layer chromatography (TLC) was run on pre-coated Merck Kieselgel 60 F254 plates (0.25 mm). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsineEDTA were purchased from Gibco/BRL (Grand Island, NY, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, IL-6, TNF-α, and PGE_2 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Antibodies against iNOS and COX-2 were obtained from Calbiochem (La Jolla, CA, USA) and BD Biosciences Pharmingen (San Jose, CA, USA), respectively. All the solvent and chemicals used in this study were of reagent grade from commercial sources.

Fungal Material

The fungal strain (Culture No. 015-2) was isolated from an unidentified marine animal collected from the Sungsan coast in Jeju Island, Korea, in 2011. The fungus was identified to be *Eurotium amstelodami*, according to a molecular biological protocol by DNA amplification and sequencing of the ITS region. A voucher specimen is deposited at Jeju National University with the code 015-2. The fugal strain was cultured (8 L) for 30 days (static) at 29°C in SWS medium containing soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and Isolation of Questinol

The cultured broth of the *E. amstelodami* strain was filtered through cheese cloth to separate into broth and mycelia. The broth was extracted with ethyl acetate (EtOAc), and the EtOAc solution was concentrated under reduced pressure to give a broth extract (015-2B) (0.7 g). The broth extract (0.7 g) was subjected to silica gel flash chromatography (*n*-hexane/EtOAc, EtOAc/methanol) to furnish 12 fractions (B1−B12) on the basis of TLC analysis. Fraction B7 (161.2 mg) was further purified on a Sephadex LH-20 column eluting with MeOH to give the compound (7.6 mg). The isolated compound was dissolved in 2% DMSO in DMEM.

The ^{1}H NMR and ^{13}C NMR spectra of the isolated compound were recorded on a JEOL JNM-ECP 400 MHz NMR spectrometer, using the DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer.

Questinol: yellow powder; ESI-MS *m/z*: 299 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 13.31 (1H, s, OH-1), 7.57 (1H, d, *J* = 1.6, H-4), 7.20 (1H, d, *J* = 1.6, H-2), 7.20 (1H, d, *J* = 2.3, H-5), 6.83 (1H, d, *J*= 2.3, H-7), 4.58 (2H, s, CH_2OH-3), 3.90 (3H, s, 6-OCH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 186.3 (C-9), 182.3 (C-10), 164.8 (C-1), 163.5 (C-6), 161.7 (C-8), 151.2 (C-3), 136.8 (C-10a), 132.1 (C-4a), 120.9 (C-2), 115.8 (C-4), 115.1 (C-8a), 112.5 (C-9a), 107.2 (C-5), 105.0 (C-7), 62.0 (CH_2OH-3), 56.3 (OCH_3-8).

Cell Culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. The cells were incubated in an atmosphere of 5% CO_2 at 37°C and were subcultured every 3 days.

MTT Assay

The RAW 264.7 cells were seeded in 96-well plates at a concentration of 1×10^5 cells/ml (180 μl). After 16 h incubation at 37°C under a humidified atmosphere, the cells were treated with 10 μl of the compound at the concentration of 50, 100, and 200 μM and LPS (1 μg/ml) , and further incubated for 30 min. Then 50 μl of MTT stock solution (2 mg/ml) was applied to the wells, to a total reaction volume of 250 μl. After 4 h of incubation, the plates were centrifuged for 5 min at 800 ×*g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured *via* ELISA at a wavelength of 540 nm. The percentage inhibitory effect was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density

of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as the mean percentages of viable cells versus the respective control.

Determination of Nitric Oxide Production

RAW 264.7 cells (1×10^5 cells/ml) were plated and incubated with the compound in the absence or presence of LPS (1 μg/ml) for 24 h. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. One hundred microliters of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Ultraspec 2100 pro) at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

Determination of PGE_2 Production

RAW 264.7 macrophages were plated on 24-well plates at a density of 1×10^5cells/ml. The cells were pre-treated for 1 h with the compound at the concentrations of 50, 100, or 200 μM prior to 24 h of stimulation with LPS (1 μg/ml). The culture supernatants were immediately utilized for PGE_2 determination. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit according to the manufacturer's instructions. The production of PGE_2 was measured relative to that following control treatment.

Measurement of Pro-Inflammatory Cytokines (TNF-α, IL-1β, and IL-6) Production

To determine the effects of the compound on production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, the RAW 264.7 macrophages were incubated with the compound (50, 100, or 200 μM) in the presence or absence of LPS (1 μg/ml) for 24 h. The inhibitory effects of the compound on the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) production from LPS-treated RAW 264.7 cells was determined as described in the Cho *et al*. [5] protocols. Supernatants were used for pro-inflammatory cytokines assay using a mouse ELISA kit.

Western Blot Analysis

Murine macrophage RAW 264.7 cells were pre-incubated for 24 h, and then stimulated with LPS (1 μg/ml) in the presence of the test compound for the indicated time. After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) and kept on ice for 30 min. Cell lysates were washed by centrifugation, and protein concentrations were determined by using a BCA protein assay kit. Aliquots of the lysates (30–50 μg of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, CA, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), and 20% MeOH (v/v)). After blocking the nonspecific site with 1% bovine serum albumin, the membrane was then incubated with specific primary antibody at 4°C for overnight. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5,000; Vector Laboratories, Burlingame, USA) at room temperature. The immune-active proteins were detected using an enhanced chemiluminescence western blotting detection kit.

Statistical Analysis

All the data were presented as the mean ± SD from three independent experiments unless stated otherwise. Statistical comparisons between different treatments were done by one-way ANOVA with Student Newman Keul's *post hoc* tests using the SPSS program (ver. 19.0). $*p < 0.05$ and $**p < 0.01$ *vs*. LPS-stimulated group.

Results and Discussion

Isolation and Identification of Bioactive Natural Product from *E. amstelodami*

Based on the results of the bioactivities of marine-derived fungi, the strain *Eurotium amstelodami* has been selected for isolation of the bioactive compounds, especially the broth extract. Therefore, 8 L of *E. amstelodami* was cultured in SWS medium for 30 days (static) at 29°C. The broth was extracted with EtOAc to obtain the B extract. Then, the B extract was purified using silica column chromatography followed by Sephadex LH-20 chromatography. The compound was successfully isolated. The structure of the isolated compound was determined by analysis of the MS and NMR spectral data as well as comparison of those data with previous reports.

Questinol was isolated as a yellow powder, and its molecular weight was determined as *m/z* 299 $[M-H]^-$, which corresponded with $C_{16}H_{12}O_6$. The ^1H and ^{13}C NMR signals

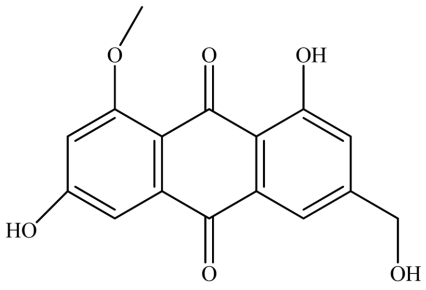

Fig. 1. Chemical structure of questinol isolated from marine-derived fungus *Eurotium amstelodami*.

indicated it might be an anthraquinone compound. In comparison of the NMR data with those of questinol in the literature [11, 14], it was determined as questinol, as shown in Fig. 1. This compound was first isolated from the marine-derived fungus *E. amstelodami*, according to our best knowledge.

Cell Viability in Raw 264.7 Cells

To investigate the cytotoxic effect of questinol in RAW 264.7 cells, MTT assays were employed. As shown in Fig. 2, treatment of LPS (1 μg/ml) alone showed cytotoxic effect to RAW 264.7 macrophages, whereas there ware no significant differences between the LPS-treated group and control group. When the cells were treated with 50, 100, and 200 μM of questinol in the presence of LPS, no significant differences of cell viability were found between these groups and the control group, respectively. The results indicated that up to the concentration of 200 μM, questinol did not affect the cell viability in RAW 264.7 cells. Thus, the concentrations of 50, 100, and 200 μM of questinol will be used in further experiments.

Effects of Questinol on NO Production in LPS-Treated RAW 264.7 Cells

To determine the inhibitory effect of questinol on NO production, RAW 264.7 cells were incubated with LPS (1 μg/ml) for 24 h. The production of NO in the culture medium was evaluated by measurement of the release of nitrite, which is a stable metabolite of NO. As shown in Fig. 3, LPS treatment could significantly elevate the production of NO. The concentration of NO in the LPS treatment group was defined as 100% of NO production. Pre-treatment with questinol could significantly inhibit LPS-induced NO production in a concentration-dependent manner, respectively. The NO production of questinol-treated groups was 80.9%, 42.1%, and 23.0% at the concentrations of 50, 100, and 200 μM, respectively. Taking together the results of cell viability and NO production inhibitory effects, the inhibitory effect of questinol on NO production was not caused by cytotoxic effects in RAW 264.7 cells.

Effect of Questinol on PGE_2 Production in LPS-Treated RAW 264.7 Cells

To determine the potential effect of questinol on the inhibition of PGE_2 production, the amount of released PGE_2 was measured using anti-PGE_2-coated ELISA plates. Conditioned media were prepared by treating the RAW 264.7 cells with questinol (50, 100, and 200 μM) for 1 h followed by 24 h of stimulation with LPS (1 μg/ml). As shown in Fig. 4, the PGE_2 production was significantly increased by the treatment of LPS. However, pre-treatment with questinol could significantly inhibit LPS-induced PGE_2 production in a concentration-dependent manner. The PGE_2 production of questinol-treated groups was

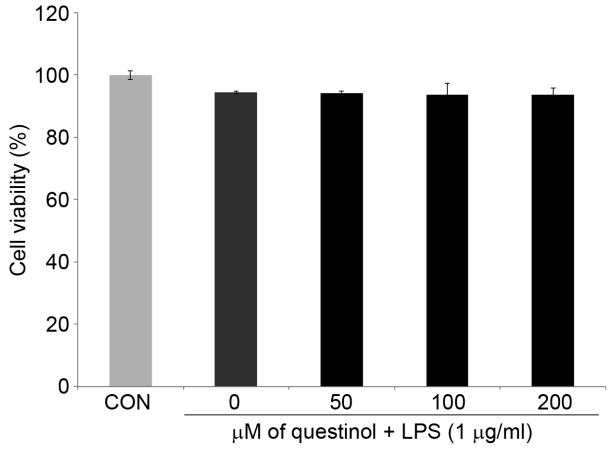

Fig. 2. Effect of questinol on cell viability in RAW 264.7 cells.
Cells were cultured with different concentrations (50, 100, and 200 μM) of questinol and LPS (1 μg/ml) for 24 h. Then, the viability of cells was determined by MTT assay. Values are the mean ± SD of triplicate experiments.

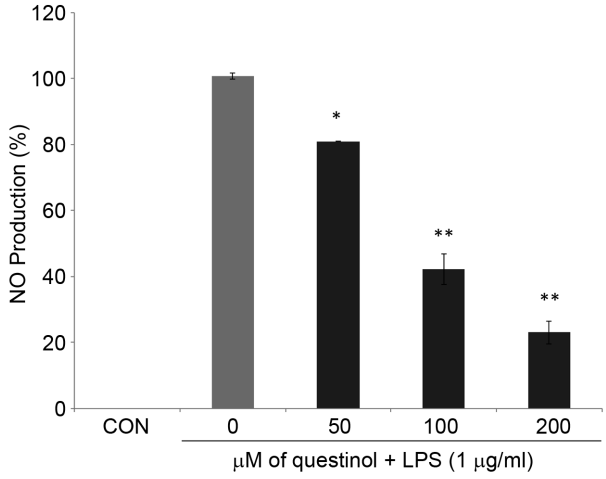

Fig. 3. Inhibitory effect of questinol on NO production in LPS-stimulated RAW 264.7 cells.
Cells (1×10^5 cells/ml) were stimulated by LPS (1 μg/ml) for 24 h in the presence of questinol (50, 100, and 200 μM). The NO production was assayed in the culture medium. Values are the mean ± SD of triplicate experiments. $*p < 0.05$ and $**p < 0.01$ indicate significant differences from the LPS-stimulated group.

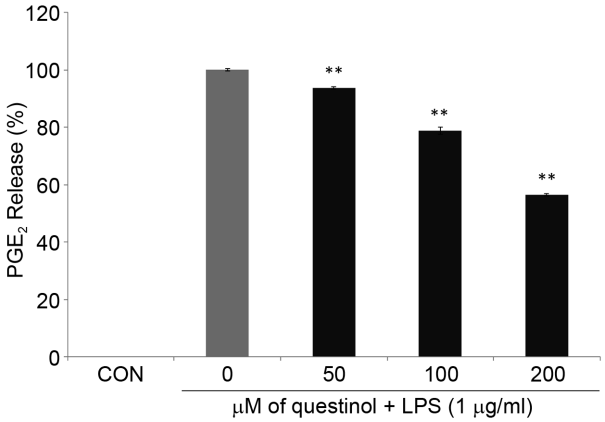

Fig. 4. Inhibitory effect of questinol on PGE_2 production in LPS-stimulated RAW 264.7 cells.

Cells (1×10^5 cells/ml) were stimulated by LPS (1 μg/ml) for 24 h in the presence of questinol (50, 100, and 200 μM). Supernatants were collected, and the PGE_2 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. $^{**}p < 0.01$ indicates significant difference from the LPS-stimulated group.

93.8%, 78.9%, and 56.5% at the concentrations of 50, 100, and 200 μM, respectively.

Effects of Questinol on Production of Pro-Inflammatory Cytokines in LPS-Stimulated RAW 264.7 Cells

The release of pro-inflammatory cytokines is an important mechanism by which the immune cells regulate the inflammatory responses and contribute to various inflammatory and autoimmune disorders. Therefore, we examined the effects of questinol on LPS-induced TNF-α, IL-1β, and IL-6 production using an ELISA kit. LPS could induce a significant increase of cytokines TNF-α (Fig. 5A), IL-1β (Fig. 5B), and IL-6 (Fig. 5C) compared with the control group. However, pre-treatment with questinol was found to reduce the TNF-α level. At the concentrations of 50, 100, and 200 μM, the levels of TNF-α were significantly reduced compared with the LPS-treated group.

The results of IL-1β production inhibitory effects by questinol are shown in Fig. 5B. The questinol could significantly reduce the production of IL-1β at the concentrations of 100 and 200 μM. However, no significant difference was found between LPS-treated and 50 μM questinol-treated RAW 264.7 cells

Similar to the results of IL-1β production inhibitory effects by questinol, pre-treatment with questinol at the concentrations of 100 and 200 μM could significantly inhibit the production of IL-6, whereas at 50 μM, questinol could not significantly inhibit the production of IL-6.

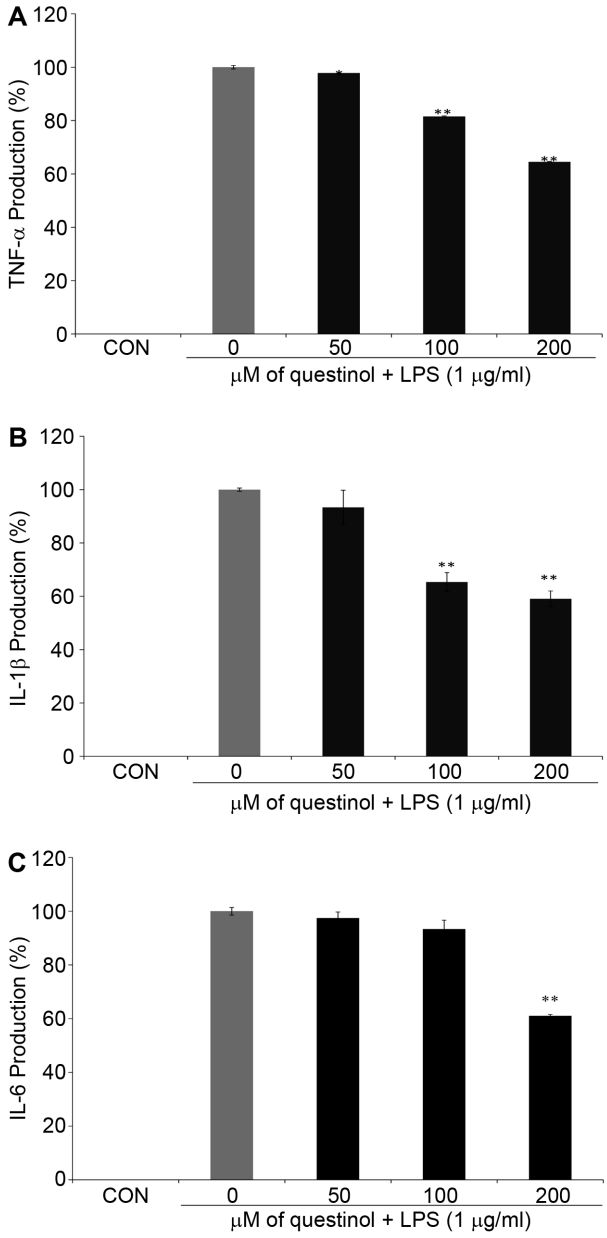

Fig. 5. Inhibitory effect of questinol on the production of TNF-α (**A**), IL-1β (**B**), and IL-6 (**C**) in LPS-stimulated RAW 264.7 cells.

Values are the mean ± SD of triplicate experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from the LPS-stimulated group.

Effect of Questinol on Expressions of iNOS and COX-2 in LPS-Stimulated RAW 264.7 Cells

To determine the mechanism by which the compound reduces LPS-induced NO and PGE_2 production, we investigated the effect of questinol on the expressions of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. As shown in Fig. 6, treatment of LPS (1 μg/ml) could

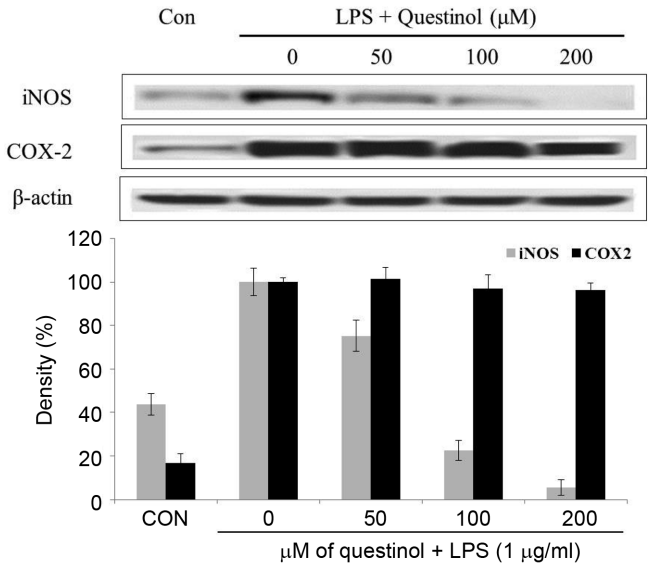

Fig. 6. Inhibitory effect of questinol on the expressions of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells.
The RAW 264.7 cells were pre-incubated for 18 h, and then stimulated with LPS (1 μg/ml) for 24 h in the presence of questinol (50, 100, and 200 μM). The expression levels of iNOS and COX-2 were determined using the immunoblotting method.

significantly increase the expression levels of iNOS and COX-2 compared with the control without LPS and questinol in RAW 264.7 cells. However, pre-treatment with questinol could inhibit the expression of iNOS in a dose-dependent manner. On the other hand, the expression of COX-2 was also found to decrease by treatment of questinol at the concentration of 200 μM (Fig. 6).

Discussion

Marine microorganisms, particularly marine-derived fungi, are fertile producers of new structurally interesting compounds, and are recognized as an important source of structurally novel and bioactive secondary metabolites for drug discovery. In this study, we have isolated questinol from a marine-derived fungus, *Eurotium amstelodami*, for the first time according to the activity-guided isolation method. Questinol was found to inhibit LPS-induced NO, PGE_2, and pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 production in RAW 264.7 cells.

NO plays a vital role in regulation of the vascular and immune system. It has been proven to be an important signaling molecule involved in regulating a wide range of biological activities in vascular, neural, and immune systems [18]. However, overproduction of NO from inflammatory cells is found to cause the pathophysiology in a variety of diseases, carcinogenesis, and inflammation. NO is formed from L-arginine by NO synthase (NOS), which is generated by many cell types [4]. Three distinct isoforms of NOS have been identified, including constitutive endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [20]. In the macrophages, iNOS is significantly induced by LPS stimulation. PGE_2 is considered one of the strongest inflammatory mediators in the inflammatory response. It is transformed from arachidonic acid *via* the COX-2 catalytic reaction. COX-2 enzymatic activity catalyzes the first committed step in prostaglandin synthesis [21]. Inhibition of iNOS, the enzyme mediating macrophage NO production, has been shown to block prostaglandin release in RAW 264.7 macrophages [1]. COX-2 also could be affected directly at its enzymatic activity by NO and iNOS [26]. In our study, questinol was found to significantly inhibit the LPS-induced NO and PGE_2 production in a concentration-dependent manner in RAW 264.7 cells. Accordingly, the levels of iNOS were also significantly suppressed by questinol at the concentration of 100 and 200 μM. However, questinol exhibited weak effect on the down-regulation of COX-2 at the concentration of 200 μM. Furthermore, the inhibitory effects of questinol on the LPS-induced NO and PGE_2 production, and iNOS and COX-2 expression were not due to the cytotoxicity, according to the results of cell viability in RAW 264.7 macrophages.

Activated macrophages secrete a number of different inflammatory mediators, including TNF-α, IL-1β, and IL-6. The overproduction of these mediators has been implicated in several inflammatory diseases and cancer [15]. TNF-α is an important pro-inflammatory cytokine and, like NO, is involved in normal physiological immune and inflammatory processes. However, when inappropriately expressed, TNF-α also plays a role in the development of chronic inflammation and associated diseases [19, 25]. IL-1β and IL-6 are also considered to be pivotal pro-inflammatory cytokines; for example, IL-1β is believed to play an important role in the pathophysiology of rheumatoid arthritis [6, 9], and IL-6 is regarded an endogenous mediator of LPS-induced fever [10]. Our finding indicated that treatment of questinol significantly inhibited the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the LPS-stimulated RAW 264.7 macrophages.

In conclusion, we have demonstrated that questinol isolated from the marine-derived fungus *E. amstelodami* inhibited the production of NO and PGE_2 through suppression of iNOS and COX-2 expresion in LPS-stimulated RAW 264.7 macrophages. The compound questinol was found to

inhibit the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 as well. Therefore, our study suggests that this compound might be selected as a promising agent for the prevention and therapy of inflammatory diseases.

References

1. Ahmed N, Chen LC, Gordon MA, Laskin JD, Laskin DL. 2002. Regulation of cyclooxygenase-2 by nitric oxide in activated hepatic macrophages during acute endotoxemia. *J. Leukoc. Biol*. **71:** 1005-1011.
2. Alderton WK, Cooper CE, Knowles RG. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J*. **357:** 593-615.
3. Bogdan C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* **2:** 907-916.
4. Chang YC, Li PC, Chen BC, Chang MS, Wang JL, Chiu WT, Lin CH. 2006. Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E_2, protein kinase A, p38 MAPK, and nuclear factor-κB pathways. *Cell Signal.* **18:** 1235-1243.
5. Cho JY, Baik KU, Jung JH, Park MH. 2000. *In vitro* anti-inflammatory effects of cynaropicrin, a sesquinterpene lactone, from *Saussurea lappa*. *Eur. J. Pharmacol.* **398:** 399-407.
6. Dayer JM. 2003. The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheumatology* (Oxford) **42(Suppl.2):** ii3-ii10.
7. Giuliano F, Warner TD. 2002. Origins of prostaglandin E2: involvements of cyclooxygenase (COX)-1 and COX-2 in human and rat systems. *J. Pharmacol. Exp. Ther*. **303:** 1001-1006.
8. Jin M, Suh SJ, Yang JH, Lu Y, Kim SJ, Kwon S, *et al*. 2010. Anti-inflammatory activity of bark of *Dioscorea batatas* DECNE through the inhibition of iNOS and COX-2 expressions in RAW264.7 cells *via* NF-κB and ERK1/2 inactivation. *Food Chem. Toxicol*. **48:** 3073-3079.
9. Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, *et al*. 2008. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW264.7 macrophages *via* the p38/ERK and NF-κB pathways. *Int. J. Biochem. Cell B* **40:** 2572-2582.
10. Kim JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT. 2008. Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression *via* the attenuation of NF-κB in RAW 264.7 macrophages. *Eur. J. Pharmacol.* **584:** 175-184.
11. Kimura Y, Kozawa M, Baba K, Hata K. 1983. New constituents of roots of *Polygonum cuspidatum*. *Planta Med*. **48:** 164-169.
12. Lawrence T, Willoughby DA, Gilroy DW. 2002. Anti-inflammatory lipid mediators and insights into resolution of inflammation. *Nat. Rev. Immunol.* **2:** 787-795.
13. Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, *et al.* 1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem*. **267:** 25934-25938.
14. Li JB, Lin M. 1993. Chemical composition of *Polygonum multiflorum*. *Zhong Cao Yao* (China) **24:** 115-118.
15. Lin WW, Karin MA. 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J. Clin. Invest*. **117:** 1175-1183.
16. MacMicking J, Xie QW, Nathan C. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15:** 323-330.
17. Mitchell JA, Larkin S, Williams TJ. 1995. Cyclooxygenase 2: regulation and relevance in inflammation. *Biochem. Pharmacol*. **50:** 1535-1542.
18. Moncada S, Palmer RM, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev*. **43:** 109.
19. Moro C, Palacios I, Lozano M, D'Arrigo M, Guillamón E, Villares A, *et al*. 2012. Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. *Food Chem*. **130:** 350-355.
20. Reddy MVB, Hwang TL, Leu YL, Chiou WF, Wu TS. 2011. Inhibitory effects of Mannich bases of heterocyclic chalcones on NO production by activated RAW 264.7 macrophages and superoxide anion generation and elastase release by activated human neutrophils. *Bioorg. Med. Chem*. **19:** 2751-2756.
21. Savonenko A, Munoz P, Melnikova T, Wang Q, Liang X, Breyer RM, *et al*. 2009. Impaired cognition, sensorimotor gating, and hippocampal long-term depression in mice lacking the prostaglandin E2 EP2 receptor. *Exp. Neurol*. **217:** 63-73.
22. Seo WG, Pae HO, Oh GS, Kim NY, Kwon TO, Shin MK, *et al*. 2001. The aqueous extract of *Rhodiola sachalinensis* root enhances the expression of inducible nitric oxide synthase gene in RAW264.7 macrophages. *J. Ethnopharmacol.* **76:** 119-123.
23. Shin KM, Kim YH, Park WS, Kang I, Ha J, Choi JW, *et al*. 2004. Inhibition of methanol extract from the fruits of *Kochia scoparia* on lipopolysaccharide-induced nitric oxide, prostaglandin E2, and tumor necrosis factor-alpha production from murine macrophage RAW 264.7 cells. *Biol. Pharm. Bull.* **27:** 538-543.
24. Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, *et al*. 1998. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res*. **58:** 717-723.
25. Szekanecz Z. 2008. Pro-inflammatory cytokines in atherosclerosis. *Israel Med. Assoc. J*. **10:** 529-530.
26. Wang QS, Cui YL, Dong TJ, Zhang XF, Lin KM. 2012. Ethanol extract from a Chinese herbal formula, "Zuojin Pill", inhibits the expression of inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 mouse macrophages. *J. Ethnopharmacol.* **141:** 377-385.
27. Woo CH, Lim JH, Kim JH. 2004. Lipopolysaccharide induces

matrix metalloproteinase-9 expression *via* a mitochondrial reactive oxygen species p38 kinase-activator protein-1 pathway in Raw 264.7 cells. *J. Immunol*. **173:** 6973-6980.

28. Zhong Y, Chiou YS, Pan MH, Shahidi F. 2012. Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages. *Food Chem*. **134:** 742-748.
29. Zhou HY, Shin EM, Guo LY, Youn UY, Bae KH, Kang SS, *et al*. 2008. Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages *via* NF-kB, JNK and p38 MAPK inactivation. *Eur. J. Pharmacol*. **586:** 340-349.