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Antiinflammatory Effects of New Chemical Compounds, HS-1580 Series (HS-1580, HS-1581, HS-1582)

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HS-1580 series (HS-1580, HS-1581, HS-1582) can produce anti-imflammatory effects were synthesized from the marine algae extraction in 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether (TDB). Raw 264.7 cells were pre-treated with 1 μg/μl lipopolysaccharide (LPS) and later treated with HS-1580 series. These cells of inflammatory mediators were tested as well. Nitric oxide (NO) is related to auto-immune disease and is produced by inducible NOS (iNOS). When treated with HS-1580 series, the product of NO will reduce in a dose-dependent manner. HS-1580 series significantly inhibit the iNOS protein expression. Cyclooxygenase (COX) involves with the various physiologic events and catalyzes in prostaglandin. HS-1580 series also inhibit the COX-2 protein expression as well as pro-inflammatory cytokines production such as tumor necrosis factor-α (TNF-α) and interluekin-1β (IL-1β). These up-coming results suggest that HS-1580 series have anti-inflammatory effects in Raw 264.7 cells by in-hibiting such as iNOS, COX-2, TNF-α and IL-1β as inflammatory mediators.

Key words – Raw 264.7, NO, COX-2, inflammation, HS-1580, HS-1581, HS-1582, TNF-α

Introduction

Macrophages exhibit a particularly vigorous response to lipopolysaccharide (LPS) and its activation by LPS which enhances the production and release of inflammatory mediators that are involves with inducible NOS (iNOS), cyclo-oxygenase-2 (COX-2) and tumor necrosis factor-α (TNF-α), interluekin-1β (IL-1β) as pro-inflammatory cytokines[7].

Nitric oxide (NO) is an inorganic free radical[9,24] substances that synthesized from amino acid L-arginine by nitric oxide synthases (inducible NOS, endothelial NOS, neuronal NOS)[12,21,25,31]. iNOS isoform is usually induced in the large quantities in response to inflammatory stimuli such as LPS and interferon-γ (IFN-γ)[5,23,32]. NO can produced from the iNOS pathway which can act as a cytotoxic agent on invading microorganisms and tumor cells and endotoxemia. However, excessive of NO levels are related to septic shock, rheumatoid arthritis and autoimmune diseases[32].

Cyclooxygenase (COX) generates prostaglandins (PGs) from arachidonic acid (AA)[8]. COX isoforms, COX-1, COX-2, COX-3, are distinguished by a pattern of expression of various types of cells and different physiological functions respectively[3,28]. The over expression of COX-2 leads to excess prostaglandin E_2 (PGE_2), which plays an important role in many pathophysiologic states such as inflammation, cancer, angiogenesis, Alzheimer's disease and several different forms of arthritis.

Pro-inflammatory cytokines, TNF-α and IL-1β, have been suggested to induce cell damage, and have been considered to be an important initiator of the inflammatory response initiator[11]. TNF-α is the one of the most important pro-inflammatory cytokines[30] and induces the secretion of cytokines as well as various biological responses[1,10]. IL-1β is produced by lymphocytes, epithelial cells, keratinocytes, mesenchymal cells, macrophages, and monocytes. Therefore, IL-1β is the best known in mediating the acute phase of inflammatory responses.

In previous study, we have extracted 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether (TDB) from marine algae, *symphyocladia latiuscula*[13,16]. Since TDB has shown $ONOO^-$ scavenging and cytoprotective activity in living cells[4,19], TDB analogs, HS-1580 series (HS-1580, HS-1581, HS-1582) were synthesized. To determine the effects of HS-1580 series on anti-inflammation, iNOS and COX-2 protein expression and pro-inflammatory cytokines production, TNF-α and IL-1 in LPS-treated Raw 264.7 macrophages can be evaluated.

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Materials and Methods

Materials

Raw 264.7 macrophages were purchased from Korean Cell Line Bank. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco-BRL-Life Technology. HS-1580 series (Figure 1) were supplied by prof. Dr. Suh's Lab (Department of Chemistry, Pusan National University). The enzyme-immunoassay (EIA) kit for PGE_2, TNF-α, and IL-1β were obtained from Amersham Bioscience and Biosource. The antibodies of iNOS, COX were purchased from Transduction Labolatories, and second antibody was purchased from Santa-Cruz.

Syntheses of HS-1580 series as TDB derivatives

Synthesis of HS-1580

Five g piperonyl alcohol and 21 g aluminium chloride ($AlCl_3$) in 80 ml of dichloromethane (CH_2Cl_2) was added 148 ml propanethiol at 0℃ for 20 min. The mixture was stirred at room temperature for 5 hr and diluted with 100 ml CH_2Cl_2 then adjusted with ammonium chloride (NH_4Cl) until pH2. The mixture layer was washed with CH_2Cl_2 solution, dried with anhydrous magnesium sulfate ($MgSO_4$). The resulting residue was purified by flash chromatography to give compound HS-1580.

Synthesis of HS-1581

By above described procedure for the preparation of HS-1580, 5 g piperonyl alcohol and 21 g aluminium chloride ($AlCl_3$) in 80 ml of dichloromethane (CH_2Cl_2), which was added 152 ml *iso*-propanethiol, gave desired product HS-1581.

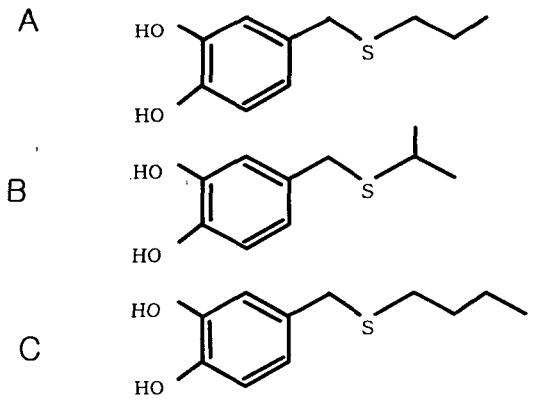

Fig. 1. The chemical structures A. HS-1580, B. HS-1581, C. HS-1582

Synthesis of HS-1582

According to the procedure for the preparation of HS-1580, 17 g piperonyl alcohol and 74 g aluminium chloride ($AlCl_3$) in 150 ml of dichloromethane (CH_2Cl_2), which was added 556 ml 1-butanethiol, for synthesis of HS-1582.

Cell culture and treatment

Raw 264.7 macrophages were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37℃ in 5% CO_2 incubator (Forma Scientific Inc., USA). These cells were incubated with HS-1580 series, which was dissolved in 100 % ethanol. 1 μg/ml LPS was pre-incubated.

Cell viability assay

The cell viability was determined by 3-[4,5-dimetylthiazoly]-2,5-diphenyltetrazolium bromide (Sigma) MTT method. The cells were plated at 1×10^5 cells/well in 96 well plates. After 24 h incubation, the cells were treated with LPS 1 h after HS-1580 series, NG-monomethyl-L-arginine (NMMA), cycloheximide (CHX) or antinomycin D (ACD) treatments. After 24 h incubation, 10 μl of MTT stock solution (5 mg/ml PBS) was added into the cells and the cells were incubated at 37℃, 5% CO_2 for 4 h. The culture supernatant was removed and converted into formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cells, that were dissolved in 150 μℓ DMSO : EtOH (1:1). A microplate reader determined absorbance at 570 nm. The assay was performed in triplicate.

Measurement of NO production

Nitrite as the end product of NO was measured by Griess reagent. The cells were seeded at 1×10^5 cells/well in 96 well plates. These cells were treated with LPS for 24 h and HS-1580 series, NMMA, CHX or ACD that were added 1 h before LPS treatment. They were placed into growth medium lacking Phenol Red. 50 μl of the culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid) and incubated at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 550 nm in 96 well plates. The standard calibration curve was prepared by using sodium nitrite as a standard.

Measurement of cytokine secretion

Cytokines were measured by an enzyme-immunoassay

(EIA kit, Biosource, International Inc. CA, USA). The cells were seeded at 1 × 10^6 cells/well in 96 well plates. After 24 h incubation, the cells were treated in growth medium (DMEM with 10% FBS and 1% antibiotics) and treated by HS-1580 series 1 h prior to LPS treatment in a volume of 100 μl/well. After 24 h incubation, the culture supernatant was obtained and stored at -70℃ until measurement.

Western blot analysis

For protein analysis, the cells plated in 10-cm-diameter dishes were washed with the phosphate-buffered saline (2.6 mM KH_2PO_4, 4.1 mM $NaHPO_4$, 135 mM NaCl, pH 7.4) and then scraped in lysis buffer (100 mM NaCl, 10 mM Hepes (pH 7.4), 0.1% Nonidet P40, 20 μg/ml Aprotinin, 10 μg/ml Leupeptin) to set on ice for 30 min. Whenever lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4 ℃ the supernatant was obtained. The protein contents of lysates were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). 30 μg/ml protein was mixed with an equal volume of electrophoresis buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating up, protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane (Amersham Corp.). After transferring, equal loading was confirmed by Ponceau S staining. The membranes were blocked for 1 h at room temperature with blocking reagent (5% non-fat milk, 0.05% Tween 20 in TNE buffer, pH 7.5). The membranes were incubated with primary antibody. After three washes, the membranes were incubated for 1 h with the second antibody and were diluted in the above blocking reagent. After the last three washes, the membranes were treated with chemiluminescence reagent (ECL, Amersharm Corp.). All procedures must be completed at room temperature.

Statistics

All experiments were repeated at least three times. Statistical analysis was performed using a Student's t-test in Minitab. A "p" value which is less than 0.05 was considered as significant.

Results

Effects of HS-1580 series on cell viability and LPS-induced NO production

HS-1580 series had little or no effect on cell viability at concentration of 50 μM and determined by MTT assay (Fig. 2). However cell viability treatment with NMMA, NOS inhibitor[18], CHX, translation inhibitor and ACD, transcriptioninhibitor, were significantly reduced. To elucidate effects of HS-1580 series on LPS-induced NO production, the cells were stimulated with LPS for 24 h and HS-1580 series. NMMA, CHX or ACD were added 1 h before LPS treatment. As shown figure 2, the cells treated with LPS showed concentration of 13.9 μM NO, that is approximately 4.6-fold increase over to control group. When treating with HS-1580 series and LPS, the cells can produced less concentration of NO according to the dose-dependent exposure. Treatment with 25 or 50 μM HS-1580 series and LPS was sufficient and efficient to show inhibition effects. Particularly, within the HS-1580 series, 50 μM HS-1582 exhibited the strongest inhibitory effects on NO production. NMMA, CHX, and ACD suppressed

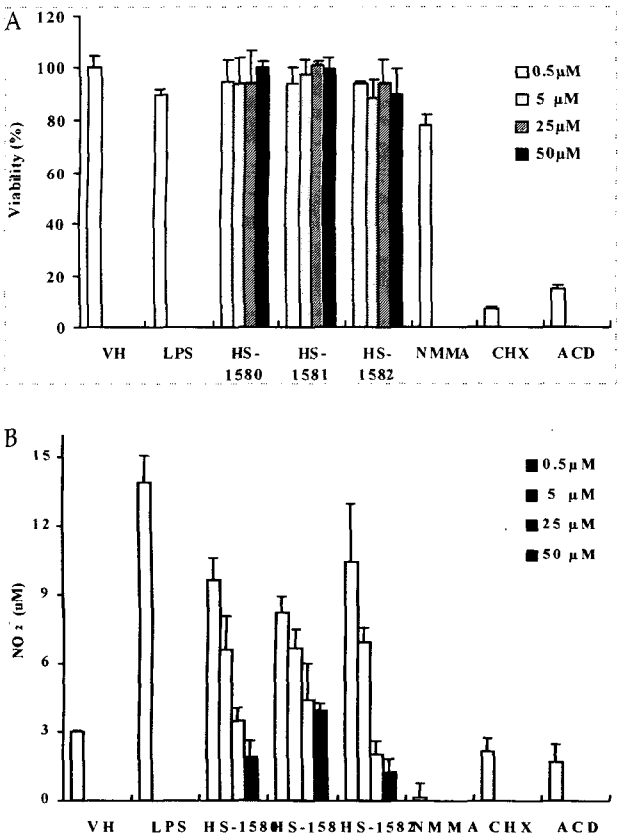

Fig. 2. Effects of HS-1580 series on cell viability and LPS-induced NO accumulation. Raw 264.7 macrophage were stimulated with LPS (1 μg/mℓ) for 24 h and HS-1580 series (0.1, 1, 5, 10 μg/mℓ), NMMA (1 mM), CHX (2 μg/mℓ) or ACD (1 μg/mℓ) was added 1 h before the stimulus of LPS. A. Cell viability, B. Nitrite was determined in the culture supernatant

LPS-induced NO production and the HS-1580 series treatment showed more inhibitory potent effects than CHX or ACD treatment.

The effects of HS-1580 series on LPS-induced iNOS and COX-2 expression

Western blot was performed in the manner of decreasing NO production that could be correlated with iNOS expression (Fig. 3). Treatment with LPS caused a significant increase of iNOS protein expression and HS-1580 series at concentration of 50 μM significantly inhibited LPS-induced iNOS protein expression. In response to LPS, COX-2 protein expression level was remarkably augmented, and HS-1580 series at concetration of 50 μM significantly inhibited LPS-induced COX-2 protein expression (Figure 3).

The effects of HS-1580 series on LPS-induced IL-1β, TNF-α, and IL-10 production by HS-1580 series

To distinguish whether HS-1580 series could inhibit LPS-induced IL-1β and IL-10 production, the cells were incubated with HS-1580 series for 1 h and treated with LPS for 24 h. As shown figure 4, treatment with LPS increased IL-1β, that is approximately 3-fold compared to the control group and the HS-1580 series treatment inhibited of IL-1β production in a dose-dependent manner. Within the HS-1580 series, 50 μM HS-1581 exhibited the strongest inhibitory effects on IL-1β production. Treatment with LPS showed 24832 pg/ml of TNF-α, an increase of approximately 7-fold compared to the control group and treatment with HS-1580 series caused a dose-dependent inhibition of TNF-α production. HS-1581 exhibited the strongest inhibitory effects on TNF-α production within the HS-1580 series.

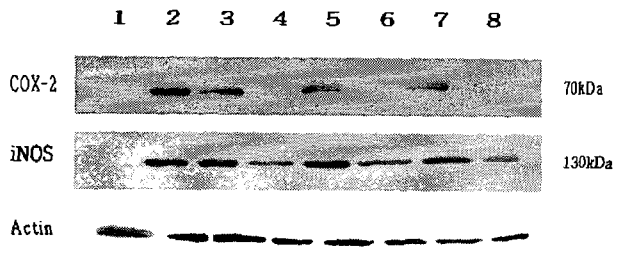

Fig. 3. Effects of HS-1580, HS-1581, HS-1582 on COX-2 and iNOS expression in Raw 264.7 macrophages The cells were treated with each compound (1 or 10 μg/mℓ) for 24 h with LPS (1 μg/mℓ). 1, untreated control; 2, LPS (1 μg/mℓ); 3, LPS+HS1580(1 μg/mℓ); 4, LPS+HS1581(1 μg/mℓ); 5, LPS+HS1582(1 μg/mℓ); 6, LPS+HS1580(10 μg/mℓ); 7, LPS+HS1581(10 μg/mℓ); 8, LPS+HS1582(10 μg/mℓ)

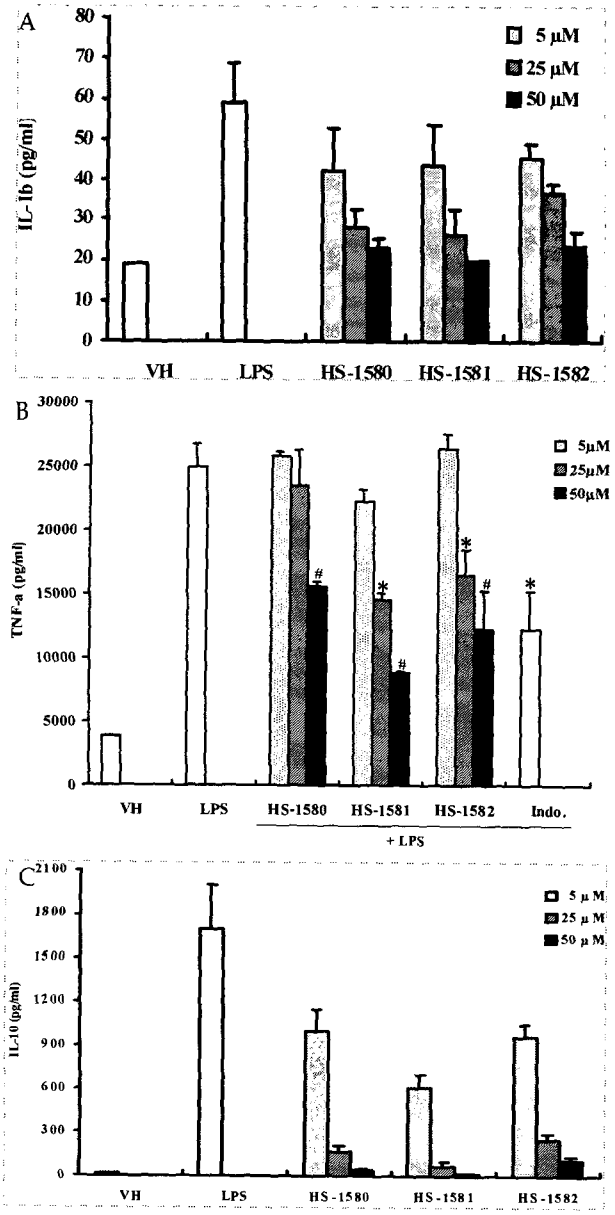

Fig. 4. Effects of HS-1580 series on LPS-induced IL-1β, TNF-α and IL-10 production in Raw 264.7 cells. A, C, The cells were treated LPS (1 μg/mℓ) for 24 h and HS-1580 series (5, 25, 50 μM) was added 1 h before LPS treatment (# $p<0.01$, * $p<0.05$ vs. LPS alone). B, The cells were treated with LPS (1 μg/mℓ) for 24 h and HS-1580 series (5, 25, 50 μM) or indomethacin (50 μM) were added 1h before LPS treatment (# $p<0.01$, * $p<0.05$ vs. LPS alone)

To confirm the effects of IL-10 on HS-1580 series-inhibited TNF-α and IL-1β production, the cells were stimulated with LPS for 24 h and the HS-1580 series was added 1 h before LPS treatment. HS-1580 series inhibited IL-10 production as well as pro-inflammatory cytokines such as TNF-α and IL-1β.

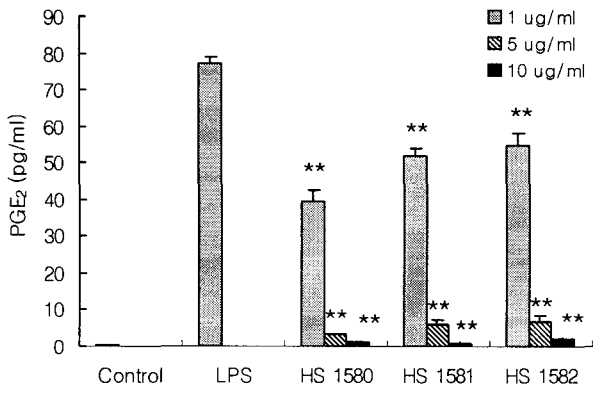

Fig. 5. Effects of HS-1580 series on PGE_2 production in Raw 264.7 cells Cells were treated with LPS (1 ㎍/㎖) for 24 h with or without each compound (1, 5, or 10 ㎍/㎖). (** $p<0.01$ from LPS)

Effect of HS-1580 series on LPS-induced PGE2 production

Raw 264.7 cells were treated with 1 μg/μl LPS and HS-1580 series for 24 h. After 24 h, PGE_2 was detected by EIA kit. As shown in figure 4, PGE_2 production was inhibited by HS-1580 series in a dose dependent manner. HS-1580 showed the highest inhibitory effect on PGE_2 production. Also, in 5 and 10 μg/μl HS-1580 series treatment, PGE_2 production was similarly inhibited.

Discussion

Macrophages exert the key functions during the immune response and exhibit a particularly vigorous response to lipopolysaccharide (LPS), which induces a variety of inflammatory modulators such as nitric oxide (NO), interluekin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interluekin-6 (IL-6) and prostaglandins (PGs)[14,15]. Because the effect of HS-1580 series on inflammation has not been reported, inflammatory modulators were examined .

Nitric Oxide (NO) has a wide and pervasive regulatory role in the inflammatory response. NO could be released by treatment with LPS[2]. HS-1580 series were examined to determine if it could suppress LPS-induced NO production and NG-monomethyl-L-arginine (NMMA), cycloheximide (CHX), and antinomycin (ACD) were used as positive control. As shown Figure 2, HS-1580 series inhibited NO production in a dose dependent manner. 10 μg/μl of HS-1580 series inhibited more than CHX and ACD. The pro-inflammatory properties of NO are attributed to the excessive production of NO by inducibel NOS (iNOS). A number of studies have shown that the chronic phase of inflammation particulary is particulary correlated with an increase in iNOS[22]. The results of this experiment showed that decreasing production of NO by HS-1580 series could have resulted from the inhibition of iNOS protein expression.

Prostaglandin E_2 (PGE_2) acts to mediate the regulation of cell proliferation by growth factors. One of the key enzymes involved in the synthesis of PG is PG endoperoxide synthase, also known as cyclooxygenase (COX)[17,20]. PGE_2 synthesis is mainly determined by the availability of the substrate arachidonic acid (AA) and the activity of enzyme COX[27,33]. The modulation of COX-2/PGE_2 pathway may play in a critical role for the pathogenesis of burn injuries and inflammation. This study suggested that HS-1580 series can regulate PGE_2 production by regulating COX-2 expression.

Pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, regulated a variety of inflammatory diseases. Although pro-inflammatory cytokines greatly increase after exposure to endotoxin and severe injury, anti-inflammtory cytokines block this process or suppress the cascade intensity. Thus, a balance between the effects of pro-inflammatory and anti-inflammatory cytokines is considered to determine the outcome of diseases[6]. In this study, HS-1580 series cause a dose-dependent inhibition of LPS-induced TNF-α and IL-1β production. The inhibitory effect of HS-1580 series on LPS-induced pro-inflammatory cytokines production, which include TNF-α and IL-1β, are similar with that of non-steroidal anti-inflammatory drug. Next, HS-1580 series was examined to determine if it could increase LPS-induced IL-10 production. IL-10 is an anti-inflammatory cytokine that is a potent down-regulator of cell-mediated immune and pro-inflammatory response. In particular, IL-10 inhibited the production of pro-inflammatory cytokines, TNF-α and IL-1β[29]. Results show that HS-1580 series greatly inhibit IL-10 production. Generally, pro-inflammatory cytokines can occur to a low level when anti-inflammatory cytokines increase in level[26] because anti-inflammatory cytokines inhibit pro-inflammatory cytokines. Hence, further study is needed to investigate the effects of HS-1580 series on LPS-induced IL-4 and IL-13 ,anti-inflammatory cytokines production, in Raw 264.7 macrophages.

In conclusion, this study demonstrates that HS-1580 series inhibit iNOS, COX-2, TNF-α and IL-1β production in LPS-treated Raw 264.7 macrophages. By inhibiting in-

flammatory mediators, these new chemical compounds, known as the HS-1580 series, have anti-inflammatory effects. Particularly, HS-1580 exhibits the most anti-inflammatory effects in LPS-treated the macrophages because the the cytotoxic effects of HS-1580 are the lowest within HS-1580 series.

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초록 : 신화학물질 HS-1580 유도체(HS-1580, HS-1581, HS-1582)의 항염증 효과

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본 연구는 염증 형성과정에 있어서, 해조류로부터 항염증 효과를 나타내는 물질을 분리하여 그 유도체인 HS-1580 series (HS-1580, HS-1581, HS-1582)를 합성하였다. Nitiric oxide (NO) 생성에 있어 Raw 264.7 cells에서 lipopolysaccharide (LPS) 단독으로 처리하였을 때는 대조군에서보다 4배 이상 NO 생성이 증가하였지만, HS-1580 series를 처리하고 LPS를 처리한 군에서는 농도 의존적으로 NO 생성이 억제되었다. HS-1580 series가 NO 생성 자체를 억제함으로서 NO 함량이 감소되었는지, inducible NOS (iNOS) 단백질 발현을 억제에 기인한 것인지 알아보기 위해서 Western blot으로 조사하였다. iNOS protein 발현이 HS-1580 series에 의해서 억제되었고 HS-1580 series가 cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α) 와 interluekin-1β (IL-1β) 생성을 농도 의존적으로 억제시켰다. 이상의 결과로 HS-1580 series가 iNOS 단백질 발현 억제에 기인한 NO 생성 억제, COX-2 발현 억제 및 pro-inflammatory cytokines인 TNF-α 와 IL-1β 생성을 억제하는 항염증 효과를 가짐을 알 수 있다.