Methyl 4-(β-D-glucopyranosyloxy)-3-hydroxy-5-methoxybenzoate, isolated from Sanguisorba officinalis, inhibits CpG-DNA-induced inflammation

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Abstract

Purpose: To evaluate the anti-inflammatory effect of methyl-4-(β-D-glucopyranosyloxy)-3-hydroxy-5-methoxybenzoate (comp-1) on immune cells.

Methods: Comp-1 was isolated from Sanguisorba officinalis. After treating with comp-1, cell viability and levels of pro-inflammatory cytokines were assessed utilizing MTT assay and ELISA, respectively. Besides, the effects of comp-1 on nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and iNOS were determined using western blotting. Moreover, nitric oxide production was assessed using the Griess reagent.

Results: Treatment of dendritic cells (DCs) with CpG DNA upregulated cytokine expression. Comp-1 markedly downregulated the expressions of IL-12 p40, IL-6, and TNF-α, with 50% inhibitory concentrations (IC50) of 1.077 ± 0.04 (p < 0.01), 0.28 ± 0.01 (p < 0.01), and 0.79 ± 0.02 μM (p < 0.01), respectively. Treatment of DCs with CpG DNA upregulated NF-κB and MAPK activation. However, pretreatment of the cells with Comp-1 suppressed CpG DNA-induced NF-κB and MAPK activation. Moreover, comp-1 exhibited a strong anti-inflammatory effect by inhibiting nitric oxide production and iNOS expression.

Conclusion: These results reveal that comp-1 has significant anti-inflammatory effect on immune cells.

Keywords: Natural compound, Inflammation, Pro-inflammatory cytokine, Toll-like receptor9

INTRODUCTION

Inflammatory responses are developed as a response to infections caused by different microorganisms [1]. Toll-like receptors (TLRs) are important receptors which play vital roles in immune responses [2]. Dendritic cells (DCs) are the highly efficient antigen presenting cells which participate in the fight against various microorganisms [3]. TLR stimulation is responsible for nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation, leading to produce pro-inflammatory mediators [1,4,5].
MAPKs play versatile roles in controlling differentiation, proliferation, growth, and apoptosis. In inflammatory pathways, pathogen-associated molecular patterns bind to TLRs. Thus, the activated MAPK pathways transmit signals from the cell surface receptor to the nucleus [6]. Inflammatory pathways activate extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs [7]. ERKs play an essential part in innate immunity via cytokines expression [8]. JNKs play an essential role in stress signaling pathways and regulate different cellular and physiological events [9]. p38 is an important MAPK pathway involved in the regulation of different cellular activities like inflammation, differentiation, apoptosis and autophagy [10].

The mammalian NF-κB signaling pathway is a well-studied inflammatory and - pathway, which is activated by IκBα phosphorylation and its subsequent degradation [11].

Unmethylated CpG DNA derived from bacteria and viruses is a tested ligand for TLR9 [1]. Stimulating immune cells with CpG DNA initiates the pro-inflammatory signaling cascade, leading to cytokines production [12].

Nitric oxide not only has anti-microbial activities, but also damages host tissues and is responsible for the development of inflammatory diseases [13]. iNOS is an important enzyme involved in nitric oxide production from L-arginine [14].

Sanguisorba officinalis L is a renowned medicinal plant. Methyl-4-(β-D-glucopyranosyloxy)-3-hydroxy-5-methoxybenzoate (comp-1) is one of the compounds extracted from S. officinalis [15]. The present investigation aimed to determine the anti-inflammatory effect of comp-1.

**EXPERIMENTAL**

**Mice**

All experiments were authenticated and conducted following the instructions of the Institutional Animal Care and Use Committee of Jeju National University (Approval no.; 2016-0059, 28 December 2016). All procedures were conducted according to international guidelines as well [16].

**Cell cultures**

According to previously described method [17], DCs were grown in RPMI 1640 medium supplementing with 10% FBS, 3 % GM-CSF, 0.1% of β-mercaptoethanol and 1% PG/SM. The cells were incubated at 37 °C in a 5 % CO₂ environment.

**Cell viability assay**

DCs (2×10^5 cells/mL) were pre-treated with comp-1 for 24 h. Thereafter, DCs were incubated with MTT solution for 4 h, and centrifuged. After discarding the supernatants, the formazan crystals produced were dissolved in DMSO (250 μL). Then the absorbance was assessed at 540 nm (A540).

**Measurement of cytokine production**

DCs were treated with comp-1 and then stimulated for 24 h with CpG DNA. The supernatant was collected and production of cytokine was assessed utilizing ELISA.

**Nitric oxide production**

Nitric oxide production was evaluated with the Griess reagent. RAW264.7 cells (2×10^5 cells/mL) were first incubated in the presence of comp-1 for 1 h and then with CpG DNA for 24 h. Then, the supernatant was collected and nitrite production was measured by assessing at A540 after treating with the Griess reagent.

**Western blot analysis**

Briefly, before stimulation with CpG DNA at different time intervals, DCs were treated with or without 10 μM of comp-1. Whole-cell lysate was extracted with a lysis buffer. Protein samples were electrophoresed on SDS-polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane. Each membrane was incubated with specific primary antibodies for phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, phospho- IκBα, IκBα, iNOS.

![Figure 1](https://example.com/figure1.png)

**Figure 1:** (a) Chemical structure of comp-1. (b) Dendritic cells (DCs) showed no cytotoxicity after treatment with different comp-1 doses (0.1, 0.5, 1, 2, 5 and 10 μM) for 24 h as shown by the cell viability that was estimated using the MTT assay.
and β-actin. Immunoreactive bands were analyzed according to the previously described procedure [18].

**Statistical analysis**

Data are shown as mean ± SD. Comparison of results between the comp-1-treated groups and controls were analyzed using One-way ANOVA. The SPSS version 16 software was used for the statistical procedure. Statistical significance was considered at $p < 0.05$.

**RESULTS**

**Comp-1 showed no cytotoxicity in DCs**

The chemical structure of comp-1 is shown (Figure 1 a). To evaluate the *in vitro* cytotoxicity of comp-1, DCs were incubated for 24 h with the mentioned comp-1 doses, and cell viability was estimated using the MTT assay. This result revealed that comp-1 concentrations used in the present investigation did not affect cell viability (Figure 1 b).

**Effect of comp-1 on CpG DNA-induced expressions of pro-inflammatory cytokines**

The cytokine levels in DCs increased upon CpG DNA stimulation. On the contrary, pre-treatment with comp-1 significantly reduced IL-12 p40, IL-6, and TNF-α levels, its 50% inhibitory concentration (IC50) being 1.077 ± 0.04, 0.28 ± 0.01 and 0.79 ± 0.02 μM, respectively (Figure 2). The results reveal that comp-1 inhibited the expression of pro-inflammatory cytokines.

**Comp-1 inhibits the MAPK signaling pathway**

The effects of comp-1 on CpG DNA-induced MAPK phosphorylation in DCs treated with or without comp-1 were analyzed using western blot assay (Figures 3a and b). All three MAPKs were phosphorylated between 15 to 30 min and returned to basal level within 60 min in CpG DNA-treated DCs. However, comp-1 treatment significantly inhibited MAPK phosphorylation in CpG DNA-treated DCs (Figures 3a and b).

**Comp-1 inhibits the NF-κB signaling pathway**

Phosphorylation and degradation of IκBα were observed between 15 to 30 min but returned to basal levels within 60 min of stimulation with CpG DNA (Figures 4a and b). Treatment with comp-1 inhibited phosphorylation and degradation of IκBα in CpG DNA-treated DCs (Figures 4a and b). Therefore, these findings indicate that comp-1 significantly blocked NF-κB activation.
Figure 4: Effect of comp-1 on NF-κB activation. (a) DCs were incubated with or without 10 µM comp-1 before 1 µM CpG DNA-stimulation. The whole-cell lysates were extracted at the given time intervals. IκBα phosphorylation and degradation were measured with respect to those of β-actin by western blotting. (b) Scanning densitometry was used to quantify IκBα phosphorylation and degradation; *p < 0.05 vs. comp-1-untreated and CpG DNA-treated DCs

Comp-1 inhibits NO and iNOS production

RAW264.7 cells were incubated with indicated doses of comp-1, then stimulated with CpG DNA, and nitric oxide release was estimated. Comp-1 pre-treatment decreased nitric oxide production dose-dependently (Figure 5a). Western blot analysis was carried out for the measurement of iNOS expression, CpG DNA treatment led to increased iNOS expression, while comp-1 treatment significantly reduced the iNOS levels (p < 0.01, Figures 5b and c). These experimental data show that comp-1 inhibited the production of NO and iNOS.

Figure 5: Effect of comp-1 on the production of NO and iNOS. Before induction with 1 µM CpG DNA, RAW264.7 cells were incubated with the given doses of comp-1 for 1 h. (a) NO production was evaluated with the Griess assay. (b) The expression levels of iNOS were measured with respect to those of β-actin using western blotting. (c) Scanning densitometry was used to quantify iNOS expression, which was normalized with the control protein; *p< 0.05, **p < 0.01 vs. comp-1-untreated and CpG DNA-treated cells

DISCUSSION

The present study illustrates that comp-1 is capable of inhibiting CpG DNA-stimulated inflammation. It also shows that comp-1 has the capability to suppress NF-κB and MAPKs signaling and inhibit production of NO and iNOS expression.

Pro-inflammatory cytokines are mainly expressed by activated DCs and play important roles in immune response [1]. IL-12 p40 plays a vital role in autoimmune response mediated by Th1 lymphocytes. Therefore, the reduction in IL-12 p40 production might be beneficial against autoimmune diseases [19]. IL-6 affects physiological processes, such as hematopoiesis, control of cell growth, inflammation, proliferation, and differentiation [20]. Treatment with comp-1 reduces IL-6 production in CpG DNA-induced DCs dose-dependently. Therefore, the attenuation of IL-6 expression by comp-1 may be helpful for developing anti-inflammatory drugs. TNF-α overexpression is responsible for autoimmune diseases like Crohn's disease and rheumatoid arthritis [21]. The present investigation shows that comp-1 has the potential to treat TNF-α-related diseases. The experimental data revealed that comp-1 inhibits cytokine production dose-dependently. Therefore, further studies focusing on the effectiveness of comp-1 in inflammatory diseases should be conducted.

CpG DNA-stimulated TLR9 activates NF-κB as well as MAPKs signaling pathways, and ultimately produce inflammatory mediators [22]. In the present investigation, MAPK phosphorylation was significantly blocked in CpG DNA-induced DCs by comp-1 treatment. Comp-1 also significantly downregulated NF-κB activation. This study reveals that comp-1 inhibits the activation of NF-κB and MAPKs signaling.

Nitric oxide regulates inflammatory responses. The cytotoxic effect of nitric oxide enables it to fight against pathogenic microorganisms. Nitric oxide also has the ability to produce reactive nitrogen species that can alter diverse cellular activities. Activated inflammatory cells produce iNOS, which catalyzes nitric oxide synthesis [13]. The present study revealed that comp-1 significantly reduced the levels of production of nitric oxide and inhibited iNOS expression.

Activated NF-κB and MAPKs participate in the transcription of different pro-inflammatory cytokines and inflammatory mediators [23]. The present research suggests that suppressive effects of comp-1 on the productions of
inflammatory cytokines, NO, and iNOS, are associated with the blockage of NF-κB and MAPKs signaling mediated by TLR9. Consequently, the anti-inflammatory properties of comp-1 might be effective in inflammatory diseases.

CONCLUSION

The findings of this study show that comp-1 inhibits the production of pro-inflammatory cytokines, which are linked to the blockage of NF-κB and MAPKs cellular signaling pathways. This compound also inhibits NO production by decreasing iNOS expression. Thus, comp-1 might be a strong drug candidate for treating inflammation as well as autoimmune diseases.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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