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REPORT

Anti-inflammatory effect of austroyunnane B on RAW264.7 cells

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Abstract: Austroyunnane B, a highly oxidized guaianolide-type sesquiterpenoid isolated from the whole plants of *Artemisia austro-yunnanensis* by our group, revealed significant inhibition against NO production on the model of lipopolysaccharide (LPS)-stimulated RAW264.7 cells. In this study, its anti-inflammatory effect on some inflammatory cytokines and proteins was further evaluated using the same model. As results, co-treatment with different concentrations (0.39-100μmol /L) of austroyunnane B on LPS-induced RAW264.7 cells displayed reduction of the nitric oxide (NO) generation in a dose dependent manner. It also showed a concentration-dependent inhibition against TNF-α, IL-1β, IL-6 and IL-10, and down-regulation of protein expressions of iNOS and COX-2 on that model at the range of 0.625-10μmol/L, suggesting that austroyunnane B can be used as a lead compound for the development of inflammatory drug.

Keywords: Austroyunnane B, anti-inflammation, RAW264.7 cells, sesquiterpenoid

INTRODUCTION

Inflammation is the primary process, through which the body itself repairs tissue damage and defends against stimuli. In physiologic condition, a regulated response protects against further injury and clears damaged tissue, whereas in pathologic situation, inflammation can lead to tissue destruction and organ dysfunction (Ikeda *et al.*, 2008). A great number of researches showed inflammation is regulated by a complex web of intercellular cytokine signals and implicated in the pathogenesis of many diseases, including cancer, diabetes, cardiovascular, neurodegenerative and other life-threating and debilitating diseases (Lawrence *et al.*, 2002).

By far, the steroidal anti-inflammatory drugs (SAID), such as Adrenal cortex hormones and non-steroidal anti-inflammatory drugs (NSAID), such as Ibuprofen, have still been the important medicines to treat inflammatory (Anti *et al.*, 2008; Gonzales *et al.*, 2013). However, the serious side effects induced by those drugs, including obesity, osteoporosis, and gastrointestinal reactions, have been confirmed (Ghosh *et al.*, 2015; Solomon *et al.*, 2017). Therefore, developing novel anti-inflammatory agents with low toxicity has been a hot issue in this research field.

Natural medicine is regarded as a crucial source of potential lead compounds, and many natural products isolated from them have been found to have significant anti-inflammatory. A lot of investigations showed many plants and sesquiterpenoids from *Artemisia* can produce anti-inflammatory activities (Ashok and Upadhyaya, 2013; Mi *et al.*, 2015). In our previous study, a series of sesquiterpenoids were isolated from *Artemisia austro-yunnanensis* Ling et Y. R. Ling, which is a special semi-herbaceous shrub, and usually distributed in the tropical and subtropical regions (Bora and Sharma, 2011). Bioassay displayed some of those sesquiterpenoids isolated from *Artemisia austro-yunnanensis* can produced significant inhibition against NO production on lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophage cells and the effect of austroyunnane B (IC_{50}=4.2±0.29μM) is better than others (Chi *et al.*, 2016). In order to reveal the potential anti-inflammatory of austroyunnane B, its effects on some inflammatory cytokines and proteins were further evaluated using the same model of LPS-stimulated RAW264.7 cells in this study.

MATERIALS AND METHODS

Austroyunnane B preparation

Austroyunnane B (1.4 mg) was stored at 4°C for further study, which was obtained from the whole plants of *Artemisia austro-yunnanensis* by our group. The details of original plant collection, the isolation and identification of austroyunnane B were described in our previous published paper (Chi *et al.*, 2016). In current study, austroyunnane B was dissolved in dimethyl sulfoxide (DMSO) to prepare the mother liquor at a concentration of 50mM and further diluted in cell culture media to obtain the test samples, and the final DMSO concentration was below 0.1% v/v.

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Cell culture

RAW264.7, a mouse macrophage cell line, was purchased from the Cell Bank of the Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China) and cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS, 100U/mL penicillin and 100μg/mL streptomycin in a 37°C humidified incubator containing 5% CO_2.

Cell viability assay

MTT assay was used to evaluate the effect of austroyunnane B on cell viability (Kwon *et al.*, 2010). In brief, RAW264.7 cells were seeded in 96-well plates (Corning Inc., Corning, NY, USA) at a density of 2×10^4 cells/well. After overnight growth, cells were treated with various concentrations of compound (0.39-100μM) for 1 h, followed in the presence or absence of LPS (1μg/mL) for the next 24 h. 20μL of MTT solution (5mg/mL) was added and the cells were further cultured for 4 h. After that, the supernatant was carefully removed and then the resulting formazan crystals were dissolved in 150μL DMSO with horizontal shaking. The absorbance at 570nm (ref. 630 nm) was measured with a microplate reader (Molecular Devices, California, USA).

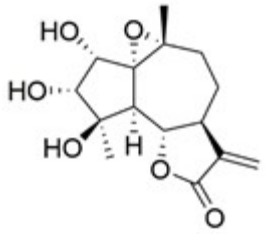

Fig. 1: Chemical structure of austroyunnane B

Assay for nitrite oxide (NO) production

To determine levels of NO in the cultured cells, RAW264.7 cells were seeded in 96-well plates (Corning Inc., Corning, NY, USA) at a density of 8×10^4 cells/well. After overnight growth, cells were treated with various concentrations of austroyunnane B (0.39-100μM), L-NMMA or Dexamethasone for 1h (Lu *et al.*, 2011; Ahn *et al.*, 2015) followed in the presence or absence of LPS (1μg/mL) for the next 12h. Griess reagents were used to determine NO levels in the media. Briefly, an equal volume (70μL) of supernatant was mixed with Griess reagent, and absorbance was measured at 540 nm against a standard sodium nitrite curve using a micro plate reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA).

Assay for TNF-α, IL-1β, IL-6 and IL-10 levels

RAW264.7 cells were seeded into 24-well plates at a density of 5×10^5 cells/mL and cultured overnight. After pretreatment with austroyunnane B at various concentrations for 1 h, the cells were stimulated with LPS (1μg/mL) for 12 h and dexamethasone (DXM) was served as positive control (Montanher *et al.*, 2007). The concentrations of the release of TNF-α, IL-1β, IL-6 and IL-10 in the cell supernatants were assayed using ELISA kits (R&D Systems, USA) according to the manufacturers' instructions. The concentrations were calculated from the standard curves.

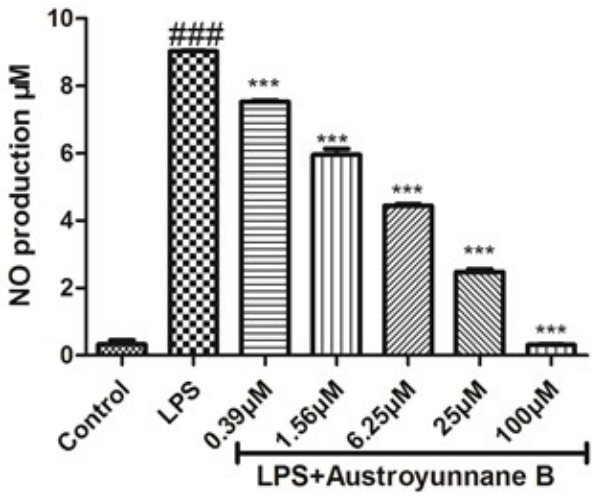

Fig. 2: Effect of austroyunnane B on the NO production in RAW 264.7 cells. Cells were pretreated with compounds for 1h and then stimulated with LPS (1μg/mL) for 12h. The release of NO was measured as described in the materials and methods. Data shown represent the mean values of three experiments ± SD. $^{###}p<0.001$ when compared with control versus LPS; $^{***}p<0.001$ as compared to the group treated with LPS alone.

Table 1: Effect of austroyunnane B on the viability of RAW264.7 cells (n = 3).

	Concentration (μmol/L)	Cell viability (%)
Control	–	100.00 ± 4.30
Austroyunnane B	0.39	98.10 ± 9.59
	1.625	102.39 ± 2.73
	6.25	97.49 ± 6.77
	25	110.50 ± 8.31
	100	92.44 ± 5.97

Western blot analysis

Aliquots of the protein samples were separated on acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred into a nitrocellulose (NC) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk the membranes were incubated with specific primary antibodies overnight at 4°C. After rinsing, the membranes were incubated with a HRP-labelled secondary antibody containing a blocking solution for 1h at room temperature. Bands were detected using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The immunosignals were

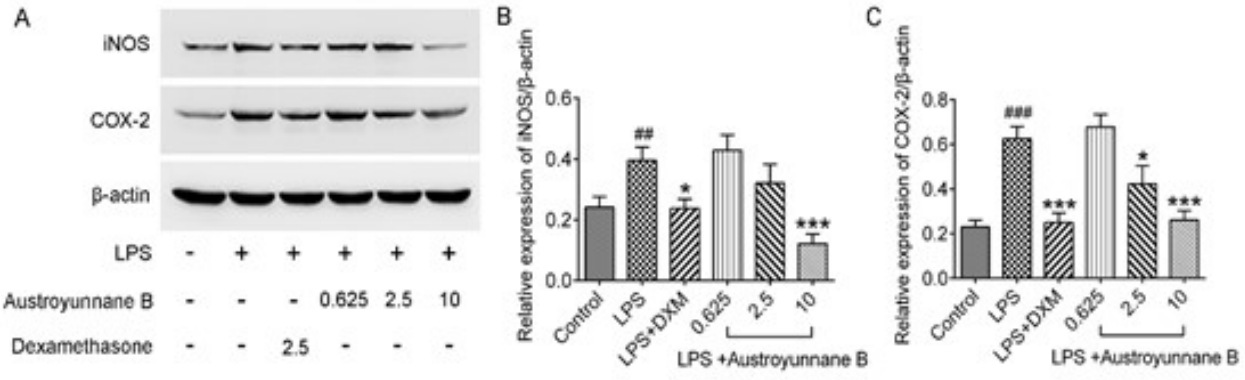

Fig. 3: Effects of austroyunnane B on iNOS and COX-2 protein expressions on LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with various concentrations of austroyunnane B for 1 h prior to LPS (1μg/mL) treatment. β-actin expression was used as an internal control. Data shown represent the mean values of three experiments ± SD. $^{###}p<0.001$, $^{##}p<0.01$ when compared with control versus LPS; ***$p<0.001$, *$p<0.05$ as compared to the group treated with LPS alone.

Table 2: Effects of Austroyunnane B on the production of cytokines TNF-α, IL-1β, IL-6 and IL-10 on LPS-induced RAW264.7 cells (n=3).

	Concentration (μmol/L)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
control	–	272.86±5.10	0.15±0.10	5.96±3.78	2.08±2.04
LPS	–	1163.21±25.84$^{###}$	18.11±0.32$^{###}$	882.07±23.67$^{###}$	38.10±0.63$^{###}$
LPS+Dexamethasone	2.5	797.22±26 ***	1.45±0.55***	184.72±7.06***	14.00±1.74***
LPS +Austroyunnane B	0.625	1151.95±58.76	12.08±0.84***	664.87±3.33***	23.40±1.01***
	2.5	1092.30±45.44	11.39±1.28***	515.11±5.00***	22.97±1.33***
	10	903.02±84.15**	9.17±1.48***	117.73 ±5.05***	18.94±1.09***

Data shown represent the mean values of three experiments ± SD. $^{###}p<0.001$ when compared with control versus LPS; ***$p<0.001$, **$p<0.01$ as compared to the group treated with LPS alone.

captured using the Gel DOC™XR +system (BioRad Laboratories, Hercules, CA, USA) and densitometric data were studied following normalization to the house-keeping loading control.

STATISTICAL ANALYSIS

Results were expressed as mean ± SD (standard deviation). Statistical analysis was performed using one-way ANOVA followed by Tukey multiple comparison tests using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Level of significance was set at 0.05.

RESULTS

Effect of austroyunnane B on cell viability

An examination of the cytotoxicity of austroyunnane B on RAW 264.7 cells by the MTT assay indicated that austroyunnane B did not affect normal cell growth at concentrations up to 100μmol/L (table1).

Effect of austroyunnane B on inhibition against NO production

The effect of austroyunnane B on inhibition against NO production was determined by measuring the level of nitrite accumulation (the stable metabolite of NO) in culture media. LPS (1μg/mL) induced significant NO production as compared with the naive control, whereas austroyunnane B produced significant inhibition of NO production with IC_{50}=4.2μM (fig. 2)

Effects of austroyunnane B on levels of TNF-α, IL-1β, IL-6 and IL-10 in RAW264.7 macrophages

ELISA assay wasused to test the levels of TNF-α, IL-1β, IL-6 and IL-10 influenced by austroyunnane B on LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with various concentrations of compound and stimulated with LPS for 12h, the 2.5μmol/L dexamethasone was used as apositive control. The inflammatory factors in LPS-induced RAW264.7 cells were strongly increased compared with the untreated control cells. As expected, austroyunnane B (0.625, 2.5 and 10μM) significantly reduced the production of TNF-α, IL-1β, IL-6 and IL-10 in dose-dependent manner (table 2).

Effects of austroyunnane B on iNOS and COX-2 proteins in LPS-induced RAW264.7 cells

In this assay, the levels of iNOS and COX-2 proteins in LPS-induced RAW264.7 cells exposed to three

concentrations of austroyunnane B were monitored, and 2.5μmol/L dexamethasone was used as a positive control. As shown in fig. 3A, the expression of the iNOS and COX-2 proteins were barely detected in the non-stimulated cells. However, the levels of those two proteins increased markedly after the LPS treatment for 12h, whereas austroyunnane B exerted a dose-dependent inhibition of iNOS and COX-2 protein expressions on the LPS-stimulated RAW264.7 cells (fig. 3B and 3C).

DISCUSSION

Inflammatory response is a complex biological reaction and regulated by a series of inflammatory factors and proteins (Melley *et al.*, 2005; Rolova *et al.*, 2014). The most common inflammatory cytokines are NO, TNF-α, IL-1β, IL-6 and IL-10, etc., which have been confirmed to play a major role in the occurrence and development process of inflammatory (Katsikis *et al.*, 1994; Zamora *et al.*, 2000; Meyer, 2003; Möller and Villiger, 2006; Nishimoto and Kishimoto, 2006; Kavanaugh, 2007; Snyder and Kim, 2008; Dick, 2010; Mcnally and Anderson, 2011). For the proteins, such as iNOS and COX-2, they are being researched more as the important enzymes associated with the inflammation (O'Banion, 1999; Leone *et al.*, 2007; Snyder and Kim, 2008; Soskić *et al.*, 2011). In this study, austroyunnane B produced inhibition against production of NO, generation of TNF-α, IL-1β, IL-6 and IL-10 on LPS-stimulated RAW264.7 cells, and protein expression of COX-2 and iNOS on LPS-induced RAW264.7 cells, indicating it has the exact role in anti-inflammatory activity. Notably, co-incubated with different concentrations (0.625, 2.5 and 10μM) of austroyunnane B on that model, a dose-dependent relationship between austroyunnane B and those above-mentioned indicators were observed.

CONCLUSION

In this study, the potential anti-inflammatory activity of austroyunnane B was evaluated on LPS-stimulated RAW264.7 cells. The results showed that it can down-regulate the levels of TNF-α, IL-1β, IL-6 and IL-10 and the protein expression of iNOS and COX-2 in *vitro*, indicating austroyunnane B is an active molecule with anti-inflammatory and can be used as a lead compound for development of anti-inflammatory agent.

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