

Inhibitory Effects of Costunolide Isolated from *Laurus nobilis* on IgE-induced Degranulation of Mast Cell-like RBL-2H3 Cells and the Growth of Y16 pro-B Cells

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This study investigated the inhibitory effects of costunolide isolated from the leaves of *Laurus nobilis* L. (Lauraceae) on basophil-mediated allergic reactions and interleukin (IL)-5-mediated B cell growth. The effects of costunolide on β -hexosaminidase (a key parameter of degranulation) release and IL-4 expression in rat basophilic leukemia (RBL-2H3) cells were determined by measuring β -hexosaminidase activity and by semi-quantitative RT-PCR, respectively. The effects of costunolide on Y16 pro-B cell viability and growth were determined by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. Costunolide was found significantly to inhibit β -hexosaminidase activity ($p < 0.01$) and IL-4 transcription in RBL-2H3 cells in a dose-dependent manner. Its 50% inhibitory concentration (IC₅₀) was 34 μ M, while that of the positive control, ketotifen, was 24 μ M (IL-4 mRNA transcription). Moreover, costunolide dose-dependently suppressed pro-B cell growth in IL-5-stimulated Y16 cells.

These results provide evidence that costunolide stabilizes mast cells by inhibiting IgE-mediated degranulation and inhibits IL-5-stimulated B cell growth. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: costunolide; *Laurus nobilis*; RBL-2H3 cells; β -hexosaminidase; interleukin-4; Y16 cells.

INTRODUCTION

The incidence of allergic diseases such as asthma, rhinitis and atopic dermatitis has increased recently, especially in developed countries. Epidemiological studies suggest that this trend may stem from exposure to environmental factors (Etzel, 2003; Bornehag *et al.*, 2004; Rönmark *et al.*, 2009). Mast cells play a key role in the development of these allergic diseases and inflammatory disorders (Fairweather and Frisancho-Kiss, 2008; Holgate *et al.*, 2009). In allergic reactions, initial exposure to an allergen triggers the production of large amounts of allergen-specific IgE antibody by B cells. These IgE molecules attach themselves to mast cells. Upon subsequent exposure to the same allergen, the IgE-primed mast cells degranulate, releasing powerful chemical mediators, including histamine and cytokines, into their local environment. These chemical mediators cause the characteristic symptoms of allergy (Stone *et al.*, 2010).

The RBL-2H3 mast cell line has been used widely as a convenient model system for studying regulated secretion in mast cells (Sur *et al.*, 2009). RBL-2H3 cells abundantly express Fc ϵ RI, a high-affinity receptor for

IgE, on their surfaces. Aggregation of IgE-Fc ϵ RI complexes by multivalent antigens activates a complex cascade of intracellular events that results in degranulation and the consequent release of chemical mediators of allergic responses including histamine (Yang *et al.*, 2009), serotonin (Hoek *et al.*, 2002) and β -hexosaminidase (Han *et al.*, 2009). These molecules play important roles in the development of some of the major symptoms of allergic diseases, including vasodilation, mucus secretion and bronchoconstriction.

Interleukin (IL)-4, which is primarily secreted by activated Th2-type cells and inflammatory cells such as mast cells and basophils, plays an important role in allergic inflammation (Steinke and Borish, 2001; Zietkowski *et al.*, 2010). It contributes to allergic reactions by inducing IgE isotype switching in B cells (Aalberse, 2006), and plays important roles in the infiltration of inflammatory cells and the induction of late-phase reactions (Metcalf *et al.*, 1997; Pearlman, 1999). Overproduction of IL-4 has been associated with allergy (Le-Buanec *et al.*, 2007).

A second cytokine, IL-5, one of the main proinflammatory mediators produced by Th2 cells and mast cells, induces eosinophilic inflammation and mucus production (Hamelmann *et al.*, 1999; Allakhverdi *et al.*, 2002). It also stimulates B cell growth, and thus immunoglobulin secretion. Notably, it is a key mediator of eosinophil activation. By increasing the release of immunoglobulin by B cells, it enhances allergic and inflammatory reactions by increasing mast cell

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activation. Glucocorticoids are used frequently to control acute allergic and/or inflammatory reactions (FERENCE and LAST, 2009; SAEKI, 2009). However, the prolonged use of high-dose glucocorticoids is associated with a variety of adverse effects (PARISER, 2009). In recent years, therefore, safer alternative approaches have been developed to control allergic reactions. Many compounds whose origins lie in herbal medicine have been developed (BYEON *et al.*, 2009; CHOI *et al.*, 2007). *Laurus nobilis* L. (Lauraceae) leaves are used frequently as a spice in cooking. Folk medicine in many countries uses infusions of this plant in stomachic and carminative remedies, as well as in the treatment of gastric disease. *Laurus nobilis* is a source of a number of polyphenolic compounds (De-Marino *et al.*, 2004; Bouaziz *et al.*, 2007), as well as monoterpenes (Yalcin *et al.*, 2007; Marzouki *et al.*, 2008) and sesquiterpenes (De-Marino *et al.*, 2005; Dall'Acqua *et al.*, 2006). It has been reported that the sesquiterpene costunolide, a constituent of *Laurus nobilis*, induces cell death (Hibasami *et al.*, 2003) and inhibits alcohol absorption (Yoshikawa *et al.*, 2000). Allergic reactions have been reported in a few individuals treated with a mixture of costunolide and other sesquiterpenes (Goulden and Wilkinson, 1998; Paulsen *et al.*, 2001). In a survey covering the 15-year period 1995–2009, 157/7163 (2.19%) individuals suffered reactions, and about 87% of those who suffered reactions were sensitive to costunolide at a concentration of 0.1% (Paulsen *et al.*, 2001; Paulsen and Andersen, 2010). In spite of these few cases of allergy, costunolide has been shown to have antiinflammatory activities (Pandey *et al.*, 2007; Pae *et al.*, 2007; Kassuya *et al.*, 2009). However, the effects of costunolide on mediators of allergic reactions have not been explored. The present study was undertaken to assess whether costunolide inhibits allergic mediators *in vitro*. Specifically, this study focused on the effects of costunolide on early- and late-phase allergic indicators including degranulation and pro-B cell growth.

MATERIAL AND METHODS

Reagents. Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA). A monoclonal anti-dinitrophenol (DNP) antibody and 4-nitrophenyl *N*-acetyl- β -D-glucosaminide were purchased from Sigma-Aldrich. One-step RT-PCR premix kits and easy-BLUE Total RNA extraction kits were purchased from iNtRON (Kyunggi, Korea).

***Laurus nobilis* extract and costunolide.** Costunolide was isolated from the leaves of *L. nobilis*, obtained from Orege Forest Agricultural and Food Products Foreign Trade, Turkey, in November, 2008. The plant was identified by Dr Yeong-Han, of Korea National Arboretum, and a voucher specimen, NPRI-Q003, has been kept in the Natural Products Research Institute, Seoul National University Herbarium. Briefly, air-dried *L. nobilis* (40.0 kg) was extracted with methanol (20 L), and then filtered. The residual marc was re-extracted in the same way with more methanol (2 $\times$ 20 L). The combined filtrates were evaporated under reduced

pressure to yield a dark green gum (2.4 kg). The sample was partitioned between *n*-hexane and 10% aqueous methanol (2 L). The sample (37.5 g) from the *n*-hexane fraction (318.7 g) was subjected to flash chromatography (Φ 10 $\times$ 50 cm; silica gel 70–230 mesh, 1 kg) with an *n*-hexane and ethyl acetate gradient. The active fractions were eluted in a solvent EtOAc/*n*-hexane fraction (2: 8) and the fraction (418.8 mg) was subjected to semi-preparative high performance liquid chromatography (HPLC; YMC-silica column, 25 $\times$ 1 cm) with an *n*-hexane–EtOAc gradient. The final fraction eluted with hexane:ethyl acetate (1.5: 8.5) resulted in a pure compound, which was recrystallized in *n*-hexane as colorless crystals. Based on electron spray ionization mass spectrometry, ^1H NMR and ^{13}C NMR spectral data, the purified compound was verified as costunolide (11.8 mg, Fig. 1) (Aifeng *et al.*, 2005).

Cell culture. Rat basophilic leukemia (RBL-2H3) cells obtained from the Korean Cell Line Bank (Seoul, Korea) were grown at 37 °C, 5% CO₂ in DMEM supplemented with 8% FBS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. Y16 pro-B cells were grown in RPMI medium supplemented with 24 mM NaHCO₃, 1X antibiotics (Sigma-Aldrich), 50 μM 2-mercaptoethanol and 8% heat-inactivated FBS.

Cell viability assay. Cell viability was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RBL-2H3 cells (5×10^4 cells/mL) were plated to 96-well plates (100 μL /well). After 24 h, they were treated with various concentrations of costunolide for another 24 h and then incubated with MTT (250 $\mu\text{g/mL}$) at 37 °C in phenol-red free medium. After 4 h, the

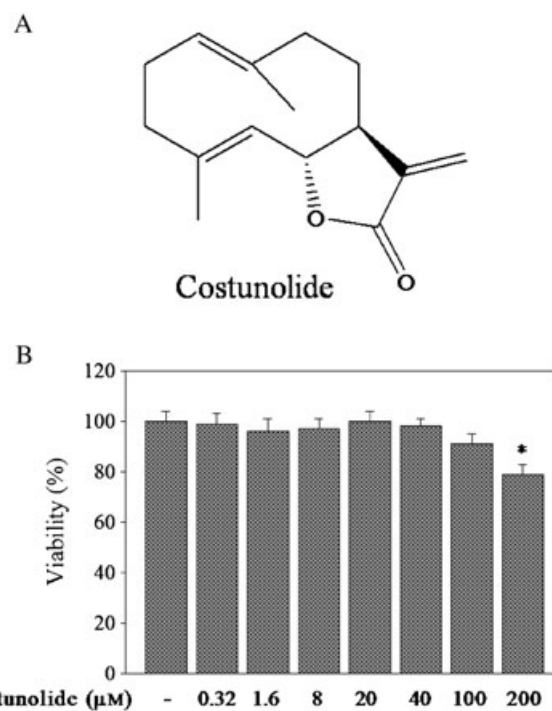


Figure 1. (A) Chemical structure of costunolide. (B) Cytotoxic effects of costunolide on RBL-2H3 cells. RBL-2H3 cells (5×10^5 cells/mL) were cultured for 48 h in growth medium containing varying amounts of costunolide. Cytotoxicity was determined by MTT assay. Data are expressed as mean $\pm$ SD. * $p < 0.05$ compared with control.

MTT solution was removed and replaced with 250 μ L of dimethyl sulfoxide (DMSO). The cell viability was determined by quantifying the production of the colored MTT metabolite formazan. This was achieved by measuring absorbances at 595 nm. Values obtained from untreated control cells were considered to represent 100% viability.

Assay for β -hexosaminidase release from RBL-2H3 cells. The release of a preformed granular enzyme, β -hexosaminidase, in mast cells correlates well with histamine release (Schwartz and Austen, 1980; Passante and Frankish, 2009). RBL-2H3 cells (5×10^4 cells/mL) were plated to 96-well plates (100 μ L/well) and then sensitized through incubation with anti-DNP-IgE (final concentration 1 μ g/mL) for 24 h at 37 °C. They were then washed twice with HEPES buffer to eliminate free IgE. After washing, the cells were incubated with costunolide [final concentration 0.32–40 μ M in 45 μ L (per well) of releasing buffer (116.9 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄·7H₂O, 5.6 mM glucose, 25 mM HEPES, 2.0 mM CaCl₂, 1.0 mg/mL BSA, pH 7.7)] at 37 °C for 20 min. The cells were next challenged through incubation with 5 μ L per well of 4 μ g/mL DNP-BSA (in PBS) at 37 °C for 1 h. Finally, 40 μ L of each supernatant was transferred to a fresh 96-well plate and mixed with 50 μ L of substrate solution (5 mM 4-nitrophenyl *N*-acetyl- β -D-glucosaminide in 50 mM citric acid buffer, pH 4.5). After incubation at 37 °C for 90 min, the reactions were terminated by the addition of 50 μ L of stop solution (0.5 M Na₂CO₃/NaHCO₃, pH 10.0). Absorbances at 405 nm were recorded using a microplate reader (Molecular Devices, USA). The second-generation of H₁-antihistamine/mast cell stabilizer ketotifen was used as a positive control (Passante *et al.*, 2009). The percentage inhibition of β -hexosaminidase release in each test sample was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (T - C) / (I - C)] \times 100$$

Control (C): Cells (+), DNP-IgE (–), test sample (–)
Test (T): Cell (+), DNP-IgE (+), test sample (+)
Inducer (I): Cell (+), DNP-IgE (+), test sample (–)

Measurement of IL-4 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

RBL-2H3 cells (5×10^5 cells/mL) were plated to 12-well plates (1 mL/well) and then sensitized through incubation with anti-DNP-IgE (final concentration 1 μ g/mL) for 24 h at 37 °C. They were then washed twice with HEPES buffer to eliminate free IgE. After washing, the cells were incubated with costunolide [70 μ L in 630 μ L (per well) of releasing buffer] at 37 °C for 3 h. The cells were next challenged through incubation with 70 μ L per well of 4 μ g/mL DNP-BSA (in PBS) at 37 °C for 1 h. Following antigen-stimulation, total RNA was isolated using an easy-BLUE Total RNA extraction kit (iNtRON). cDNA was synthesized from 1 μ g of total RNA using a ONESTEP RT-PCR premix kit (iNtRON). Based on their previous reported efficacy, the following primer pairs were used: IL-4, forward 5'-ACCTTGCTGTCACCCTGTTC-3' and reverse 5'-TTGTGAGCGTGGACTCATTC-3'; and

β -actin, forward 5'-GACCCAGATCATGTTTGAGA-3' and reverse 5'-GCTTGCTGATCCACATCTGC-3'. The thermal conditions for denaturation, annealing and extension, respectively, were as follows: IL-4: 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s (35 cycles); β -actin: 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s (25 cycles). PCR reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Seoul, Korea). The PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen, Seoul, Korea). Detection and densitometric analysis of bands were performed using a LAS 1000 Plus image analyser in conjunction with Multi-Gauge software (both Fuji Photo Film, Japan). Product molecular weights were determined through comparison with molecular size markers (100 bp DNA Ladder Marker, Takara). For each sample, the IL-4 mRNA value was normalized to the β -actin signal.

Y16 pro-B cell growth assay. Y16 cells (5×10^4 cells/well) were plated to a 96-well microplate (Nunc, Denmark). IL-5 (final concentration 10 U/mL) was added, with or without serially diluted costunolide or tyrphostin AG-490 (positive control), to each well. As a positive control, tyrphostin AG490 (Sigma-Aldrich), 2-cyano-3-(3,4-dihydroxyphenyl)-*N*-(benzyl)-2-propenamide inhibiting Janus kinase-2 critical for the anti-apoptotic effect of IL-5 (Pazdrak *et al.*, 1998) was used. After incubation for 48 h at 37 °C, 5% CO₂, the cells were incubated with fresh culture medium containing MTT (250 μ g/mL) for a further 4 h. The culture medium was then replaced with 250 μ L of DMSO to ensure complete solubilization of the colored MTT metabolite formazan. Relative rates of cellular metabolism were determined by measuring absorbances at 540 nm (*A*₅₄₀) using a microplate reader. The percentage inhibition of IL-5-stimulated growth in each test sample was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (\text{Sample } A_{540} - \text{Control } A_{540}) / (\text{Inducer } A_{540} - \text{Control } A_{540})] \times 100$$

Statistical analysis. All data are expressed as mean $\pm$ SD. Statistical significance was determined by Student's *t*-test using Sigma Plot 10.0 software (SPSS, USA). A value of *p* < 0.05 was considered to be statistically significant.

RESULTS

Lack of cytotoxic effect of costunolide on RBL-2H3 cells

The viability of RBL-2H3 cells treated for 24 h with various concentrations of costunolide (0.32–200 μ M) was determined by the MTT assay. There was a significant reduction in viability in cells treated with costunolide at a concentration of 200 μ M. In contrast, over 98% of cells treated with concentrations of costunolide ranging from 0.32 to 40 μ M remained viable (Fig. 1B). The non-cytotoxicity of costunolide in Y16 cells was confirmed by staining with 0.4% trypan blue (data not shown).

Inhibition of β -hexosaminidase release from RBL-2H3 cells by costunolide

Degranulation of RBL-2H3 cells sensitized with IgE and challenged with DNP-BSA was evaluated by assaying β -hexosaminidase release. Treatment with costunolide dose dependently reduced the release of β -hexosaminidase. Treatment with 0.32, 1.6, 8 and 40 μ M of costunolide inhibited the release of β -hexosaminidase by $2.8 \pm 3.2\%$, $7.9 \pm 1.1\%$, $14.7 \pm 3.2\%$, and $37.5 \pm 4.4\%$, respectively (Fig. 2). Costunolide was as effective as ketotifen (the positive control) at inhibiting degranulation.

Effect of costunolide on IgE-induced IL-4 expression in RBL-2H3 cells

IL-4 plays an important role in allergic diseases. The effect of costunolide on the expression of IL-4 mRNA

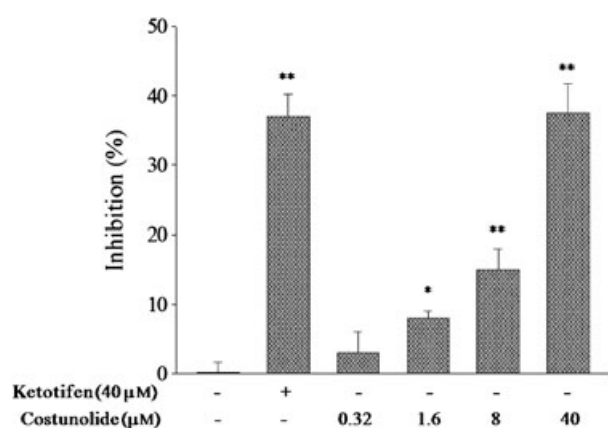


Figure 2. Inhibitory effect of costunolide on β -hexosaminidase release from RBL-2H3 cells. Degranulation was dose-dependently inhibited by both costunolide and ketotifen (positive control). Data are expressed as mean $\pm$ SD of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$ compared with control.

was examined. IgE-sensitized RBL-2H3 cells were treated with costunolide for 3 h and then challenged with DNP-BSA. Costunolide dose-dependently reduced IL-4 mRNA expression ($IC_{50} = 34 \mu$ M) (Fig. 3A). The study also examined the effects of ketotifen, which has been used as an antiallergic drug. It strongly suppressed IL-4 mRNA expression in RBL-2H3 cells ($IC_{50} = 24 \mu$ M) (Fig. 3B). Costunolide was thus slightly less potent than ketotifen as an inhibitor of RBL-2H3 cell IL-4 mRNA.

Inhibitory effect of costunolide on IL-5-dependent proliferation of Y16 pro-B cells

The cytokine IL-5 is known to play a significant role in allergic diseases. Costunolide dose-dependently inhibited the growth of IL-5-stimulated Y16 pro-B cells. The IC_{50} values of costunolide and tyrphostin AG-490 (a positive control) were 31 and 24 μ M, respectively (Fig. 4). Costunolide was thus a slightly less potent inhibitor of IL-5-stimulated Y16 pro-B cell growth than was tyrphostin AG-490.

DISCUSSION

This study examined the effects of costunolide on the degranulation of IgE sensitized mast cells and the growth of IL-5-stimulated Y16 pro-B cells to test whether costunolide might be useful in treating early- and late-phase allergic reactions. Allergen binding triggers the aggregation of complexes formed between IgE and its high-affinity receptor Fc ϵ RI, and thereby induces mast cell degranulation and the release of allergic mediators including histamine, serotonin and β -hexosaminidase (Siraganian, 2003). Costunolide inhibited the secretion of these allergic mediators (measured

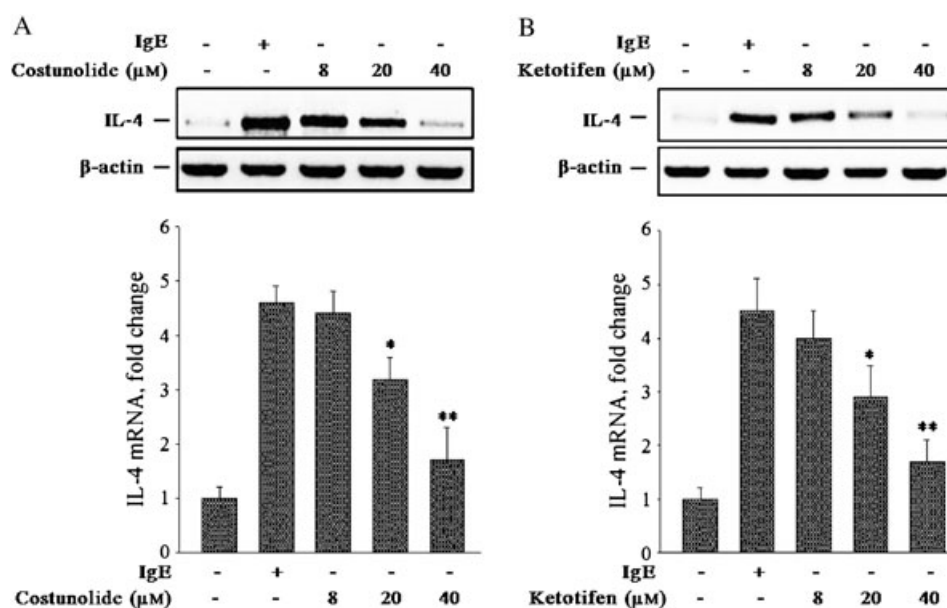


Figure 3. Effect of costunolide and ketotifen on IL-4 mRNA expression in RBL-2H3 cells. RBL-2H3 cells were treated costunolide for 3 h and then challenged with NPBSA for 1 h. IL-4 mRNA expression was quantified by RT-PCR. (A) Costunolide and (B) the antiallergic drug ketotifen dose-dependently reduced IL-4 mRNA expression. Expression was measured using Multi-Gauge software. Data are expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$, compared with control.

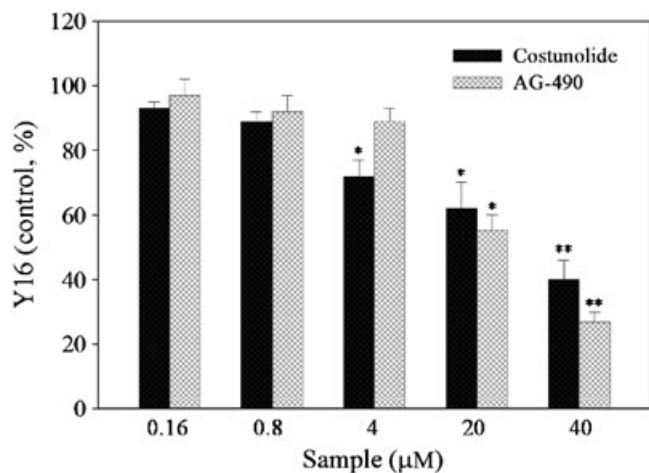


Figure 4. Inhibitory effects of costunolide on IL-5-induced proliferation of Y16 cells. Y16 cells were treated with IL-5 with or without costunolide for 48 h. Cell growth was measured by MTT assay. Data are expressed as mean $\pm$ SD of at least three independent experiments. * p < 0.05; ** p < 0.01 compared with control.

through β -hexosaminidase release) dose-dependently and as effectively as the positive control ketotifen. These results suggest that costunolide inhibits the release of histamine and other inflammatory mediators. In Th2 immune responses, mast cells and T cells secrete IL-4, IL-5 and IL-13. IL-4 maintains cell activation, thereby reinforcing Th2 responses. Moreover, it induces the growth of basophils and mastocytes, and the differentiation of lymphocytes (Ricci *et al.*, 1997). It is therefore an important factor in facilitating mast cell activation (via effects on B cells), as well as in inducing the secretion of other Th2 cytokines. It was found that costunolide dose-dependently decreased the expression of IL-4 mRNA in anti-DNP-IgE-treated mast cell-like cells. This inhibitory effect was similar to that observed with ketotifen, a well-known inhibitor of IL-4 expression. These results provide evidence that costunolide

inhibits anti-DNP-IgE-induced mast cell activation, measured in terms of β -hexosaminidase activity and IL-4 mRNA expression. Mast cells are activated following the binding of IgE to receptors on their cell surfaces. Activated mast cells secrete proinflammatory cytokines including IL-4 and IL-5. Mast cell-derived IL-5 stimulates B cell growth and thus increases immunoglobulin secretion. Costunolide inhibited the growth of IL-5-stimulated Y16 pro-B-cells with an IC_{50} value of 31 μ M (tyrphostin AG-490, used as a positive control, exhibited an IC_{50} value of 24 μ M). This potent inhibition of IL-5-stimulated growth of Y16 pro-B cells by costunolide suggests that it controls not only the early phases of mast cell activation, but also subsequent IgE amplification.

CONCLUSION

It was shown that costunolide limits the ability of IgE to induce β -hexosaminidase activity and IL-4 mRNA expression in RBL-2H3 cells without causing significant cytotoxicity. Furthermore, costunolide dose-dependently inhibited IL-5-dependent growth of Y16 pro-B cells. These findings reveal the pharmacological potential of costunolide as a mast cell stabilizer and inhibitor of pro-B cell proliferation in allergic and/or inflammatory disorders.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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