Arch Pharm Res Vol 22, No 4, 348-353, 1999

Eudesmin Inhibits Tumor Necrosis Factor-α Production and T cell Proliferation

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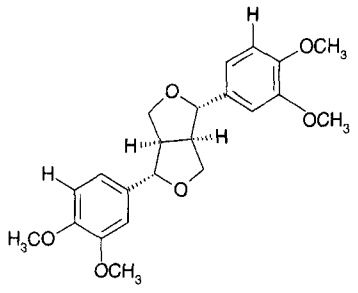
(Received March 25, 1999)

Possible antiinflammatory effects of eudesmin were examined by assessing the effects on tumor necrosis factor (TNF)-α production and lymphocyte proliferation as well as cytotoxicity against murine and human macrophages. The compound significantly inhibited TNF-α production by lipopolysaccharide (LPS)-stimulated murine macrophage RAW264.7 without displaying cytotoxicity suggesting that eudesmin may inhibit TNF-α production without any interference of normal cell function. It also significantly attenuated T cell proliferation stimulated by concanavalin A (Con A) in a dose-dependent manner.

Key words : Eudesmin, *Magnolia fargessi*, Tumor necrosis factor (TNF)-α, Lymphocyte proliferation.

INTRODUCTION

Lignan compounds are known as the primary materials having various biological activities including antioxidative, bactericidal, fungicidal, antiviral, phytotoxic, antiinflammatory and anticancer effects (Ayres and Loike, 1990; Hirano *et al.*, 1994; Kumaravelu *et al.*, 1995; Cho *et al.*, 1998d; Vlietinck *et al.*, 1998). The compounds can be divided into several different structural subfamilies (Ayres and Loike, 1990).



Eudesmin

Fig. 1. The chemical structure of eudesmin.

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Even though eudesmin, a representative furofuran type of lignan (Fig. 1), was generally identified and purified in several medicinal plants, there have been few reports on its biological activities. It was reported that the compound is a potent platelet activating factor antagonist (Pan *et al.*, 1987), antioxidant (Cavin *et al.*, 1998), and inhibitor of cAMP phosphodiesterase (Nishibe *et al.*, 1986). In recent studies (Chae *et al.*, 1998; Cho *et al.*, 1999), we have reported that eudesmin, in a form of an ethanol of *Magnolia fargessi*, is an active inhibitory principle of TNF-α production, by possessing *in vivo* TNF-α inhibitory activity.

In this study, we report several biological activities of eudesmin on cotreatment with well-known TNF-α inhibitors as well as TNF-α inhibition, cytotoxicity against human and murine macrophage cell, and inhibitory effect of Con-A-stimulated T cell proliferation.

MATERIALS AND METHODS

Animals

Eight-week-old BALB/c male mice were purchased from B & K Universal (Fremont, CA, USA). The BALB/c mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied ad *libitum*.

Materials

Eudesmin was isolated from *Magnolia fargessi* (Chae *et al.*, 1998). Cynaropicrin, used as a positive naturally-occurring standard, was a kind gift from Prof. Jee H. Jung (Pusan National University, Pusan, Korea). A77,1726 was obtained from Department of Chemistry in Daewoong R & D Center (Sungnam, Korea). Dibutyryl cyclicAMP (dbcAMP), pentoxifylline, theophylline, chloroquine3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), concanavalin A (Con A), and lipopolysaccharide (LPS, *E. coil* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roliparm was obtained from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS), penicillin, streptomycin, and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 and U937 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of Sigma grade.

Cell culture

RAW264.7 and U937 cells were maintained in RPMI-1640 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% FBS. Cells were grown at 37°C cand 5% CO_2 in humidified air.

TNF-α production *in vitro*

The inhibitory effect of eudesmin on TNF-α production was determined as previously described (Cho *et al.*, 1998c). Eudesmin solution (89.9% propylene glycol, 10% ethanol, and 0.1% dimethyl sulfoxide), was diluted with RPMI1640. The final concentration of vehicle never exceeded 0.1% in the culture medium. Under these conditions, none of the solubilization solvents altered TNF-α production in RAW264.7 cells. Before stimulation with LPS (1 mg/ml) and testing samples, RAW264.7 cells (210^6 cells/ml) were incubated for 18 h in 24 well plates with the same conditions. Stimuli and the various concentrations of testing samples were then added to the wells for 5 h. Supernatants were then collected and assayed for TNF-α content using mouse TNF-α ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Splenocyte preparation

Mice were sacrificed by cervical dislocation under sterile condition. Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions described previously (Cho *et al.*, 1998b). Briefly, splenocytes were released by teasing into RPMI1640 medium supplemented with 20μM HEPES buffer. After removing red blood cells using 0.83% NH_4Cl-Tris buffer (pH 7.4), splenocytes were washed three times in Ca^{2+}-Mg^{2+} free Hanks balanced salt solution and resuspended to 5×10^6 cells/ml in RPMI1640 with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% FBS.

Splenocyte proliferation assay

Splenocytes (5×10^6 cells/ml) were cultured in flat bottom 96 well microtiter plates (Corning Glass, Corning, NY, USA) in the presence and absence of T lymphocyte mitogen (Con A) with eudesmin and positive control in a total volume of 200 μl/well at the same conditions for 48 h (Cho *et al.*, 1998b). The proliferation was assessed by MTT assay.

Cytotoxicity and cell proliferation assay

Cytotoxicity of eudesmin was evaluated by MTT assay with a minor modification as reported previously (Cho *et al.*, 1998a). The cell suspension of 10^6 cells/ml was plated in 96-well plate. After 18 h culture, varying concentrations of testing compounds were added to each well and cultured for another 5 h or 24 h. Finally, 10 μl of MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4) was added to each well and incubated for another 4 h. Culture was stopped by addition of 15% sodium dodesyl sulfate (SDS) into each well for solubilization of formazan. The optical density (OD) at 570 nm ($OD_{570-630}$) was measured by a microplate Spectramax 250 microplate reader. The mean value of OD content of 4 wells was used for calculating the viability (% of control).

Statistical analysis

All values expressed as mean ± SEM from 4 observations. The Student's *t* - test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; p values of 0.05 or less were considered as statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity of eudesmin against macrophage cell line

It was necessary to assess the cytotoxic effect of eudesmin before further *in vitro* tests were carried out. MTT assay using RAW264.7 and U937 cells is a suitable model for this purpose, as the cells are representative macrophage which can release TNF-α. Fig. 2 shows that viability of neither RAW264.7 nor U937 cells was suppressed by eudesmin during incubation for 24 h. In contrast, cynaropicrin inhibited cell proliferation with IC_{50} of 17.1 and 20.3 μM, respec-tively. However, these compounds did not affect cell viability within 6 h incubation (data not shown). From the results, it was suggested that eudesmin may inhibit TNF-α production without any interference of normal

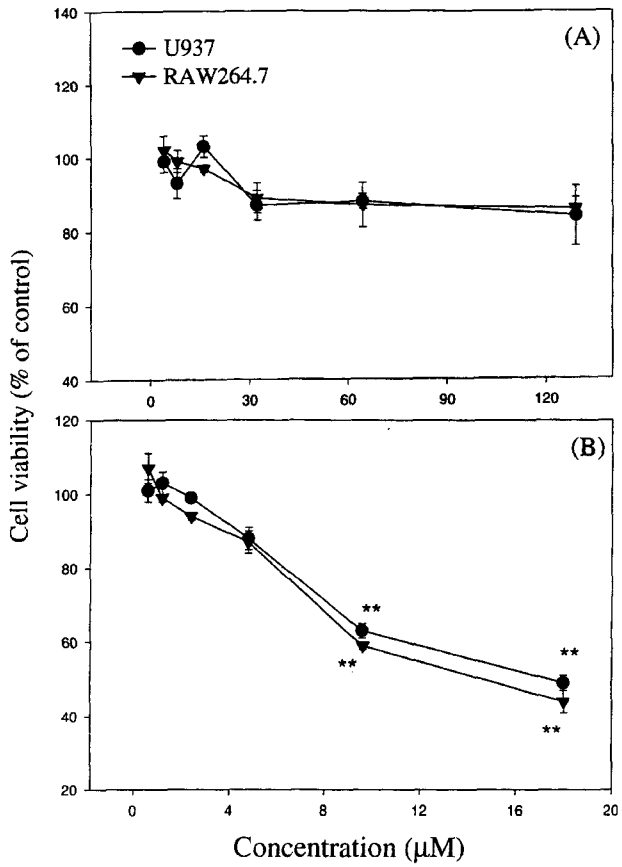

Fig. 2. Effect of eudesmin (A) and cynaropicrin (B) on cell viability of murine and human macrophage cell lines (RAW264.7 and U937). RAW264.7 and U937 ($0.5 10^6$ cells/ml) cells were incubated with various concentrations of eudesmin and cynaropicrin for 24 h. Cell viability was assessed by conventional MTT assay. Data represent mean ±SEM of 4 observations.

cell function.

Effect on TNF-α production

TNF-α is one of the important proinflammatory cytokines which are mainly produced by monocytes and macrophages (Vileek and Lee, 1991). It is secreted at the early stage of inflammatory diseases such as asthma, rheumatoid arthritis, septic shock, and other allergic diseases, and triggers diverse inflammatory cascades such as the secretion of other cytokines including interleukin-1, -6 and -8, and the activation of T cells (Rossi *et al.*, 1985; Dayer *et al.*, 1986; Hensel *et al.*, 1987). Therefore, it is regarded that anti-TNF-α therapy is a potential tool for treatment of acute and chronic inflammatory diseases.

As in the previous reports, we used LPS-activated RAW264.7 cells incubated for 5 h for optimal TNF-α production. Fig. 3 and Table I display that eudesmin significantly inhibited the TNF-α production in a dose-dependent manner with IC_{50} value of 51.3 μM as reported in the previous report (Chae *et al.*, 1998). Positive controls (cynaropicrin, pentoxifylline, and theophylline) used in this experiment also significantly suppressed TNF-α production in a dose-dependent manner with IC_{50} values of 8.2, 243.1, and 448.9 μM, respectively. The inhibitory potency of eudesmin was comparable to or higher than those of nonspecific cAMP phosphodiesterase inhibitors (pentoxifylline and theo-phylline), protopanaxadiol ginsenosides (Rb_2 and Rb_1) (Cho *et al.*, 1998b), pinoresinol (Cho *et al.*, 1998c), thalidomide (Shannon *et al.*, 1997), tyrphostin-related tyrosine kinase inhibitors (Ruetten and Thiemermann, 1997), but lower than those of sesquiterpene lactones (cynaropicrin, parthenolide, and reynosine: IC_{50}, 1~30 μM) (Hwang *et al.*, 1996; Cho *et al.*, 1998a) and bisbenzylisoquinoline alkaloids (fanchino-line and isotetrandrine: IC_{50}= 5~20 μM) (Onai *et al.*, 1995).

Although the inhibitory potency of eudesmin on TNF-α production was less than that of cynaropicrin (Table I), it showed less cytotoxic effect than cynaropicrin (Fig. 2), suggesting that eudesmin may be more useful as a model compound in new drug development. We also evaluated co-treatment effect of well-known TNF-α inhibitors on the pharmacology of eudesmin to determine the possible mechanism by which eudesmin attenuates TNF-α production, as mentioned in several papers (Lang *et al.*, 1995; Joseph and Isaacs, 1996; Sajjadi *et al.*, 1996). As shown in Table 3 all tested inhibitors, including protein kinase C (PKC)

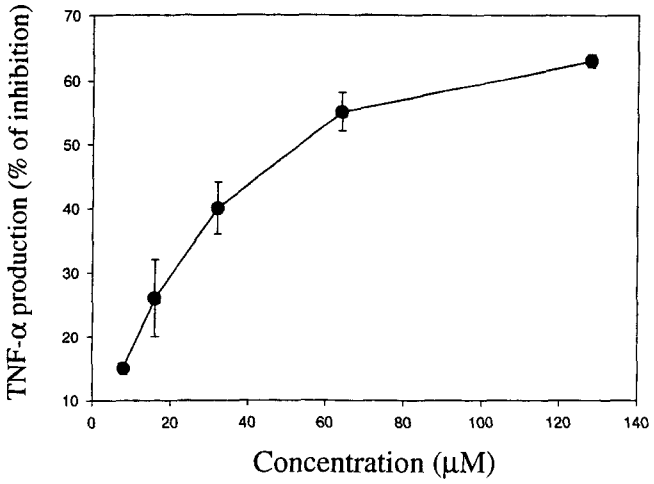

Fig. 3. The inhibitory effect of eudesmin on TNF-α production in RAW264.7 stimulated by 1 μg/ml of LPS for 5 h. RAW264.7 cells (110^6 cells/ml) were stimulated by 1 mg/ml of LPS with various concentrations of eudesmin. Supernatants were collected after 5 h and assayed by ELISA. Data represent mean ±SEM of 4 observations. Basal and stimulated level of TNF-α were 0.51.0 ng/ml and 25~30 ng/ml, respectively.

Table I. The molar concentrations of eudesmin producing 50% inhibition (IC_{50}) of TNF-α and lymphocyte proliferation.

Parameter	IC_{50} (μM)
TNF-α production	
Eudesmin	51.3±4.1
Cynaropicrin	8.2±1.6
dbcAMP	28.9±2.7
Pentoxifylline	243.1±17.8
Theophylline	448.9±27.4
Lymphocyte proliferation	
Eudesmin	154.2±12.5
A77,1726	6.7±1.4
dbcAMP	603.5±34.2

Data represent mean ±SEM of 4 observations.

Table II. Effect of known TNF-a inhibitor on TNF-α inhibitory activity of eudesmin in LPS-stimulated RAW264.7 cells.

Treatment	% of inhibition	
	alone	with eudesmin
None		40.3±5.1
Herbimycin A	38.7± 3.3	78.0±4.9
Staurosporin	44.9±10.1	83.6±7.2
Rolipram	44.7± 4.1	82.1±4.7
dbcAMP	52.6± 4.6	98.1±0.4
2-chloroadenosine	51.1± 1.8	90.8±7.3
Chloroquine	37.4± 5.5	48.9±9.1

PKC inhibitors (staurosporin, 25 nM), PTK inhibitors (herbimycin A, 500 μM), PKA activator (dbcAMP, 25 μM), cAMP PDE IV inhibitor (rolipram, 50 nM), pro-TNF-α processing inhibitor (chloroquine, 200 μM), and A3 receptor agonist (2-chloroadenosine) were tested alone or in combination with eudesmin (30 μM) on TNF-α production in LPS-stimulated RAW264.7 cells. Supernatants were collected after 5 h and assayed by ELISA. Data represent mean ±SEM of 4 observations.

inhibitor (staurosporin, 25 nM), protein tyrosine kinase (PTK) inhibitor (herbimycin A, 500 nM), cAMP phosphodiesterase IV inhibitors (rolipram 50 nM), protein kinase A (PKA) activator (dbcAMP, 25 μM), and A3 receptor agonist (2-chloroadenosine, 2.5 μM), showed additive inhibitory effects. There were no inhibitor showing a synergistic inhibition. Thus, these results suggest that these inhibitors may increase the therapeutic potential of eudesmin clinically against TNF-α mediated diseases. Additionally it is likely that none of these enzymes including PKC, PTK and PKA acts as the pharmacological target of eudesmin in the inhibition of TNF-α. Interestingly, however, the total inhibitory effect of the combination treatment with chloroquine and eudesmin was not additive suggesting that they may interact with the same enzyme(s). Since it has been demonstrated that chloroquine suppressed pro-TNF-α processing (Jung and Ju, 1997), the pathway, which requires detailed study, may be possibly related in inhibitory action by eudesmin.

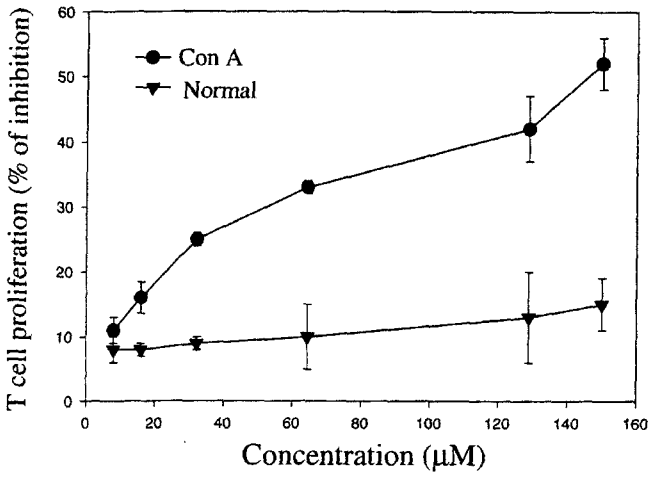

Fig. 4. Effect of eudesmin on T lymphocyte proliferation from mouse splenocytes in response to Con A. Splenocytes (5×10^6 cells/ml) were incubated with various concentrations of eudesmin in the presence or absence of 1 μg/ml of Con A or LPS for 48 h. Cell proliferation was assayed by conven-tional MTT method. Data represent mean ±SEM of 4 observations. Basal and stimulated OD values of lymphocyte were 0.200.21 (basal) and 1.401.45 (Con A), respectively.

Effect on lymphocyte proliferation

One method to control the progression of rheumatoid arthritis is to attenuate the proliferation or activation of T cells, since they play a central role in the pathophysiological state of the diseases (Panayi *et al.*, 1992). Therefore, we examined the inhibitory effects of eudesmin on lymphocyte proliferation from splenocyte in the presence of common T cell mitogen by MTT assay. In this assay, Con A was added at a concentration of 1 μg/ml to splenocytes. The proliferation of T lymphocytes treated by Con A was significantly increased by three to four compared to untreated cells. As shown in Fig. 4 and Table I, eudesmin significantly reduced the ability of lymphocytes to respond to Con A in a dose-dependent manner whereas t Con A slightly affected normal cell viability without any significance at concentrations tested (Fig. 4). Therefore, it was suggested that the inhibitory effect on T cell proliferation may increase the anti-inflammatory activity of eudesmin, although the effect was much lower than that of common immunosuppressants such as cyclosporin, leflunomide, and FK506 (Fruman *et al.*, 1992; Cherwinski *et al.*, 1995). Under the condition we have demonstrated that, A77,1726, an active metabolite of leflunomide, strongly suppressed T cell prol-

iferation from Con A-stimulated splenocytes with an IC_{50} values of 6.7 μM, although it did not inhibit TNF-α release (data not shown). However, dbcAMP also showed a lower inhibitory activity compared to A77,1726 as shown in eudesmin. Therefore, in terms of dual action against major pathological factors in inflammatory diseases it seems that eudesmin may have a beneficial effect for treatment of the diseases.

CONCLUSION

In summary, we have shown that eudesmin significantly inhibited either TNF-α production or T lymphocyte proliferation without any interference of normal cell function, suggesting that eudesmin may possess anti-inflammatory effect. Since the pathophysiological consequences of several diseases are caused by the excessive release of TNF-α and proliferation of T lymphocyte (Moncada *et al.*, 1991; Panayi *et al.*, 1992; Firestein, 1994; Sekut and Conolly 1996), it is conceivable that eudesmin may have beneficial effects clinically although activities were lower than those of other drugs. By the co-treatment experiment with several known TNF-α inhibitors, it was suggested that some TNF-α inhibitors may enhance the therapeutic effect of eudesmin.

ACKNOWLEDGEMENTS

We are grateful to Prof. Jee H. Jung (Pusan National University, Pusan, Korea) for providing cynaropicrin. We are also grateful to Ms. Sook Hee Chae, Mr. Ho Jung Son and Mr. Jisoo Park (R & D Center, Daewoong Pharm., Sungnam, Korea) for providing eudesmin and A77,1726, and for technical assistance.

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