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Potentiation of 1,25-dihydroxyvitamin D_3-induced differentiation of human promyelocytic leukemia cells into monocytes by costunolide, a germacranolide sesquiterpene lactone

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Abstract

Costunolide, a germacranolide sesquiterpene lactone that exists in several medicinal plants, is known to be a possible anti-cancer and chemopreventive agent for tumorigenesis. In this report, we investigated the effect of costunolide on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. Costunolide markedly increased the degree of HL-60 leukemia cell differentiation when simultaneously combined with 5 nM 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). Costunolide by itself had very weak effects on the differentiation of HL-60 cells. Cytofluorometric analysis and cell morphologic studies indicated that costunolide potentiated 1,25-$(OH)_2D_3$-induced cell differentiation predominantly into monocytes. Inhibitors for PKC, PI3-K, and ERK markedly inhibited HL-60 cell differentiation induced by costunolide in combination with 1,25-$(OH)_2D_3$. In addition, pretreatment of HL-60 cells with costunolide before the 1,25-$(OH)_2D_3$ addition also potentiated cell differentiation in a concentration- and time-dependent manner, and the enhanced levels of cell differentiation closely correlated with the inhibitory levels of NF-κB-binding activity by costunolide. These results indicate that PKC, PI3-K, ERK and NF-κB may be involved in 1,25-$(OH)_2D_3$-mediated cell differentiation enhanced by costunolide.

Keywords: Costunolide; Cell differentiation; 1,25-Dihydroxyvitamin D_3; Extracellular signal-regulated kinase; Nuclear factor-κB; Protein kinase C

1. Introduction

Most cancer cells exhibit a defect in their capacity to mature into non-replicating adult cells, thereby existing in a highly proliferating state, which results in outgrowing their normal cellular counterparts. The induction of terminal differentiation represents an alternative approach to the treatment of cancer by conventional anti-neoplastic agents since cells exposed to chemical or biological inducers of differentiation do not undergo the cytodestruction produced by cytotoxic agents. Instead they acquire the phenotypic characteristics of endo-stage adult cell forms with no replicative capacity and ultimately undergo programmed cell death. Leukemia cells can be induced to undergo terminal differentiation by a variety of chemical and biological agents, indicating that the malignant state is not an irreversible process. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cytodestructive agents [1].

Human promyelocytic leukemia HL-60 cells are differentiated into monocytic lineage when treated with 1,25-$(OH)_2D_3$ [2,3]. HL-60 cell culture has been employed as an excellent model system for studying cellular differentiation *in vitro*. 1,25-$(OH)_2D_3$ has been shown to be one of the most potent initiators of the differentiation of HL-60 cells

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Abbreviations: EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NBT, nitroblue tetrazolium; NF-κB, nuclear factor-kappaB; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

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as well as other hematopoietic cell lines and to activate a variety of protein kinases including PKC [4], MAPK [5] and PI3-K [6,7]. Other studies have demonstrated that interference with the activation of NF-κB appears to be a common feature for agents that enhance the differentiation of HL-60 cells induced by 1,25-$(OH)_2D_3$ [8,9].

Several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic and anti-inflammatory activity [10–13]. Cytostatic and cytocidal effects of sesquiterpenes against tumor cells have also been reported [14,15]. Costunolide, a well-known germacranolide sesquiterpene lactone, was isolated from many species such as *Saussurea lappa* [16], *Magnolia grandiflora* [17] and *Magnolia sieboldii* [18]. Costunolide exerts an anti-inflammatory activity by inhibiting the expression of inducible nitric oxide synthase [19] and the DNA-binding activity of NF-κB [20], an anti-viral activity by suppressing the expression of hepatitis B virus surface antigen [21], an anti-bacterial activity [22] and an anti-fungal activity [23]. Costunolide inhibits tyrosine phosphorylation induced by cross-linking of CD3 molecules in cytotoxic T lymphocytes [24]. These activities may be explained by effects on several of various protein kinase involved in the activation of cellular process such as PKC, MAPK, PI3-K and NF-κB. Costunolide is also known to be a possible chemopreventive agent for colon tumorigenesis [11] and human intestinal neoplasia [10] and is related to suppression of cell proliferation [12].

In this report, we investigated the effect of costunolide on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. We also investigated the effects of combinations of costunolide with 1,25-$(OH)_2D_3$ on HL-60 cell differentiation. 1,25-$(OH)_2D_3$ was chosen for this study because 1,25-$(OH)_2D_3$ has been widely used as an endogenous stimulator of differentiation in HL-60 cells, and analogues of vitamin D_3 including 1,25-$(OH)_2D_3$ are used clinically for the treatment of psoriasis [25].

2. Materials and methods

2.1. Materials

The HL-60 cell line was obtained from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. A stock solution of 1 mM 1,25-$(OH)_2D_3$ was dissolved in absolute ethanol and stored at −80°. Costunolide was dissolved in dimethylsulfoxide to make a stock solution of 20 mM. The solutions were diluted at least 1000-fold in the growth medium such that the final concentration of ethanol or dimethylsulfoxide had no effect on the differentiation and proliferation of HL-60 cells. All manipulations were performed in subdued light.

2.2. Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay as previously described [26]. Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μL of MTT (5 mg/mL) was added to each well in 96-well plates. After incubation for 4 hr at 37°, the crystals of viable cells were dissolved with 100 μL of 0.04 N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

2.3. Determination of cell differentiation

HL-60 cell differentiation was assessed by the NBT reduction assay as previously described [27]. This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with tissue plasminogen activator. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 0.1% NBT dissolved in PBS containing 200 ng/mL of freshly diluted tissue plasminogen activator at 37° for 30 min in the dark. Cytospin slides were prepared and examined for blue–black nitroblue diformazan deposits, indicative of a tissue plasminogen activator-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

2.4. Morphologic studies

Single-cell suspensions were prepared and 2×10^5 cells were loaded into a cyto-funnel and spun at 27 *g* in a cytospin centrifuge. The slides were fixed with methanol and dried. The slides were stained with Giemsa staining solution for 20 min and rinsed in deionized water, air dried, and observed under a microscope with a camera. The stained cells were assessed for size, regularity of the cell margin, and morphological characteristics of the nuclei.

2.5. Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic XL flow cytofluorograph equipped with a multi-parameter data acquisition and display system as previously described [28]. Briefly, single-cell suspensions were collected from the various cultures and washed twice with ice-cold PBS (pH 7.4). Thereafter, fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 mAb was added, followed by incubation at 4° for 1 hr. After incubation, the cells were washed and fixed in PBS containing 1% paraformaldehyde, after which cytofluorometric analysis was performed. Background staining was determined by substituting cells stained with FITC-conjugated isotype control mAb. One-parameter

fluorescence histograms were generated by analyzing at least 1×10^4 cells.

2.6. Preparation of cell lysates and Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5 containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/mL leupeptin, 50 μg/mL aprotinin, and 50 μg/mL PMSF) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 *g* at 4° for 10 min. The proteins in 15 μg of the supernatants were separated using a 10% SDS–PAGE and transferred to the pre-wetting PVDF membrane. The blots were probed with mouse anti-pERK and rabbit anti-ERK2 antibodies, washed and exposed to horseradish peroxidase-conjugated anti-mouse IgG2a and rabbit IgG antibodies, respectively. Immunoreactive bands were visualized by the enhanced chemiluminescence system.

2.7. Electrophoretic mobility shift assay

The nuclear extracts were prepared from the cells, as previously described [29]. An oligonucleotide containing an NF-κB-binding site within the Ig κ-chain (5′-CCG GTT AAC AGA GGG GGC TTT CCG AG-3′) was used as a probe. Labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 μg of nuclear extracts, in 20 μL of binding buffer (10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI-dC), and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris–borate buffer. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

2.8. Statistical analysis

The Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between values for various experimental and control groups. *P*-values <0.05 were considered significant.

3. Results

3.1. Effect of costunolide on 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation

To determine the effect of costunolide on 1,25-$(OH)_2D_3$-induced cell differentiation, the HL-60 cells were simultaneously treated with 5 nM of 1,25-$(OH)_2D_3$ in combination with various concentrations of costunolide, and the numbers of differentiated cells, as measured by NBT positivity, were determined. As controls, the cells were treated with costunolide alone. As shown in Fig. 1B, the

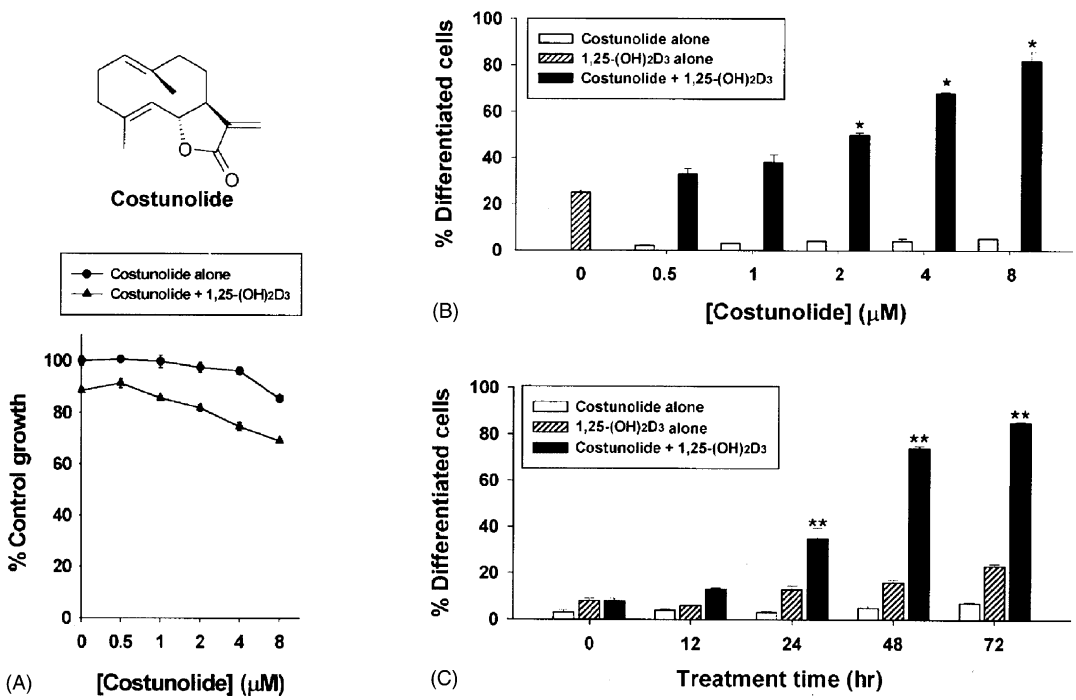

Fig. 1. Effect of costunolide on 1,25-$(OH)_2D_3$-induced HL-60 cell proliferation and differentiation. HL-60 leukemia cells were treated with 5 nM 1,25-$(OH)_2D_3$ alone or in combination with various concentrations of costunolide for 72 hr (A and B), or with 8 μM costunolide for various periods (C), and the cell proliferation was determined by the MTT assay (A) and the cell differentiation was assessed by the NBT reduction assay (B and C). Each value represents the mean ± SD of triplicate determinations from one representative experiment. The experiment was repeated more than three times with similar results. (*) $P < 0.001$, relative to a group treated with 1,25-$(OH)_2D_3$ alone. (**) $P < 0.05$, relative to the sum of the individual treatments.

addition of costunolide to cultures exposed to a suboptimal concentration of 1,25-$(OH)_2D_3$ (5 nM), which by itself caused a relatively low level of differentiation (<25%), resulted in a marked increase in the degree of cell differentiation. Costunolide strongly enhanced 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation in a concentration- and a time-dependant manner. The effects were maximal at 8 μM of costunolide, with greater than 81% of the treated cells attaining a differentiated state. Costunolide by itself did not induce significant cell differentiation, with less than 7% of the cells attaining a differentiated phenotype, suggesting that costunolide was a weak inducer of differentiation in HL-60 cells.

The cell proliferation and viability for each treatment group were determined. As shown in Fig. 1A, treatment with 8 μM costunolide inhibited cell proliferation by 10%, as determined by the MTT assay. Treatment with costunolide in combination with 5 nM 1,25-$(OH)_2D_3$ inhibited cell proliferation approximately by 11–30%. For all treatment, cells' viability was greater than 97% throughout the incubation period, as demonstrated by the trypan blue exclusion assay (data not shown).

To further confirm the cell differentiation enhanced by costunolide, the morphologic phenotypes and the expression of cell surface antigens on HL-60 cells were analyzed. As shown in Fig. 2, Giemsa-stained undifferentiated control HL-60 cells (Fig. 2A) were predominantly promyelocytes with round and regular cell margins, and large nuclei, suggesting that the cells were highly active in DNA synthesis and were rapidly proliferating. Costunolide- or 1,25-$(OH)_2D_3$-treated cells (Fig. 2B or C) showed relatively small changes in cell morphology such as irregular cell margins. Combined treatment of HL-60 cells with 1,25-$(OH)_2D_3$ plus costunolide (Fig. 2D) resulted in significantly denser chromatin and an increased cytoplasm to nuclear ratio, which suggested less DNA synthesis. As shown in Fig. 2D, some cells showed a horseshoe-shaped nucleus, which is a sign of cell differentiation into a monocytic lineage.

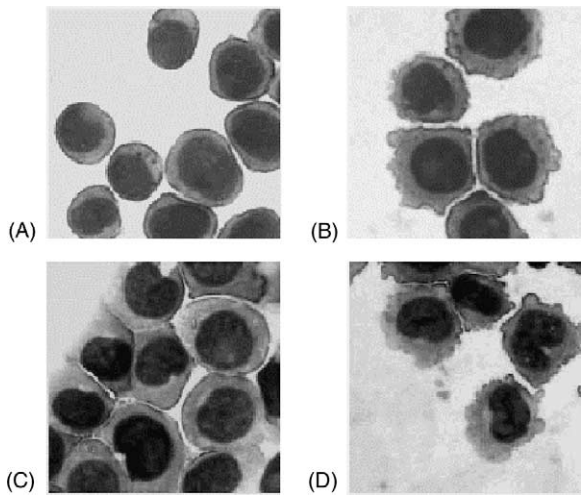

Fig. 2. Morphologic analysis of HL-60 cells treated with costunolide alone or in combination with 1,25-$(OH)_2D_3$. HL-60 cells were treated for 72 hr with medium alone (A), 8 μM costunolide (B), 5 nM 1,25-$(OH)_2D_3$ (C), or 8 μM costunolide plus 5 nM 1,25-$(OH)_2D_3$ (D). The cells were assessed by morphologic analysis using Giemsa stain. The data are representative of three separate experiments.

3.2. Effects of costunolide and 1,25-$(OH)_2D_3$ on differentiation pathways of HL-60 leukemia cells

To determine the differentiation pathway taken by HL-60 cells after treatment with costunolide and 1,25-$(OH)_2D_3$, HL-60 cells were first treated with costunolide alone or in combination with 1,25-$(OH)_2D_3$, and cytofluorometric analysis using mAb for the monocytic surface antigen CD14 was determined. The CD14 antigen is exclusively expressed when cells are differentiated into monocytes [30]. As shown in Fig. 3, HL-60 cells treated with a mixture of costunolide and 1,25-$(OH)_2D_3$ reacted very strongly with anti-CD14 mAb. Cells treated with 1,25-$(OH)_2D_3$ alone also reacted with anti-CD14 mAb, but to a lesser extent than did the cells treated with a mixture of costunolide and 1,25-$(OH)_2D_3$. These results indicate that costunolide stimulated 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation along the monocytic pathway.

3.3. Effect of inhibitors for PKC, PI3-K and ERK on HL-60 cell differentiation induced by costunolide in combination with 1,25-$(OH)_2D_3$

Previous studies have provided evidence that activation of PKC is necessary for the differentiation of HL-60 cells

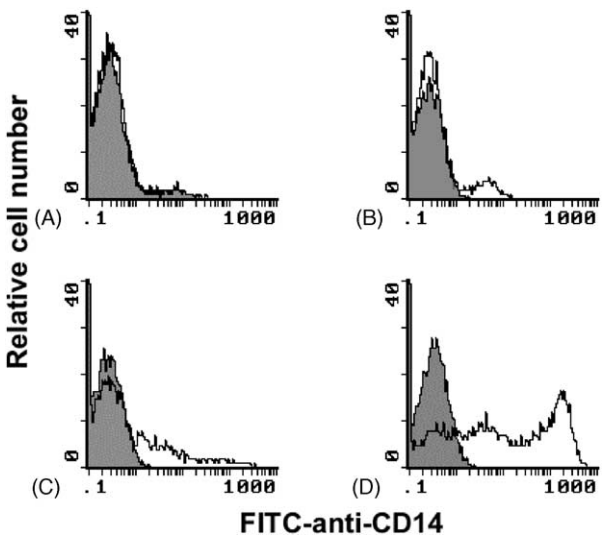

Fig. 3. Cytofluorometric analysis of costunolide-induced HL-60 cell differentiation. HL-60 cells were treated with medium alone (A), 8 μM costunolide (B), 5 nM 1,25-$(OH)_2D_3$ (C), or 8 μM costunolide in combination with 5 nM 1,25-$(OH)_2D_3$ (D) for 72 hr. The cells were assessed by cytofluorometric analysis using FITC-conjugated anti-CD14 mAb (unshaded area), or isotype control mAb (shaded area). The data are representative of three separate experiments.

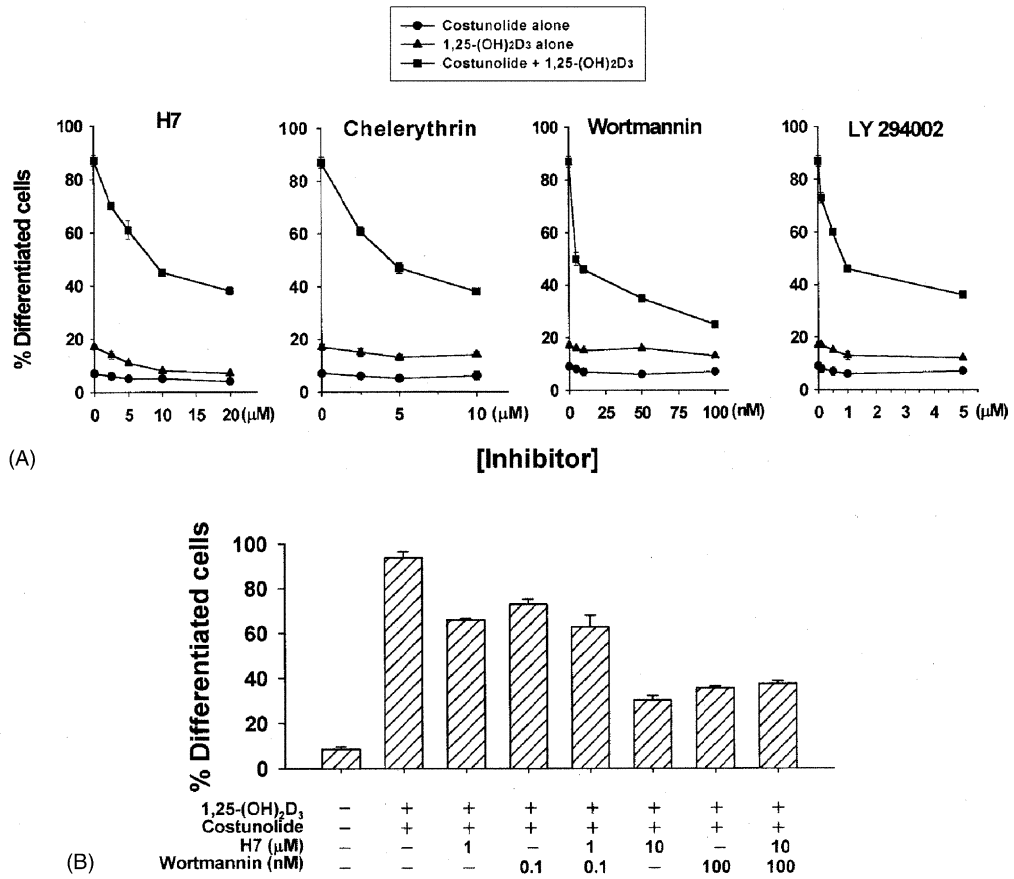

Fig. 4. Effect of PKC and PI3-K inhibitors on HL-60 cell differentiation induced by costunolide alone or in combination with 1,25-$(OH)_2D_3$. HL-60 cells were treated with varying concentrations of PKC inhibitors (H7, chelerythrine) or PI3-K inhibitors (wortmannin, LY 294002) for 40 min, followed by incubation with 8 μM costunolide, 5 nM 1,25-$(OH)_2D_3$, or 8 μM costunolide plus 5 nM 1,25-$(OH)_2D_3$ for 72 hr (A). HL-60 cells were treated with 1 μM H7, 0.1 nM wortmannin, 1 μM H7 plus 0.1 nM wortmannin for 40 min, followed by incubation with 8 μM costunolide in combination with 5 nM 1,25-$(OH)_2D_3$ for 72 hr (B). The cellular differentiation was assessed by the NBT reduction assay. The data are presented as a percentage of differentiated cells with the mean ± SEM (N = 3).

[4,31,32]. To determine any relationship between the effect of costunolide on cellular differentiation and PKC activation, HL-60 cells were treated with specific PKC inhibitors, 1,2-methoxy-12-methyl-[1,3]benzodioxolo[5,6-c]phenanthridinium (chelerythrine) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), in the presence of costunolide alone or in combination with 1,25-$(OH)_2D_3$. Afterwards, the degree of cellular differentiation was assessed by the NBT reduction assay. As shown in Fig. 4A, both PKC inhibitors significantly inhibited HL-60 cell differentiation induced by costunolide in combination with 1,25-$(OH)_2D_3$. The PKC inhibitors were not toxic to the HL-60 cells at concentrations used in the experiments, as demonstrated by the trypan blue exclusion assay (data not shown).

PI3-K activity has been also known to play an essential role in differentiation of HL-60 cells [7,33]. To determine any role of PI3-K in the 1,25-$(OH)_2D_3$-induced cell differentiation enhanced by costunolide, HL-60 cells were treated with specific PI3-K inhibitors, 3*H*-furo[4,3,2-de]indeno[4,5-h]-2-benzopyran-3,6,9-trione (wortmannin) or 2-(4-morpholinyl)-8-phenyl-1(4*H*)-1-benzopyran-4-one (LY 294002), in the presence of costunolide alone or in combination with 1,25-$(OH)_2D_3$. As shown in Fig. 4A, both PI3-K inhibitors significantly inhibited HL-60 cell differentiation treated with costunolide in combination with 1,25-$(OH)_2D_3$. The combinations of the inhibitors did not induce the additive effect of inhibition on the costunolide-mediated cell differentiation (Fig. 4B). In addition, the combinations could not reduce the enhanced cell differentiation to the baseline value at nontoxic concentrations of inhibitors on HL-60 cells.

ERK is one of the MAPKs and is a downstream element in the PKC signaling pathway of HL-60 cells [34]. To determine the involvement of ERK in 1,25-$(OH)_2D_3$-induced cell differentiation enhanced by costunolide, HL-60 cells were treated with 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059), a specific ERK kinase inhibitor, in the presence of costunolide alone or in combinations of 1,25-$(OH)_2D_3$. The synthetic compound, PD 98059, inhibits the ERK pathway by preventing the activation of ERK kinase by c-Raf [35]. As shown in Fig. 5A, PD 98059 inhibited HL-60 cell differentiation induced by costunolide in combination with 1,25-$(OH)_2D_3$. In contrast, p38 MAPK inhibitors, SB 203580 and SB 202190, did not inhibit the cell differentiation but

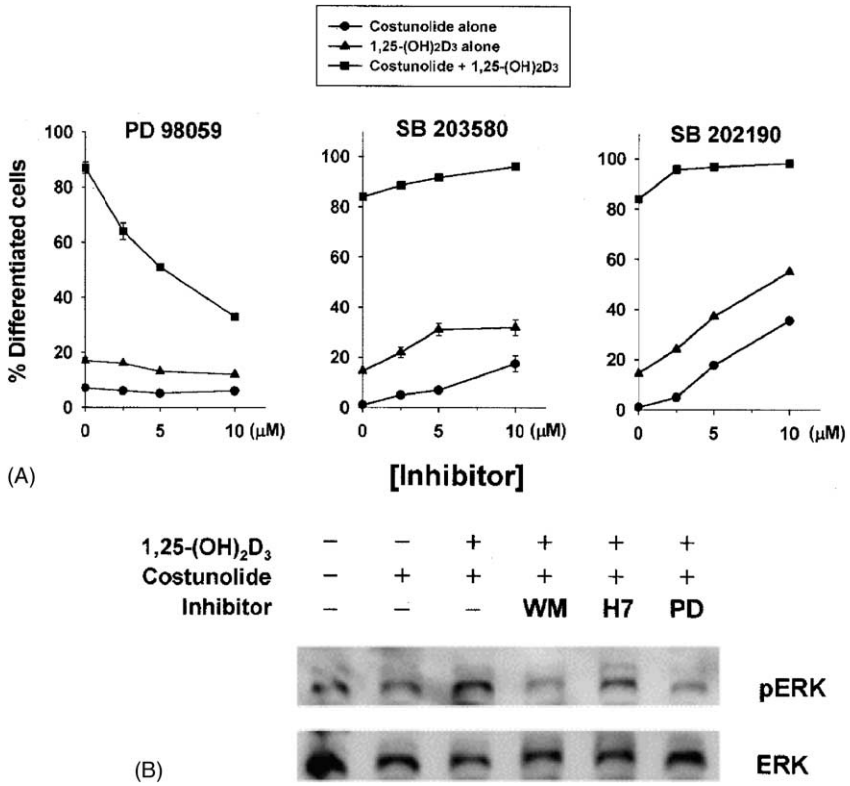

Fig. 5. Involvement of ERK in HL-60 cell differentiation induced by costunolide in combination with 1,25-$(OH)_2D_3$. (A) HL-60 cells were treated for 40 min with varying concentrations of ERK inhibitor (PD 98059) or p38 MAPK inhibitors (SB 203580, SB 202190), followed by incubation with 8 μM costunolide, 5 nM 1,25-$(OH)_2D_3$, or 8 μM costunolide in combination with 5 nM 1,25-$(OH)_2D_3$ for 72 hr. The cellular differentiation was assessed by the NBT reduction assay. The results are presented as a percentage of differentiated cells with the mean ± SEM (N = 3). (B) HL-60 cells were treated with 100 nM wortmannin (WM), 20 μM H7, or 10 μM PD 98059 (PD) for 40 min. The levels of ERK MAPK were determined by Western blot analysis at 1 hr after treatment with costunolide alone or in combination with 5 nM 1,25-$(OH)_2D_3$. The experiment was repeated twice with similar results.

increased HL-60 cell differentiation induced with costunolide alone or in combination with 1,25-$(OH)_2D_3$. Inhibitors for PKC and PI3-K inhibited the ERK activation stimulated by costunolide in combination with 1,25-$(OH)_2D_3$ (Fig. 5B).

Therefore, costunolide may potentiate 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation, and PKC, PI3-K and ERK may be involved in the cell differentiation synergistically enhanced by costunolide.

3.4. NF-κB is active in HL-60 leukemia cells and the inhibition of NF-κB activity by costunolide may be involved in the enhanced induction of HL-60 cell differentiation

Costunolide was shown to inhibit activation of NF-κB by preventing phosphorylation and degradation of IκBα and IκBβ in LPS-stimulated RAW 264.7 cells [36]. To ascertain whether the levels of costunolide employed were capable of causing the inhibition of NF-κB in unstimulated HL-60 leukemia cells, we analyzed nuclear extracts of HL-60 cells for NF-κB activity by an EMSA, using a probe specific for the κB DNA-binding motif of NF-κB. As shown in Fig. 6A, HL-60 cells showed relatively high levels of NF-κB DNA-binding activity in an untreated control condition. The binding was specific since it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, non-specific oligonucleotide. A 24 hr exposure to costunolide caused a marked reduction in the NF-κB DNA-binding activity both in the presence and absence of 1,25-$(OH)_2D_3$, while exposure of the cells to 5 nM 1,25-$(OH)_2D_3$ alone for 24 hr had no effect on the level of NF-κB complex. Interestingly, pretreatment with costunolide for 1 day, followed by washing and treatment with 1,25-$(OH)_2D_3$ alone in the absence of costunolide for 2 days, gave similar levels of enhancing effect on cellular differentiation, compared with those simultaneously treated with 1,25-$(OH)_2D_3$ plus costunolide for 2 days (Fig. 6B), suggesting that the inhibition of NF-κB-binding activity before the 1,25-$(OH)_2D_3$ addition was important for enhancing cell differentiation induced by low levels of 1,25-$(OH)_2D_3$. Furthermore, pretreatment with costunolide enhanced 1,25-$(OH)_2D_3$-induced cell differentiation in both a time- and dose-dependent manner, in which the enhanced levels of cell differentiation closely correlated with the inhibitory levels of NF-κB-binding activity by costunolide (Fig. 7).

To determine the effects of inhibitors for PKC, PI3-K and ERK on NF-κB activation in the costunolide-treated HL-60 cells, HL-60 cells were treated with costunolide,

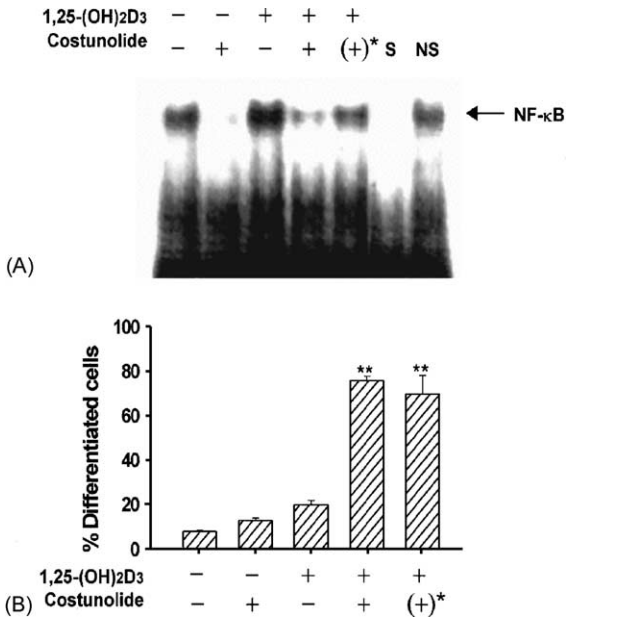

Fig. 6. Effect of costunolide on NF-κB binding to κB sites in HL-60 cells. HL-60 cells were treated with dimethylsulfoxide as a vehicle (control), 8 μM costunolide, 5 nM 1,25-$(OH)_2D_3$, or 8 μM costunolide in combination with 5 nM 1,25-$(OH)_2D_3$ for 24 hr (A) or 48 hr (B). Nuclear extracts from HL-60 cells were analyzed by EMSA for NF-κB DNA-binding activity using a labeled oligonucleotide containing a consensus Ig-κB site (A). The degree of cell differentiation was also assessed by the NBT assay (B). $(+)^*$ indicates that the cells were pretreated with 8 μM costunolide for 24 hr, then washed with RPMI medium before the incubation with 5 nM 1,25-$(OH)_2D_3$ for 24 hr (A) or 48 hr (B). S and NS indicate the presence of an unlabeled, identical oligonucleotide and non-specific oligonucleotide, respectively. Data are representative of two independent experiments. (**) $P < 0.01$, relative to a treated group with 5 nM 1,25-$(OH)_2D_3$ alone.

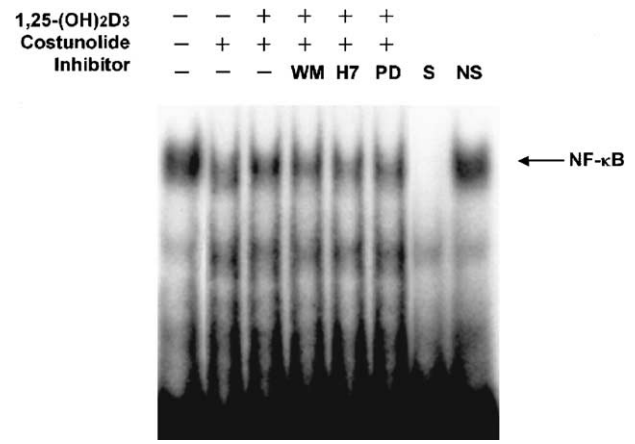

Fig. 8. Effect of PI3-K, PKC and ERK inhibitors on NF-κB activation induced by costunolide in combination with 1,25-$(OH)_2D_3$. HL-60 cells were pretreated with 8 μM costunolide for 24 hr. After washing with RPMI medium, the cells were treated with 100 nM wortmannin (WM), 20 μM H7, or 10 μM PD 98059 (PD) for 40 min, followed by incubation with 5 nM 1,25-$(OH)_2D_3$ for 48 hr, and analyzed by the EMSA for NF-κB DNA-binding activity. S and NS indicate the presence of an unlabeled, identical oligonucleotide and non-specific oligonucleotide, respectively. Data are representative of two independent experiments.

followed by treatment with each inhibitor. The levels of NF-κB DNA-binding activity were determined by the NF-κB EMSA. As shown in Fig. 8, costunolide inhibited NF-κB-binding activity in HL-60 cells. The inhibitors for PKC, PI3-K and ERK could not recover the NF-κB-binding activity inhibited by costunolide.

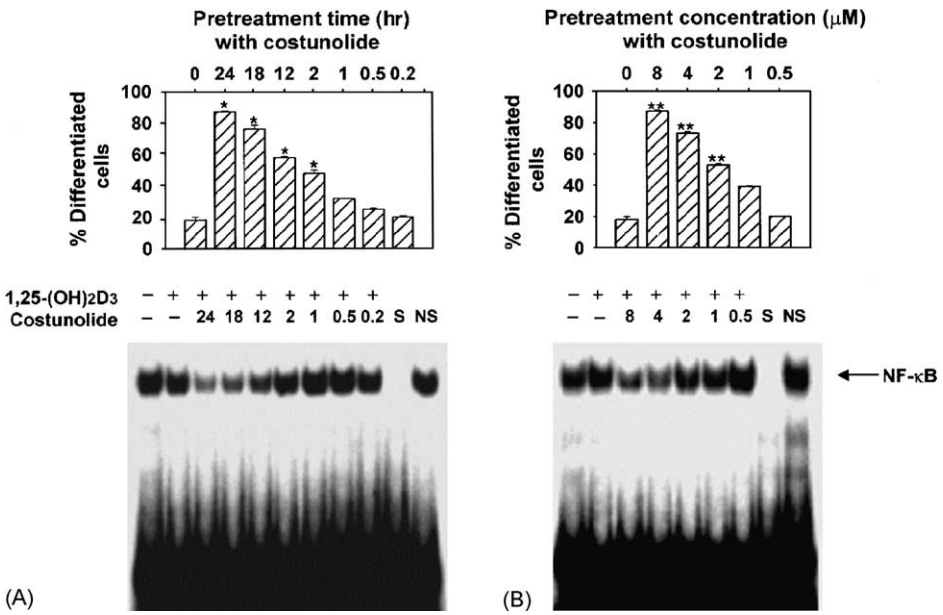

Fig. 7. Pretreatment effect of costunolide on 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation. HL-60 cells were pretreated with 8 μM costunolide for various periods (24, 18, 12, 2, 1, 0.5, 0.2 hr, respectively) or not pretreated (A), or with costunolide at various concentrations (8, 4, 2, 1, 0.5 μM, respectively) for 24 hr (B). After washing with RPMI, the cells were treated with 5 nM 1,25-$(OH)_2D_3$ for 48 hr, and analyzed by the NBT reduction assay for cellular differentiation and by the EMSA for NF-κB DNA-binding activity. S and NS indicate the presence of an unlabeled, identical oligonucleotide and non-specific oligonucleotide, respectively. Data are representative of two independent experiments. (*) $P < 0.001$, relative to a treated group with 5 nM 1,25-$(OH)_2D_3$ alone. (**) $P < 0.005$, relative to a treated group with 5 nM 1,25-$(OH)_2D_3$ alone.

4. Discussion

In the present study, we have demonstrated that costunolide potentiated 1,25-$(OH)_2D_3$-induced differentiation in HL-60 promyelocytic leukemia cells that are widely used as a model system for differentiation studies. HL-60 cells were synergistically differentiated into monocytes when treated with costunolide in combination with 1,25-$(OH)_2D_3$. Many previous studies have shown some chemical combinations which exerted an additive or synergistic effect on HL-60 cell differentiation. These combinations include hexafluoro-1,25-$(OH)_2D_3$ with sodium butyrate [37], 1,25-$(OH)_2D_3$ and silibinin or capsaicin [38,39], 1,25-$(OH)_2D_3$ and tumor necrosis factor-α [40], 1,25-$(OH)_2D_3$ and tretinoin tocoferil [41].

The mechanism by which costunolide potentiates 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation is not clear. 1,25-$(OH)_2D_3$ may mediate biological responses including the cell differentiation as a consequence of its interaction with nuclear receptors to regulate gene transcription [42] and with a putative cell membrane receptor to generate rapid non-genomic effects [43], the opening of voltage-gated calcium and chloride channels [44], and the activation of PKC, MAPK and PI3-K [4,7,45]. In our study, inhibitors for PKC, ERK and PI3-K significantly decreased HL-60 cell differentiation induced with costunolide in combination with 1,25-$(OH)_2D_3$, suggesting that PKC, PI3-K and ERK may be, at least in part, involved in the potentiation of 1,25-$(OH)_2D_3$-mediated cell differentiation by costunolide. Importantly, inhibitors for PKC and PI3-K inhibited ERK activation stimulated by costunolide in combination with 1,25-$(OH)_2D_3$ (Fig. 5B), indicating that PKC and PI3-K may be upstream components of ERK activation induced by costunolide. In contrast, p38 MAPK inhibitors, SB 203580 and SB 202190, increased HL-60 cell differentiation induced with costunolide alone or in combination with 1,25-$(OH)_2D_3$, in consistent with a previous report [46].

Many previous studies have demonstrated that interference with the activation of NF-κB appears to be involved in the enhanced differentiation of HL-60 cells induced by 1,25-$(OH)_2D_3$ [8,9]. Our NF-κB EMSA indicated that HL-60 leukemia cells showed relatively high levels of NF-κB activity that was not affected by 1,25-$(OH)_2D_3$ treatment. Costunolide, a sesquiterpene lactone with an inhibitory activity of NF-κB, enhanced 1,25-$(OH)_2D_3$-induced cell differentiation (Fig. 6). The pretreatment experiments showed that the enhanced levels of cellular differentiation by costunolide closely correlated with the inhibitory levels of NF-κB-binding activity (Fig. 7), suggesting possible involvement of NF-κB inhibition in the enhanced cell differentiation by costunolide. However, the role of NF-κB inhibition in the costunolide-mediated enhancement of cell differentiation should be further investigated. The inhibitors for PKC, PI3-K and ERK did not change the NF-κB-binding activity although the inhibitors significantly suppressed the enhanced cell differentiation by costunolide (Fig. 8). Furthermore, the combination of costunolide and 1,25-$(OH)_2D_3$ was likely to show less inhibitory effect on NF-κB-binding activity than costunolide alone although the combination enhanced the cell differentiation (Fig. 6).

Another possibility of the costunolide-mediated enhancing effect on cell differentiation is that costunolide may enhance cell differentiation, in part, by decreasing the catabolism of 1,25-$(OH)_2D_3$ *via* inhibition of cytochrome P450 enzymes. Some sesquiterpene lactones such as helenalin are known to inhibit cytochrome P450 enzymes [47] and isoforms of cytochrome P450 families are expressed in human myeloid leukemia cell lines [48]. The inhibition of cytochrome P450 enzymes may be mediated *via* inhibitory effect of NF-κB activity since many compounds induced cytochrome P450 enzymes by enhancing NF-κB activation [49,50].

1,25-$(OH)_2D_3$ and some of its analogues are also used for the treatment of psoriasis [51]. The results presented here suggest that treatment of patients with combinations of costunolide and 1,25-$(OH)_2D_3$ may produce a greater therapeutic response than 1,25-$(OH)_2D_3$ alone, possibly with less toxicity. Clinical studies are needed to evaluate this possibility, especially at concentrations of costunolide that do not induce known side-effects. It is possible that many dietary chemicals such as curcuminoids, tocopherols, carotenoids, and other edible plants can prevent human cancer, in part by synergizing with endogenously produced stimulators of differentiation such as 1,25-$(OH)_2D_3$ and retinoic acids. Epideminological studies suggest that people who eat large amounts of fruits and some vegetables have a lower risk of many kinds of cancer [52].

In summary, we have shown that costunolide potentiates 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation *via* the PKC, MAPK or PI3-K signaling pathway and that inhibition of NF-κB activation by costunolide could be a prerequisite to the efficient entry of promyelocytic leukemia cells into a differentiation pathway. These results may explain some known activities of costunolide, including its anti-carcinogenic effects and suggest a possible use of costunolide in the treatment of neoplastic diseases.

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References

[1] Beere HM, Hickman JA. Differentiation: a suitable strategy for cancer chemotherapy? Anti-Cancer Drug Design 1993;8:299–322.

[2] Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 1980;77:2936–40.
[3] Tanaka H, Abe E, Miyaura C, Shiina Y, Suda T. 1α,25-dihydroxyvitamin D_3 induces differentiation of human promyelocytic leukemia cells (HL-60) into monocytic macrophage, but not into granulocytes. Biochem Biophys Res Commun 1983;117:86–92.
[4] Pan Q, Granger J, O'Connell TD, Somerman MJ, Simpson RU. Promotion of HL-60 cell differentiation by 1,25-dihydroxyvitamin D_3 regulation of protein kinase C levels and activity. Biochem Pharmacol 1997;54:909–15.
[5] Marcinkowska E. Evidence that activation of MEK 1,2/erk 1,2 signal transduction pathway is necessary for calcitriol-induced differentiation of HL-60 cells. Anticancer Res 2001;21(1A):499–504.
[6] Marcinkowska E, Wiedlocha A, Radzikowski C. Evidence that phosphatidylinositol 3-kinase and p70S6K protein are involved in differentiation of HL-60 cells induced by calcitriol. Anticancer Res 1998;18:3507–14.
[7] Zakaria H, Devki N, Laura S, Keith LK, Patricia HV, Neil E. 1,25-Dihydroxyvitamin D_3-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. J Exp Med 1999;190:1583–94.
[8] Sokoloski JA, Shyam K, Sartorelli AC. Induction of the differentiation of HL-60 promyelocytic leukemia cells by curcumin in combination with low levels of vitamin D_3. Oncol Res 1997; 9:31–9.
[9] Sokoloski JA, Narayanan R, Sartorelli AC. Enhancement by antisense oligonucleotides to NF-κB of the differentiation of HL-60 promyelocytic leukemia cells induced by vitamin D_3. Cancer Lett 1998;125:157–64.
[10] Mori H, Kawamori T, Tanaka T, Ohnishi M, Yamahara J. Chemopreventive effect of costunolide, a constituent of oriental medicine, on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett 1994;83:171–5.
[11] Kawamori T, Tanaka T, Hara A, Yamahara J, Mori H. Modifying effects of naturally occurring products on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. Cancer Res 1995;55:1277–82.
[12] Ohnishi M, Yoshimi N, Kawamori T, Ino N, Hirose Y, Tanaka T, Yamahara J, Miyata H, Mori H. Inhibitory effects of dietary protocatechuic acid and costunolide on 7,12-dimethylbenz[*a*]anthracene-induced hamster cheek pouch carcinogenesis. Jpn J Cancer Res 1997;88:111–9.
[13] Hehner SP, Hofmann TG, Droge W, Schmitz ML. The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-κB by targeting the IκB kinase complex. J Immunol 1999;163:5617–23.
[14] Hall IH, Williams Jr WL, Grippo AA, Lee KH, Holbrook DJ, Chaney SG. Inhibition of nucleic acid synthesis in P-388 lymphocytic leukemia cells in culture by sesquiterpene lactones. Anticancer Res 1988;8:33–42.
[15] Ross JJ, Arnason JT, Birnboim HC. Low concentrations of the feverfew component parthenolide inhibit *in vitro* growth of tumor lines in a cytostatic fashion. Planta Med 1999;65:126–9.
[16] Lee MG, Lee KT, Chi SG, Park JH. Costunolide induces apoptosis by ROS-mediated mitochondrial permeability transition and cytochrome C release. Biol Pharm Bull 2001;24:303–6.
[17] el-Feraly FS, Chan YM. Isolation and characterization of the sesquiterpene lactones costunolide, parthenolide, costunolide diepoxide, santamarine, and reynosin from *Magnolia grandiflora* L. J Pharm Sci 1978;67:347–50.
[18] Park SH, Choi SU, Lee CO, Yoo SE, Yoon SK, Kim YK, Ryu SY. Costunolide, a sesquiterpene from the stem bark of *Magnolia sieboldii*, inhibits the RAS-farnesyl-protein transferase. Planta Med 2001;67:358–9.
[19] Park HJ, Jung WT, Basnet P, Kadota S, Namba T. Syringin 4-*O*-β-glucoside, a new phenylpropanoid glycoside, and costunolide, a nitric oxide synthase inhibitor, from the stem bark of *Magnolia sieboldii*. J Nat Prod 1996;59:1128–30.
[20] Castro V, Murillo R, Klaas CA, Meunier C, Mora G, Pahl HL, Merfort I. Inhibition of the transcription factor NF-κB by sesquiterpene lactones from *Podachaenium eminens*. Planta Med 2000;66: 591–5.
[21] Chen HC, Chou CK, Lee SD, Wang JC, Yeh SF. Active compounds from *Saussurea lappa* Clarks that suppress hepatitis B virus surface antigen gene expression in human hepatoma cells. Antiviral Res 1995;27:99–109.
[22] Fischer NH, Lu T, Cantrell CL, Castaneda-Acosta J, Quijano L, Franzblau SG. Antimycobacterial evaluation of germacranolides. Phytochemistry 1998;49:559–64.
[23] Wedge DE, Galindo JC, Macias FA. Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. Phytochemistry 2000;53: 747–57.
[24] Taniguchi M, Kataoka T, Suzuki H, Uramoto M, Ando M, Arao K, Magae J, Nishimura T, Otake N, Nagai K. Costunolide and dehydrocostus lactone as inhibitors of killing function of cytotoxic T lymphocytes. Biosci Biotechnol Biochem 1995;59:2064–7.
[25] Orfanos CE, Ehlert R, Gollnick H. The retinoids: a review of their clinical pharmacology and therapeutic use. Drugs 1987;34:459–503.
[26] Coligan JE, Kruisbeck AM, Margulies DH, Shevach EM, Strober W. Current protocols in immunology. New York: Wiley; 1995.
[27] Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. J Exp Med 1979;149:969–74.
[28] Kim TS, Kang BY, Lee MH, Choe YK, Hwang SY. Inhibition of interleukin-12 production by auranofin, an anti-rheumatic gold compound, deviates $CD4^+$ T cells from the Th1 to the Th2 pathway. Br J Pharmacol 2001;134:571–8.
[29] Chung SW, Kang BY, Kim SH, Pak YK, Cho D, Trinchieri G, Kim TS. Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB. J Biol Chem 2000;275:32681–7.
[30] Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431–3.
[31] Martell RE, Simpson RU, Hsu T. Effects of protein kinase inhibitors 1(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) and *N*-[2-quanidinoethyl]-5-isoquinolinesulfonamide hydrochloride (HA1004) on calcitriol-induced differentiation of HL-60 cells. Biochem Pharmacol 1988;37:635–40.
[32] Wu XZ, Shao GY, Chen S, Wang XW, Wang ZY. Studies on the relationship between protein kinase C and differentiation of human promyelocytic leukemia cells induced by retinoic acid. Leuk Res 1989;13:869–74.
[33] Bertagnolo V, Neri LM, Marchisio M, Mischiati C, Capitani S. Phosphoinositide 3-kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells. Cancer Res 1999;59:542–6.
[34] Marcinkowska E, Wiedlocha A, Radzikowski C. 1,25-Dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. Biochem Biophys Res Commun 1997;241:419–26.
[35] Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. J Biol Chem 1995;270:27489–94.
[36] Koo TH, Lee JH, Park YJ, Hong YS, Kim HS, Kim KW, Lee JJ. A sesquiterpene lactone, costunolide, from *Magnolia grandiflora* inhibits NF-κB by targeting I kappa B phosphorylation. Planta Med 2001;67:103–7.
[37] Yoshida M, Tanaka Y, Eguchi T, Ikekawa N, Saijo N. Effect of hexafluoro-1,25-dihydroxyvitamin D_3 and sodium butyrate combina-

tion on differentiation and proliferation of HL-60 leukemia cells. Anticancer Res 1992;12:1947–52.

[38] Kang SN, Lee MH, Kim K-M, Cho D, Kim TS. Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by silibinin: involvement of protein kinase C. Biochem Pharmacol 2001;61:1487–95.

[39] Kang SN, Chung SW, Kim TS. Capsaicin potentiates 1,25-dihydroxyvitamin D3- and all-*trans* retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Eur J Pharmacol 2001;420:83–90.

[40] Wang SY, Chen LY, Wang SJ, Lin CK, Ho CK. Growth inhibition and differentiation in HL-60 leukemia cells induced by 1α,25-dihydroxyvitamin D_3 and tumor necrosis factor α. Exp Hematol 1991; 19:1025–30.

[41] Makishima M, Kanatani Y, Yamamoto-Yamaguchi Y, Honma Y. Enhancement of activity of 1α,25-dihydroxyvitamin D_3 for growth inhibition and differentiation induction of human myelomonocytic leukemia cells by tretinoin tocoferil, a α-tocopherol ester of all-trans retinoic acid. Blood 1996;87:3384–94.

[42] Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 1998;13:325–49.

[43] Norman AW, Okamura WH, Hammond MW, Bishop JE, Dormanen MC, Bouillon R, van Baelen H, Ridall AL, Daane E, Khoury R, Farach-Carson MC. Comparison of 6-s-*cis*- and 6-s-*trans*-locked analogs of 1α,25-dihydroxyvitamin D_3 indicates that the 6-s-*cis* conformation is preferred for rapid nongenomic biological responses and that neither 6-s-*cis*- nor 6-s-*trans*-locked analogs are preferred for genomic biological responses. Mol Endocrinol 1997; 11:1518–31.

[44] Zanello LP, Norman AW. Stimulation by 1α,25$(OH)_2$-vitamin D_3 of whole cell chloride currents in osteoblastic ROS 17/2.8 cells. A structure–function study. J Biol Chem 1997;272:22617–22.

[45] Song X, Bishop JE, Okamura WH, Norman AW. Stimulation of phosphorylation of mitogen-activated protein kinase by 1α,25-dihydroxyvitamin D_3 in promyelocytic NB4 leukemia cells: a structure–function study. Endocrinology 1998;139:457–65.

[46] Wang X, Rao J, Studzinski GP. Inhibition of p38 MAPK kinase activity up-regulates multiple MAP kinase pathways and potentiates 1,25-dihydroxyvitamin D_3-induced differentiation of human leukemia HL60 cells. Exp Cell Res 2000;258:425–37.

[47] Chapman DE, Holbrook DJ, Chaney SG, Hall IH, Lee KH. *In vivo* and *in vitro* effects of helenalin on mouse hepatic microsomal cytochrome P450. Biochem Pharmacol 1991;41:229–35.

[48] Nagai F, Hiyoshi Y, Sugimachi K, Tamura HO. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. Biol Pharm Bull 2002;25:383–5.

[49] Gruebele A, Zawaski K, Kaplan D, Novak RF. Cytochrome P450E1- and cytochrome P4502B1/2B2-catalyzed carbon tetrachloride metabolism: effects on signal transduction as demonstrated by altered immediate-early (c-*fos* and c-*jun*) gene expression and nuclear AP-1 and NF-κB transcription factor levels. Drug Metab Dispos 1996;24:15–22.

[50] Puga A, Barnes SJ, Chang C, Zhu H, Nephew KP, Khan SA, Shertzer HG. Activation of transcription factors activator protein-1 and nuclear factor-κB by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Biochem Pharmacol 2000;59:997–1005.

[51] Kragballe K. Vitamin D_3 and skin diseases. Arch Dermatol Res 1992;284:30–6.

[52] Negri E, La Vecchia C, Franceschi S, D'Avanzo B, Parazzini F. Vegetable and fruit consumption and cancer risk. Int J Cancer 1991;48:350–4.