Biochemical Pharmacology 77 (2009) 1694–1701

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Nargenicin enhances 1,25-dihydroxyvitamin D_3- and all-*trans* retinoic acid-induced leukemia cell differentiation via PKCβI/MAPK pathways

Seung Hyun Kim [a], Jin Cheol Yoo [b,*], Tae Sung Kim [a,**]

[a] School of Life Sciences and Biotechnology, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea
[b] Department of Pharmacy, College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea

ARTICLE INFO

Article history:
Received 4 December 2008
Accepted 3 March 2009

Keywords:
Nargenicin
Cell differentiation
1,25-Dihydroxyvitamin D_3
All-*trans* retinoic acid
Protein kinase C
Mitogen-activated protein kinase

ABSTRACT

A major goal in the treatment of acute myeloid leukemia (AML) is to achieve terminal differentiation and prevent drug resistance and side effects. Combined treatment with low doses of ATRA or 1,25-$(OH)_2D_3$ that do not induce toxicity with another drug is one useful strategy for the treatment of AML. Actinomycetes are the well known sources of antibiotics and bioactive molecules. Previously, we isolated nargenicin from the culture broth of an actinomycete isolate, *Nocardia* sp. CS682. In this study, we evaluated the effects of nargenicin on cellular differentiation in a human myeloid leukemia HL-60 cell system. Nargenicin inhibited cell proliferation and induced HL-60 cell differentiation when administered in combination with 1,25-$(OH)_2D_3$ or ATRA. In addition, western blot analyses and kinase inhibitor studies demonstrated that nargenicin primarily enhanced leukemia cell differentiation via PKCβ1/MAPK pathways. The results of this study indicate that nargenicin has the ability to induce differentiation and suggest that it may be useful for the treatment of neoplastic diseases.

1. Introduction

The majority of leukemia cells exhibit deficiencies with regard to their capacity to mature into non-replicating adult cells and remain in a highly proliferative state, thereby resulting in their characteristic tendency to outgrow their normal cellular counterparts. The induction of terminal differentiation represents an alternative approach to the treatment of leukemia using conventional anti-neoplastic agents because cells exposed to chemical or biological inducers of cellular differentiation do not undergo the cytodestruction in response to cytotoxic agents [1–3]. 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and all-*trans* retinoic acid (ATRA) are known to induce terminal differentiation in leukemic cell lines including HL-60 and U937 cells [4]. Moreover, ATRA has been found to induce complete remission in almost all patients with acute promyelocytic leukemia (APL) via in vivo induction of the differentiation of APL blasts. Although ATRA can bring about complete remission in cases of APL, treatment with ATRA alone is associated with certain severe side effects, including ATRA syndrome and the induction of secondary ATRA resistance [5,6]. Therefore, it is necessary to isolate or synthesize new anticancer drugs to overcome these limitations and improve the effectiveness of drugs used to treat leukemia [7].

Human myeloid leukemia HL-60 cells are differentiated into the monocytic lineage or granulocytic lineage when treated with 1,25-$(OH)_2D_3$ or ATRA, respectively [8,9]. Accordingly, treatment with these compounds has been employed as a model system for studying cellular differentiation in vitro. In addition, vitamin D_3 has been shown to be one of the most potent initiators of the differentiation of HL-60 cells as well as other hematopoietic cell lines. Furthermore, vitamin D_3 is known to activate a variety of protein kinases including protein kinase C (PKC) [10,11], mitogen-activated protein kinase (MAPK) [12] and phosphatidylinositol 3-kinase (PI3-K) [13,14], which are significantly inhibited by their specific inhibitors [11–13]. Moreover, retinoids are also known to induce cell differentiation in cells with increased levels of PKC [10], MAPK [15] and PI3-K [9,13].

Natural products represent an important source of drugs such as anti-infectives and compounds used in cancer therapy. In addition, natural products are considered to be biologically validated lead structures, and it is anticipated that many compounds with novel or enhanced therapeutic effects will be developed from compounds present in natural product libraries [16]. Actinomycetes, which are widely distributed in the natural environment, are the source of various natural drugs, including rapamycin, lipstatin and thienamycin [17]. Furthermore, numerous anti-tumor metabolites with

** Corresponding author. Tel.: +82 2 3290 3416; fax: +82 2 3290 3921.
* Corresponding author.
E-mail addresses: jcyu@chosun.ac.kr (J.C. Yoo), tskim@korea.ac.kr (T.S. Kim).

Abbreviations: AML, acute myeloid leukemia; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NBT, nitroblue tetrazolium; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.03.004

a variety of structures are produced by actinomycetes [18]. Recently, we purified nargenicin from the novel actinomycete strain CS682, which was isolated from soil in Jeonnam, Korea. Nargenicin has strong antibacterial activity against methicilin-resistant *Staphylococcus aureus* [19].

In this study, we evaluated the use of nargenicin to induce leukemia cell differentiation, and further determined the underlying mechanisms involved in nargenicin-induced HL-60 cell differentiation when combined with low doses of 1,25-$(OH)_2D_3$ or ATRA. We found that nargenicin profoundly enhanced 1,25-$(OH)_2D_3$- or ATRA-induced cell differentiation via PKCβ1/MAPK pathways.

2. Materials and methods

2.1. Cell line and materials

HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% FBS (Gibco BRL, Grand Island, NY, USA). Nargenicin used in these experiments was characterized via FTIR spectroscopy, ESI-MS, ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H and ^{1}H–^{13}C COSY NMR to confirm its identity and purity, as previously described [19]. Nargenicin was then dissolved in DMSO to generate a 0.5 M stock solution. The solutions were diluted at least 1000-fold in the growth medium so that the final DMSO concentration had no effect on the differentiation and proliferation behavior of the HL-60 cells. P-nitroblue tetrazoliumchloride was purchased from the USB Co. (Cleveland, OH, USA). Chelerythrine, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H 7) and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from Tocris Cookson Ltd. (Bristol, UK). 1,25-$(OH)_2D_3$, all-*trans* retinoic acid (ATRA), phorbol 12-myristate 13-acetate (PMA), 2-[4-morpholinyl]-8phenyl-1[4H]-benzopyran-4-one (LY 294002), wortmannin, anthrapyrazolone (SP 600125), and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The rabbit anti-human PKC isoforms, mouse anti-pJNK, rabbit anti-JNK, mouse anti-pERK and rabbit anti-ERK2 antibodies, horseradish peroxidase-conjugated anti-mouse IgG2a and rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and phycoerythrin (PE)-conjugated anti-human CD11b, fluorescein isothiocyanate (FITC)-conjugated anti-human CD14, PE- or FITC-conjugated isotype control monoclonal antibodies were purchased from BD Bioscience (San Jose, CA, USA).

2.2. Determination of cell viability and growth

Cell viability was determined by a trypan blue exclusion assay, as previously described [8]. Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. Briefly, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. The samples were then incubated at 37 °C for 4 h, after which the formazan crystals formed in viable cells were dissolved with 100 μl of DMSO. The absorbance of each well at 540 nm was then read using a kinetic microplate reader.

2.3. Determination of cell differentiation

HL-60 cell differentiation was assessed by a nitroblue tetrazolium reduction assay as previously described [8]. This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, cells were harvested by centrifugation and then incubated with an equal volume of 1% nitroblue tetrazolium dissolved in PBS containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in the dark. Cytospin slides were then prepared and examined for blue–black nitroblue diformazan deposits, which are indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

2.4. Morphologic studies

Single-cell suspensions were prepared and loaded into a cytofunnel and spun at 500 rpm in a cytospin centrifuge. Next, the slides were fixed with methanol and then dried. The slides were stained with Giemsa staining solution for 20 min, 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

The expression of cell surface molecules was analyzed by cytofluorometry using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Briefly, a single-cell suspension was collected from each of the various cultures and washed twice with ice-cold PBS (pH 7.4). Next, PE-anti-human CD11b or FITC-anti-human CD14 monoclonal antibodies (BD Bioscience, San Jose, CA, USA) were added, and the samples were then incubated at 4 °C for 1 h. After incubation, the cells were washed with PBS, fixed in PBS containing 1% paraformaldehyde, and then analyzed by flow cytometry. Background staining was conducted by staining the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. 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 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 phenylmethylsulphonyl fluoride) by incubation on ice for 30 min. The lysates were then centrifuged at 13,000 rpm at 4 °C for 10 min, after which the proteins (10 μg) of the supernatants were separated by 10% SDS–PAGE and then transferred to a nitrocellulose membrane. Next, the blots were probed with rabbit anti-human PKC isoforms, mouse anti-pJNK, rabbit anti-JNK, mouse anti-pERK and rabbit anti-ERK2 antibodies, washed and then exposed to horseradish peroxidase-conjugated anti-mouse IgG2a or rabbit IgG antibodies (Santa Cruz Biotechnology. Inc., Santa Cruz, CA, USA). Immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

2.7. Statistical analysis

A Student's *t*-test and one-way analysis of variance were used to determine if significant differences existed between the values of various experimental and control groups. A P value <0.05 was considered to indicate statistical significance.

3. Results

3.1. Enhancing effect of nargenicin on 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation

To determine the effect of nargenicin on 1,25-$(OH)_2D_3$- and ATRA-induced cell differentiation, HL-60 leukemia cells were treated with nargenicin in combination with either 1,25-$(OH)_2D_3$

or ATRA, after which cellular differentiation was assessed by an NBT reduction assay. As controls, the cells were treated with nargenicin alone. As shown in Fig. 1D, treatment with nargenicin alone induced a relatively small increase of HL-60 cell differentiation approximately by 31%, whereas the combined treatments with a suboptimal concentration of 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA resulted in a marked increase in the degree of cell differentiation approximately by 82–85%. Furthermore, the cell proliferation and viability of each treatment group were determined. As shown in Fig. 1C, treatment with 200 μM nargenicin inhibited cell proliferation by 37–47%, as determined by an MTT assay. Treatment with nargenicin in combination with 1,25-$(OH)_2D_3$ or ATRA inhibited cell proliferation approximately by 6–9%. For all treatment, the cell viability was greater than 96% throughout the incubation period, as demonstrated by a trypan blue exclusion assay (Fig. 1B).

To further determine the cell differentiation enhanced by nargenicin, the morphologic phenotypes of HL-60 cells were analyzed. As shown in Fig. 1E, Giemsa-stained undifferentiated control HL-60 cells (upper left) were predominantly comprised of promyelocytes with round and regular cell margins, and large nuclei, suggesting that the cells were highly active in DNA synthesis and were rapidly proliferating. Cells that were treated with 200 μM nargenicin, 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA exhibited relatively small changes in cell morphology such as irregular cell margins. However, combined treatments of HL-60 cells with 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA plus 200 μM nargenicin resulted in a significantly decreased cell size, denser chromatin and an increased cytoplasm to nuclear ratio.

3.2. Dissection of differentiation pathways of HL-60 leukemia cells enhanced by nargenicin

Cytofluorometric analysis was performed to determine if differentiation-associated surface antigens were expressed on HL-60 cells. CD11b is a membrane constituent of specific granules, gelatinase granules and secretory vesicles [20]. HL-60 leukemia cells express CD11b, when differentiated into monocytes and granulocytes [8]. As shown in Fig. 2A, treatment with nargenicin resulted in a marked increase in the number of cells with a high fluorescence intensity, as well as a synergistic increase in the number of CD11b-positive cells when combined with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA. These findings confirm that nargenicin potentiated 1,25-$(OH)_2D_3$- or ATRA-induced differentiation of HL-60 cells. The CD14 antigen is exclusively expressed when cells are differentiated into monocytes [21]. Therefore, to determine the differentiation pathway following treatment with nargenicin and 1,25-$(OH)_2D_3$ or ATRA, cytofluorometric analysis was conducted in HL-60 cells treated with nargenicin alone or in combination with 1,25-$(OH)_2D_3$ or ATRA. As shown in Fig. 2B, HL-60 cells treated with a mixture of nargenicin and 1,25-$(OH)_2D_3$ reacted very strongly with anti-CD14 monoclonal antibody. Cells treated with 1,25-$(OH)_2D_3$ alone also reacted with anti-CD14 monoclonal antibody, but to a lesser extent than did the cells

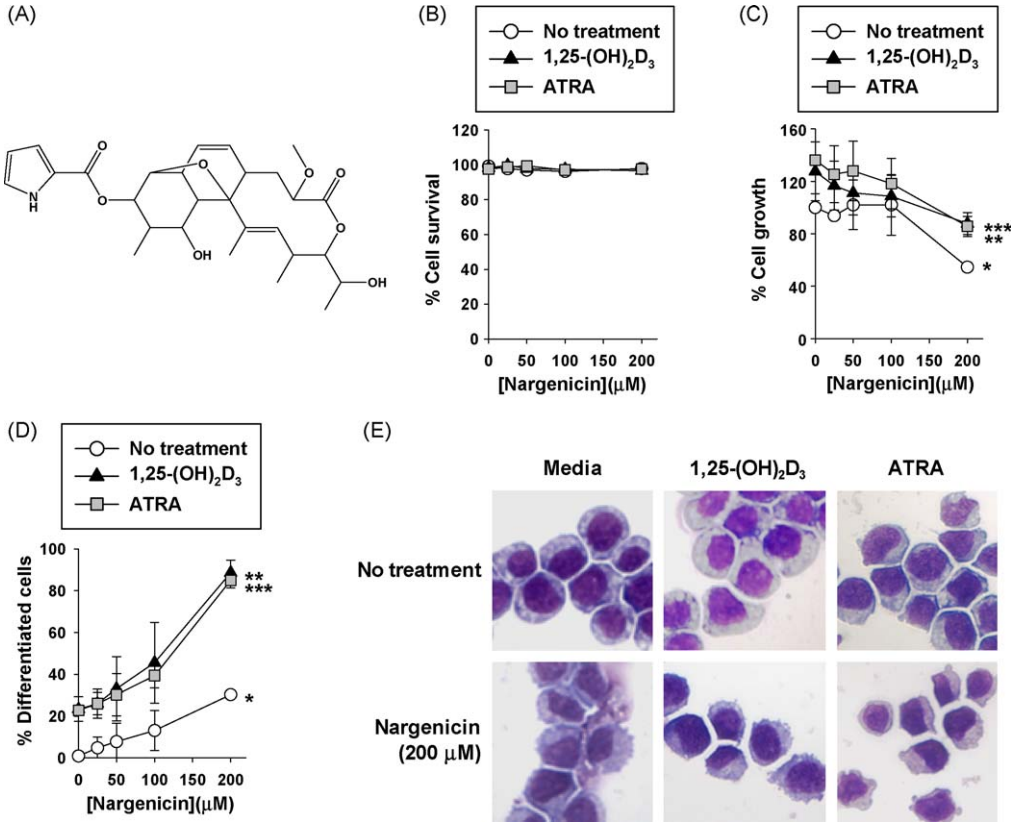

Fig. 1. Chemical structure of nargenicin and the effects of nargenicin on 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell proliferation and differentiation.
HL-60 leukemia cells were treated with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, or in combination with various concentrations of nargenicin (chemical structure, A) for 72 h. The viability of HL-60 cells was then measured by trypan blue assay (B), and cellular proliferation and differentiation were evaluated by an MTT assay (C) and NBT reduction assay (D), respectively. Data are expressed as the means ± standard deviations of triplicate determinations from one representative experiment. The differentiation experiment was repeated more than three times with similar results. $^{*}P < 0.01$, relative to an untreated group; $^{**}P < 0.01$, relative to a group treated with 5 nM 1,25-$(OH)_2D_3$ alone; $^{***}P < 0.01$, relative to a group treated with 50 nM ATRA alone. (E) HL-60 cells were treated for 72 h with media alone, 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone (top panel), 200 μM nargenicin alone, nargenicin plus 1,25-$(OH)_2D_3$, or nargenicin plus 50 nM ATRA (bottom panel). The cells were then assessed by morphologic analysis using Giemsa stain. The data are representative of three independent experiments.

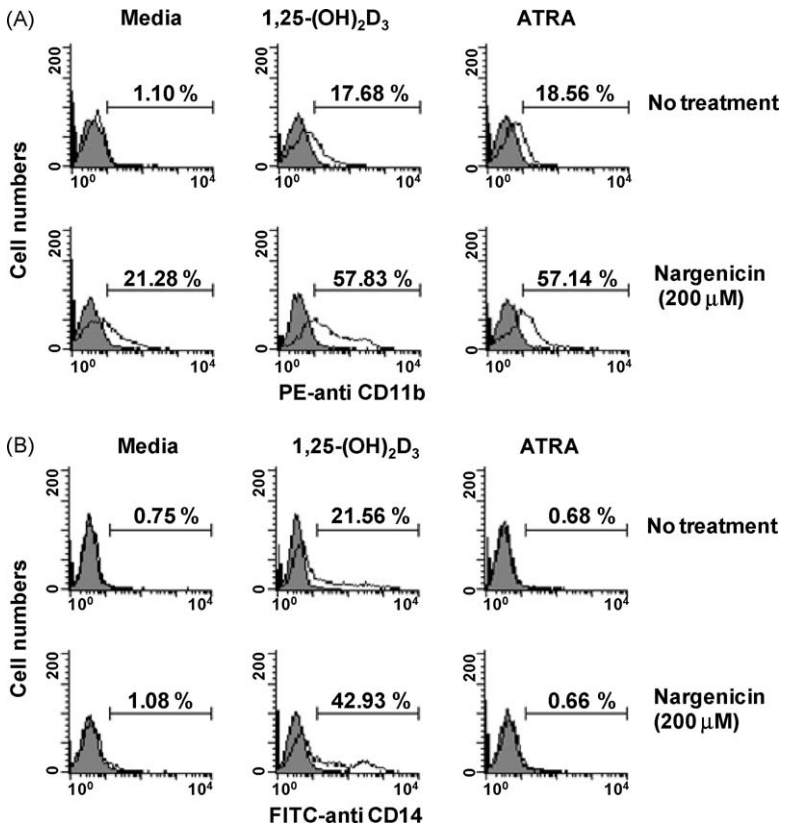

Fig. 2. Cytofluorometric analysis of HL-60 cells treated with nargenicin alone or in combination with 1,25-$(OH)_2D_3$ or ATRA.
HL-60 cells were treated for 72 h with media alone, 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, 200 μM nargenicin alone, 200 μM nargenicin plus 5 nM 1,25-$(OH)_2D_3$, or 200 μM nargenicin plus 50 nM ATRA. The cells were then assessed via cytofluorometric analysis using PE-conjugated anti-CD11b mAb (A) or FITC-conjugated anti-CD14 (B) (unshaded area), or with the PE- or FITC-conjugated isotype control mAb (shaded area). Data are representative of three independent experiments.

treated with a mixture of nargenicin and 1,25-$(OH)_2D_3$. These results indicate that nargenicin stimulated 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation along the monocytic pathway. In contrast, HL-60 cells treated with a mixture of nargenicin and ATRA exhibited little staining with anti-CD14 monoclonal antibody, although the synergistic induction of cell differentiation was observed in HL-60 cells treated with a mixture of nargenicin and ATRA as determined by an nitroblue tetrazolium reduction assay and a cytofluorometric analysis using a monoclonal antibody against HL-60 cell differentiation marker CD11b (Fig. 2B). These results indicate that nargenicin stimulated ATRA-induced HL-60 cell differentiation along the granulocytic pathway.

3.3. Sensitizing effects of nargenicin on 1,25-$(OH)_2D_3$- or ATRA-mediated HL-60 cell differentiation

Some differentiation-enhancing agents sensitize leukemia cells to other differentiation-inducing agents, resulting in a strong increase of cell differentiation [22]. In addition, it has been reported that the sequence of administration is important when combining drugs with conventional chemotherapy for the treatment of AML [23]. Previously, we simultaneously treated nargenicin with 1,25-$(OH)_2D_3$ or ATRA. To confirm if the sequence of exposure to nargenicin affected the levels of cell differentiation, HL-60 cells were pre-, co-, or post-treated with nargenicin, followed by treatment with 1,25-$(OH)_2D_3$ or ATRA. As shown in Fig. 3, pretreatment and simultaneous treatment with nargenicin significantly increased the levels of 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation, while post-treatment with nargenicin had smaller effects. Moreover, removing nargenicin prior to treatment with 1,25-$(OH)_2D_3$ or ATRA had no effects on

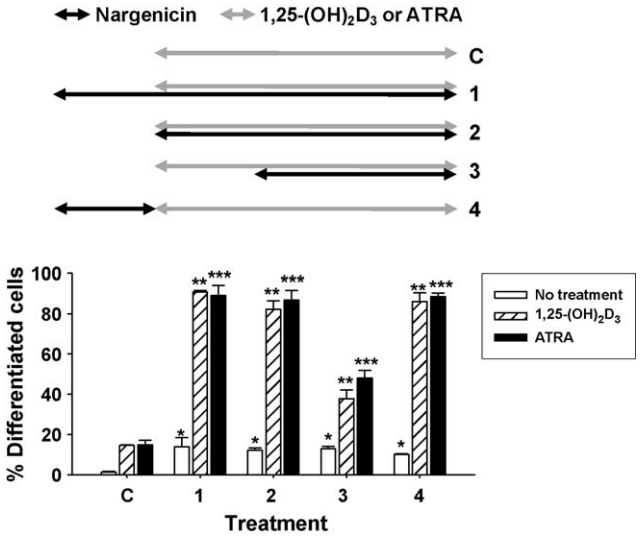

Fig. 3. Pretreatment effect of nargenicin on 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation.
HL-60 cells were incubated in the absence or presence of 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA (1–4) for 72 h with the addition of 200 μM nargenicin at 24 h before (group 1), at the time of (group 2), or 24 h after treatment with 1,25-$(OH)_2D_3$ or ATRA treatment (group 3). Alternatively, HL-60 cells were incubated for 24 h with nargenicin, followed by incubation with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone (group 4). The cell differentiation was then assessed by an NBT assay. $^{*}P < 0.01$, relative to an untreated group; $^{**}P < 0.01$, relative to a group treated with 5 nM 1,25-$(OH)_2D_3$ alone; $^{***}P < 0.01$, relative to a group treated with 50 nM ATRA alone.

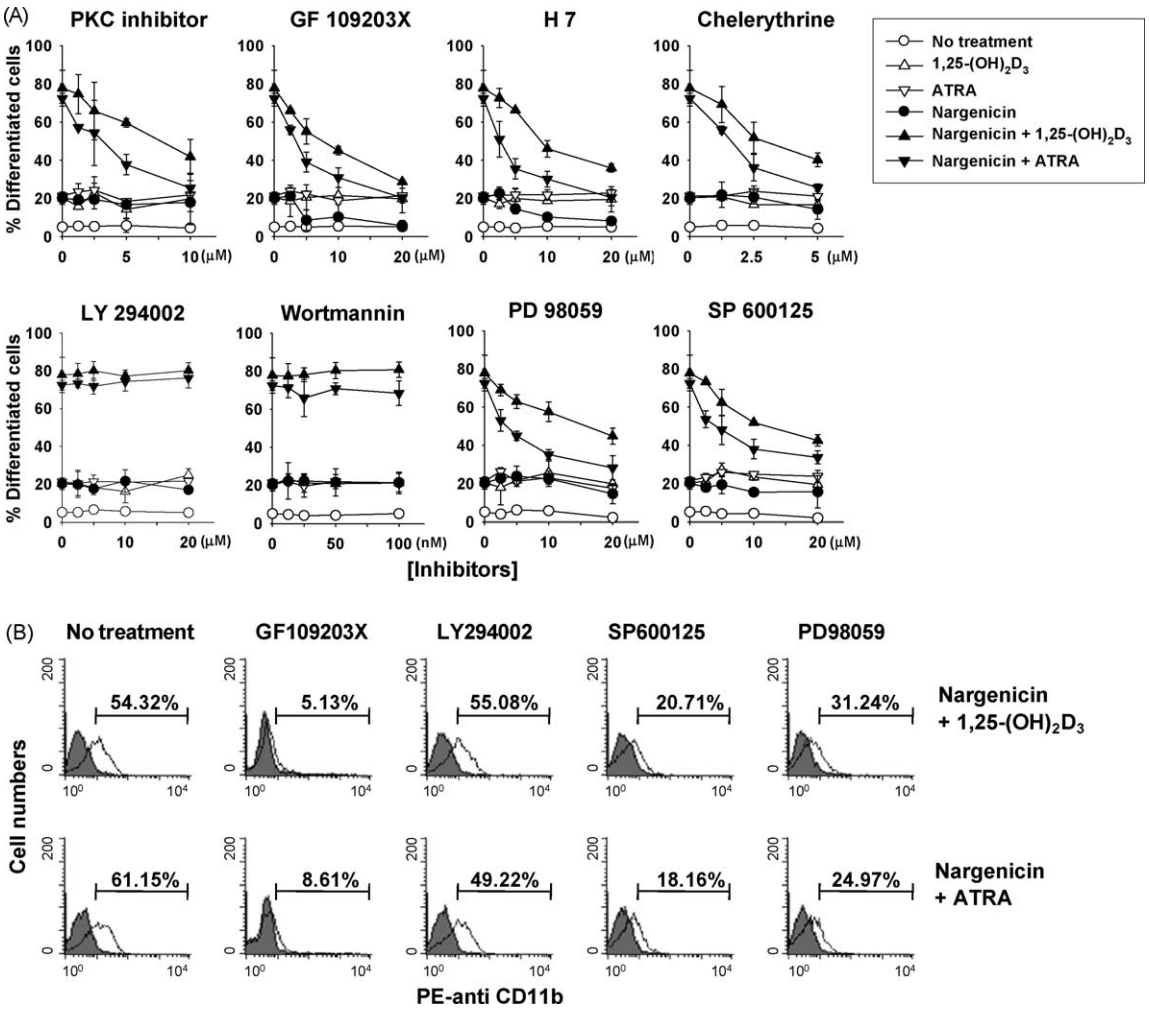

Fig. 4. Effect of PKC, JNK, PI3-K and ERK inhibitors on HL-60 cell differentiation induced by nargenicin alone or in combination with 1,25-$(OH)_2D_3$ or ATRA. (A) HL-60 cells were treated for 1 h with various concentrations of PKC inhibitors (PKC peptide inhibitor, GF 109203X, H 7, chelerythrine), JNK inhibitor (SP 600125), PI3-K inhibitors (wortmannin, LY 294002) or ERK inhibitor (PD 98059), followed by incubation with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, or in combination with 200 μM nargenicin. Cellular differentiation was then assessed by an NBT reduction assay. The differentiation data are expressed as a percentage of differentiated cells with the mean ± S.E. ($n = 3$). (B) Following the treatment with 10 μM of inhibitors (GF 109203X, LY 294002, PD 98059 or SP 600125) for 1 h, CD11b surface expression at 72 h in the treatment with 200 μM nargenicin plus 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA was assessed by cytofluorometric analysis using PE-conjugated anti-CD11b mAb (unshaded area) or with the PE-conjugated isotype control mAb (shaded area). Data are representative of two independent experiments.

HL-60 cell differentiation. These results indicate that nargenicin potentiated leukemia cell differentiation and sensitized the cells to 1,25-$(OH)_2D_3$ or ATRA, which resulted in enhancement of the cell differentiation.

3.4. Involvement of PKC, ERK and JNK in HL-60 cell differentiation enhanced by nargenicin

Previous studies have provided evidence that the activation of PKC is necessary for the differentiation of HL-60 cells [10,11] and that mitogen-activated protein kinases (MAPK) are downstream elements in the PKC signaling pathway of HL-60 cells [12]. In addition, the c-Jun N-terminal kinase (JNK) signaling module is also known to participate in myeloid cell differentiation [22,24]. Furthermore, phosphatidylinositol 3-kinase (PI3-K) activity plays an essential role in the differentiation of HL-60 cells [13,14].

To determine if there was a relationship between the enhancing effect of nargenicin on 1,25-$(OH)_2D_3$- or ATRA-induced cell differentiation and the activation of PKC, ERK, JNK and PI3-K, HL-60 cells were pretreated with specific inhibitors, followed by incubation for 72 h in the presence of nargenicin alone or in combination with either 1,25-$(OH)_2D_3$ or ATRA. The degree of cell differentiation was then assessed by a nitroblue tetrazolium reduction assay. As shown in Fig. 4, PKC, ERK and JNK inhibitors significantly inhibited HL-60 cell differentiation in cells that were treated with nargenicin in combination with 1,25-$(OH)_2D_3$ or ATRA, whereas PI3-K inhibitors did not inhibit cell differentiation enhanced by nargenicin.

To further investigate the involvement of PKC in HL-60 cell differentiation enhanced by nargenicin, HL-60 cells were treated with 200 μM nargenicin, after which the protein levels of PKC isoforms were determined by Western blot analysis using mAbs for each PKC isoform. In this study, we focused on conventional PKC isoforms such as α, βI, and βII because they are known to be most abundantly expressed in leukemia cells and their expression levels have been found to be closely correlated with cell differentiation in HL-60 cells. As shown in Fig. 5A, treatment with nargenicin led to increased levels of total PKC in HL-60 cells, which peaked at 12 h and then gradually decreased. Particularly, the protein levels of PKCβI were also elevated after 3 h and peaked at 12 h. Finally, combined treatment with nargenicin increased the total PKC and PKCβI levels in 1,25-$(OH)_2D_3$- or ATRA-treated HL-60 cells (Fig. 5B).

In determine if ERK and JNK were involved in the 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation enhanced by nargen-

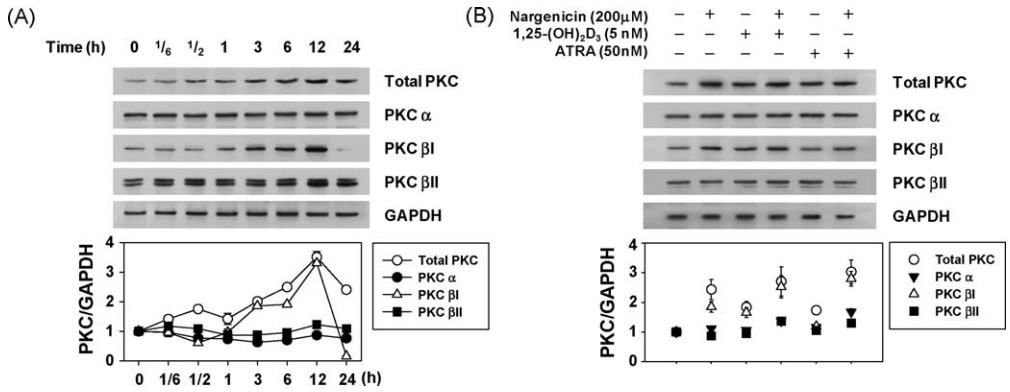

Fig. 5. Effect of nargenicin on the protein levels of conventional PKC isoforms in HL-60 cells.
HL-60 cells were treated with 200 μM nargenicin alone for the indicated time (0–24 h) (A). Cells were treated with 200 μM nargenicin alone or in combination with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA for 1 h (B). The protein levels of total PKC and conventional PKC isoforms (α, βI and βII) were then determined by Western blot analysis. The experiment was repeated twice with similar results.

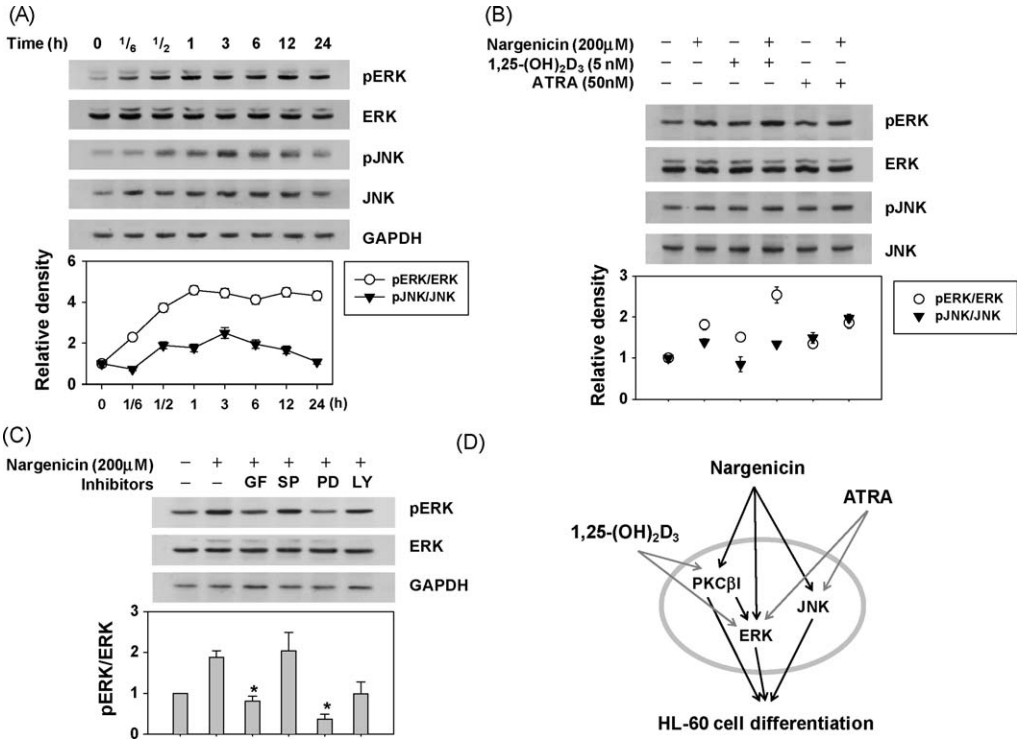

Fig. 6. Involvement of ERK and JNK in HL-60 cell differentiation enhanced by nargenicin.
HL-60 cells were treated with 200 μM nargenicin alone for the indicated time (0–24 h) (A). Cells were treated with 200 μM nargenicin alone or in combination with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA for 1 h (B). The protein levels of ERK and JNK were then determined by Western blot analysis. (C) HL-60 cells were pretreated with 10 μM GF 109203X (GF), 10 μM SP 600125 (SP), 10 μM PD 98059 (PD), or 10 μM LY 294002 (LY) for 1 h, followed by incubation with 200 μM nargenicin for 1 h, and then analyzed by Western blot analysis. Data are representative of two independent experiments. (D) A proposed mechanism of HL-60 cell differentiation in response to treatment with nargenicin administered in combination with 1,25-$(OH)_2D_3$ or ATRA.

icin, the protein levels of ERK and JNK were determined by Western blot analysis. As shown in Fig. 6A and B, the levels of pERK and pJNK were found to increase in response to treatment with nargenicin. To further investigate the involvement of PKC, JNK and ERK in 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation enhanced by nargenicin, HL-60 cells were treated with specific inhibitors for PKC, JNK and ERK in the presence of nargenicin alone or in combination with 1,25-$(OH)_2D_3$ or ATRA, after which the ERK activity in the treated cells was determined by Western blot analysis. As shown in Fig. 6C, inhibitors for PKC inhibited the activation of ERK stimulated by treatment with nargenicin in combination with 1,25-$(OH)_2D_3$ or ATRA in HL-60 cells, whereas inhibitors for JNK did not. Taken together, these findings indicate that PKC, JNK and ERK are involved in nargenicin-induced cell differentiation, and that PKCβI may be an upstream component of the ERK pathway in 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation enhanced by nargenicin.

4. Discussion

Many previous studies have revealed some chemical combinations that exerted an additive or synergistic effect on the differentiation of HL-60 cells. These combinations include plant-derived compounds and ATRA or 1,25-$(OH)_2D_3$ [25], arsenic trioxide and ATRA [7], COX inhibitors and 1,25-$(OH)_2D_3$ [26], histone deacetylase inhibitors and RA [27], and AM-80 and ATRA

[28]. In this study we demonstrated that nargenicin potentiated 1,25-$(OH)_2D_3$- and ATRA-induced differentiation of HL-60 leukemia cells, which are widely used as a model system in studies designed to evaluate differentiation. HL-60 cells were differentiated into either monocytes or granulocytes when treated with nargenicin in combination with 1,25-$(OH)_2D_3$ or ATRA, respectively.

The mechanism by which nargenicin potentiates 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation is not clear. However, it is believed that 1,25-$(OH)_2D_3$ and ATRA mediate biological responses such as cell differentiation as a result of their interaction with nuclear receptors to regulator gene transcription [29] and with a putative cell membrane receptor to generate rapid non-genomic effects, including the opening of voltage-gated calcium and chloride channels [30] and the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) [11–14].

The induction of differentiation in HL-60 cells requires the activation of a variety of signal transduction pathways, including the PI3-K, PKC and MAPK pathways [11–14]. Previous reports demonstrated the involvement of the MEK/ERK/MAP kinase pathway in PMA-, RA- and G-CSF-induced myeloid differentiation [31]. Additionally, the results of another study indicated that PMA-induced MAPK activation was dependent on PKC signaling in myeloid differentiation [32]. In the present study, inhibitors for PKC, JNK or ERK significantly inhibited the differentiation of HL-60 cells induced by nargenicin in combination with 1,25-$(OH)_2D_3$ or ATRA, suggesting that the potentiation of cell differentiation by nargenicin may occur via the PKC-, JNK- and ERK-mediated signaling pathway, at least when it is administered in combination with 1,25-$(OH)_2D_3$ or ATRA. In contrast, treatment with PI3-K inhibitors did not decrease the enhanced cell differentiation by nargenicin in the presence of low levels of 1,25-$(OH)_2D_3$ or ATRA. Indeed approximately 60–80% of the cells treated with nargenicin plus 1,25-$(OH)_2D_3$ or ATRA were still differentiated in the presence of the inhibitors, suggesting that PI3-K may not be involved in the enhancement of the 1,25-$(OH)_2D_3$ or ATRA-induced HL-60 cell differentiation by nargenicin.

Terminally differentiated cells undergo growth arrest. Several studies suggested that the differentiation inducing compounds could induce cell growth arrest [15,22]. HL-60 cells undergo G_0 cell cycle arrest and myeloid differentiation in response to ATRA or monocytic differentiation in response to 1,25-$(OH)_2D_3$ [33]. As shown in Fig. 1B and C, nargenicin inhibited HL-60 cell proliferation without toxic cell death, indicating that these drug combinations might induce the growth arrest of HL-60 cells.

1,25-$(OH)_2D_3$ is a secosteroid hormone that regulates calcium and bone metabolism, controls cell proliferation and differentiation, and plays an important role as an immunomodulator. Recently, 1,25-$(OH)_2D_3$ and its analogs were shown to have the potential for the treatment of diseases such as psoriasis, multiple sclerosis and rheumatoid arthritis [34,35]. ATRA, which is the first model of efficient differentiation therapy for malignant disease, induces terminal differentiation, thereby leading to the natural apoptosis of malignant cells. As a result, ATRA is the leading drug used for modification of the biological process involved in malignant cells [36]. Moreover, its analogs have been used for the treatment of psoriasis [37]. The results presented here suggest that treatment of patients with combinations of nargenicin and 1,25-$(OH)_2D_3$, or nargenicin and ATRA may produce a greater therapeutic response than treatment with 1,25-$(OH)_2D_3$ or ATRA alone, possibly with less toxicity. However, clinical studies are needed to evaluate this possibility, especially at concentrations of nargenicin that do not induce known side effects.

In conclusion, we have shown that nargenicin potentiates 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation via PKC/MAPK signaling pathways. These results may explain some of the known activities of nargenicin, including its anti-carcinogenic effects.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A010385) and Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea, 2007.

References

[1] Warner JK, Wang JC, Hope KJ, Jin L, Dick JE. Concepts of human leukemic development. Oncogene 2004;23:7164–77.
[2] Sell S. Leukemia: stem cells, maturation arrest, and differentiation therapy. Stem Cell Rev 2005;1:197–205.
[3] Zhou GB, Zhang J, Wang ZY, Chen SJ, Chen Z. Treatment of acute promyelocytic leukaemia with all-trans retinoic acid and arsenic trioxide: a paradigm of synergistic molecular targeting therapy. Philos Trans R Soc Lond B Biol Sci 2007;362:959–71.
[4] James SY, Williams MA, Newland AC, Colston KW. Leukemia cell differentiation: cellular and molecular interactions of retinoids and vitamin D. Gen Pharmacol 1999;32:143–54.
[5] Tallman MS. Differentiating therapy in acute myeloid leukemia. Leukemia 1996;10:1262–8.
[6] Patatanian E, Thompson DF. Retinoic acid syndrome. J Clin Pharm Ther 2008;33:331–8.
[7] Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008;111:2505–15.
[8] Kim SH, Kang SN, Kim HJ, Kim TS. Potentiation of 1,25-dihydroxyvitamin D_3-induced differentiation of human promyelocytic leukemia cells into monocytes by costunolide, a germacranolide sesquiterpene lactone. Biochem Pharmacol 2002;64:1233–42.
[9] Matkovic K, Brugnoli F, Bertagnolo V, Banfic H, Visnjic D. The role of the nuclear Akt activation and Akt inhibitors in all-*trans*-retinoic acid-differentiated HL-60 cells. Leukemia 2006;20:941–51.
[10] Zimber A, Chedeville A, Abita JP, Barbu V, Gespach C. Functional interactions between bile acids, all-*trans* retinoic acid, and 1,25-dihydroxyvitamin D_3 on monocytic differentiation and myeloblastin gene down-regulation in HL60 and THP-1 human leukemia cells. Cancer Res 2000;60:672–8.
[11] 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.
[12] Marcinkowska E, Garay E, Gocek E, Chrobak A, Wang X, Studzinski GP. Regulation of C/EBPbeta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D_3. Exp Cell Res 2006;312:2054–65.
[13] Yamada O, Ozaki K, Nakatake M, Akiyama M, Kawauchi K, Matsuoka R. Multistep regulation of telomerase during differentiation of HL60 cells. J Leukoc Biol 2008;83:1240–8.
[14] Hughes PJ, Lee JS, Reiner NE, Brown G. The vitamin D receptor-mediated activation of phosphatidylinositol 3-kinase (PI3Kalpha) plays a role in the 1alpha,25-dihydroxyvitamin D_3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cell lines. J Cell Biochem 2008;103:1551–72.
[15] Wang J, Yen A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. J Biol Chem 2008;283:4375–86.
[16] Weist S, Süssmuth RD. Mutational biosynthesis—a tool for the generation of structural diversity in the biosynthesis of antibiotics. Appl Microbiol Biotechnol 2005;68:141–50.
[17] Ganesan A. The impact of natural products upon modern drug discovery. Curr Opin Chem Biol 2008;12:306–17.
[18] Paradkar A, Trefzer A, Chakraburtty R, Stassi D. Streptomyces genetics: a genomic perspective. Crit Rev Biotechnol 2003;23:1–27.
[19] Sohng JK, Yamaguchi T, Seong CN, Baik KS, Park SC, Lee HJ, et al. Production, isolation and biological activity of nargenicin from *Nocardia* sp. CS682. Arch Pharm Res 2008;31:1339–45.
[20] Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 2003;5:1317–27.
[21] Warren MK, Rose WL, Beall LD, Cone J. CD34+ cell expansion and expression of lineage markers during liquid culture of human progenitor cells. Stem Cells 1995;13:167–74.
[22] Wang Q, Salman H, Danilenko M, Studzinski GP. Cooperation between antioxidants and 1,25-dihydroxyvitamin D3 in induction of leukemia HL60 cell differentiation through the JNK/AP-1/Egr-1 pathway. J Cell Physiol 2005;204:964–74.
[23] Levis M, Pham R, Smith BD, Small D. In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects. Blood 2004;104:1145–50.
[24] Wang Q, Harrison JS, Uskokovic M, Kutner A, Studzinski GP. Translational study of vitamin D differentiation therapy of myeloid leukemia: effects of the

combination with a p38 MAPK inhibitor and an antioxidant. Leukemia 2005;19:1812–7.
[25] Kim SH, Danilenko M, Kim TS. Differential enhancement of leukaemia cell differentiation without elevation of intracellular calcium by plant-derived sesquiterpene lactone compounds. Br J Pharmacol 2008;155: 814–25.
[26] Jamshidi F, Zhang J, Harrison JS, Wang X, Studzinski GP. Induction of differentiation of human leukemia cells by combinations of COX inhibitors and 1,25-dihydroxyvitamin D_3 involves Raf1 but not Erk 1/2 signaling. Cell Cycle 2008;7:917–24.
[27] Savickiene J, Treigyte G, Borutinskaite V, Navakauskiene R, Magnusson KE. The histone deacetylase inhibitor FK228 distinctly sensitizes the human leukemia cells to retinoic acid-induced differentiation. Ann N Y Acad Sci 2006;1091: 368–84.
[28] Ishida S, Shigemoto-Mogami Y, Shinozaki Y, Kagechika H, Shudo K, Ozawa S, et al. Differential modulation of PI3-kinase/Akt pathway during all-trans retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis. Biochem Pharmacol 2004;68: 2177–86.
[29] Si J, Mueller L, Schuler A, Simon J, Collins SJ. The retinoic acid receptor/CaMKII interaction: pharmacologic inhibition of CaMKII enhances the differentiation of myeloid leukemia cells. Blood Cells Mol Dis 2007;39:307–15.
[30] Norman AW, Bishop JE, Bula CM, Olivera CJ, Mizwicki MT, Zanello LP, et al. Molecular tools for study of genomic and rapid signal transduction responses initiated by 1 alpha,25$(OH)_2$-vitamin D_3. Steroids 2002;67:457–66.
[31] Miranda MB, McGuire TF, Johnson DE. Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia 2002;16:683–92.
[32] Schultz H, Engel K, Gaestel M. PMA-induced activation of the p42/44ERK- and p38RK-MAP kinase cascades in HL-60 cells is PKC dependent but not essential for differentiation to the macrophage-like phenotype. J Cell Physiol 1997;173: 310–8.
[33] Shen M, Yen A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. Cancer Res 2008;68:8761–9.
[34] Mathieu C, Jafari M. Immunomodulation by 1,25-dihydroxyvitamin D_3: therapeutic implications in hemodialysis and renal transplantation. Clin Nephrol 2006;66:275–83.
[35] Wolters M. Diet and psoriasis: experimental data and clinical evidence. Br J Dermatol 2005;153:706–14.
[36] Degos L, Wang ZY. All *trans* retinoic acid in acute promyelocytic leukemia. Oncogene 2001;20:7140–5.
[37] van de Kerkhof PC. Update on retinoid therapy of psoriasis in: an update on the use of retinoids in dermatology. Dermatol Ther 2006;19:252–63.