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Differential involvement of protein kinase C in human promyelocytic leukemia cell differentiation enhanced by artemisinin

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Abstract

Artemisinin, a sesquiterpene lactone endoperoxide that exists in several medicinal plants, is a well-known anti-malarial agent. In this report, we investigated the effect of artemisinin on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. Artemisinin markedly increased the degree of HL-60 leukemia cell differentiation when simultaneously combined with low doses of 1α,25-dihydoxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid (all-*trans* RA). Artemisinin by itself had very weak effects on the differentiation of HL-60 cells. Cytofluorometric analysis and cell morphologic studies indicated that artemisinin potentiated 1,25-$(OH)_2D_3$-induced cell differentiation predominantly into monocytes and all-*trans* RA-induced cell differentiation into granulocytes, respectively. Extracellular-regulated kinase (ERK) inhibitors markedly inhibited HL-60 cell differentiation induced by artemisinin in combination with 1,25-$(OH)_2D_3$ or all-*trans* RA, whereas phosphatidylinositol 3-kinase (PI3-K) inhibitors did not. Particularly, protein kinase C (PKC) inhibitors inhibited HL-60 cell differentiation induced by artemisinin in combination with 1,25-$(OH)_2D_3$ but not with all-*trans* RA. Artemisinin enhanced PKC activity and protein level of PKCβI isoform in only 1,25-$(OH)_2D_3$-treated HL-60 cells. Taken together, these results indicate that artemisinin strongly enhanced 1,25-$(OH)_2D_3$- and all-*trans* RA-induced cell differentiation in which PKC is differentially involved in arteminisin-mediated enhancement of leukemia cell differentiation.

Keywords: Artemisinin; Leukemia; Cell differentiation; PKC (protein kinase C); ERK (extracellular-regulated kinase)

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 end-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 irreversible process. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cytodestructive agents (Beere and Hickman, 1993).

Human promyelocytic leukemia HL-60 cells are differentiated into monocytic lineage or granulocytic lineage when treated with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid (all-*trans* RA), respectively (Breitman et al., 1980; Tanaka et al., 1983). 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 as well as other hematopoietic cell lines and to activate a variety of protein kinases including protein kinase C (PKC) (Pan et al., 1997), mitogen-activated protein kinase (MAPK) (Kharbanda et al., 1994) and phosphatidylinositol 3-kinase (PI3-K) (Marcinkowska et al., 1998), which were significantly inhibited by their inhibitors (Martell et al., 1987; Zakaria et al., 1999).

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Retinoids are also induced cell differentiation with the increased levels of PKC (Wu et al., 1989), MAPK (Yen et al., 1998) and PI3-K (Bertagnolo et al., 1999).

Several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic and anti-inflammatory activity (Mori et al., 1994; Kawamori et al., 1995; Ohnishi et al., 1997; Hehner et al., 1999). Cytostatic and cytocidal effects of sesquiterpenes against tumor cells have also been reported (Hall et al., 1988; Ross et al.,1999). Artemisinin, a sesquiterpene lactone endoperoxide, is isolated from *Artemisia annua* (sweet or annual wormwood) that has been used as a herbal remedy for fever and malaria. Artemisinin and its derivatives have impressive activity against multidrug-resistant forms of *Plasmodium falciparum* both in vivo and in vitro (Klayman, 1985; Trigg, 1989; Sowumni and Oduola, 1994; White, 1994; Sowumni and Oduola, 1998; van Vugt et al., 1998). The mechanism of artemisinin appears to involve the intraparasitic iron- or heme-catalyzed cleavage of the endoperoxide bridge to generate toxic free radicals or intermediates (Meshnick et al., 1991; Posner and Oh, 1992; Zhang et al., 1992; Meshnick et al., 1993). Recent studies suggested that artemisinin and the derivatives enhance the reactive oxygen response of neutrophils but depress their phagocytic ability at therapeutic blood levels (Wenisch et al., 1997) and have cytotoxicity against EN2 tumor cells (Beekman et al., 1997).

In this report, we investigated the effect of artemisinin on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. We also investigated the effects of combinations of artemisinin with 1,25-$(OH)_2D_3$ or all-*trans* RA on HL-60 cell differentiation. 1,25-$(OH)_2D_3$ and all-*trans* RA were chosen for this study because they have been widely used endogenous stimulators of differentiation in HL-60 cells.

2. Materials and methods

2.1. 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

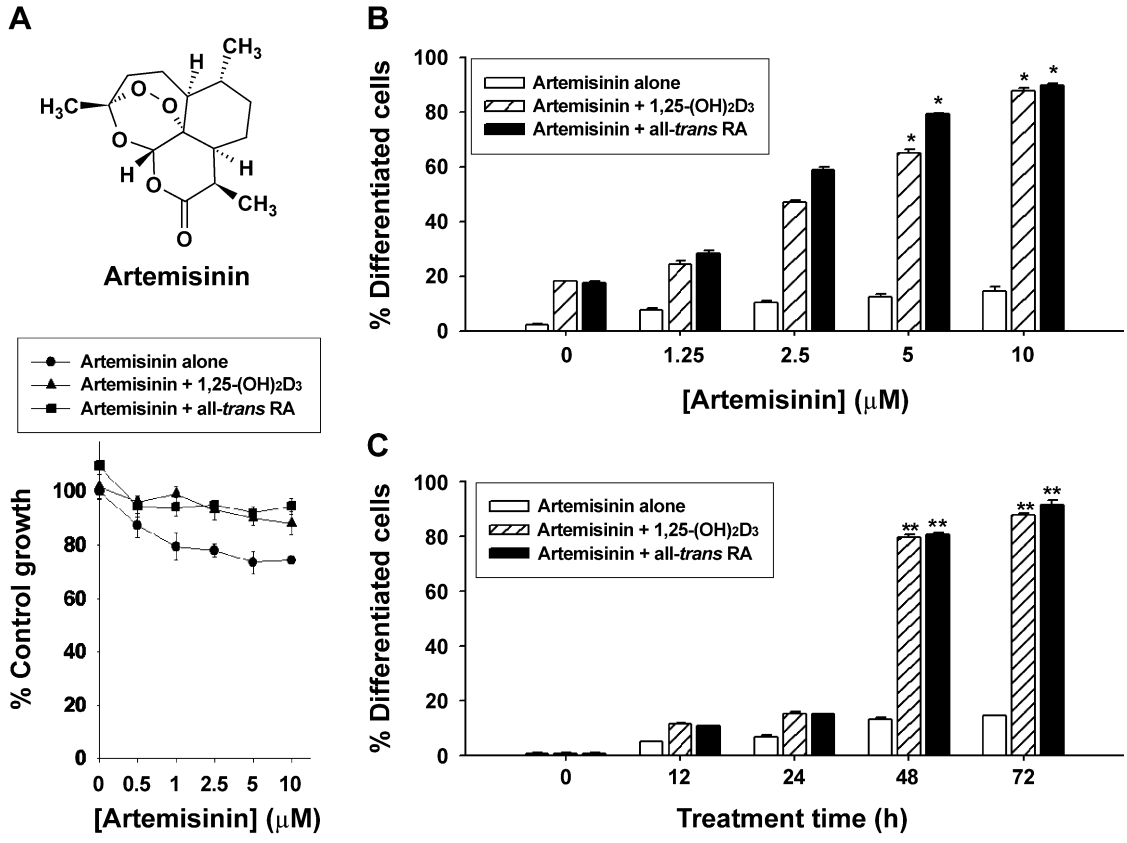

Fig. 1. Effect of artemisinin on 1,25-$(OH)_2D_3$- and all-*trans* RA induced HL-60 cell proliferation and differentiation. HL-60 leukemia cells were treated with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* RA in combination with various concentrations of artemisinin for 72 h (A, B), or with 10 μM artemisinin for various periods (C), and the cell proliferation was determined by the MTT assay (A) and the cell differentiation was assessed by nitroblue tetrazolium reduction assay (B, C). Each value represents the mean ± standard deviations 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 either 1,25-$(OH)_2D_3$ or all-*trans* RA alone. **$P < 0.001$, relative to a group treated with artemisinin alone.

10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Artemisinin was purchased form the Aldrich Chemical (St. Louis, MO, USA). 1,25-$(OH)_2D_3$, all-*trans* RA, phorbol 12-myristate 13-acetate (PMA), 2-[4-Morpholinyl]-8phenyl-1[4H]-benzopyran-4-one (LY 294002) and wortmannin, ethanol, Giemsa staining solution, methanol-free paraformaldehyde, and all other reagents were purchased from the Sigma (St. Louis, MO, USA). Chelerythrine, bisindolylmaleimide (GF 109203X), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H 7) and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from the Tocris Cookson (UK). A stock solution of 1 mM 1,25-$(OH)_2D_3$ and all-*trans* RA were dissolved in absolute ethanol and dimethylsulfoxide, respectively. Artemisinin 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 (Coligan et al., 1995). 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 h at 37 °C, 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 nitroblue tetrazolium reduction assay as previously described (Collins et al., 1979). This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in the dark. Cytospin slides were prepared and were examined for blue–black nitroblue diformazan deposits, 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 2×10^5 cells were loaded into a cyto-funnel and spun at 500 rpm 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 (Coulter Electronics, Hialeah, FL, USA) equipped with a multiparameter data acquisition and display system (MDADS) as previously described (Kim et al., 2001). Briefly, a single-cell suspension was collected from the various cultures and washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4). Afterwards, phytoerythrin (PE)-conjugated anti-

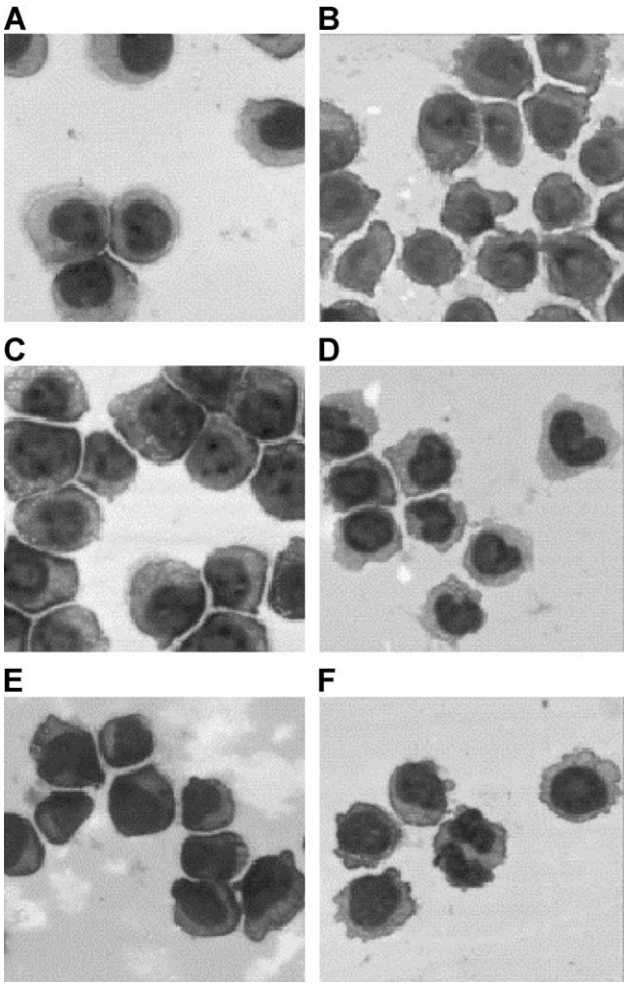

Fig. 2. Morphologic analysis of HL-60 cells treated with artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. HL-60 cells were treated for 72 h with medium alone (A), 10 μM artemisinin (B), 5 nM 1,25-$(OH)_2D_3$ (C), 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$ (D), 50 nM all-*trans* RA (E), or 10 μM artemisinin plus 50 nM all-*trans* RA (F). The cells were assessed by morphologic analysis using Giemsa stain. The data are representative of three separate experiments.

human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) were added, followed by incubation at 4 °C for 1 h. After incubation, the cells were washed with PBS and were fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was performed. Background staining was determined 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 lyzed in lysis buffer (50 mM Tris buffer, pH 7.5 containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM ethylenediaminetetraacetic acid, 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 rpm at 4 °C for 10 min. The proteins in 15 μg of the supernatants were separated using a 10% SDS-PAGE and transferred to the nitrocellulose membrane. The blots were probed with rabbit anti-human PKC isoforms, mouse anti-pERK and rabbit anti-ERK2 antibodies, washed and exposed to horseradish peroxidase-conjugated anti-mouse IgG2a or rabbit IgG antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

2.7. Protein kinase C activity assay

Protein kinase C activity was determined as previously described (Rojnuckarin et al., 2001). In brief, HL-60 cells were lyzed in lysis buffer containing 50 mM Tris (pH 7.5), 2 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′ ,*N*′ -tetraacetic acid, 1% Triton X-100, 150 mM NaCl, 1 μM dithiothreitol, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 50 μg/ml aprotinin by incubation on ice for 30 min. Lysates were then centrifuged at 14,000 rpm at 4 °C for 20 min. The supernatants were incubated with PKC antibody at 4 °C for 2 h. After protein A was added, the mixture was shaken at 4 °C for 1 h and washed with lysis buffer. The antibody-coupled proteins were centrifuged at 5000 rpm for 1 min and reacted with 5 μg myelin basic protein and 0.5 μl $^{32}\gamma$ P-ATP in reaction buffer (0.5 mM

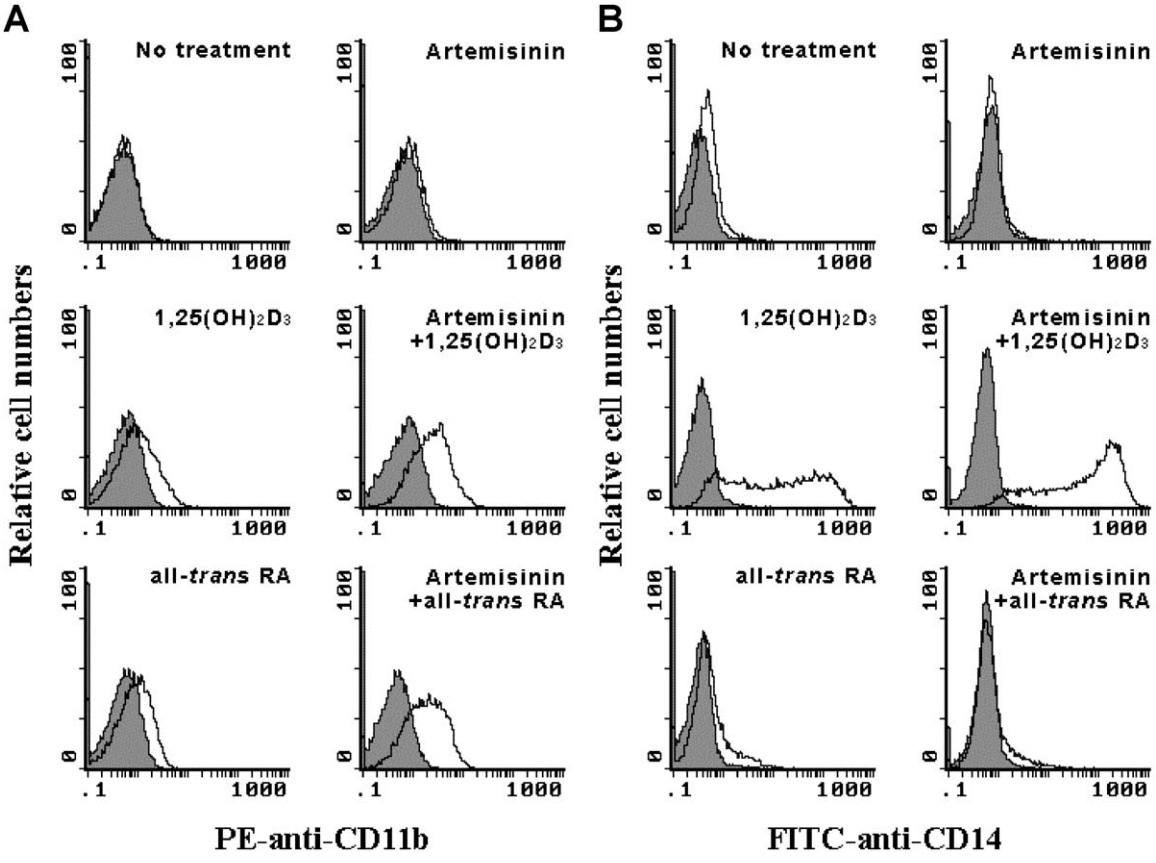

Fig. 3. Cytofluorometric analysis of artemisinin-mediated HL-60 cell differentiation. HL-60 cells were treated with medium alone, 10 μM artemisinin, 5 nM 1,25-$(OH)_2D_3$, 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* RA, or 10 μM artemisinin plus 50 nM all-*trans* RA for 72 h. The cells were assessed by cytofluorometric analysis using PE-conjugated anti-CD11b mAb (A) or FITC-conjugated anti-CD14 mAb (B) (unshaded area), or each isotype control mAb (shaded area). The data are representative of three separate experiments.

ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 50 mM ATP, 2 mM dithiothreitol, 2 mM NaF, and 2 mM sodium orthovanadate) at room temperature for 30 min. The reaction mixture was analyzed by electrophoresis on a 15% SDS-PAGE.

2.8. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between values for various experimental and control groups. A *P* value of <0.05 was considered as significant.

3. Results

3.1. Effect of artemisinin on 1,25-$(OH)_2D_3$- and all-trans RA-induced HL-60 cell differentiation

We examined the effect of artemisinin on 1,25-$(OH)_2D_3$- and all-*trans* RA-induced cell differentiation. HL-60 leukemia cells were treated with artemisinin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA, and cellular differentiation was assessed by nitroblue tetrazolium reduction assay. As controls, the cells were treated with artemisinin alone. As shown in Fig. 1B and C, the addition of artemisinin to cultures exposed to a suboptimal concentration of 1,25-$(OH)_2D_3$ (5 nM) or all-*trans* RA (50 nM), which by itself caused a relatively low level of differentiation, resulted in a marked increase in the degree of cell differentiation. Artemisinin strongly enhanced 1,25-$(OH)_2D_3$- and all-*trans* RA-induced HL-60 cell differentiation in both dose- and time-dependant manners. The effects were maximal at 10 μM of artemisinin, with greater than 87.7% of the treated cells attaining a differentiated state. Artemisinin by itself induced low degree of cell differentiation, with less than 15% of the cells attaining a differentiated phenotype, suggesting that artemisinin 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 10 μM artemisinin inhibited cell proliferation by 13–25%, as determined by MTT assay. Treatment with artemisinin in combination with 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* RA inhibited cell proliferation approximately by 6–12%. For all treatment, cells' viability was greater than 97% throughout the incubation period, as demonstrated by the trypan blue exclusion assay (data not shown).

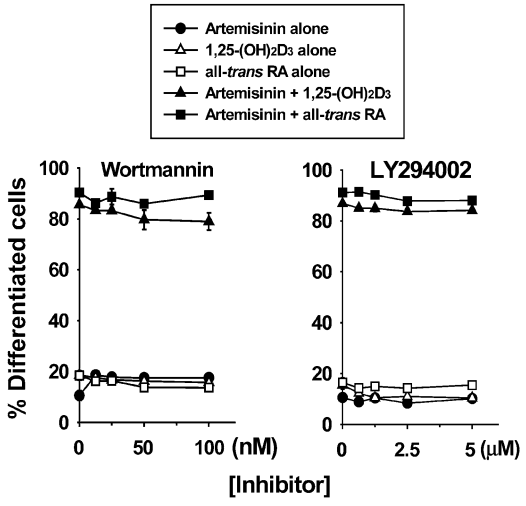

Fig. 4. Effect of inhibitors for PI3-K on HL-60 cell differentiation induced by artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. HL-60 cells were treated with varying concentrations of PI3-K inhibitors (wortmannin, LY 294002) for 40 min, followed by incubation with 10 μM artemisinin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* RA, 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$ or 10 μM artemisinin plus 50 nM all-*trans* RA for 72 h. The cellular differentiation was assessed by NBT reduction assay. The data are presented as a percentage of differentiated cells with the mean ± S.E.M. ($n=3$).

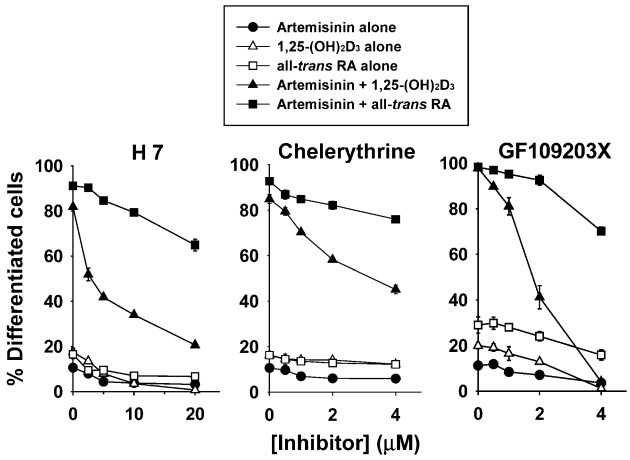

Fig. 5. Effect of inhibitors for PKC on HL-60 cell differentiation induced by artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. HL-60 cells were treated with varying concentrations of PKC inhibitors (H 7, chelerythrine, GF 109203X) for 40 min, followed by incubation with 10 μM artemisinin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* RA, 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$ or 10 μM artemisinin plus 50 nM all-*trans* RA for 72 h. The cellular differentiation was assessed by NBT reduction assay. The data are presented as a percentage of differentiated cells with the mean ± S.E.M. ($n=3$).

To further determine the cell differentiation enhanced by artemisinin, 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. 10 μM Artemisinin-, 5 nM 1,25-$(OH)_2D_3$- or 50 nM all-*trans* RA-treated cells (Fig. 2B, C and E) exhibited relatively small changes in cell morphology such as irregular cell margins. Combined treatment of HL-60 cells with 5 nM 1,25-$(OH)_2D_3$ or 50

nM all-*trans* RA plus 10 μM artemisinin (Fig. 2D and F) resulted in significantly decreased cell size, denser chromatin and an increased cytoplasm to nuclear ratio, which suggested less DNA synthesis. As shown in Fig. 2D and F, some cells showed a horseshoe-shaped nucleus, which is a sign of cell differentiation into a monocytic lineage and some cells showed a multilobed nucleus, which is a sign of cell differentiation into a granulocytic lineage.

Cytofluorometric analysis was also performed to determine the expression of specific surface antigens on HL-60 cells. CD11b (Mac-1) is expressed on activated monocytes, granulocytes, lymphocytes, and a subset of NK cells. HL-60 leukemia cells express a cell surface marker, CD11b, when differentiated into monocytes/macrophages and granulocytes by high concentration of 1,25-$(OH)_2D_3$ and all-*trans* RA, respectively (Kansas et al., 1990). As shown in Fig. 3A, artemisinin synergistically increased the number of CD11b-positive cells when combined with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* RA, confirming that artemisinin potentiated 1,25-$(OH)_2D_3$- or all-*trans* RA-induced HL-60 cell differentiation.

3.2. Effects of artemisinin and 1,25-$(OH)_2D_3$ or artemisinin and all-trans RA on differentiation pathways of HL-60 leukemia cells

To determine the differentiation pathway that HL-60 cells have followed after treatment with artemisinin and 1,25-$(OH)_2D_3$ or with artemisinin and all-*trans* RA, HL-60 cells were treated with artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA, and flow cytometric analysis using monoclonal antibody for the monocytic surface antigen CD14 was determined. CD14 antibody reacts with a glycosyl phosphatidyl inositol-anchored single chain glycoprotein (CD14) expressed on monocytes (Wright et al., 1990). The CD14 antigen is expressed exclusively when HL-60 leukemia cells are differentiated into monocytes (van der Schoot et al., 1987). As shown in Fig. 3B, HL-60 cells treated with a mixture of artemisinin 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 treated with a mixture of artemisinin and 1,25-$(OH)_2D_3$. These results indicate that artemisinin 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 artemisinin and all-*trans* RA showed little staining with anti-CD14 monoclonal antibody, although synergistic induction of cell differentiation was observed as shown by nitroblue tetrazolium reduction assay. In addition, HL-60 cells treated with a mixture of artemisinin and all-*trans* RA stained strongly with a monoclonal antibody against HL-60 cell differentiation marker CD11b (Fig. 3), indicating that artemisinin stimulated all-*trans* RA-induced HL-60 cell differentiation along the granulocytic pathway.

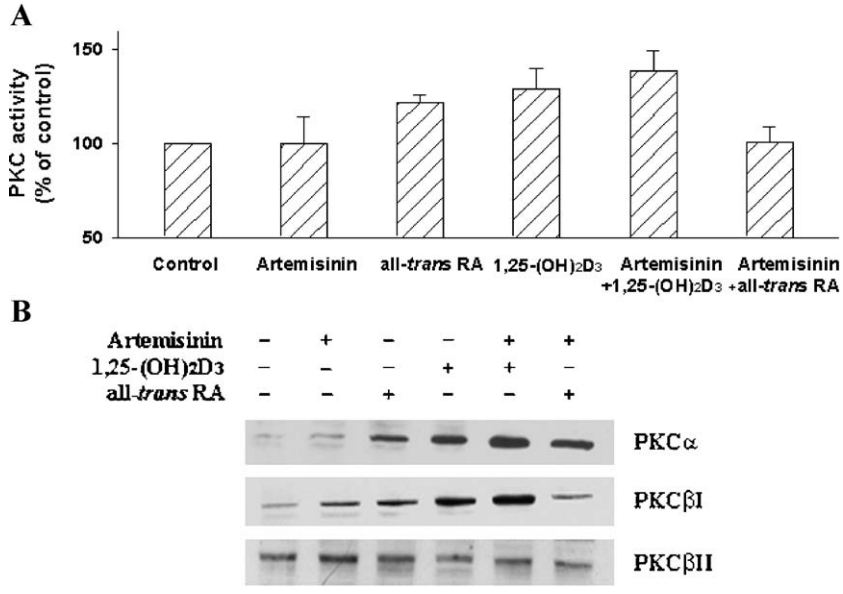

Fig. 6. Effect of artemisinin on PKC activity and protein levels of PKC isoforms in 1,25-$(OH)_2D_3$- or all-*trans* RA-induced HL-60 cells. (A) HL-60 cells were treated with 10 μM artemisinin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* RA, 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$, 10 μM artemisinin plus 50 nM all-*trans* RA for 2 h, and total PKC activity in the treated cells was determined. PKC activity represents the percentage of PKC activity of each treated group relative to the untreated control group. The values represent the means ± S.E.M. ($n = 3$). (B) HL-60 cells were treated with 10 μM artemisinin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* RA, 10 μM artemisinin plus 5 nM 1,25-$(OH)_2D_3$, 10 μM artemisinin plus 50 nM all-*trans* RA for 48 h, and PKC isoforms were determined by Western blot analysis. The experiment was repeated twice with similar results.

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

Previous studies have provided evidence that phosphatidylinositol 3-kinase (PI3-K) activity plays an essential role in differentiation of HL-60 cells (Bertagnolo et al., 1999; Zakaria et al., 1999). To determine any relationship between the effect of artemisinin on cellular differentiation and PI3-K activation, HL-60 cells were treated with specific PI3-K inhibitors, wortmannin or LY 294002, in the presence of artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. Afterward, the degree of cellular differentiation was assessed by nitroblue tetrazolium reduction assay. As shown in Fig. 4, both PI3-K inhibitors did not inhibit HL-60 cell differentiation after treatment with artemisinin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA.

Other studies have provided evidence that activation of PKC has been shown to be necessary for differentiation of HL-60 cells (Martell et al., 1988; Wu et al., 1989; Pan et al., 1997). To determine any relationship between the effect of artemisinin on cellular differentiation and PKC activation, HL-60 cells were treated with specific PKC inhibitors, H 7, chelerythrine or GF 109203X, in the presence of artemisinin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. Afterward, the degree of cellular differentiation was assessed by nitroblue tetrazolium reduction assay. As shown in Fig. 5, PKC inhibitors significantly inhibited HL-60 cell differentiation treated with artemisinin in combination with 1,25-$(OH)_2D_3$ in a dose-dependent manner. In contrast, PKC inhibitors repressed the enhanced HL-60 cell differentiation only at low degree when HL-60 cells were treated with artemisinin in combination with all-*trans* RA.

To further investigate the involvement of PKC in 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation enhanced by artemisinin, HL-60 cells were treated with artemisinin alone or in combination with 1,25-$(OH)_2D_3$ or all-*trans* RA, and PKC activity in the treated cells was determined. As shown in Fig. 6A, artemisinin increased PKC activity in 1,25-$(OH)_2D_3$-induced HL-60 cells, but not in all-*trans* RA-induced HL-60 cells.

In addition, to determine PKC isoforms induced by artemisinin combined with 1,25-$(OH)_2D_3$ or all-*trans* RA, HL-60 cells were treated with artemisinin alone or in combination with 1,25-$(OH)_2D_3$ or all-*trans* RA, and the protein levels of PKC isoforms were determined by Western blot analysis using mAbs for each PKC isoform. As shown in Fig. 6B, artemisinin increased the protein levels of PKCα and PKCβI in 1,25-$(OH)_2D_3$-treated HL-60 cells, whereas artemisinin only increased the levels of PKCα not PKCβI in all-*trans* RA-induced HL-60 cells.

Mitogen-activated protein kinases (MAPKs) are downstream elements in the PKC signaling pathway of HL-60 cells (Marcinkowska et al., 1997). To determine the involvement of extracellular signal-regulated kinase (ERK), which is a MAPK, in 1,25-$(OH)_2D_3$- and all-*trans* RA-induced cell differentiation enhanced by artemisinin, HL-60 cells were treated with 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059), a specific ERK inhibitor, in the presence of artemisinin alone or in combinations of either 1,25-$(OH)_2D_3$ or all-*trans* RA. The synthetic compound, PD 98059, inhibits the ERK pathway by preventing the activation of ERK kinase by c-Raf (Alessi et al., 1995). As shown in Fig. 7, PD 98059 significantly inhibited HL-60 cell differentiation after treatment with artemisinin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA.

To further investigate the relation of PI3-K, PKC and ERK in 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation enhanced by artemisinin, inhibitors for PI3-K, PKC and ERK were treated with artemisinin alone or in combination

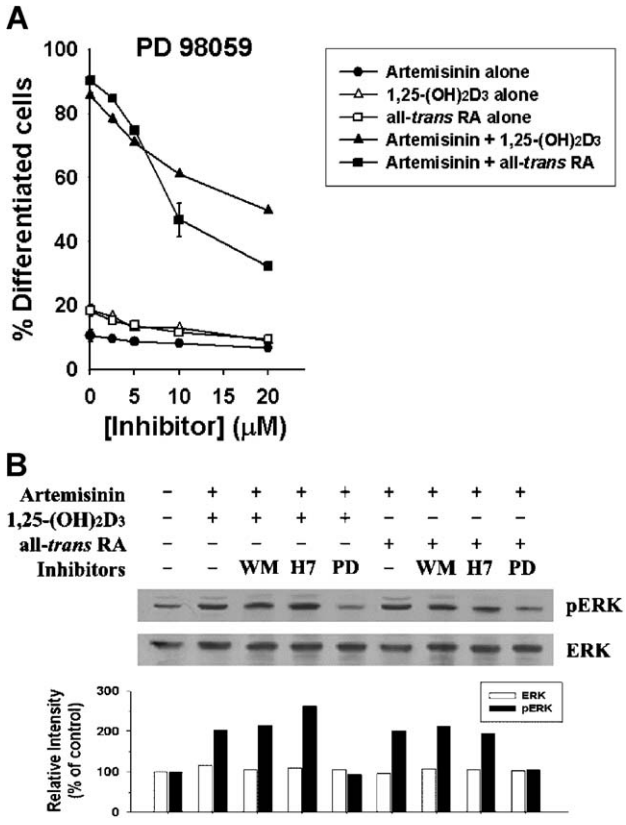

Fig. 7. Involvement of ERK in HL-60 cell differentiation induced by artemisinin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* RA. (A) HL-60 cells were treated for 40 min with varying concentrations of ERK inhibitor (PD 98059), followed by incubation with for 72 h. The cellular differentiation was assessed by NBT reduction assay. The results are presented as a percentage of differentiated cells with the mean ± S.E.M. ($n=3$). (B) HL-60 cells were treated with 100 nM wortmannin (WM), 20 μM H 7, or 20 μM PD 98059 (PD) for 40 min. The levels of ERK were determined by Western blot analysis at 1 h after treatment with artemisinin alone or in combination with 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* RA. The band intensity of each treatment group was measured by densitometric analysis and represented as relative intensity to that of untreated control HL-60 cells. The experiment was repeated twice with similar results.

with 1,25-$(OH)_2D_3$- or all-*trans* RA in HL-60 cells, and ERK activity in the treated cells was determined by Western blot analysis. As shown in Fig. 7B, inhibitors for PKC and PI3-K did not inhibit the ERK activation stimulated by artemisinin in combination with 1,25-$(OH)_2D_3$- or all-*trans* RA in HL-60 cells.

Therefore, artemisinin potentiates 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation, and PKC and ERK but not PI3-K may be involved in the enhanced cell differentiation. In contrast, artemisinin potentiates all-*trans* RA-induced HL-60 cell differentiation, and ERK but not PI3K and PKC may be involved in the enhanced cell differentiation.

4. Discussion

In the present study, we have demonstrated that artemisinin potentiates 1,25-$(OH)_2D_3$- and all-*trans* RA-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 and granulocytes when treated with artemisinin in combination with low doses of 1,25-$(OH)_2D_3$ and all-*trans* RA, respectively. Many previous studies have shown some chemical combinations that exerted an additive or synergistic effect on HL-60 cell differentiation. These combinations include hexafluoro-1,25-$(OH)_2D_3$ with sodium butyrate (Yoshida et al., 1992), 1,25-$(OH)_2D_3$ and silibinin or capsaicin (Kang et al., 2001a,b), 1,25-$(OH)_2D_3$ and tumor necrosis factor-α (Wang et al., 1991), 1,25-$(OH)_2D_3$ and tretinoin tocoferil (Makishima et al., 1996), and retinoic acid with sodium butyrate, dimethyl sulfoxide, or hexamethylen bisacetamide (Breitman and He, 1990).

The mechanism by which artemisinin potentiates 1,25-$(OH)_2D_3$- or all-*trans* RA-induced HL-60 cell differentiation is not clear. 1,25-$(OH)_2D_3$ and all-*trans* RA are believed to mediate biological responses including cell differentiation as a consequence of their interaction with nuclear receptors to regulator gene transcription (Haussler et al., 1998) and with a putative cell membrane receptor to generate rapid non-genomic effects (Norman et al., 1997), including the opening of voltage-gated calcium and chloride channels (Zanello and Norman, 1997), and activation of PI3-K, PKC, and MAPK (Pan et al., 1997; Song et al., 1998; Zakaria et al., 1999).

In our study, inhibitors for ERK, which is a MAPK, strongly inhibited the HL-60 cell differentiation induced by artemisinin in combination with 1,25-$(OH)_2D_3$ or all-*trans* RA, whereas PI3-K inhibitors did not, indicating that ERK may be a common signaling component involved in the artemisinin-enhanced HL-60 cell differentiation into granulocytic and monocytic lineage. 1,25-$(OH)_2D_3$ is known to activate MAPK in HL-60 and NB4 cells (Marcinkowska et al., 1997; Song et al., 1998) and all-*trans* RA is also known to activate ERK in HL-60 human leukemia cells (Yen et al., 1998).

However, it is likely that PKC is differentially involved in 1,25-$(OH)_2D_3$- and all-*trans* RA-mediated differentiation enhanced by arteminisin. PKC inhibitors significantly inhibited the HL-60 cell differentiation induced by artemisinin in combination with 1,25-$(OH)_2D_3$, suggesting that potentiation of cell differentiation by artemisinin may be, at least in combination with 1,25-$(OH)_2D_3$, via a PKC-mediated signaling pathway. In contrast, PKC inhibitors decreased the enhanced cell differentiation at low degree when the cells were treated with artemisinin in the presence of low levels of all-*trans* RA. Approximately 65–80% of the cells treated with artemisinin plus all-*trans* RA were still differentiated into granulocytes in the presence of the inhibitors, suggesting that PKC may not be involved in the all-*trans* RA-induced HL-60 cell differentiation enhanced by artemisinin. PKC is known to be an essential mediator of commitment to monocytes differentiation in diverse leukemia cell models. 1,25-$(OH)_2D_3$ or phorbol ester can increase PKC expression and stimulate translocation of PKC in several differentiated leukemia cell systems. 1,25-$(OH)_2D_3$ increases the expression of PKCα and PKCβ mRNA in HL-60 cells (Obeid et al., 1990). 12-Myristate 13-acetate (PMA) increases the translocation of PKCα and PKCβ from the cytosol to the membrane of HL-60 cells (Aihara et al., 1991).

1,25-$(OH)_2D_3$ and some of its analogues are also used for the treatment of psoriasis (Kragballe, 1992). All-*trans* RA has been used for the treatment of leukemia patients (Warrell et al., 1991) and its analogues have been used for the treatment of psoriasis (Posner and Oh, 1992). The results presented here suggest that treatment of patients with combinations of artemisinin and 1,25-$(OH)_2D_3$, or artemisinin and all-*trans* RA may produce a greater therapeutic response than 1,25-$(OH)_2D_3$ or all-*trans* RA alone, possibly with less toxicity.

In clinical studies, several hundred thousands of patients have been treated with artemisinin derivatives. In addition, artemisinin and its derivatives have cured attacks of malaria more rapidly and with fewer unwanted effects than other anti-malarial agents. No neurological abnormalities in patients have been seen until now (White, 1994). 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 retinoic acids and 1,25-$(OH)_2D_3$. Epideminology studies suggest that people who eat large amounts of fruit and some vegetables have a lower risk of many kinds of cancer (Negri et al., 1991).

In conclusion, artemisinin potentiates 1,25-$(OH)_2D_3$- and all-*trans* RA-induced HL-60 cell differentiation in which PKC is differentially involved. In addition, these results suggest a possible use of artemisinin in the treatment of leukemic diseases.

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References

Aihara, H., Asaoka, Y., Yoshida, K., Nishizuka, Y., 1991. Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage. Proc. Natl. Acad. Sci. U. S. A. 88, 11062–11066.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Beekman, A.C., Barentsen, A.R., Woerdenbag, H.J., Van Uden, W., Pras, N., Konings, A.W., el-Feraly, F.S., Galal, A.M., Wikstrom, H.V., 1997. Sterochemistry-dependent cytotoxicity of some artemisinin derivatives. J. Nat. Prod. 60, 325–330.

Beere, H.M., Hickman, J.A., 1993. Differentiation: a suitable strategy for cancer chemotherapy? Anti-Cancer Drug Des. 8, 299–322.

Bertagnolo, V., Neri, L.M., Marchisio, M., Mischiati, C., Capitani, S., 1999. Phosphoinositide 3-kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells. Cancer Res. 59, 542–546.

Breitman, T.R., He, R.Y., 1990. Combinations of retinoic acid with sodium butyrate, dimethyl sulfoxide, or hexamethylene bisacetamide synergistically induce differentiation of the human myeloid leukemia cell line HL60. Cancer Res. 50, 6268–6273.

Breitman, T.R., Selonick, S.E., Collins, S.J., 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. U. S. A. 77, 2936–2940.

Coligan, J.E., Kruisbeck, A.M., Margulies, D.H., Shevach, E.M., Strober, W., 1995. Current Protocols in Immunology. Wiley, New York.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E., Gallo, R.C., 1979. Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. J. Exp. Med. 149, 969–974.

Hall, I.H., Williams Jr., W.L., Grippo, A.A., Lee, K.H., Holbrook, D.J., Chaney, S.G., 1988. Inhibition of nucleic acid synthesis in P-388 lymphocytic leukemia cells in culture by sesquiterpene lactones. Anticancer Res. 8, 33–42.

Haussler, M.R., Whitfield, G.K., Haussler, C.A., Hsieh, J.C., Thompson, P.D., Selznick, S.H., Dominguez, C.E., Jurutka, P.W., 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J. Bone Miner. Res. 13, 325–349.

Hehner, S.P., Hofmann, T.G., Droge, W., Schmitz, M.L., 1999. The anti-inflammatory sesquiterpene lactone parthenolide inhibits NF-κB by targeting the IκB kinase complex. J. Immunol. 163, 5617–5623.

Kang, S.N., Chung, S.W., Kim, T.S., 2001a. Capsaicin potentiates 1,25-dihydroxyvitamin D_3- and all-*trans* retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Eur. J. Pharmacol. 420, 83–90.

Kang, S.N., Lee, M.H., Kim, K.-M., Cho, D., Kim, T.S., 2001b. Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by silibinin: involvement of protein kinase C. Biochem. Pharmacol. 61, 1487–1495.

Kansas, G.S., Muirhead, M.J., Dailey, M.O., 1990. Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. Blood 76, 2483–2492.

Kawamori, T., Tanaka, T., Hara, A., Yamahara, J., Mori, H., 1995. Modifying effects of naturally occurring products on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. Cancer Res. 55, 1277–1282.

Kharbanda, S., Saleem, A., Emoto, Y., Stone, R., Rapp, U., Kufe, D., 1994. Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. J. Biol. Chem. 269, 872–878.

Kim, T.S., Kang, B.Y., Lee, M.H., Choe, Y.K., Hwang, S.Y., 2001. 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. 134, 571–578.

Klayman, D.L., 1985. Qinghaosu (artemisinin): an antimalarial drug from China. Science 228, 1049–1055.

Kragballe, K., 1992. Vitamin D_3 and skin diseases. Arch. Dermatol. Res. 284, 30–36.

Makishima, M., Kanatani, Y., Yamamoto-Yamaguchi, Y., Honma, Y., 1996. 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 87, 3384–3394.

Marcinkowska, E., Wiedlocha, A., Radzikowski, C., 1997. 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. 241, 419–426.

Marcinkowska, E., Wiedlocha, A., Radzikowski, C., 1998. Evidence that phosphatidylinositol 3-kinase and p70S6K protein are involved in differentiation of HL-60 cells induced by calcitriol. Anticancer Res. 18, 3507–3514.

Martell, R.E., Simpson, R.U., Taylor, J.M., 1987. 1,25-Dihydroxyvitamin D_3 regulation of phorbol ester receptors in HL-60 leukemia cells. J. Biol. Chem. 262, 5570–5575.

Martell, R.E., Simpson, R.U., Hsu, T., 1988. Effects of protein kinase inhibitors 1(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and *N*-[2-quanidinoethyl]-5-isoquinolinesulfonamide hydrochloride (HA1004) on calcitriol-induced differentiation of HL-60 cells. Biochem. Pharmacol. 37, 635–640.

Meshnick, S.R., Thomas, A., Ranz, A., Xu, C.-M., Pan, H.-Z., 1991. Artemisinin qinghaosu: the role of intracellular hemin in its mechanism of anti-malarial action. Mol. Biochem. Parasitol. 48, 181–190.

Meshnick, S.R., Yang, Y.-L., Lima, V., Kuypers, F., Kamchonwongpaisan, S., Yuthavong, Y., 1993. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). Antimicrob. Agents Chemother. 37, 1108–1114.

Mori, H., Kawamori, T., Tanaka, T., Ohnishi, M., Yamahara, J., 1994. Chemopreventive effect of costunolide, a constituent of oriental medicine, on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett. 83, 171–175.

Negri, E., La Vecchia, C., Franceschi, S., D'Avanzo, B., Parazzini, F., 1991. Vegetable and fruit consumption and cancer risk. Int. J. Cancer 48, 350–354.

Norman, A.W., Okamura, W.H., Hammond, M.W., Bishop, J.E., Dormanen, M.C., Bouillon, R., van Baelen, H., Ridall, A.L., Daane, E., Khoury, R., Farach-Carson, M.C., 1997. 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. 11, 1518–1531.

Obeid, L.M., Okazaki, T., Karolak, L.A., Hannun, Y.A., 1990. Transcriptional regulation of protein kinase C by 1,25-dihydroxyvitamin D_3 in HL-60 cells. J. Biol. Chem. 265, 2370–2374.

Ohnishi, M., Yoshimi, N., Kawamori, T., Ino, N., Hirose, Y., Tanaka, T., Yamahara, J., Miyata, H., Mori, H., 1997. Inhibitory effects of dietary protocatechuic acid and costunolide on 7,12-dimethylbenz[*a*]anthracene-induced hamster cheek pouch carcinogenesis. Jpn. J. Cancer Res. 88, 111–119.

Pan, Q., Granger, J., O'Connell, T.D., Somerman, M.J., Simpson, R.U., 1997. Promotion of HL-60 cell differentiation by 1,25-dihydroxyvitamin D_3 regulation of protein kinase C levels and activity. Biochem. Pharmacol. 54, 909–915.

Posner, G.H., Oh, C.H., 1992. Regiospecifically oxygen-18 labelled 1,2,4-trioxane: a simple chemical model system to probe the mechanism(s) for the antimalarial activity of artemisinin (qinghaosu). J. Am. Chem. Soc. 114, 8328–8329.

Rojnuckarin, P., Miyakawa, Y., Fox, N.E., Deou, J., Daum, G., Kaushansky, K., 2001. The roles of phosphatidylinositol 3-kinase and protein kinase Cξ for thrombopoietin-induced mitogen-activated protein kinase activation in primary murine megakaryocytes. J. Biol. Chem. 276, 41014–41022.

Ross, J.J., Arnason, J.T., Birnboim, H.C., 1999. Low concentrations of the feverfew component parthenolide inhibit in vitro growth of tumor lines in a cytostatic fashion. Planta Med. 65, 126–129.

Song, X., Bishop, J.E., Okamura, W.H., Norman, A.W., 1998. Stimulation of phosphorylation of mitogen-activated protein kinase by 1α,25-dihydroxyvitamin D_3 in promyelocytic NB4 leukemia cells: a structure–function study. Endocrinology 139, 457–465.

Sowumni, A., Oduola, A.M., 1994. Efficacy of artemether in severe falciparum malaria in African children. Acta Trop. 61, 57–63.

Sowumni, A., Oduola, A.M., 1998. Viability of *Plasmodium falciparum* ex vivo: comparison of the effects of artemether and sulfadoxine–pyrimethamine. Eur. J. Clin. Pharmacol. 54, 221–226.

Tanaka, H., Abe, E., Miyaura, C., Shiina, Y., Suda, T., 1983. 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. 117, 86–92.

Trigg, P.I., 1989. Qinghaosu (artemisinin) as an antimalarial drug. Econ. Med. Plant Res. 3, 19–55.

van der Schoot, C.E., von dem Borne, A.E., Tetteroo, P.A., 1987. Characterization of myeloid leukemia by monoclonal antibodies, with an emphasis on antibodies against myeloperoxidase. Acta Haematol. 78, 32–40.

van Vugt, M., Brockman, A., Gemperli, B., Luxemberger, C., Gathmann, I., Royce, C., Slight, T., Looareesuwan, S., White, N.J., Nosten, F., 1998. Randomised comparison of artemether–benflumetol and artesunate–mefloquine in treatment of multidrug-resistant falciparum malaria. Antimicrob. Agents Chemother. 42, 135–139.

Wang, S.Y., Chen, L.Y., Wang, S.J., Lin, C.K., Ho, C.K., 1991. Growth inhibition and differentiation in HL-60 leukemia cells induced by 1α,25-dihydroxyvitamin D_3 and tumor necrosis factor. Exp. Hematol. 19, 1025–1030.

Warrell Jr., R.P., Frankel, S.R., Miller Jr., W.H., Scheinberg, D.A., Itri, L.M., Hittelman, W.N., Vyas, R., Andreeff, M., Tafuri, A., Jakubowski, A., 1991. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). N. Engl. J. Med. 324, 1385–1393.

Wenisch, C., Parschalk, B., Zedwitz-Liebenstein, K., Wernsdorfer, W., Graninger, W., 1997. The effect of artemisinin on granulocyte function assessed by flow cytometry. J. Antimicrob. Chemother. 39, 99–101.

White, N.J., 1994. Artemisinin; current status. Trans. R. Soc. Trop. Med. Hyg. 88, 3–4.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433.

Wu, X.Z., Shao, G.Y., Chen, S., Wang, X.W., Wang, Z.Y., 1989. Studies on the relationship between protein kinase C and differentiation of human promyelocytic leukemia cells induced by retinoic acid. Leuk. Res. 13, 869–874.

Yen, A., Roberson, M.S., Varvayanis, S., Lee, A.T., 1998. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Cancer Res. 58, 3163–3172.

Yoshida, M., Tanaka, Y., Eguchi, T., Ikekawa, N., Saijo, N., 1992. Effect of hexafluoro-1,25-dihydroxyvitamin D_3 and sodium butyrate combination on differentiation and proliferation of HL-60 leukemia cells. Anticancer Res. 12, 1947–1952.

Zakaria, H., Devki, N., Laura, S., Keith, L.K., Patricia, H.V., Neil, E., 1999. 1,25-Dihydroxyvitamin D_3-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. J. Exp. Med. 190, 1583–1594.

Zanello, L.P., Norman, A.W., 1997. 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. 272, 22617–22622.

Zhang, F., Gosser, D.K., Meshnick, S.R., 1992. Hemin-catalysed decomposition of artemisinin (qinghaosu). Biochem. Pharmacol. 43, 1805–1809.