

Full-length article

Anticancer effect of aloe-emodin on cervical cancer cells involves G₂/M arrest and induction of differentiation¹

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Key words

HeLa cells; cell cycle; aloe-emodin; alkaline phosphatase; c-myc

¹ Project supported by grants from the Ningbo Natural Science Foundation (No. 2006A610047); the “151 Personal Training Project” of Zhejiang Province, China; the “Famous Professor Training Project” of Ningbo, China; the “Key Subjects Fund of Ningbo University (No. XK0614052), and the KC Wong Magna Fund at Ningbo University.

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Received 2007-03-26
Accepted 2007-07-09

doi: 10.1111/j.1745-7254.2007.00707.x

Abstract

Aim: The aim of this study was to investigate the effects of aloe-emodin, a natural compound from the root and rhizome of *Rheum palmatum*, on the growth of human cervical cancer cells, HeLa. **Methods:** HeLa cells were treated with various concentrations of aloe-emodin for 1–5 d, and cell growth was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. The long-term growth effect was investigated by crystal violet assay. The distributions of the cell cycle and apoptosis were analyzed by flow cytometry. The alkaline phosphatase (ALP) activity was analyzed by a chemical analyzer. Finally, Western blotting was used to indicate the abundant changes of protein kinase C (PKC), c-myc, cyclins, cyclin-dependent kinases (CDK), and proliferating cell nuclear antigen (PCNA). **Results:** Aloe-emodin inhibited the growth of HeLa cells in a dose-dependent manner at concentrations ranging between 2.5 and 40 μmol/L. The flow cytometric analysis showed that HeLa cells were arrested at the G₂/M phase. This effect was associated with the decrease in cyclin A and CDK2, and the increase in cyclin B1 and CDK1. More importantly, the ALP activity was found to be increased by aloe-emodin treatment, and accompanied by the inhibition of PCNA expression. In addition, aloe-emodin suppressed the expression of PKCα and c-myc. **Conclusion:** These findings provide a possible mechanistic explanation for the growth inhibitory effect of aloe-emodin on HeLa, which includes cell cycle arrest and inducing differentiation.

Introduction

There is an increasing demand for natural compounds that improve humans' health. Many nutritive and non-nutritive phytochemicals with diversified pharmacological properties have shown promising responses for the prevention and/or intervention of various cancers^[1]. Aloe-emodin (1,8-dihydroxy-3-hydroxymethyl-9,10-anthracenedione) is a herbal anthracenedione derivative from *Rhei rhizoma*, a traditional Oriental herb commonly used in laxation, antiviral, and hepatoprotection practice^[2–4]. Recent reports have shown that aloe-emodin possesses antiproliferation effects on some types of cancer cells, such as lung squamous, glioma, and neuroectodermal cancer cells^[5–7]. The anticancer mechanisms of aloe-emodin involve the induction of caspase-dependent apoptosis, where the greater sensitivity of neuroectodermal tumor cell lines could be related to an energy-dependent

pathway^[5,7]. Furthermore, the antiglioma action of aloe-emodin involves extracellular signal-regulated kinases (ERK) 1 and 2-independent induction of both apoptosis and autophagy, as well as the ERK inhibition-mediated differentiation of glioma cells^[6]. The inhibitory effect of aloe-emodin on the activity and gene expression of N-acetyltransferase, which plays an initial role in the metabolism of arylamine carcinogens, was found in human malignant melanoma cells^[8]. Recently, Lin *et al* found that aloe-emodin-induced apoptosis in T24 human bladder cancer cells was mediated through the activation of p53, p21, Fas/Apo-1, Bax, and caspase-3^[9]. However, the anticancer molecular mechanisms of aloe-emodin are largely unclear, especially for cervical cancer cells.

Cervical cancer continues to be a major public health problem in the world. Of all neoplasms found in females around the world, cervical cancer has the third highest incidence and is the number four cause of death^[10,11]. In this

study, in order to probe the mechanisms underlying the chemopreventive potential of aloe-emodin on cervical cancer, its effects on cell growth, cell cycle, alkaline phosphatase (ALP) activity, protein kinase C (PKC), and c-myc expressions in HeLa cells were investigated. The results of the present study demonstrated the ability and detail mechanisms of aloe-emodin with the potential anticancer therapeutic activity of cervical cancer.

Materials and methods

Materials and cell line Aloe-emodin (N₀A7687), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, N₀M5655), and crystal violet (N₀C3886) were purchased from Sigma (St Louis, MO, USA). RPMI-1640 medium was purchased from Life Technologies (Grand Island, NY, USA), and human cervical cancer cell line HeLa was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Cell culture The HeLa cells were cultured in plastic flasks or multi-well plates at 37 °C in a humidified atmosphere of 5% CO₂ with RPMI-1640 medium containing 10% fetal calf serum, 50 000 U/L penicillin, and 50 mg/L streptomycin. The medium was changed every other day. Exponentially growing cells were used in the experiments. For the quantitative assays of proliferation, 1×10⁴ cells were seeded in 96-well plates in regular growth medium and incubated for 24 h. The cells were then incubated in medium at different concentrations of aloe-emodin dissolved in dimethylsulphoxide (Me₂SO). The concentrations of aloe-emodin used were 2.5, 5, 10, 20, and 40 μmol/L, respectively. The cells were then treated for 1–5 d and monitored for cell growth using the MTT assay. In all the assays, the vehicle (Me₂SO) was present at less than 0.1% and the controls with the vehicle (0.1% Me₂SO) were carried out in parallel.

MTT assay Sets of 12 wells were used for each dose in this assay. In total, 30 μL MTT solution [2 g/L in phosphate-buffered saline (PBS)] was added into each of the 96 wells. After the cells were incubated at 37 °C for 4 h, the medium was removed and 150 μL of Me₂SO was added to solubilize the formazan. The microplate was shaken on a rotary platform for 10 min. Finally, the optical density (OD) values were measured at 550 nm using a Wellscan reader (Labsystems, Santa Fe, NM, USA). The inhibitive rate was used to indicate the suppressive effect of aloe-emodin on the HeLa cells. Growth inhibition was calculated as a percentage as follows: $(OD_{\text{control}} - OD_{\text{experiment}}) / OD_{\text{control}} \times 100\%$ ^[12].

Crystal violet assay The intensity of crystal violet staining is directly proportional to the number of adherent cells^[13]. In order to observe the long-term antiproliferation effect, the

HeLa cells were seeded in flat-bottom 6-well plates at 1×10⁴ cells/well and treated with various concentrations of aloe-emodin for 8 d. Then the medium was removed very carefully by mild suction, and 2 mL/well of 1% glutaraldehyde solution in PBS was added. The plates were incubated for 15 min at room temperature to fix the cells. The fixative was removed and replaced by the same amount of PBS. The PBS was removed and the same amount of 0.02% aqueous solution of crystal violet was added. After incubation at room temperature for 30 min, the crystal violet solution was poured and the plates were washed gently with water. Then 2 mL 70% ethanol was used to release crystal violet. Finally, the absorbance was measured at 570 nm using a Wellscan reader.

Cell cycle analysis and apoptosis measurement A total of 1×10⁶ HeLa cells were treated with various concentrations of aloe-emodin for 1–5 d. The cells were harvested with 0.25% trypsin and sedimented by centrifugation at 937×g for 5 min at room temperature. After the supernatant was removed, ice-cold 70% ethanol was added. Finally, the cell cycle was analyzed with a Coulter flow cytometer (Beckman Coulter, Miami, FL, USA). The cell cycle distribution was estimated according to standard procedures^[14]. The percentage of cells in the different cell cycle phases (G₀/G₁, S, or G₂/M phase) was calculated using Coulter Epicx XL-MCL DNA analysis software (Beckman Coulter, USA). The sub-G₁ peak was considered a measure of apoptosis^[15,16].

Determination of relative ALP activities The HeLa cells were seeded at a density of 1×10⁴ and treated with 2.5, 5, 10, 20, and 40 μmol/L aloe-emodin for 1–5 d before being assayed for ALP activity. The cells were then dissolved with 0.25% sodium deoxycholate. Finally, the ALP activities were measured by dynamics assay with a Screen Master 3000 semi-automatic biochemistry analyzer (Hospitex Diagnostics, Florence, Italy). The relative enzyme activity was expressed as U/g^[17,18]. Six wells of a 12-well plate were used for each dose and treatment time. Three independent experiments were performed in this analysis.

Western blotting Total cell lysates from 2×10⁵ cells were prepared by lysing the washed cell pellet directly in radioimmunoprecipitation buffer. The lysates were clarified by centrifugation at 13 000×g for 15 min at 4 °C. The lysates were boiled and separated by SDS-PAGE in 10% polyacrylamide gels, blotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and analyzed by Western blotting with the antibodies from Boster Bioengineering (Wuhan, China).

Statistics The statistical analysis was performed using SPSS version 10.0 (SPSS, Chicago, IL, USA). The Student's *t*-test was used to make a statistical comparison between the groups. The level of significance was set at *P*<0.05.

Results

Growth inhibitory effect of aloe-emodin on human cervical cancer cells The effect of aloe-emodin on cell growth was evaluated by MTT assay (Figure 1A). Aloe-emodin inhibited the growth of HeLa cells in a time- and dose-dependent inhibitory manner at concentrations of 2.5–40 $\mu\text{mol/L}$ (at 2.5 $\mu\text{mol/L}$ –20 $\mu\text{mol/L}$, $P < 0.01$; at 40 $\mu\text{mol/L}$, $P < 0.001$). Next, to observe the long-term effect, the crystal violet assay was used. The results showed that the number of adherent cancer cells was decreased by aloe-emodin ($P < 0.001$, Figure 1B). These data imply that aloe-emodin has a significant growth inhibitory effect on HeLa cells *in vitro*.

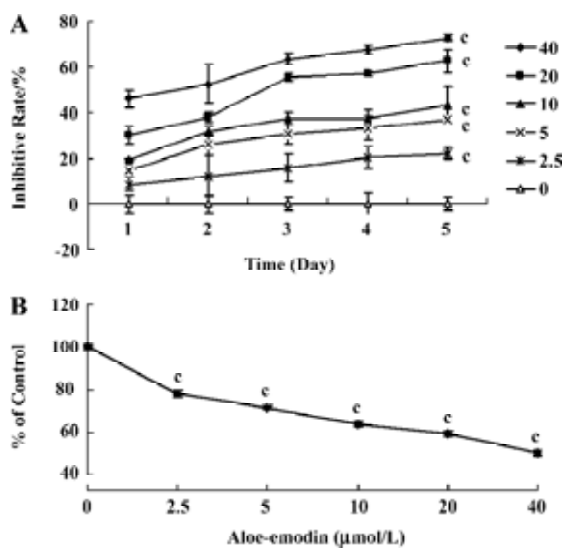


Figure 1. Effect of aloe-emodin on the growth of human cervical cancer HeLa cells. (A) MTT assay. HeLa cells were treated with aloe-emodin at concentrations ranging from 2.5 to 40 $\mu\text{mol/L}$ for 1–5 d. Cells growing in 0.1% Me_2SO were used as the control group. Each concentration had 12 independent wells, and experiments were done in triplicate. Bars represent mean \pm SEM ($^cP < 0.01$). (B) crystal violet assay. HeLa cells treated by 0, 2.5, 5, 10, 20, and 40 $\mu\text{mol/L}$ aloe-emodin for 8 d. Mean \pm SEM represent 3 independent experiments ($^cP < 0.01$).

Effect of aloe-emodin on the cell cycle progression of HeLa cells

In order to decipher the suppressive mechanisms of aloe-emodin on HeLa cells, changes in the cell cycle distribution were monitored by flow cytometry. The treatment of aloe-emodin resulted in a time-dependent increase in the distribution of cells at the G_2/M phase (Table 1). Furthermore, the sub- G_1 peak (apoptosis peak) was not observed (data not shown). By using DNA fragmentation analysis, the DNA ladder was not obviously observed (data not shown). Next, the levels of the cell cycle-associated proteins were determined by Western blotting. As shown in Figure 2, aloe-emodin decreased the abundance of cyclin A and cyclin-dependent kinase (CDK) 2, while increased cyclin B1 and CDK1 in the protein levels in a dose-dependent manner. Such data were consistent with arresting cells in the G_2/M boundary. Cyclins function as regulators of CDK. Cyclin A binds and activates CDK2, and thus promotes both cell cycle G_1/S and G_2/M transitions^[19]. Cyclin B1 binds and activates CDK1, and is expressed predominantly during the G_2/M phase^[20]. The results obtained from this study suggest that one of the mechanisms of the growth inhibitory effect of aloe-emodin on HeLa cells is through cell cycle arrest, but not by apoptosis induction, at least at the concentrations observed.

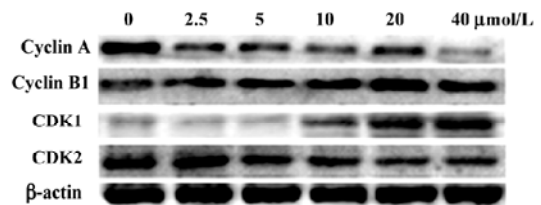


Figure 2. Effects of aloe-emodin on cell cycle-associated proteins in HeLa cells. Cells were treated with various concentrations of aloe-emodin for 72 h.

Increase of ALP activity by aloe-emodin In order to determine whether aloe-emodin is capable of affecting ALP activity in HeLa cells, a dynamics assay was used. The ALP

Table 1. The cell cycle effect of aloe-emodin on HeLa cells. $n=3$. Mean \pm SD. $^bP < 0.05$, $^cP < 0.01$ vs control group.

Group	48 h			72 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	65.10 \pm 0.42	31.70 \pm 0.14	3.20 \pm 0.57	66.55 \pm 2.47	31.05 \pm 0.49	2.40 \pm 3.04
Aloe-emodin 10 $\mu\text{mol/L}$	65.70 \pm 1.84	28.95 \pm 0.49 ^b	5.35 \pm 1.34 ^b	66.45 \pm 0.07	27.60 \pm 0.49 ^b	5.95 \pm 0.57 ^b
Aloe-emodin 20 $\mu\text{mol/L}$	65.30 \pm 0.14	25.45 \pm 0.35 ^c	9.25 \pm 0.35 ^c	65.90 \pm 3.18	24.20 \pm 0.28 ^c	9.90 \pm 2.90 ^c
Aloe-emodin 40 $\mu\text{mol/L}$	62.20 \pm 0.49	20.10 \pm 0.07 ^c	17.70 \pm 0.57 ^c	65.30 \pm 1.84	20.05 \pm 0.64 ^c	14.65 \pm 2.47 ^c

activity was increased by aloe-emodin and reached its peak level at 5 d (Figure 3A). The statistical significance between the ALP of the control and treated cells was observed (5 $\mu\text{mol/L}$, $P < 0.05$; 10, 20, and 40 $\mu\text{mol/L}$, $P < 0.01$). As the proliferating cell nuclear antigen (PCNA) is a commonly used marker of both cell proliferation and differentiation^[21], its changes in the protein level was studied by Western blotting. As shown in Figure 3B, aloe-emodin decreased the abundance of PCNA.

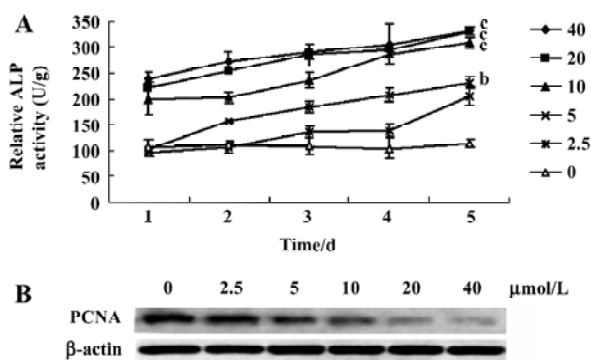


Figure 3. Effects of aloe-emodin on differentiation of HeLa cells. (A) effects of aloe-emodin on ALP activity. HeLa cells treated with 2.5, 5, 10, 20, and 40 $\mu\text{mol/L}$ aloe-emodin for 1–5 d. Cells growing in 0.1% Me_2SO were used as the control group. Mean \pm SEM represent 3 independent experiments. ^b $P < 0.05$, ^c $P < 0.01$ compared to the controls. (B) effects of aloe-emodin on the expression of the differentiation-associated protein. HeLa cells were treated for 3 d. PCNA protein level was investigated by Western blotting.

Aloe-emodin decreases PKC α and c-myc in HeLa cells

As both the PKC pathway and c-myc participate in a wide range of cellular programs controlling proliferation, differentiation, and survival, the influence of aloe-emodin on the activation of these important signaling molecules in HeLa cells was examined. Western blotting results showed that the PKC α and c-myc protein levels in the control group was high, while after treatment with aloe-emodin, both decreased (Figure 4). A clear dose-dependent decrease was

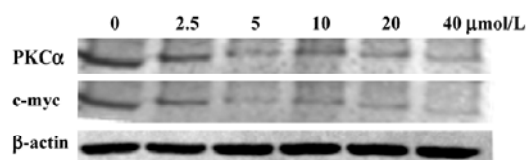


Figure 4. Effects of aloe-emodin on the expression of PKC α and c-myc. HeLa cells were treated with various concentrations of aloe-emodin for 3 d. Levels of PKC α and c-myc were determined by Western blotting. Level of β -actin was used as a loading control.

found in cancer cells treated by aloe-emodin (Figure 4).

Discussion

Phytochemicals present in medicinal herbs and dietary plants are one of the most attractive approaches in cancer chemotherapy. Aloe-emodin, a hydroxyanthraquinone from *Rhei rhizoma* leaves, has been found to have anticancer effects in several cancer cell lines^[5–7]. More importantly, aloe-emodin was found to have no appreciable toxic effects *in vivo*^[7]. Until now, the effect of aloe-emodin on cervical cancer has been largely unknown.

The results of the present study clearly demonstrate the anticancer activity of aloe-emodin on human cervical cancer HeLa cells. Aloe-emodin inhibited the growth of HeLa cells in a dose-dependent manner from 2.5 $\mu\text{mol/L}$ to 40 $\mu\text{mol/L}$ (Figure 1A). At the same time, crystal violet assays indicated that aloe-emodin has a durable growth inhibition on cervical cancer cells (Figure 1B).

To examine the mechanism responsible for cell growth inhibition, cell cycle distribution was evaluated using flow cytometry. The loss of the proliferative capacity of cervical cancer cells treated by aloe-emodin was associated with the G₂/M phase arrest (Table 1). At the same time, the cell cycle-associated proteins were obviously involved (Figure 2). Similar to our observations, several other studies found that aloe-emodin blocked human glioma U251 cells and promyelocytic leukemia HL60 cells at the G₂/M phase^[6,22], and hepatoma cells at the G₀/G₁ phase^[23]. These suggest that multiple mechanisms may be responsible for the anticancer effects of aloe-emodin on different types of cancers.

Cellular ALP are increasingly recognized as important markers for monitoring tumor cell behavior in human malignancies. The measurement of ALP activity is usually used to determinate the effect of inducing the differentiation of anticancer reagents^[24]. Early *in vitro* studies showed that some drugs, such as Ara C, peptichemio, or hydrocortisone, inhibited HeLa cell growth with the elevation of ALP activity^[25]. In this study, the ALP activity in HeLa cells treated by aloe-emodin increased in a time- and dose-dependent manner (Figure 3A). This is one of the first studies to focus on the expression of ALP in human cervical carcinomas cells treated by aloe-emodin.

Cell signal pathways have been become the targets for many drugs. Among them, the PKC pathway is gaining more and more attention for cancer chemotherapy. Several studies have shown that PKC α plays a role in tumor proliferation and survival^[26,27]. In this study, we found that PKC α was suppressed by aloe-emodin (Figure 4). These data strongly suggest that PKC α is one of the key targets of the antitumor action of aloe-emodin. To test this hypothesis, we observed

the expression change of c-myc, a target gene of PKC α ^[28]. As shown in Figure 4, c-myc was also decreased by aloe-emodin. As we know, c-myc is an immediate early gene encoding transcription factors expressed in the G₁ phase of the cell cycle and has a DNA-binding property. Furthermore, the inhibition of c-myc by antisense oligomers has been shown to inhibit cell proliferation^[29]. From this experiment, it is well established that aloe-emodin has a downregulatory effect on the expression of c-myc in human cervical cancer cells (Figure 4). Since c-myc is the downstream target of the PKC pathway, this effect may be through the PKC α pathway.

Taken together, aloe-emodin can affect cell growth, cell cycle, and ALP activity of human cervical cancer cells *in vitro*.

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